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یافته‌های نوین در علوم زیستی
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جداسازی و شناسایی باکتری تولید کننده پروتئاز قلیایی در حضور شوینده از چشمه آب گرم در ایران

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چکیده. پروتئازها مهم ترین آنزیم های صنعتی هستند که کاربردهای متعددی در حوزه های مختلف تجاری و صنعتی از جمله شوینده، صنایع غذایی و چرم سازی دارند. این مطالعه با هدف جداسازی سویه ی تولیدکننده پروتئاز قلیایی از چشمه آبگرم قینرجه و ارزیابی فعالیت پروتئازی به منظور استفاده در صنایع شوینده انجام شد. باکتری جداسازی شده در محیط پایه ی معدنی شامل ۲٪ اسکیم میلک کشت داده شد. فعالیت پروتئازی با استفاده از روش هیدرولیز کازئین اندازه گیری شد. تاثیر عوامل مختلف از جمله pH، دما، SDS، توین ۸۰ و EDTA بر پایداری و فعالیت آنزیمی مورد سنجش قرار گرفت. سازگاری پروتئاز در حضور شوینده های مختلف صنعتی مورد ارزیابی قرار گرفت. نتایج حاصل از بررسی های فیلوژنتیک، مورفولوژیک و بیوشیمیایی نشان داد که باکتری جداسازی شده، سویه ای جدید از *Brevibacillus borstelensis* است که قادر به تولید پروتئاز قلیایی برون سلولی است. مطالعات آنزیمی نیز نشان داد که بیشترین فعالیت پروتئازی در دمای 60 °C و pH ۹ است. علاوه بر این، آنزیم پایداری بالایی در حضور توین ۸۰، SDS، H_2O_2 و سازگاری بالایی با انواع شوینده های تجاری دارد. پروتئازهایی که قادر به هیدرولیز پروتئین در شرایط محیطی سخت (همانند شرایط قلیایی، دمای بالا و حضور سورفکتانت) هستند، اهمیت والایی در فرایندهای بیوتکنولوژیکی و صنعتی دارند. بنابراین از این آنزیم می توان در آینده، در ترکیبات شوینده ها استفاده بهینه نمود.

واژه های کلیدی. بروی باسیلوس، پایداری، پروتئاز قلیایی، شوینده، فعالیت

Isolation and identification of alkaline protease-producing bacterium in the presence of washing-powder from Geinarje hot spring, Ardabil, Iran

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Abstract. Proteases are important industrial enzymes used in different areas of industry, mainly detergent, food and leather industries. In this study, novel alkaline protease-producing bacterium was isolated from Geinarje hot spring and examined for maximum protease activity to be utilized in washing-powder. The isolated bacterium was cultured in mineral salt medium including 2% Skim Milk. Proteolytic activity of supernatant was measured by caseinolytic method. The effects of pH, temperature, SDS, Tween 80 and EDTA on protease stability and activity were investigated. The detergent compatibility of protease was assayed. On the basis of phylogenetic analysis and morphological as well as biochemical tests, the isolate was identified as a new strain of *Brevibacillus borstelensis* capable of generating extracellular alkaline protease. The generated protease was determined as alkaline metallo-protease having high activity at 60 °C and pH 9. Moreover, the alkaline protease was stable in the presence of SDS, Tween 80 and H_2O_2. It is compatible with commercial detergents. Finding proteases capable of degrading proteins in extreme environment (i.e. alkaline pH, high temperature and presence of surfactants) is valuable in biotechnological and industrial practices. Therefore, it can be utilized in detergent formulation in the future.

Keywords. alkaline protease, *Brevibacillus* sp., activity, stability, laundry detergent

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INTRODUCTION

Proteases are the most significant and cost-efficient industrial enzymes (Maharaja *et al.*, 2018). Microbial (*i.e.* bacterial and fungal) sources are leading suppliers of these enzymes. Proteases were widely present as key enzymes for processing important therapeutics. *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Halomonas*, *Pseudomonas* and *Serratia* are the most important bacteria in the production of proteases (Pena- Montes *et al.*, 2008). *Bacillus* species, such as *B. licheniformis*, *B. subtilis*, *B. amyloliquifaciens*, and *B. mojavensis*, are the main sources of alkaline proteases (Nguyen *et al.*, 2013).

Proteases may be classified by the optimal pH in which they are active: Acidic proteases, Neutral proteases and Basic proteases (or Alkaline proteases) (Dorra *et al*., 2018). Alkaline proteases possess wide application in industrial plans. The use of enzymes in detergents started in 1914 when two German scientists, Rohm and Haas, utilized pancreatic proteases in detergents (Jisha *et al*., 2013). In 1956, scientists utilized a bacterial alkaline protease (called Bio-40) in detergent for the first time. Nowadays, *Bacillus*-based alkaline proteases have more practical applications than those derived from other species (Kuberan *et al*., 2010). Rai and Mukherjee produced detergent-stable alkaline $^{\beta}$-keratinase from *Brevibacillus* sp. strain AS-S10-II (Rai & Mukherjee, 2011). *B. brostelensis* generated enzymes which were active at high temperatures (Bisht & Panda, 2011). *Brevibacillus* sp. PLI-1 produced extracellular alkaline proteases (Wang *et al*., 2012) Seifzadeh demonstrated that proteolytic enzymes produced *by Bacillus* sp. GUS1 were alkaline-stable and active in a wide range of pH (Seifzadeh *et al*., 2008).

The vast variety of proteases, with specific actions and applications, have attracted worldwide attention due to their physiological as well as biotechnological applications (Fekadu, 2015). To be beneficial in industry, proteases must be stable and active in a vast range of pH, temperature and surfactants (Gupta & Khare, 2007). Most biological laundry detergents contain protease enzymes, while proteases work to break down protein chains. Their ability to break down these compounds makes them excellent for stain removal (Padmapriya *et al*., 2011). Biological detergents are effective at a wide range of temperatures. The ability to wash at lower temperatures also means that a wide variety of materials such as wool and silk, which would be damaged at high temperatures, can be washed in the washing machine. Lower temperatures are also desirable for dyed clothing such as denim jeans because it reduces color transfer (Kumar & Bhalla, 2005).

The goal of this research is to isolate alkaline protease-producing bacteria and optimize the condition for maximum stability and activity of the extracellular alkaline protease to be used in washing powders.

MATERIALS AND METHODS

Isolation of alkaline protease-producing bacteria

Bacteria with potent alkaline proteases were obtained from water samples collected at Geinarje hot spring, Ardabil, Iran. The Isolation of the bacteria was conducted by the new strategy of using hand washing powder in enrichment medium. The salinity, temperature and pH of water were measured in field. 5 ml of water samples were added to a 250 ml baffled Erlenmeyer flask containing 100 ml mineral salt medium containing 2% Skim Milk, 0.05% K_2HPO_4, 0.02% $MgSO_47H_2O$, 0.001% $FeSO_4.7H_2O$, 0.001% $CaCl_2.2H_2O$ and 1% hand washing powder in tap water at pH 9. The culture medium was incubated at 50 °C for 72 h in shaker incubator operating at 120 rpm. After 5 passages, 1ml of culture medium was added to a 15-ml test tube containing 9 ml of 0.85% saline solution and diluted to 10^{-8} by serial dilution method. The diluted samples were spread onto agar plates containing Skim Milk (10% W/V), agar (1.5% W/V), 0.05% KH_2PO_4, 0.02% $MgSO_4$. $7H_2O$, 0.001% $CaCl_2.2H_2O$ and 0.001% $FeSO_4.7H_2O$ in 100 ml tap water at pH 9. Plates were incubated at 50 °C for 24 hours. Clear zones around the colonies are indicative of protease production by bacteria. Colonies with large halo zones were selected and were cultured in nutrient broth medium for subsequent studies.

Identification of isolated strain

Identification of isolated strain was conducted on the basis of 16S rDNA gene sequences, phylogenetic analysis and morphological and physiological tests by Bergey's Manual of Systematic Bacteriology. DNA of selected strain was extracted using high pure PCR product kit. PCR amplifications were performed on a Primus 25 PCR system (PEQLab, Germany). The universal 16S rDNA primers; 27F (5-AGAGTTTGA TCCTGGCTCAG-3) and 1492R (5-GGTTACCTTG TTACGACTT-3) primers were utilized in this procedure. Amplification of purified DNA was divided into stages: 1. initialization step (heating the reaction to temperature of 94-96 °C for 5 minutes), 2. Denaturation step (30 cycles of heating the reaction to 94 °C for 1 minute), 3. Annealing step (decrease reaction temperature to 53 °C for 40 seconds), 4. Extension step (heating the reaction to

72 °C for 1 minutes), 5. Final extension step (heating the reaction to 72 °C for 10 minutes). To check whether the PCR generated the anticipated DNA, 1% agarose gel electrophoresis was employed for size separation of the PCR products. Nucleotide sequencing analysis was performed by dideoxy chain termination method (SEQLAB, Germany) (Kumar *et al*., 2018). The 16SrRNA gene sequence of strain was compared with similar sequences of reference organisms by BLAST algorithm (www.ncbi.nlm.nich.-gov/BLAST/).

Protease assay

The strain A was cultured in alkaline Skim Milk broth medium at 50 ºC and 120 rpm for 48 h in a shaker incubator. Then the supernatant was harvested by centrifugation at 10000 ×g for 10 minutes. Proteolytic activity of supernatant was measured by the modified caseinolytic assay (Chan *et al*., 2014). In this way, 0.5 ml of supernatant was combined with 3 ml of the reaction mixture containing 0.7% casein in 10 mM borax buffer (pH 9) and the mixture was incubated at 60 ºC for 10 minutes. Then, 3.2 ml of a terminating solution containing 0.11 M trichloro acetic acid, 0.22 M sodium acetate and 0.33 M acetic acid was added to terminate the reaction. The resulting mixture was incubated at room temperature for 20 min and centrifuged at 10000 ×g for 4 minutes in order to remove precipitation. The activity of alkaline protease was distinguished by measuring the amount of tyrosine released from casein at 275 nm. Similarly, a control sample was prepared by adding terminating solution prior to the addition of the enzyme. One unit of enzyme activity was the amount of enzyme which liberated 1 µg tyrosine per minute under experimental condition. Enzyme units were calculated using tyrosine standard curve made by 0-100 µg/ ml tyrosine in 20 mM borax buffer, pH 9. All assays were performed in triplicate and the average of the three was taken to evaluate the activity units.

Effect of pH on Protease Stability and Activity

The effect of pH on protease activity was measured at pH 5 to 11, using casein as the substrate at 60 °C. 0.5 ml of diluted crude protease was mixed with 3 ml of 0.7% casein in specific buffers. The mixtures were incubated for 10 minutes at 60 °C and protease activity was measured by the aforementioned method. The pH stability of the crude protease was examined by incubating the crude enzyme in different buffers with various pH (5-11) for 1 hour. Then, the remaining proteolytic activity was assayed in the mentioned manner. Buffer systems used in this assay included sodium acetate (pH 5-6), phosphate buffer (pH 7), glycin-NaOH (pH 8), borax (pH 9) and carbonate buffer (pH 10-11). The enzyme with high proteolytic activity was considered to be 100% active (Li *et al*., 2011).

Effect of Temperature on Protease Stability and Activity

In order to determine the optimum temperature of protease for maximum catalytic activity, the enzyme assay was performed at different incubation temperatures in a range of 20 to 90 °C at constant reaction mixture and pH 9 for 10 minutes. Thermal stability was studied by incubating the crude enzyme at different temperatures ranging from 20 to 90 °C for 1 hour in screw capped tube in water bath (Memmert, Germany). The remaining enzyme activity was evaluated by standard assay condition. The enzyme with high activity was assumed to be 100% active. All experiments were performed in triplicates (Anandharaj *et al*., 2016).

Effect of chelator, surfactants and oxidants on protease stability

The effect of the chelating agent, EDTA (1, 5 and 10 mM), surfactants such as SDS, Tween 80 (0.05, 0.1, 1% w/v) and oxidants like H_2O_2 (0.5, 1% w/v) on the stability of crude protease was investigated. The crude enzyme was pre-incubated by the aforementioned chelator and surfactants for 1 h at room temperature. The remaining activity of protease was measured on the basis of standard enzyme assay. The activity of enzyme without the aforementioned agents was taken to be 100% (Annamalai *et al*., 2014).

Stability and adaptability of alkaline protease in detergent solution

The compatibility of crude protease in the presence of commercial laundry detergents like Barf®, Persil®, Darya® and Tage® were studied by the mentioned method. At first, the laundry detergent was heated to eliminate native protease activity, if any (Rai *et al*., 2010). Detergents were then added to the reaction mixture at the concentration of 1% and enzyme activity was assayed by the previously explained method. To determine the stability of alkaline protease in detergents, 4 heated detergents were applied and incubated in crude enzyme with the concentration of 1% for 1 h at room temperature. After the incubation, the remaining proteolytic activity was assayed by activity was assayed by the standard method. Simultaneous control was performed without detergent (100%).

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Table. 1. The comparison of protease activity of isolates.

Isolate No	Optical density	Enzyme activity (U/ml)
A	0.489	0.99
P	0.498	0.68
X	0.491	0.12

Fig. 1. Clear zones around alkaline protease-producing bacteria isolated in Skim Milk Agar medium after 24 hour incubation.

RESULTS

Isolation and screening of alkaline protease producing bacteria

In this study, we used washing powder for selective enrichment of alkaline bacteria in the isolation media. After five passages, 3 strains (named A, X and P) with the ability to produce alkaline protease were isolated. Among them, strain A produced large transparent halo in Skim Milk Agar and was selected for subsequent research (Fig. 1), (Table 1).

Characterization and identification of strain A

The sequence of 16SrDNA was obtained after DNA extraction and PCR amplification (Fig. 2). The sequence was reversed, aligned and compared with similar database sequences using the Bioedit software (Ibis Biosciences, USA). BLAST analysis demonstrated 99% similarity with *Brevibacillus borstelensis*, *Brevibacillus* sp., *Bacillus* sp., *Actinobacterium* sp. and *Mycobacterium* sp.

Morphological and biochemical properties of the isolated bacterium were investigated by Bergey's Manual of Systematic Bacteriology. The physiological-biochemical characteristics of strain A were similar to *Brevibacillus borstelensis* as confirmed by phylogenetic analysis of the 16SrRNA gene. *Brevibacillus borstelensis* AMN was recorded in NCBI by the KY933393 accession number.

Characterization of the protease

Effect of pH on activity and stability of protease

To characterize optimum pH of protease, the activity of the enzyme was analyzed in different buffers with various pH (5-11) (Fig. 3). Protease was active at pH 7, 8, 9, 10 and 11 with the remaining activity being about 49.54%, 72.48%, 100%, 80.73% and 67.89%, respectively. The maximum activity of enzyme was considered to be pH 9 while its minimum activity was determined to be at pH 5. Since protease possesses alkaline stability in various range of pHs, it can effectively be

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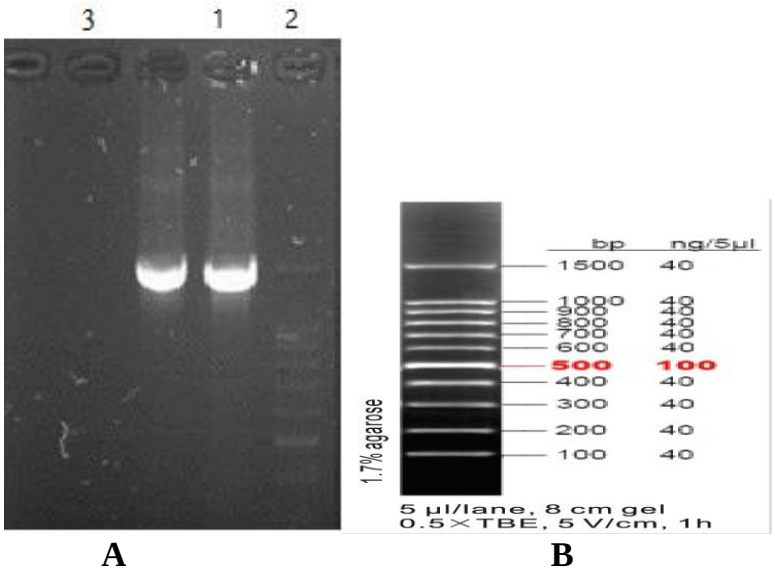

Fig. 2. A: Separation of PCR product of 16S rDNA on 1% agarose gel. [1: Strain A, 2: 100bp, DNA ladder, 3: negative control]. **B:** DNA ladder 100 bp.

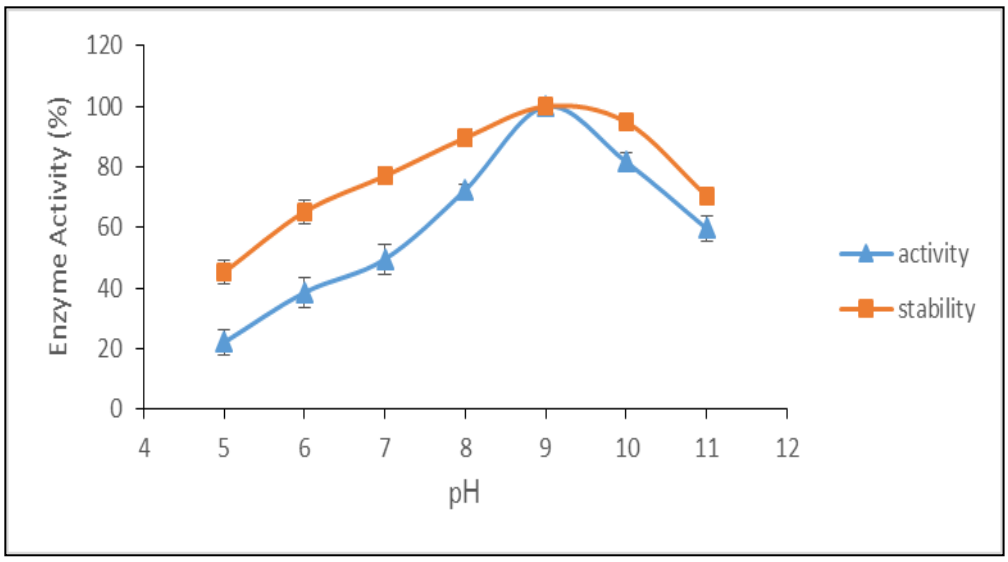

Fig. 3. Effect of pH on proteplytic activity (▲) and stability (■). Protease activity were assayed at 60 °C in buffers: sodium acetate (pH 5-6), phosphate buffer (pH 7), glycin-NaOH (pH 8), borax (pH 9) and carbonate buffer (pH 10-11). The maximum activity of the protease was assumed as 100% activity. Each value represents the means of three experiments and the error bars indicate ±SD.

used in several industries. This is an important feature for use in the commercial detergent formulation as the pH of laundry detergents is in the range of 9–12 (Suribabu *et al*., 2014).

Effect of temperature on the activity and stability of protease

Temperature has wide effects on industrial applications. Protease activity of *Brevibacillus borstelensis* AMN was at maximum level at 60 °C and slowly decreased at 70 °C and 80 °C (Fig. 4). Its activity was lowest at 20 °C. The results showed that protease from the strain AMN was active at high temperature. The heat stability of the crude enzyme showed the considerable stability of enzyme at temperatures between 40 °C to 90 °C after 1 hour of incubation. The thermostability of protease was at maximum rate at 60 °C (100%); it maintained with 71.86% activity at 80 °C and 59.88% at 90 °C. Results suggested that this protease could be utilized in mild and harsh heat condition.

Most of the clothes are susceptible to high temperatures. Using high temperature settings on clothes dryers can damage cotton fabrics and silk

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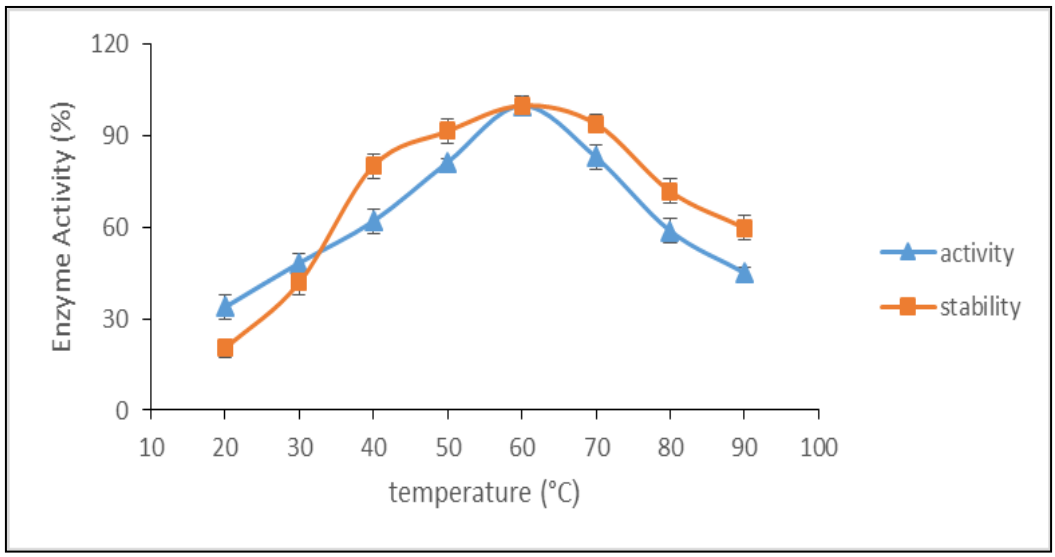

Fig. 4. Effect of temperature on caseinolytic activity (▲) and stability (■) of protease. The maximum activity of the protease was assumed as 100% activity. Each value represents the means of three experiments and the error bars indicate ±SD.

clothes while some stains demand high temperatures for removal. So enzymes used in detergents should be active in thermal ranges of 30-80 ºC. Therefore, alkaline protease from *Brevibacillus borstelensis* AMN is competent for application in laundry detergent and for industrial utilization.

Effect of surfactants, inhibitor and oxidizing agent on protease activity

The activity of enzyme in the presence of Tween 80 (0.05%) and SDS (0.05%) was 88% but the activity slowly reduced to 71% and 59%, respectively at the concentration of 1%. Also, various concentrations of H_2O_2 didn't make a big difference in the activity of protease and the enzyme fully (100%) maintained activity in the presence of H_2O_2 (1%) (Table 2).

Stability and adaptability of alkaline protease in detergent solution

In order to consider laundry detergent compatibility, the enzyme activity was assayed in relation with laundry detergents. Washing powder was added to the reaction mixture at a concentration of 1%. Results showed that the protease activity increased in the presence of Darya® and Tage® to 121% and 114%, respectively, while it decreased in the presence of Persil® and Barf® to 85% and 92%, respectively. The crude protease was pre-incubated towards different commercial detergents for 1 hour. The results are presented in Fig. 5. The catabolic activity of protease in the presence of different detergents is extremely stable. The residual activity of protease in the presence of Persil®, Barf®, Tage® and Darya® was 74%, 89%, 96% and 136% respectively (Fig. 5).

DISCUSSION

In this study, 3 alkaline protease-producing bacteria were isolated, of which one was chosen on the basis of the size of clear zone on Skim Milk Agar medium and high enzyme activity. The isolated strain was Gram-Positive, rod-shaped, aerobic and spore-forming. According to morphological, biochemical, physiological and molecular features, the strain AMN was extremely analogous to *B. borstelensis*. The genus of *Brevibacillus* includes strains which are capable of generating applicable enzymes such as protease, amylase, lipase and cellulase, some of which can degrade polymers (Liang *et al*., 2009). *Brevibacillus* genus has many strains with biological importance like *B. laterosporus* with larvicidal activities (Tian *et al*., 2006) and antimicrobial activities (Hassi *et al*., 2012), *B. brevis* and *B. laterosporus* (De Oliveira *et al*., 2004) with applications in bio-control activities (Edwards & Seddon, 2001).

Maximum enzyme activity was attained at 60 °C. However, heat stability data showed that the protease was fully stable up to 90 °C and displayed high resistance at 60 °C. A previous research showed that the optimal temperatures for proteases activity were ranged from 45 °C to 90 °C (Rai *et al*., 2010). High temperature is an important factor in increasing reaction rate, inhibiting microbial growth and decreasing the possibility of microbial contamination (Kamran *et al*., 2015). Optimum enzyme activity of *Brevibacillus* sp. AK-P2 was measured at 75 °C (Panda *et al*., 2016). Nattokinase of

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Table 2. Stability of alkaline protease in the presence of inhibitor, surfactants and oxidizing agents. The crude enzyme was pre-incubated against detergents components for 1 hour and the remaining activity was assayed by standard method. Each value represents the means of three experiments and the error bars indicate ±SD.

Substrate	Concentration	Enzyme Stability (%)
Blank	–	100
EDTA	1 mM	76 ± 1.32
	5 mM	59 ± 1.43
	10 mM	47 ± 2.14
SDS	0.05 ٪	88 ± 0.87
	0.1 ٪	76 ± 1.41
	1 ٪	59 ± 0.45
Tween 80	0.05 ٪	88 ± 1.03
	0.1 ٪	82 ± 1.38
	1 ٪	71 ± 1.13
H_2O_2	0.5 ٪	106 ± 1.01
	1 ٪	100 ± 0.98

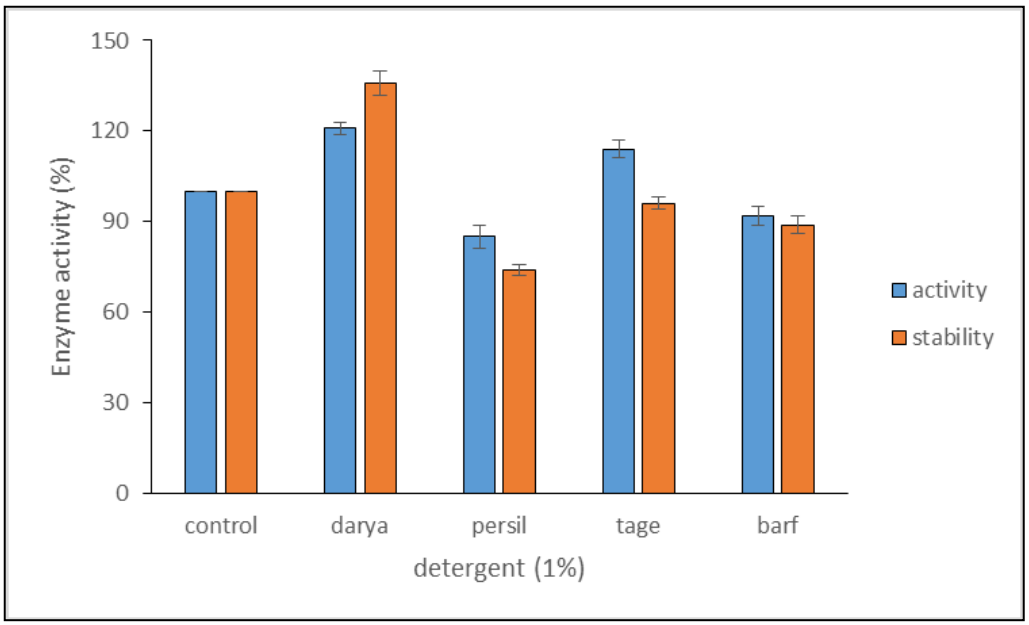

Fig. 5. Laundry detergent compatibility and stability of crude protease. Values were mean ± SD of triplicate determinations.

B. subtilis VTCC-DVN-12-01 showed maximum activity at 65 °C (Suribabu *et al*., 2014). *B. licheniformis* NH1 generated protease with 100% activity at 60 °C (El Hadj-Ali *et al*., 2007). *Bacillus halotolerants* strain CT2 showed maximum protease activity at pH 9 (Dorra *et al*., 2018). Protease from *Bacillus subtilis* AKAL7 and *Exigubacterium indicum* AKAL11showed similar pH activity (Hakim *et al*., 2018).

Adding chemical agent to enzyme solution can enhance or decline enzyme activity, so they act as stimulator or suppressor, respectively (Singh & Banal, 2013). Also, for protease to be used in laundry detergent, it must be firm against surfactant and oxidizing agents. Stability of enzyme against various surfactant and oxidizing agents such as Tween 80, SDS and H_2O_2 was analyzed. The activity of the enzyme in the presence of Tween 80 (0.05%) and SDS (0.05%) was 88% but the activity

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slowly reduced to 71% and 59%, respectively. Constancy of protease in the presence of SDS and other surfactants demonstrated that it was a good choice to be used in washing detergents (Rai *et al*., 2010). The protease obtained from *Pseudomonas aeruginosa* PD100 maintained its activity against SDS and Tween 80 (Najafi *et al*., 2005).

The alkaline protease of *Bacillus subtilis* and *Pseudomonas aeruginosa* showed highest activity in the presence of Tween 20 (Marathe *et al*., 2018).

As shown in table 2, EDTA reduces the activity of protease, so it belongs to metallo-protease family. A previous research illustrated that different halotolerant bacteria produced alkaline metallo-protease like *B. aquimaris* VITP4 (Stoner *et al*., 2004), *Jeotgalicoccus sp*. (Manni *et al*., 2010), *B. alveayuensis* CAS5 (Padmapriya *et al*., 2011) and *Bacillus cereus* (Rai *et al*., 2009). Reese and Maguire showed that cellulase activity of *Brevibacillus* increased in the presence of Tween 80 (Reese & Maguire, 1969). Tween 80 increased the nattokinase activity from *B. subtilis* (Suribabu *et al*., 2014).

Detergent component, stability in high temperature, alkaline pH, oxidizing agent and bleaching agent are in direct relationship with selecting the appropriate enzyme for industrial use. It is important to focus on heat resistance and high activity in alkaline situation for having advantage in washing detergent (Gupta *et al*., 2002). *Bacillus subtilis* PE-11 produced enzyme for cleaning blood clots (Adinarayana *et al*., 2003). The protease of *Bacillus licheniformis* N-2 was utilized in detergent formulation because of its consistency in harsh situations. Alkaline protease of *B. licheniformis* NK displayed 97% activity in the presence of Arial® and Bahar® (Ramkumar *et al*., 2018). *Bacillus thuinginsis*-SH-II-1A exhibited good compatibility in the presence of detergent Surf excel® (Harer *et al*., 2018). The effects of multiple laundry detergent on the studied protease activity are illustrated in fig. 5. It was shown that protease activity ranged from 74% to 136% and protease was stable in the presence of laundry detergent at a concentration of 1%.

CONCLUSION

This research was reported characterization of alkaline protease from *Brevibacillus borstelensis* AMN. It showed high catalytic efficiency in different situations. The protease was stable in the presence of surfactant, inhibitor and oxidizing agents and laundry detergent at 60 °C and pH 9. It can be concluded that the alkaline protease of *Brevibacillus borstelensis* AMN is a heat stable metalloenzyme. The constancy of protease from *B. borstelensis* with respect to optimal temperature and alkaline pH was detected to be different from other proteases. It was shown that protease from *Brevibacillus borstelensis* AMN could be properly utilized in production of laundry powders as well as other commercial and industrial uses.

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