



Alkaline protease producing *Bacillus* isolation and identification from Iran

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Abstract. Although several microorganisms are known to produce proteases, *Bacillus* strains are often preferred as major sources for commercial alkaline proteases due to their exceptional ability to secrete large amounts of highly active enzymes. The present study aimed at isolation and molecular identification of alkaline protease producing *Bacillus* spp. from soil samples of Damghan County in Iran. This research was conducted in the laboratory complexes in the school of pharmacy of Islamic Azad University Damghan Branch. Soil samples were collected from 3 different regions. Streaking plate was done and 9 colonies were selected on the basis of appearance. To identify isolates, biochemical tests were performed. 16SrRNA gene duplicated with PCR and sent to Sinagene Company for sequencing. All isolates were oxidase-negative and catalase-positive. The ONPG tests were negative. 16SrRNA analysis showed that the isolated bacteria belonged to genus *Bacillus*. The results confirmed that four bacterial isolates had alkaline protease production capacity. It appears that after complementary examinations, the isolated *Bacillus* strains can be used for production of alkaline protease.

Keyword: alkaline protease, *Bacillus*, enzyme, biochemical tests.

Introduction

The vast majority of genus *Bacillus* are Gram-positive, rod-shaped, aerobic and endospore-forming bacteria, which are found in diverse environments such as soil, rocks, aquatic environments, clays, dust, vegetation, food and the gastrointestinal tracts of various animals. Commercial products including enzymes, amino acids, antibiotics and insecticides are produced by *Bacillus* [XIN ZHAO, *et al.*, 2013, BUTNARIU, *et al.*, 2015a, BUTU, *et al.*, 2014b].

In the recent decades there is a raising application of microbial enzymes replacing chemical catalysts in manufacturing chemicals, pharmaceuticals, leather goods, paper, food and textiles. Microbial enzymes are relatively more active and stable than the corresponding enzymes derived from animals or plants [RAMAMOORTHY SATHISHKUMAR, *et al.*, 2014]. Proteases are primarily considered as the enzymes of digestion.

Proteases (EC 3.4.21) are a large group of hydrolytic enzymes that catalyze the hydrolysis of the proteins by cleavage of the peptide bonds between the amino acid residues in other proteins [BUTU, *et al.*, 2014c, BUTNARIU, *et al.*, 2015b]. Proteases are also envisaged as having extensive

applications in development of ecofriendly technologies as well as in several bioremediation processes [ABDELNASSER, *et al.*, 2015; GULERIA and SHIWANI, *et al.*, 2016].

Proteases constitute one of the most important groups of industrial enzymes, accounting for more than 65 percent of total industrial enzyme market.

Moreover, microbial proteases constitute approximately 40 percent of the total worldwide production of enzymes.

Alkaline proteases, with high activity and stability in high alkaline range, are interesting for several biotechnological and bioengineering applications. However, their main application is in the detergent industry, because the pH of laundry detergents is in the range of 9 to 12 [BUTU, *et al.*, 2015, BUTNARIU, *et al.*, 2016].

Alkaline proteases are used in detergent formulations, with other hydrolytic enzymes, as cleaning additives to facilitate the breakdown and release of proteins. In addition, alkaline proteases have various other industrial applications including diagnostic reagents, protein processing, pharmaceuticals, foods, soy processing, peptide synthesis industries, and extraction of silver from used X-ray film [BUTU, *et al.*, 2014a, BUTNARIU, 2012].



Therefore, the industrial demand for highly active alkaline proteases with high specificity and stability of pH, temperature, and organic solvents continues to enhance the search for new enzymes [ABDELNASSER, *et al.*, 2015].

Although several microorganisms are known to produce proteases, *Bacillus* strains are often preferred as major sources for commercial alkaline proteases due to their exceptional ability to secrete large amounts of highly active enzymes.

Therefore, the isolation and screening of new proteolytically active *Bacillus* strains from natural habitats or alkaline wastewater could open new opportunities for the discovery and production of novel keratinolytic protease for use in highly alkaline conditions.

Bacillus licheniformis, *Bacillus subtilis* and *Bacillus pumilus* are the most frequently used species in the industrial production of alkaline protease [AMINA BENKIAR, *et al.*, 2013; ARASTOO BADOEI, *et al.*, 2015].

The present study aimed at the isolation and molecular identification of alkaline protease produced by *Bacillus* spp. isolated from soil samples of Damghan County in Iran.

Material and methods

The greater part of the trials was performed in Laboratory Complex of Faculty of Pharmacy, Islamic Azad University, Damghan Branch, Damghan, Iran. The culture media used included: Plate Count Skim Milk Agar, Lauryl Sulphate Broth, and Nutrient Agar, Q-Lab, Canada. The specimens were taken from the soils of three regions in Damghan County in Iran.

The soil at the initial 2 centimeters depth of collection sites was eliminated and then, the sampling was done up to the depth of 10 centimeters [BUTNARIU, *et al.*, 2012, RODINO, *et al.*, 2014]. The samples were then transported to the laboratory in sterile plastic bags. Serial dilutions of 10^{-3} to 10^{-1} were prepared in order to screen the microorganisms present in the soil [NASERI, *et al.*, 2009; JASIM, 2016]. 1-cc aliquots of dilutions 10^{-2} and 10^{-3} were transferred to sterile plates by sampler. Then, 15 mL of sterile 45 °C melted Nutrient Agar

medium (kept in water bath) was dispensed in the plates. The sample and medium were completely mixed and the plates were put upside down and incubated at 37 °C for 24 hours [NASERI, *et al.*, 2009]. After 24 hours, streak culture was done using colonies grown on each plate.

Some streaks were gently done in four areas of the plates containing Nutrient Agar medium. All of the plates were incubated at 37 °C for 24 hours. The biochemical tests and Gram staining were then conducted. The base Nutrient Agar medium was supplemented with 10 g of Skim Milk Agar and the pH was set at 7.5 and autoclaved [NASERI, *et al.*, 2009].

Cinnagen DNA extraction kit (Cat.No.DN81 5C, Cinnagen, Iran) was exploited to identify the isolated bacteria via sequencing methods and to extract DNA. First, five selected colonies with enzyme production capacity were harvested from 24-hour cultures under sterile conditions and inoculated in 10 mL of Lauryl Sulphate Broth and incubated at shaking incubator at 37 °C.

Then to prepare the sample and isolate bacteria from the liquid phase, the samples were centrifuged at 5000 rpm for 10 minutes. At OD600 of 0.8–1.0 harvest cells was removed by centrifuged (10 minutes, 5000 rpm, room temperature) and the resultant pellet was washed by 400 µL of TEN⁺ (Tris hydrochloride 10 mM, with pH 8.0, ethylenediamine tetraacetate 10 mM, sodium chloride 150 mM) and transferred to a 2-mL microtube. 20 µL of lysozyme was applied to loosen the bacterial cell wall and incubation was done at 37 °C for 20 minutes. Then, 2 µL of RNase A was added and incubated at 65 °C for 3 minutes. 40 µL of sodium dodecyl sulfate solution (10 % w/v) and a small amount of proteinase K was mixed with 550 µL of TEN⁺ (Tris hydrochloride 10 mM, with pH: 8.0, ethylenediamine tetraacetate 1 mM, sodium chloride 150 mM) and the mixture was incubated at 60 °C for 2 hours. 900 µL of pheno (PH: 7.5–8.0, in equilibrium with TE buffer) was added and mixed through inverting the test tube. The resultant solution was centrifuged at 3000 rpm for 5 minutes and then, the



supernatant was transferred to 1.5–mL microtubes. 1:1 mixture of phenol and chloroform causes protein sedimentation, but DNA and RNA remain in the supernatant, causing the formation of a bilayer in centrifugation with the accumulation of proteins in the form of a white coat between two layers.

The transparent supernatant contains DNA and RNA and by removing this part in another tube, DNA purification can be achieved. However when the protein content is very high in cellular extract, it is necessary to repeat phenol–chloroform treatment to remove all of protein content. To do so, the extraction was repeated one time with phenol and two times with chloroform: isoamyl alcohol (1:24). To precipitate DNA in the transparent solution, the aquatic supernatant was transferred into 10 mL of cold ethanol in a micro tube.

The precipitated DNA was removed by curled tip of Pasteur pipette. Then, the DNA was dried in vacuum and dissolved

in 100 mL of TED* and incubated at 4 °C overnight. The quality of extracted DNA was evaluated through gel electrophoresis on agarose gel.

Then, to determine the primer annealing temperature, thermal OD gradient was conducted with thermal range of 63–73 °C [ANNAPURNA, *et al.*, 2013; SAEIDI, *et al.*, 2014]. 5 µL of the extracted DNA was mixed with 1 µL of loading buffer and loaded at 1 % agarose gel. Then, the samples were run or 20–40 minutes with voltage of 35 V in Tris borth ethylenediamine tetraacetate buffer.

Finally, the quality of bands was assessed using electrophoresis. After determining the proper temperature, PCR was conducted for the samples on desired temperature [SAEIDI *et al.*, 2014, BUTNARIU, 2014, BARBAT, *et al.*, 2013]. PCR Master Mix 2 kit was purchased from Viragene company, containing PCR buffer, MgCl₂, dNTPs, Taq DNA polymerase, reverse and forward primers, (16SrRNA), and template DNA (Table 1 and 2).

Table 1.

The program gene amplification on the device thermo cycler

Stage	Temp	Time	Cycles
Initial denaturation	95	3min	1
denaturation	95	30sec	32
Annealing	55	30 sec	32
Extension	72	45 sec	32
Final extension	72	5min	1

PCR procedure up to the volume of 25 µL (DW 11 µL, Master Mix 11 µL, Primer Reverse 1 µL, Primer Forward 1 µL and DNA Template 1 µL). The number of copies amplified at the end of reaction was calculated according to Formula 1:

$$Y=X(\text{efficiency}+1)^n \quad (1)$$

Where, X is the copy number of initial template DNA and n is the number of PCR cycles.

The ideal efficiency is 1, but some limitations decrease it. 16S rRNA gene was amplified by PCR using the primers summarized in Table 2.

Table 2.

Specifications primers16srRNA

1	2	3	4	5	6	7	8
Primer Name	OD (1000µl)	MW	N mol	Water/tube (µL)	TM	Seq.(5–3)	mer
8F	3	6148	16.10	161.03	58.4	AGAGTTTGATCCTGGCTCAG	20
154IR	3	6176	16.03	160.30	62.5	AAGGAGGTGATCCAGCCGCA	20

The PCR conditions were as follows: 94 °C for 3 minutes, 30 cycles for 1 minute at 94 °C, 1minute at 56 °C and 2 minutes at 72 °C, 10 minutes at 72 °C and stop at 4 °C. The PCR products were cloned in pMD–18T plasmid (Tarkara, DaLian, China) and sequenced. Then, the BLAST analysis was conducted for the

determined sequence (<http://www.ncbi.nlm.nih.gov/BLAST>).

All of experiments were done in triplicates. Quality of PCR product evaluated with gel agarose. Finally, the PCR products were sent to Cinnagen Company, Iran for sequencing [CHENG and LI, 2009].



Results and discussion

After examination of the appearance of colonies grown by pour plate method, streak plate method was performed for the samples related to the

soils of three regions in Damghan County, Iran.

Eventually, nine single colonies were selected to perform the next steps of investigation (Table 3).

Table 3.

As a result, after the blast sequences on NCBI

Sample Name	Strain	Query Cover	E value
1	<i>Bacillus subtilis</i> strain RP242	100%	0.0
2	<i>Bacillus</i> sp. strain M08	100%	0.0
3	<i>Bacillus</i> sp. hswX18	100%	0.0
4	<i>Bacillus cereus</i> strain HASD 01	100%	0.0

The results of Gram staining included four different morphologies: bacilli with central spores, bacilli with terminal spores, swollen spore-forming bacilli, and non-spore-forming bacilli, all Gram-positive.

All of the colonies related to these three samples were oxidase-negative and catalase-positive. No color change

was observable in culture media related to the samples. Negative results of ONPG test are indicative of lack of beta-galactosidase in the selected colonies.

The results showed that of the isolated strains, the strains below are capable of alkaline protease production. The optimum PCR temperature was 55 °C. Three bands appeared (Figure 1).

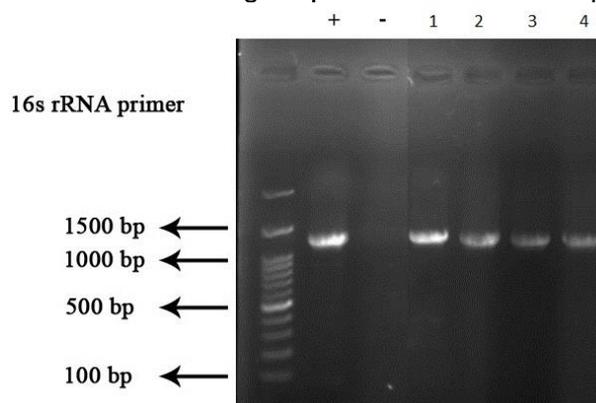


Figure 1. PCR results for gene.

The partially sequenced 16S rRNA gene from isolates was uploaded to the (NCBI) National Center for Biotechnology Information website to search for similarity to known DNA sequences and to confirm the species of these local isolates.

The BLAST query revealed that isolate number 1 (sample 1) was similar to *Bacillus licheniformis* strain WSE-KSU302 with 100 % sequence similarity; isolate number 2 was similar to *Bacillus subtilis* strain amyP216 CICC10034 with 100 % sequence similarity; isolate number 3 was similar to *Bacillus subtilis* strain LT617063.1 with 100 % sequence similarity and isolate number 4 was similar to *Bacillus* sp. strain B2.IIPR with 100 % sequence similarity.

Phylogenetic tree was constructed for these isolates (Figure 2).

Right now, numerous industries are welcome to join the eco-design approach; its point is to create eco-friendly strategies to lessen ecological effects confronting increased pollutants produced by industrial activities.

In such manner, the presenting a supportable cleaner technologies as the utilization of industrial enzymes turns into an imperative resource since they offer less contaminating procedures than synthetic catalysts and perform responses with high specificity. Among these enzymes, proteases, likewise called proteolytic enzymes or proteinases are the most imperative enzymes of the total



industrial enzyme market. Proteases are found in different sources, for example, microorganisms, plants and animals.

The selection of the right organism a key part in high return of alluring enzymes. These days, the significant extent of the commercial protease is

gotten from *Bacillus* strains because of their wide temperature and pH resilience and stability. Proteases from *Bacillus* sp are broadly utilized in waste treatment, food, pharmaceutical, synthesis of oligopeptides, detergent and leathers industries [BRIKI, *et al.*, 2016].

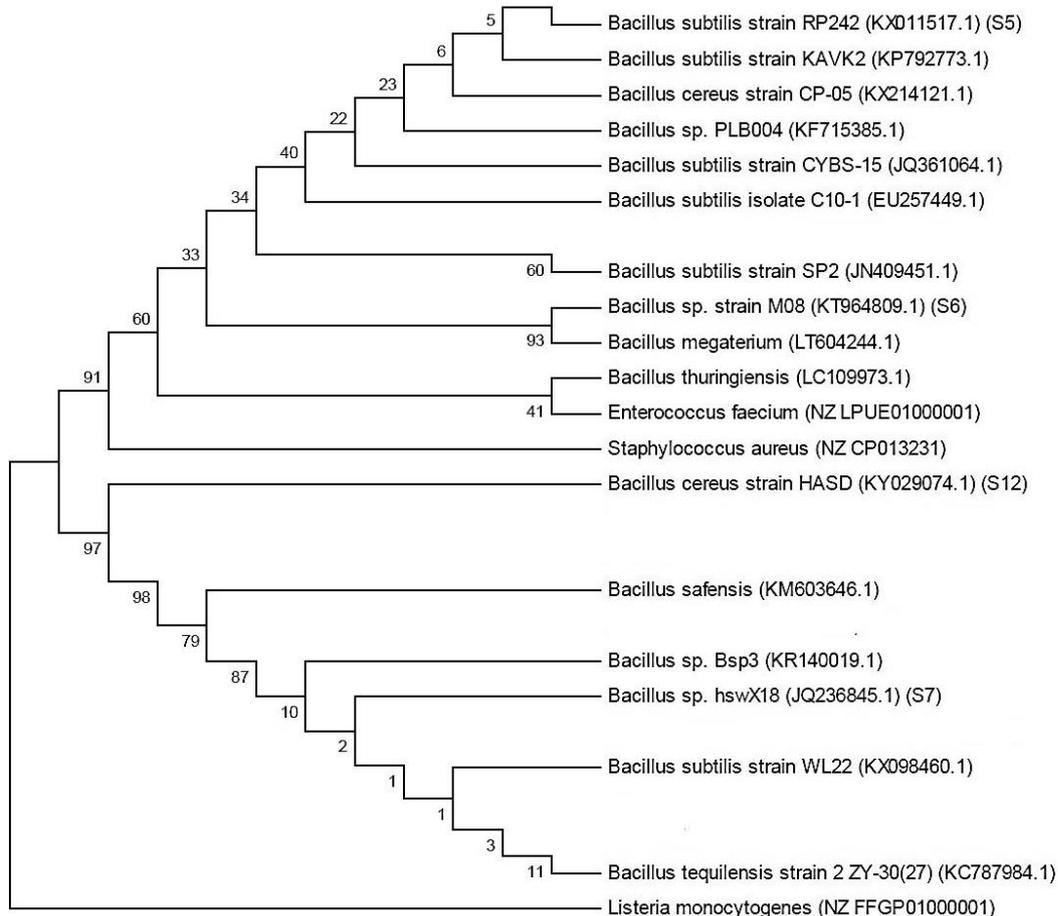


Figure 2. Phylogenetic tree isolated *Bacillus* strains 16srRNA gene sequence

Streak plate technique was directed for the samples gathered in the present project lastly, nine colonies were chosen for the following stages of investigation, after examination of colony appearance for the colonies grown colonies grown by pour plate method. Biochemical and identification methods assisted to identify the strains isolated from soil samples three districts in Damghan County.

All of the results gained from biochemical tests were indicative of genus *Bacillus*. To confirm the results of biochemical tests, molecular identification tests were also carried out. DNA was extracted according to the specific directions. The optimum temperature for PCR was 55 °C. In a project similar to the

present work, a sectional study was conducted on slaughterhouse wastewater specimens of Jiroft in Iran.

The outcomes of this study showed that the isolated bacterial strain had Gram-positive, catalase positive, motile cells and rod shaped. The protease-producing isolate was screened on Skim Milk Agar medium. 16SrRNA gene was PCR amplified and sequenced. Then, biochemical properties of the purified protease were investigated [BUTNARIU and CORADINI, 2012, PETRACHE, *et al.*, 2014].

The results of sequencing demonstrated that the isolated strains belonged to species *Bacillus tequilensis* with proximity of 100 %. The purified protease had the maximum activity and



stability at pH: 9.0 and 46 °C [BADOOYI, et al., 2015], which was consistent with our results. In 1971, Horikoshi studied the alkaline proteases produced by a haloalkalophilic *Bacillus* species isolated from Gujarat State in India. Like the present examination, they utilize nucleotide sequence and 16DrRNA amplification methods for the identification of *Bacillus* taxon. Their results showed that the isolated *Bacillus* strain had the maximum protease production in the presence of casamino acids; ammonia and glucose stopped alkaline protease production [HORIKOISH, 1971].

In a similar project Joe et al. reported extraction of an alkaline protease from *Bacillus clausii* I-52 [JOO and CHANG, 2005].

Solvent resistant alkaline protease was extracted from *Bacillus circulans* MTCC strain residing hydrocarbon polluted home grounds, which was able to grow properly in the presence of organic solvents such as dimethyl sulfoxide, cyclohexane, butanol, benzene, xylene, acetone, and toluene. Heat- and organic solvent-resistant alkaline protease extraction from *Bacillus Coagulans* PSB-07 was also reported, which retained its activity in the presence of 70 % methanol [OLAJUYIGBE and EHIOSUN, 2013]. In 2003 Ammar and coworkers were reported a protease with optimum stability at 55 °C, purified from *Bacillus brevis* [AMMAR, et al., 2003].

Sen and collab. also reported that the alkaline protease extracted from *B. pseudofirmus* SVB showed 91, 95, 98, and 87-percent activity enhancement in the presence of detergents Nirma, Surf, Rin, and Ariel, respectively [SEN, et al., 2011].

In an examination by Asghari and collab. a native strain of *Bacillus licheniformis* isolated from industrial zones was selected for amylase and protease production. Their results showed that the native strain *Bacillus licheniformis* GLU113 had the capacity to simultaneously produce the two industrially important enzymes amylase and protease [ASGARI, et al., 2012], which is in conformity with our findings.

In the results of a research work published by Tebyanian and collab. in 2016, members of the genus *Bacillus*

were isolated from alkaline soils of Guilan Province in the north of Iran and after screening; enzyme production was evaluated concurrently through closed culture and semi-continuous culture methods [TABYANIYAN, et al., 2016].

The applied method and the genera of isolated bacteria were identical to those of our findings. Many of proteases isolated from genus *Bacillus* are active at alkaline pH values and are suitable for industrial utilizations. In a survey similar to this work, a protease extracted from *B. licheniformis* BBRC 100053 with optimum activity at pH range of 8–10 was also reported. This bacterium was also isolated and its protease production was also proven [RAHMAN, et al., 2007]. In the study published by Rajablu and colleagues, the alkaline protease produced by strain *Bacillus* RZ1 isolated from hot springs of Sabalan in Iran was documented [RAJABLOO, et al., 2010], which was accordant with the findings of the present study.

Conclusions

Here we report the isolation and identification of alkaline protease producing *Bacillus*. On the basis of the above mentioned results, four isolated species belonged to genus *Bacillus*.

These findings confirm the production of alkaline protease by the identified strains. It appears that after complementary examinations, the isolated *Bacillus* strains can be used for production of alkaline protease.

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