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

Mahnaz Nasre Taheri, Gholamhosein Ebrahimimopour & Hosein Sadeghi

Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran.

Correspondent author: Mahnaz Nasre Taheri, m_nasretaheri@sbu.ac.ir

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 H₂O₂. 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

Received 09.05.2017/ Revised 12.02.2019/ Accepted 04.03.2019/ Published 20.10.2019
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 β-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) Seifzadheh demonstrated that proteolytic enzymes produced by Bacillus sp. GUS1 were alkaline-stable and active in a wide range of pH (Seifzadheh 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% K₂HPO₄, 0.02% MgSO₄7H₂O, 0.001% FeSO₄7H₂O, 0.001% CaCl₂:2H₂O 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 10⁶ 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% KH₂PO₄, 0.02% MgSO₄, 7H₂O, 0.001% CaCl₂:2H₂O and 0.001% FeSO₄:7H₂O 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'-GTTACCTTG 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
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%).
Table 1. The comparison of protease activity of isolates.

<table>
<thead>
<tr>
<th>Isolate No</th>
<th>Optical density</th>
<th>Enzyme activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.489</td>
<td>0.99</td>
</tr>
<tr>
<td>P</td>
<td>0.498</td>
<td>0.68</td>
</tr>
<tr>
<td>X</td>
<td>0.491</td>
<td>0.12</td>
</tr>
</tbody>
</table>

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 KY93393 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...
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...
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 $\text{H}_2\text{O}_2$ didn’t make a big difference in the activity of protease and the enzyme fully (100%) maintained activity in the presence of $\text{H}_2\text{O}_2$ (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
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.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Enzyme Stability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>76 ± 1.32</td>
</tr>
<tr>
<td></td>
<td>5 mM</td>
<td>59 ± 1.43</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>47 ± 2.14</td>
</tr>
<tr>
<td>SDS</td>
<td>0.05%</td>
<td>88 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>0.1%</td>
<td>76 ± 1.41</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>59 ± 0.45</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.05%</td>
<td>88 ± 1.03</td>
</tr>
<tr>
<td></td>
<td>0.1%</td>
<td>82 ± 1.38</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>71 ± 1.13</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.5%</td>
<td>106 ± 1.01</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>100 ± 0.98</td>
</tr>
</tbody>
</table>

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 *Exiguabacterium indicum* AKAL11 showed 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<sub>2</sub>O<sub>2</sub> was analyzed. The activity of the 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. 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 metalloprotease like *B. aquimaris* VTP4 (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 thiingensis*-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.

**ACKNOWLEDGEMENT**

The authors wish to thank Dr. Mohammad Yaghoobi and Ms. Zahra Khosravi for their assistance with the experiments.

**REFERENCES**


How to cite this article: