Alcaline protease from *Bacillus subtilis* IMRUZ -7 and its properties

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ABSTRACT

In the present study, we report the isolation from the soil of one of the slaughterhouses in the Tashkent region, the proteolytic bacterium *Bacillus subtilis* IMRUZ-7, which was chosen to optimize its cultivation conditions for protease production. This strain can be used for large-scale alkaline protease production to meet the needs of current industrial applications. Starch and corn broth are cheap sources of carbon and nitrogen, which led us to offer an inexpensive nutrient medium to maximize protease production. The optimum pH and temperature for enzyme activity were determined to be 9.0 and 60°C, respectively. Since the thermoactivity and pH stability of proteases are of great importance for industrial use, the enzymatic properties of *Bacillus subtilis* IMRUZ-7 protease indicate the potential use of this bacterium and its protease for various industrial purposes.

Key words: alkaline protease, *Bacillus subtilis*, optimal conditions, production, enzyme.

1. INTRODUCTION

Proteases are the most important industrial enzymes that perform a wide range of functions and have various important biotechnological applications [1]. They make up two-thirds of all enzymes used in various industries and account for at least a quarter of the total world production of enzymes [2]. Among various proteases, microbial proteases play an important role in biotechnological processes. The resulting alkaline proteases are of particular interest because they can be used in the production of detergents, foodstuffs, pharmaceuticals, and leather [3]. Most alkaline proteases used for industrial purposes have limitations, firstly, many of the alkaline proteases exhibit low activity and low stability over a wide range of pH and temperature, and secondly, 30-40% of the production cost of industrial enzymes is due to the cost nutrient medium [4]. It is known that the amount of enzyme produced is highly dependent on the strain and growth conditions. The use of an economical nutrient medium for the production of alkaline proteases from an alkaliphilic strain of Bacillus sp. especially important [5]. Among the bacteria Bacillus spp. are specific producers of extracellular alkaline proteases [6]. Microbial proteases, especially from species of the genus Bacillus, have traditionally occupied the predominant market share of industrial enzymes in the world sales of enzymes, being widely used in detergent formulations [7]. It was found that various bacteria that have been tested for use in various industrial purposes, representatives of the genus Bacillus, mainly strains of *B. subtilis* and *B. licheniformis*, are the predominant and rich source of alkaline proteases [8]. Therefore, there is a need to search for new Bacillus strains producing proteolytic enzymes with new properties and to develop inexpensive media.

The aim of our studies was to isolate and select the most active alkaline protease-producing bacterial strain from the soil of slaughterhouses and to study the optimal conditions for protease production by the isolated bacterial strain.

2. MATERIALS AND METHODS

2.1. Sample Collection

To isolate protease-producing bacterial strains, soil samples were obtained from slaughterhouses located on private farms.

2.2. Isolation of Bacteria Producing Alkaline Proteases

All soil samples were sequentially diluted and added to the screening medium (g/l) (skimmed milk powder - 10; yeast extract - 3.0; ammonium sulfate - 6.7; NaCl - 0.5; K_2HPO_4 - 0.7; $MgSO_4x7H_2O$ - 0.5) [9] with pH 8.5 and incubated at 55°C for 48 h [10]. The formation of a clear zone around the colonies confirms the production of alkaline protease. Colonies of strains that formed a clear zone around their boundaries were chosen as enzyme producers. Positive isolates were further screened for better enzyme production by assaying protease activity in liquid nutrient culture using casein as a substrate at 45°C. The strains that showed the maximum activity were selected for further study.

2.3. Bacteria Identification

DNA was isolated by heat treatment as described by Dashti et al. [11]. The isolated 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the following primers: 27F 5'-GAGTTTGATCCTGGCTCAG-3' (Sigma-Aldrich, St. Louis, Missouri, USA) and 1492R 5'-GAAAGGAGGTGATCCAGCC-3' (Sigma-Aldrich, St. Louis, Missouri, USA) [12]. The PCR products were purified using the USB® ExoSAP-IT® PCR Product Cleanup Kit (Affymetrix, USB® Products, USA). Purified PCR products were sequenced using the ABI PRISM BigDye 3.1 Terminator Cycle Sequencing Ready kit (Applied Biosystems, United States). Nucleotide sequences were evaluated, corrected and combined using Chromas software (v. 2.6.5) and EMBOSS Explorer (http://emboss.bioinformatics.nl/). The Basic Local Alignment Search Tool was used to identify 16S rRNA gene sequences and compared with the closest relatives registered in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/).

The phylogenetic tree was constructed using the Neighbor-Joining method [13]. Shown above the branches is the percentage of repetitive trees in which associated taxa are grouped together in a bootstrap test (500 repetitions) [14]. The tree is drawn to scale with branch lengths in the same units as the

evolutionary distances used to derive the phylogenetic tree. Evolutionary distances were calculated using the Maximum Composite Likelihood method [15] and expressed in terms of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. All ambiguous positions were removed for each pair of sequences (pairwise removal option). In total, there were 1563 positions in the final dataset. Evolutionary analysis was carried out using the MEGA X program [16].

2.4. Study of Morphological-Cultural and Physiological-Biochemical Properties of Bacteria

The nature of bacterial growth was assessed when grown on meat-peptone broth (MPB) and meat-peptone agar (MPA). Mobility, size and shape of cells were determined in a daily culture of bacteria grown on MPB medium using a crushed drop preparation using phase contrast microscopy at x900 magnification.

To test the fermentation of carbohydrates by bacteria, a liquid Hiss medium was used with the addition of the following carbohydrates: sucrose, glucose, fructose, lactose, galactose, maltose, raffinose, rhamnose, inositol, sorbitol, dulcite. Catalase activity was tested according to Smibert and Krieg [17]. To check the formation of indole and hydrogen sulfide, indicator paper was used, which was placed under the stopper of a test tube with a bacterial culture growing on the BCH. To detect the reduction of nitrates, the bacteria were cultured for 48 h on BCH with nitrates (KNO_3). 1 ml of the reagent (an equal mixture of 10% chemically pure H_2SO_4 and a solution of 1% potato starch and 0.5% KI) was added to the test tubes with the inoculated broth. To detect casein hydrolysis, bacteria were grown on milk agar. Seeded Petri dishes were incubated at 30°C and observed for the appearance of transparent zones of hydrolysis. To determine the ability to hydrolyze starch, the bacteria were sown on potato-peptone agar. The seeded cups were incubated for 48 h at 30°C and filled with Lugol's solution. The formation of light zones around bacterial colonies indicated starch hydrolysis.

2.5. Protease Production

The culture medium used in this study for protease production contained (g/L distilled water): saturated corn broth, 4.0; starch - 10.0; KCl - 0.3; $MgSO_4$ - 0.5; K_2HPO_4 - 0.87; $CaCl_2$ - 0.29. The pH was adjusted to 7.0-8.0 with 1% Na_2CO_3. This medium was sterilized by autoclaving at 121°C for 15 min. The above medium (50 ml in 250 ml Erlenmeyer flasks) was inoculated with

1 ml of a one-day culture and incubated at 45°C in a rotating shaker at 150 rpm for 24 h. At the end of the fermentation period, the contents were centrifuged at 15500 g for 15 min at °C, and the cell-free supernatant was used as crude enzyme to test it.

2.6. Protease Activity Test

Protease activity was assessed in triplicate by measuring the release of trichloroacetic acid soluble peptides from 0.5% (w/v) casein in 50 mM glycine with NaOH (pH 9.0) at 60°C for 10 min. The reactions were stopped by adding 0.5 ml of 10% trichloroacetic acid to 1 ml of the mixture. The mixture was left for 15 min and then centrifuged at 14000 g for 10 min. One unit of enzyme activity was taken as the amount of enzyme required to release 1 µg of tyrosine/min under standard conditions [5].

2.7. Influence of Nutrient Medium Components on Protease Production

The effect of 1% (w/v) carbon sources and 0.5% (w/v) nitrogen sources on enzyme production was determined by growing the isolate in production environments with various carbon and nitrogen sources. This study used various carbon sources such as glucose, galactose, maltose, lactose, starch, sucrose, and fructose. Nitrogen sources included yeast extract, beef extract, corn broth, whey protein, peptone, tryptone, and urea.

2.8. Effect of pH on Protease Production

The effect of pH on protease production was determined by growing the isolate in nutrient medium with an initial pH range of 6.0 to 10.0 using 1% Na_2CO_3.

2.9. Effect of Agitation Rate on Protease Production

The effect of agitation speed on enzyme production was investigated by incubating culture flasks at different agitation speeds: 115, 135, 155 and 185 rpm.

2.10. Effect of pH on Protease Activity

The optimum pH for enzyme activity was determined with 0.5% casein (w/v) as substrate dissolved in various buffers (sodium phosphate, pH 6.0-7.0, Tris-HCl, pH 8.0-9.0, and glycine with NaOH, pH 9.0-11.0).

2.11. Effect of Temperature on Protease Activity

The effect of temperature on enzyme activity was determined by performing a standard assay procedure at pH 9.0 over a temperature range of 40°C to 75°C.

2.12. Statistical Analysis

The statistical significance of the data was checked by variance analysis using Microsoft Excel 2010. Mean comparisons were made using the least significant difference test (P = 0.05). Mean values and standard deviation were calculated based on multiple replicates.

3. RESULTS AND DISCUSSION

3.1. Isolation and Screening of Protease-Producing Bacteria

Soil from slaughterhouses is one potential source of isolation of protease-producing bacteria. In this work, to isolate protease-producing bacteria, we used soil samples taken from private slaughterhouses located in the Tashkent and Bukhara regions.

In the present study, 50 bacterial isolates were isolated, of which 18 isolates had proteolytic activity. Proteolytic activity was assessed using skimmed milk agar and expressed as clear zone diameter in mm. The strain IMRUZ-7 showed the highest proteolytic activity with a net zone diameter of 55 mm after 72 hours, while other isolates showed a net zone diameter of 52 mm (IMRUZ-5), 49 mm (IMRUZ-11), 45 mm (IMRUZ- 2) and below.

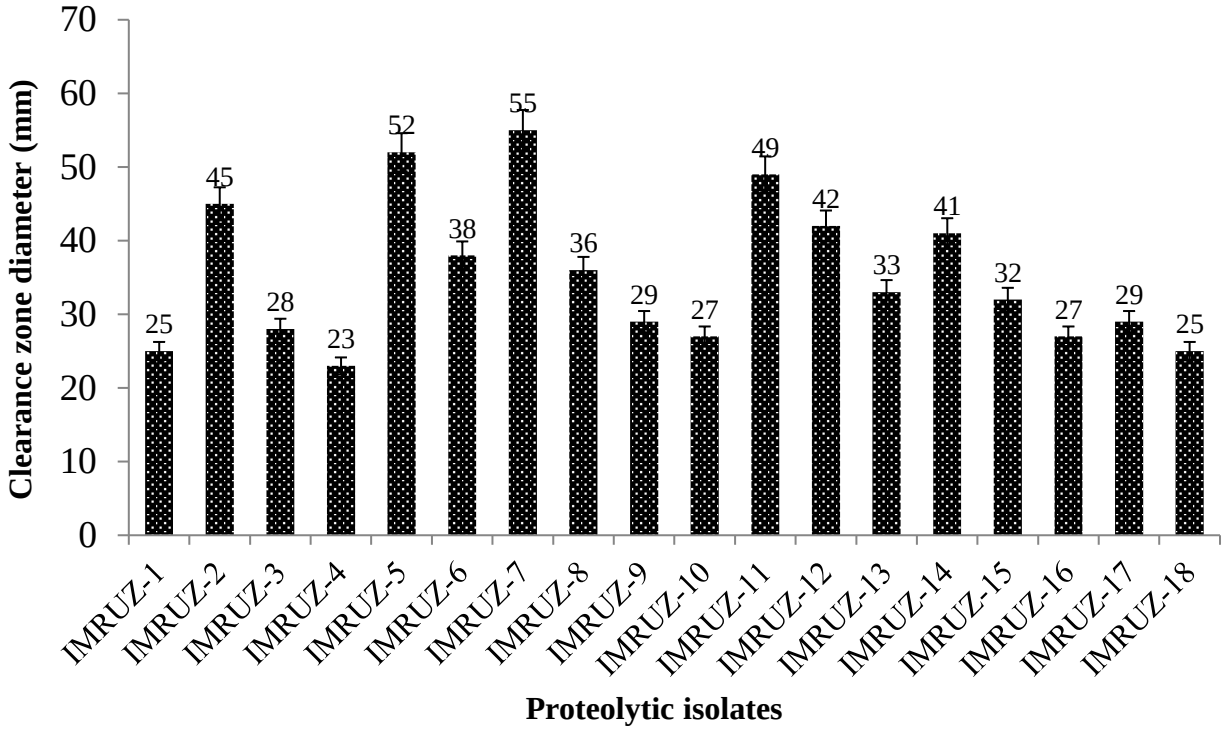


Fig.1. Diameter of clearing zones of proteolytic bacterial isolates on skimmed milk agar.

Figure 1 compares the clear zones of proteolytic bacterial isolates from different soil samples on defatted milk agar.

The IMRUZ-7 isolate was selected for further studies to optimize protease production.

3.2. Identification of the Bacterial Isolate IMRUZ-7

The bacterial isolate IMRUZ-7 was identified as belonging to the species *Bacillus subtilis* based on the determination of the nucleotide sequence of the 16S rRNA gene. The 16S rRNA gene sequence of the isolated bacterium was deposited in GenBank under the number OP023837, and the strain itself was named *Bacillus subtilis* IMRUZ-7.

Figure 2 shows a phylogenetic tree showing the evolutionary relationships of the *B. subtilis* IMRUZ-7 strain with the closest related strains from the GenBank collection (NCBI) based on a comparison of the nucleotide sequence of the 16S rRNA gene.

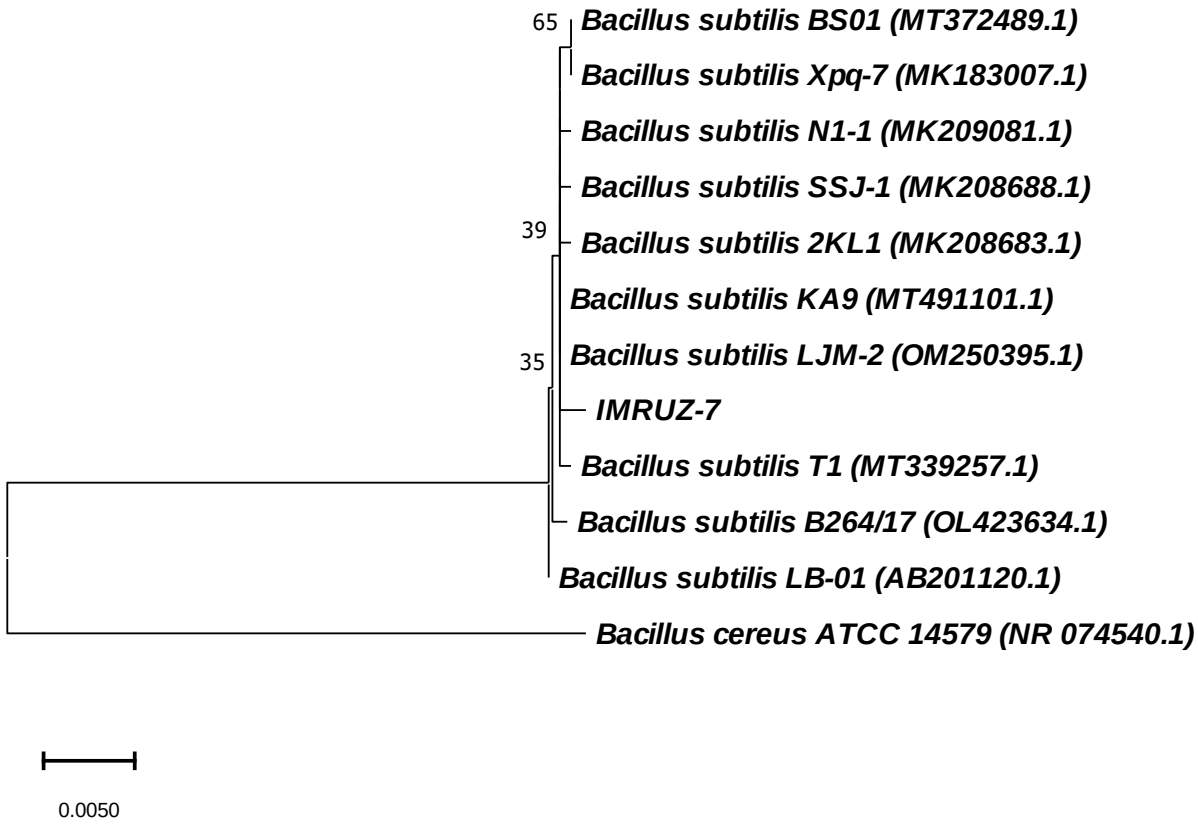


Fig. 2. Phylogenetic tree of the *B. subtilis* IMRUZ-7 strain and the closest relative bacterial strains registered in GenBank (NCBI).

3.3. Morphological-Cultural and Physiological-Biochemical Properties of *B. subtilis* IMRUZ-7

The nature of the growth of the *B. subtilis* IMRUZ-7 strain on the BCH: the medium becomes cloudy, and a dense grayish-white film forms on the surface. In this case, growth is accompanied by the formation of a specific odor.

The nature of the growth of the strain *B. subtilis* IMRUZ-7 on MPA: the daily culture is grayish-white colonies, the edges of the colonies are uneven, the surface is bumpy, the consistency is viscous. Microscopic examination revealed Gram-positive motile rods 0.5-0.6 x 3-5 μm.

The strain ferments sucrose, lactose, glucose, fructose, galactose, maltose, does not ferment raffinose, rhamnose, inositol, sorbitol, dulcite.

The strain hydrolyzes starch, casein, produces catalase, reduces nitrates, forms hydrogen sulfide,

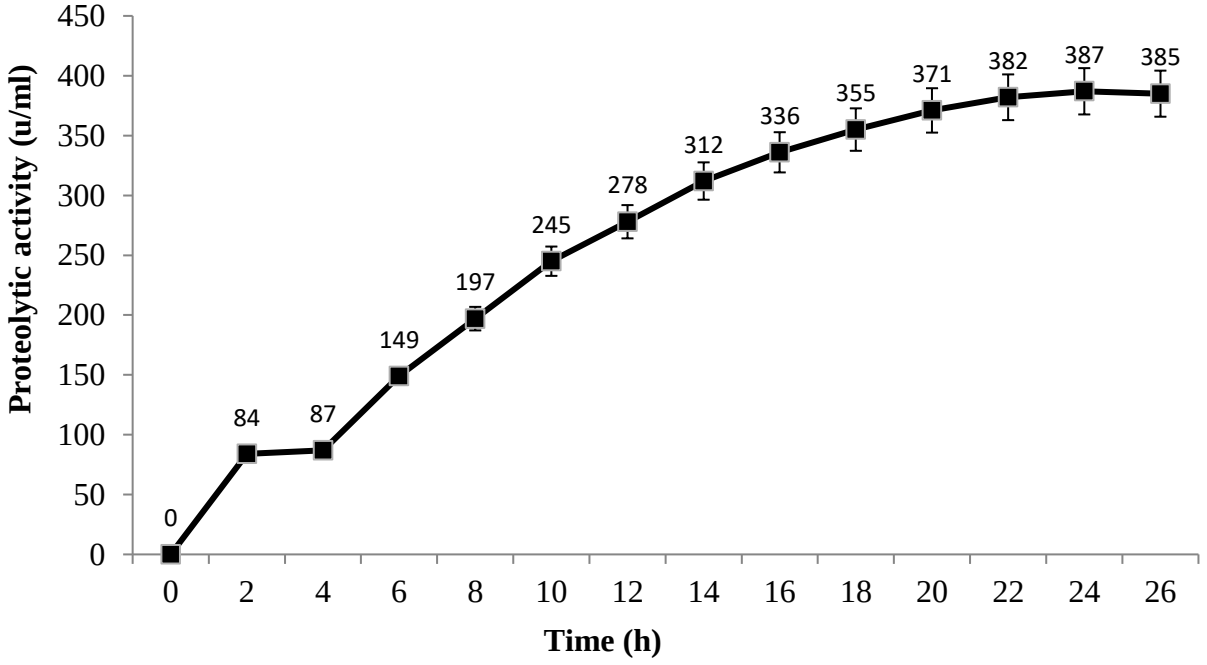
indole does not form.

3.4. Protease Production by *B. subtilis* IMRUZ-7

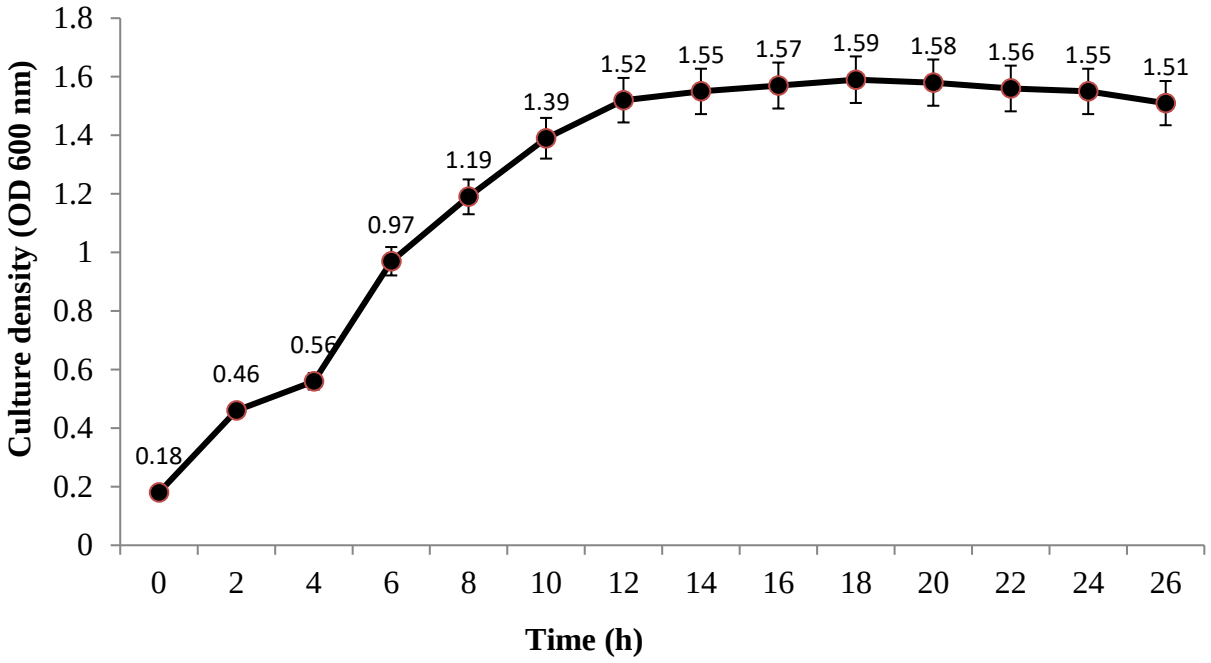
Figure 3 shows the dynamics of protease production by the *B. subtilis* IMRUZ-7 strain in a liquid medium containing starch (1%) as a carbon source and corn broth (0.5%) in 250 ml Erlenmeyer flasks.

Protease production largely started from the early stationary phase and peaked at 24 hours at 387 U/mL and then began to decline. In a similar study, the maximum activity of the protease was determined at the 18th hour, which was observed in the late stationary phase, when most bacteria formed spores [18]. Bacillus sp. are spore-forming bacteria; thus, during sporulation as well as spore germination, protease activity is increased. Scientists recognized that during sporulation and germination, hydrolyzed proteins were used to create proteins for endospores or vegetative cells. This process requires an increase in protease production.

Previously, Khan et al. [19] reported that in his study, the maximum protease activity was reached after 48 hours of fermentation, after which the activity began to decline.



(A)



(B)

Fig. 3. Protease production as a function of cultivation time (A) and culture density (B) of *B. subtilis* IMRUZ-7 growing on starch (1%) and corn broth (0.5%) in shaking flasks at initial pH 8.0 and temperature 45°C.

3.5. Influence of Nutrient Medium Components on Protease Production by *B. subtilis* IMRUZ-7

The use of an economical nutrient medium for the production of alkaline proteases from alkaliphilic Bacillus sp. especially important. Therefore, there is a need to search for new strains of bacteria capable of producing proteolytic enzymes with new properties and to develop inexpensive media. The present study was aimed at optimizing the components of the environment, which play an important role in increasing the production of alkaline proteases. The *B. subtilis* IMRUZ-7 strain was able to use a wide range of carbon sources, but protease production varied depending on the carbon source (Table 1).

Table 1. Effect of a carbon source on the growth and protease activity of the *B. subtilis* IMRUZ-7 strain. Culture density and extracellular protease activity were determined after 24 hours of incubation at 45°C and pH 8.0

Carbon source	**Culture density (OD 600 nm)**	**Maximum protease activity (u/ml)**
Starch	1.323±0.06	351.624±15
Lactose	1.217±0.05	242.511±11
Fructose	1.020±0.05	91.389±6
Galactose	1.407±0.07	312.428±14
Maltose	1.514±0.08	318.571±14
Glucose	1.015±0.05	65.773±5
Sucrose	1.010±0.05	62.256±5

In our study, starch was the best substrate for enzyme production, followed by maltose, galactose, and lactose, while glucose, fructose, and sucrose were less effective. This is consistent with a previous report which showed that starch induces high levels of enzyme expression in Bacillus species [20]. However this contradicts the report of Bhatiya and Jadeja [21], who showed that maltose and starch contribute to low protease production.

The use of maltose and galactose has also been shown to result in better growth than starch consumption, although starch was the best substrate for enzyme production. This suggests that the optimal conditions for protease production do not necessarily coincide with the best conditions for growth. This observation contrasts with a previous study by da Silva et al. [22], who showed that starch is the best carbon source for both growth and protease production.

Organic nitrogen sources such as corn broth, beef extract, yeast extract, whey protein, tryptone and peptone were tested for growth and production of *B. subtilis* IMRUZ-7 protease (Table 2).

Table 2. Effect of a nitrogen source on the growth and protease activity of the *B. subtilis* IMRUZ-7 strain. Culture density and extracellular protease activity were determined after 24 hours of incubation at 45°C and pH 8.0

Nitrogen source	Culture density (OD 600 nm)	Maximum protease activity (u/ml)
Corn broth	1.311±0.06	354.628±15
Whey protein	1.016±0.05	264.751±11
Beef extract	1.281±0.06	218.378±10
Yeast extract	1.292±0.06	323.853±14
Tryptone	0.857±0.04	205.964±10
Peptone	0.943±0.04	134.153±8
Urea	0.521±0.03	62.749±5

The results obtained showed that corn broth, which is cost effective, resulted in both maximum protease production and growth. Thus, by using corn broth in a nutrient medium, the cost of enzyme production can be reduced. However, in some organisms, organic nitrogen sources proved to be the best sources of nitrogen for both growth and protease production [23]. In a similar study, Singh et al. [24] used cheap nitrogen sources such as corn broth to produce a thermostable acid protease with *Aspergillus niger* F2078.

This conclusion contradicts the data of Shafee et al. They found that beef extract is the best substrate for protease production [20].

3.6. Dependence of the Activity of Proteases on the Density of the Culture of Bacteria Grown at Different pH Values

Protease activity varied depending on the initial pH value of the culture medium (Table 3). The highest levels of protease activity were found in cultures grown at pH 8.0. Growth was also maximum at pH 8.0. This suggests that *B. subtilis* IMRUZ-7 can be classified as alkaliphilic bacilli, since alkaliphiles are defined as organisms that grow optimally at alkaline pH, with an optimum pH for growth greater than pH 8.0, and some of them are able to grow at pH>11.0 [25].

Table 3. The effect of pH on the growth and protease activity of *B. subtilis* IMRUZ-7 strain cultivated in a liquid nutrient medium containing starch (1%) and corn broth (0.5%) in rotating flasks for 24 h at 45°C

Initial pH	**Culture density (OD 600 nm)**	**Maximum protease activity (u/ml)**
6.0	0.886±0.04	173.379±9
7.0	1.241±0.06	275.635±11
8.0	1.308±0.06	343.236±15
9.0	0.918±0.05	268.861±11
10.0	0.642±0.04	159.482±9

3.7. Influence of the Rate of Mixing of the Culture Medium with Bacteria on the Activity of the Protease

Microorganisms have different requirements for oxygen. In particular, O_2 acts as the final electron acceptor in oxidative reactions, providing energy for cellular activity. Changing the agitation rate has been found to affect the degree of agitation in shake flasks as well as nutrient availability [26]. In the present study, the *B. subtilis* IMRUZ-7 strain grown in culture medium

containing starch and corn broth showed maximum protease activity at a stirring speed of 155 rpm after 24 hours of incubation (Table 4).

Table 4. The effect of rotation rate on the growth and protease activity of the *B. subtilis* IMRUZ-7 strain during cultivation in a liquid nutrient medium containing starch (1%) and corn broth (0.5%) in rotating flasks for 24 h at 45°C

Rotation speed (rpm)	**Culture density (OD 600 nm)**	**Maximum protease activity (u/ml)**
115	1.233±0.06	316.589±14
135	1.295±0.06	335.132±15
155	1.323±0.07	369.621±15
185	1.236±0.06	323.361±14

At this rate, the aeration of the culture medium increased, which could lead to a sufficient supply of dissolved oxygen to the medium. The uptake of nutrients by the bacteria will also increase, leading to an increase in protease production. At 185 rpm, protease activity decreased. This may have been due to enzyme denaturation caused by the high agitation speed. High agitation speeds can also damage bacterial cells, so that a decrease in protease producers will result in a decrease in protease production. The stirring speed from 115 to 135 rpm significantly affected the growth of the organism. At this agitation rate, insufficient aeration and nutrient uptake may have caused the bacteria to fail to grow effectively. A similar study reported a marked increase in protease production at high agitation speed (>200 rpm). It was also found that reducing the stirring rate dramatically reduces the overall yield of proteases [27].

3.8. Effect of pH on Protease Activity

From an industrial point of view, the protease should exhibit significant activity at high pH and temperature. The pH range from 6.5 to 11.0 was used to study the effect of pH on protease activity (Figure 4).

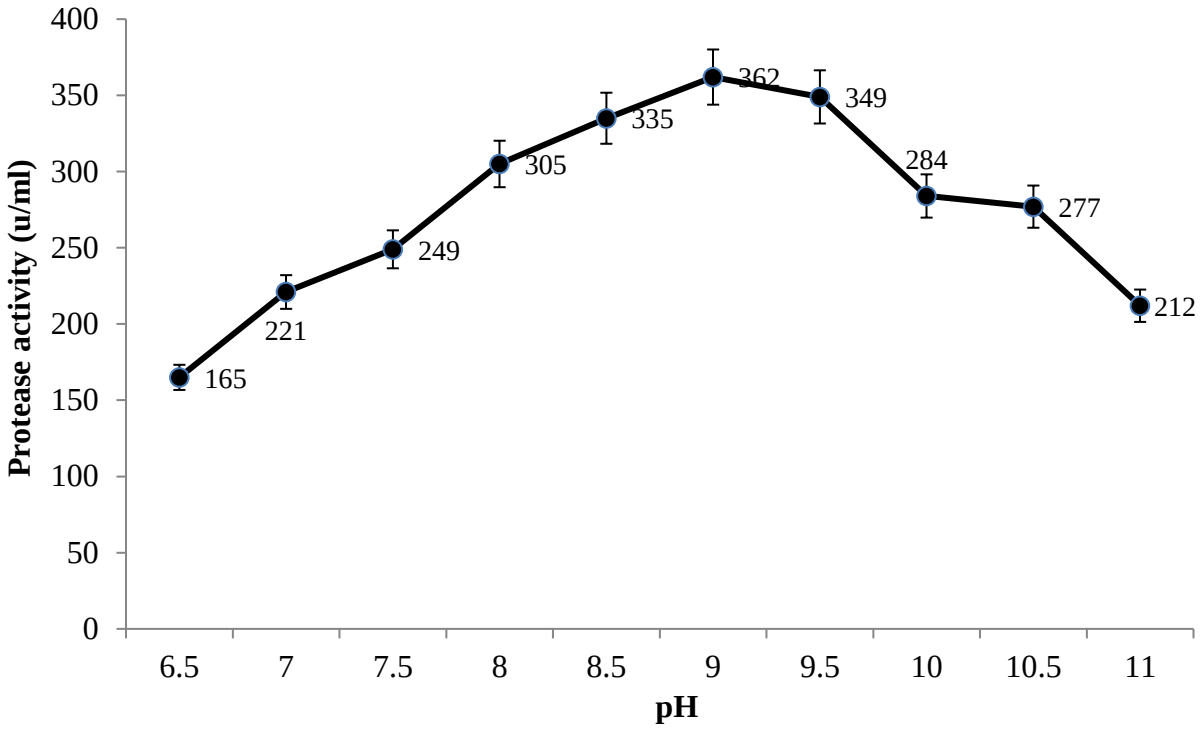


Fig. 4. Effect of pH value on protease production by *B. subtilis* IMRUZ-7 strain during growth at 45°C for 24 h.

The crude protease had a relatively wide pH range of 8.0 to 10.5 with maximum enzymatic activity at pH 9.0. Proteases with an optimum pH in the range of 8.0–11.0 are categorized as alkaline proteases. Maximum activity at pH 9.0 indicates that the enzyme is an alkaline protease, so this protease can be used as an industrially and economically viable enzyme. In a similar study, Ibrahim et al. found an alkaline protease that was maximally active at pH 10.0 [28].

3.9. Effect of Temperature on Protease Activity

Protease activity was assessed at various temperatures ranging from 40°C to 75°C at a constant pH of 9.0 (Fig. 5).

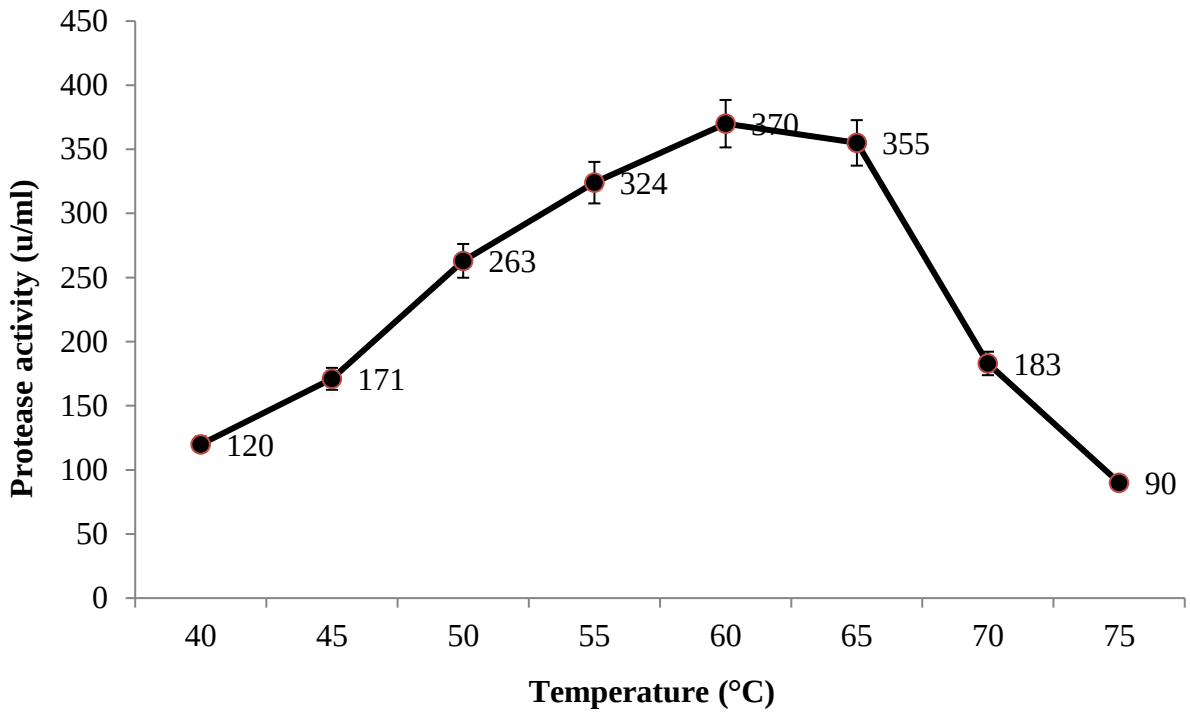


Fig. 5. Effect of temperature on protease production by Bacillus sp. IMRUZ-7 at pH 9.0 for 24 hours.

Enzyme activity increased with increasing temperature in the range from 40°C to 60°C and was high in the temperature range from 50°C to 65°C with maximum activity at 60°C. A decrease in enzyme activity was observed at values above 60°C. Maximum activity at 60°C indicates that the enzyme is a thermoprotease. This observation is similar to the report by Nascimento and Martins [26]. They worked on a thermophilic Bacillus sp., which produced a protease with maximum activity at 60°C. The maximum protease activity at high temperatures is a very suitable characteristic for its industrial application.

4. CONCLUSION

In the present study, we report the isolation of proteolytic bacteria from the soil of slaughterhouses in Tashkent and Bukhara regions. One of the best alkaline protease producers, the *B. subtilis* IMRUZ-7 strain, was chosen to optimize the culture conditions for enzyme production. This strain can be used for large scale production of alkaline protease to meet current industrial application needs. Starch and corn broth are cheap sources of carbon and nitrogen, which led us to offer an inexpensive nutrient medium to maximize protease production. The optimum pH and temperature for enzyme activity were determined to be 9.0 and 60°C, respectively. Since the thermoactivity and pH stability of proteases are of great importance for industrial use, the enzymatic properties of the *B. subtilis* strain IMRUZ-7 protease indicate the potential use of this bacterium and its protease for various industrial purposes.

5. AUTHORS' CONTRIBUTION

VS performed the experiments and wrote draft of manuscript. UR analyzed data including statistical analysis. KD worked out concept and design, and performed critical revision of manuscript. KD and AS supervised the experiments. AS funded the experiments, supported technically and materially. All the authors contributed equally in this manuscript and agree to submit it for publication.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Mohsen FN, Dileep D, Deepti D. Potential application of protease isolated from *Pseudomonas auriginosa* PD100. Electron J Biotechnol 2005; 8(2):197–203.

2. Kumar A, Sachdev A, Balasubramanyam SD, Saxena AK, Lata A. Optimization of conditions for production of neutral and alkaline protease from species of *Bacillus* and *Pseudomonas*. Ind J Microbiol 2002; 42:233–236.
3. Saeki K, Ozaki K, Kobayashi T, Ito S. Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. J Biosci Bioeng 2007; 6:501–508; doi: 10.1263/jbb.103.501
4. Giarrizzo J, Bubis J, Taddei A. Influence of the culture medium composition on the excreted/secreted proteases from *Streptomyces violaceoruber*. World J Microbiol Biotechnol 2007; 23:553–558; doi: 10.1007/s11274-006-9260-z
5. Joo H.S., Kumar C.G., Park G.C., Kim K.T., Paik S.R., Chang C.S. Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. Process Biochem 2002; 38:155–159; doi: 10.1016/S0032-9592(02)00061-4
6. Godfrey TA, Reichelt J. Industrial enzymology - the application of enzymes in industry. USA: The Nature Press, 582 p 1984.
7. Beg QK, Gupta R. Purification and characterization of an oxidation stable thiol-dependent serine alkaline protease from *Bacillus mojavenesis*. Enzyme Microbial Technol 2003; 32:294–304; doi: 10.1016/S0141-0229(02)00293-4
8. Kumar CG, Takagi H. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 1999; 17(7):561–594; doi: 10.1016/s0734-9750(99)00027-0
9. Lee SH, Chung CW, Yu YJ, Rhee YH. Effect of alkaline protease producing *Exiguobacterium* sp. YS1 inoculation on the solubilisation and bacterial community of waste activated sludge. Bioresour Technol 2009; 100(20):4597–4603; doi: 10.1016/j.biortech.2009.04.056
10. Bayoumi RA, Bahobil AS. Production of thermoalkaliphilic protease by *Shewanella putrefaciens* EGKSA21 under optimal conditions for application in biodetergent technology. J Basic Appl Sci Res 2011; 1(2):95–107.
11. Dashti A, Jadaon MM, Abdulsamad AM, Dashti H. Heat treatment of bacteria: a simple method of DNA extraction for molecular techniques. Kuwait Med J 2009; 41(2):117–122.
12. Lane DJ. 16S/23S rRNA Sequencing. In: Stackebrandt E, Goodfellow M (eds.). Nucleic Acid Techniques in Bacterial Systematic, John Wiley and Sons, NY, pp 115–175, 1991.
13. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4(4):406–425; doi: 10.1093/oxfordjournals.molbev.a040454
14. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985; 39(4):783–791; doi: 10.1111/j.1558-5646.1985.tb00420.x

15. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 2004; 101(30):11030–11035; doi: 10.1073/pnas.0404206101
16. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 2018; 35(6):1547–1549; doi: 10.1093/molbev/msy096
17. Smibert RM, Krieg NR. Phenotypic characterization. In: Gerhardt P (ed.). Methods for general and molecular bacteriology, American Society for Microbiology, Washington DC, pp 607–654, 1994.
18. Ozgur K, Nilufer C. Isolation of protease producing novel *Bacillus cereus* and detection of optimal conditions. Afr J Biotechnol 2011; 10(7):1160–1164; doi: 10.5897/AJB10.164
19. Khan MA, Ahmad N, Zafar AU, Nasir IA, Qadir MA. Isolation and screening of alkaline protease producing bacteria and physio-chemical characterization of the enzyme. Afr J Biotechnol 2011; 10(33):6203–6212; doi: 10.5897/AJB11.413
20. Shafee N, Norariati Aris S, Noor Zaliha Abd Rahman R, Basri M, Salleh AB. Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus cereus* strain 146. J Appl Sci Res 2005; 1(1):1–8.
21. Bhatiya R, Jadeja GR. Optimization of environmental and nutritional factors for alkaline protease production. Elec J Env Agricult Food Chem 2010; 9(3):594–599.
22. da Silva CR, Delatorre AB, Martins MLL. Effect of the culture conditions on the production of an extracellular protease by thermophilic *Bacillus* sp and some properties of the enzymatic activity. Braz J Microbiol 2007; 38(2):253–258; doi: 10.1590/S1517-83822007000200012
23. Phadatare SU, Deshpande VV, Srinivasan MC. High activity alkaline protease from *Conidiobolus coronatus (NCL 86.8.20)*: enzyme production and compatibility with commercial detergents. Enzyme Microb Technol 1993; 15(1):72–76; doi: 10.1016/0141-0229(93)90119-M
24. Singh A, Ghosh VK, Ghosh P. Production of thermostable acid protease by *Aspergillus niger*. Lett Appl Microbiol 1994; 18(3):177–180; doi: 10.1111/j.1472-765X.1994.tb00839.x
25. Grant WD, Jones BE. Alkaline environments. In: Lederberg J (ed.). Encyclopedia of Microbiology 2nd ed., Academic Press, NY, 1:126–133, 2000.
26. Nascimento WCA, Martins MLL. Production and properties of an extracellular protease from thermophilic *Bacillus* sp. Braz J Microbiol 2004; 35(1-2):91–96; doi: 10.1590/S1517-83822004000100015

27. Saurabh S, Jasmine I, Pritesh G, Rajendra Kumar S. Enhanced productivity of serine alkaline protease by *Bacillus* sp. using soybean as substrate. Malays J Microbiol 2007; 3(1):1–6; doi: 10.21161/mjm.00107
28. Ibrahim ASS, EI-Shayeb NMA, Mabrouk S. Isolation and identification of alkaline protease producing alkaliphilic bacteria from an Egyptian soda lake. J Appl Sci Res 2007; 3(11):1363–1368.