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Modern Journal of Microbial Biology

Original Article

Biochemical and molecular characterization of proteolytic bacterial strains isolated from Jazan region, KSA with the application as an antibacterial agent

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Received : 03 February 2021
Accepted : 01 March 2021
Published : 07 April 2020

DOI
10.25259/MJMB_1_2021

Quick Response Code:

ABSTRACT

Objectives: Biochemical and molecular characterization of proteolytic bacterial strains isolated from Jazan region, KSA with the application as an antibacterial agent.

Materials and Methods: Three samples were collected from extreme environment, Jazan, KSA. Skim milk nutrient agar medium was used for protease screening for several colonies by streaking method at 37°C. API biochemical kit was used to characterize the three isolates using some selective media. The genetic identification was done using 16S rRNA gene sequencing. The sensitivity of the tested strains; *Staphylococcus aureus, Escherichia coli,* and *Klebsiella pneumoniae* against the extracellular crude protease enzyme produced from the three isolated bacteria and different antibiotics was done.

Results: The isolates were identified as *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus cereus. B. cereus* and *B. licheniformis* recorded high sensitivity (71%) against most antibiotics, in addition, *B. subtilis* showed resistance to Aztreonam only. It was found a considerable increase in the level of both of protease activity (units/ml) and bacterial growth (colony-forming units/ml) of the cultures that were directed by the *B. subtilis* and *B. licheniformis* up to 37°C then decreased at 45°C. On the contrary, the growth of *B. cereus* and its activity gradually increased up to 45°C. The enzyme activity and bacterial growth of *B. subtilis* and *B. cereus* strains were increased at alkaline medium. However, *B. licheniformis* gave the highest growth and activity at neutral pH. In addition, it was found that the enzyme activity and bacterial growth of *B. subtilis* were reached to the maximum at 5% NaCl. However, the maximum bacterial growth and enzyme activity for *B. licheniformis* and *B. cereus* was at 2% NaCl. It was found high effect on inhibiting the growth of pathogenic bacteria using 5 µl of crude enzyme with specific enzyme activity 73, 76, and 92 (units/ml)/(mg protein/ml) for *B. subtilis, B. licheniformis,* and *B. cereus,* respectively. All pathogenic bacteria were totally inhibited with 10 µl of crude enzyme.

Conclusion: The potential *Bacillus* proteases can promote new industry as antimicrobial agents.

Keywords: Proteolytic *Bacillus* strains, Pathogenic bacteria, Antibacterial agent

INTRODUCTION

Proteolysis considers one of the most important biological reactions. These enzymes are of wide distribution, and they perform significant biological processes.[1] Proteases are produced in animals, plants, bacteria, archaea, and viruses. Among the different sources of proteases, bacterial

protease was the most significant compared with animal, fungi, and plant protease. *Bacillus* species were specific producers of extracellular protease.[2]

Bacterial diseases become a serious problem in public health. Antibiotic resistance as become one of the largest worldwide anxieties. Resistance to antibiotics occurs because of the changing nature of bacteria that no longer can be killed. Efficacy of the drug lost. Bacterial strains that are resistant to different antibiotics will multiply then spread so that they become more serious.[3] Recent reports indicated that various microbial enzymes might be used as potential matrix-degrading therapeutic agents.[4]

Proteases are involved in many aspects of human biology. For example, in the small intestine, proteases digest dietary proteins to allow absorption of amino acids. Other processes mediated by proteases include blood coagulation, immune function, maturation of prohormones, bone formation, programmed cell death, and the recycling of cellular proteins that are no longer needed. Proteases also offer a valuable target in many therapeutic settings, including cancer, Alzheimer's, and viral infection. A matrix metallopeptidase-9 plays a role in angiogenesis and is a therapeutic target for cancer. Because of their significance in the pathology of disease, proteases are a relevant drug target class. Proteases activity is central to diverse physiological cascades throughout biology. Some are essential for coagulation, while others contribute to cancer pathology.[5,6]

Bacterial diseases become a serious problem in public health. Antibiotic resistance becomes one of the largest worldwide anxieties. Resistance to antibiotics occurs because of the changing nature of bacteria that no longer can be killed. Efficacy of the drug lost. Bacteria that are resistant to antibiotics will multiply and spread so that they become more dangerous.[3] Recent reports indicated that various microbial enzymes might be used as potential matrix-degrading therapeutic agents.[4]

MATERIALS AND METHODS

Samples collection

Three samples were collected from different extreme environment [Figure 1], Jazan, Kingdom of Saudi Arabia. Samples were assembled in 500 mL glass screw cap bottles and brought to the laboratory. These sources of the bacterial isolation were, Salty soil from Farasan island (S), Al-Harth Hot spring soil (H), and Salty water, Red Sea, Farasan Island (W).

Isolation, purification, and screening of proteolytic bacteria

One milliliter of the bacterial sample was taken and diluted with pre-sterilized physiological saline solution (under aseptic conditions) to reach to appropriate dilutions. A 100 µl of each dilution was plated on milk agar (M.A.) in triplicates. The plates were incubated O.N. at 37°C and the number of developed colonies was processed. After incubation, the total bacterial count was recorded as colony-forming units (CFU) mL^{-1} as described earlier. The developed colonies were repeatedly streaked on NA plates to isolate of pure cultures.[7]

Skim milk nutrient agar medium was used for protease screening for several colonies by streaking method using sterile toothpick and incubated at 37°C. Colonies forming transparent zones, because of partial hydrolysis of milk casein, were selected.[8]

The gelatin hydrolysis test was used to detect the ability of isolated microorganism to produce the enzyme gelatinase. The procedures were done by inoculation a heavy inoculum by stabbing on the tube containing nutrient gelatin medium. After that, incubation the inoculated tube along with an uninoculated medium which utilized as a control tube at 37°C for 14 days. Removing the tubes daily from the incubator and placing in refrigerator (4°C) for 15–30 min until control is gelled. Every day the same method repeats to check for gelatin liquefaction.[9]

Characterization of bacterial isolates

Gram staining was done using a commercially kit (Quimica Clinica Aplicada, S.A., Amposta, Spain) according to the standard method.[10]

Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, a small inoculum of a bacterial isolate is mixed into hydrogen peroxide solution (3%) and is observed for the rapid elaboration of oxygen bubbles.[11]

Biochemical characterization of bacterial isolates

Biochemical characterization of the proteolytic bacterial isolates was performed using commercially available identification systems API, BioMérieux.[12]

Antibiotics susceptibility test

A single colony of the isolates was picked using a sterile loop and streaked on the Muller-Hinton agar plate. A filter paper disk contained the antibiotics; Cefoxitin (FOX, 30 µg), Aztreonam (ATM, 30 µg), Cefotaxime (CTX, 30 µg), Ceftazidime (CAZ, 30 µg), Amoxicillin/Clavulanate (AUG, 30 µg), Meropenem (MEM, 10 µg), Amikacin (AK, 30 µg), Cefepime (CPM, 30 µg), Piperacillin/Tazobactam (PTZ, 110 µg), Imipenem (IMI, 10 µg), Tetracycline (T, 30 µg), Teicoplanin (TEC, 30 µg), Neomycin (NE, 30 µg), Mupirocin (MUP, 200 µg), Mupirocin (MUP, 5 µg), Rifampicin (RP, 5 µg), Erythromycin (E, 15 µg), Vancomycin (VA, 30 µg),

Figure 1: Sources locations for the isolated samples.

Clindamycin (CD, 2 µg), Trimethoprim/Sulfamethoxaz (TS, 25 µg), Cefuroxime (CXM, 30 µg), Trimethoprim (TM, 5 µg), Ciprofloxacin (CIP, 5 µg), Amoxicillin (AX, 10 µg), Cefazolin (CZ, 30 µg), Penicillin (PG, 10 µg), Fucidin Acid (FC, 10 µg), and Nitrofurantoin (NI, 300 µg) separately were dispensed onto the plate. Using sterilized forceps, each disk was placed on the agar medium to ensure that the disk is attached and fixed on the agar. Muller-Hinton agar medium containing antibiotic disks was incubated O.N. at 35°C in incubator.[13]

Identification using 16S rRNA

The selected proteolytic bacterial isolates were identified using 16S rRNA gene sequencing technique. The genomic DNA was extracted using TIANamp genomic DNA kit (Tiagen, Korea), according to its manufacturer instructions. The primers used in amplification of the 16S rRNA gene were forward primer (F; AGA GTT TGA TCC TGG CTC AG) and reverse primer (R; GGT TAC CTT GTT ACG ACT T).[14] PCR was performed according to Sambrook and Russel, 2001,[15] with some modifications for 35 cycles under the following conditions: Denaturation step at 94°C for 40 s, annealing step at 55°C for 1 min, extension step at 72°C for 2 min, and final extension at 72°C for 10 min. Sequencing of the amplified fragments was done at Macrogen biotechnology company. Cycle Sequence PCR fragments were purified by cycle sequencing purification, using big dye X terminator (applied Bio system). Sequence analysis was performed using the sequence alignment software BLASTn with the national center for biotechnology information (NCBI) database, http://www.ncbi.nlm.nih.gov/.

Effect of some factors on the bacterial growth and protease activity

Bacterial cells were activated by growing them O/N on M.A. plate at 37°C. Ten milliliter of Casein soyabean broth medium were inoculated with several recently growing colonies (fresh colonies) after which cells were allowed to grow for 24 h at 30°C with shaking at 150 rpm. To study the effect of temperature on the bacterial growth and protease activity, three flasks containing 100 ml of fresh Casein soyabean broth medium were inoculated with 100 µl of the above culture, for each. The new culture was allowed to grow at 30, 37, and 45°C with shaking for 24 h. Activated bacterial cells were used to inoculate Casein soyabean broth medium with different pH (4, 7, and 9). Cultures were grown at 30°C with shaking at 150 RPM for 24 h. In another experiment to study the effect of salinity on the bacterial growth and protease activity, Casein soyabean broth media were supplemented with 2, 5, and 10% Na Cl. In addition, all cultures were grown at 30°C with shaking at 150 RPM for 24 h. For all cultures, at the indicated time, 2 ml of the growing culture was taken and measured the O.D. at Ab. 600 nm. To get cells free supernatant, 1.5 ml of the culture was centrifuged in a microcentrifuge at 8000 rpm for 5 min. The supernatants were then used as crude enzyme to determine the protease activity.[16]

Determination of the protease activity

The activity of the protease was determined according to the method of Sutar *et al.* 1986.[17] Reaction mixtures (2 ml) contained 10 mg casein, 200 µmol sodium carbonate buffer, pH 9.7 and 0.1 ml of the supernatants. Reactions were carried out at for 30 min then were terminated by the addition of 2.6 ml 5% (w/v) trichloroacetic acid (TCA), and 0.4 ml 3.3 M HCl. Reactions were then kept on ice for at least 1 h after which they were centrifuged for 30 min at 4000 rpm. The absorbance of the TCA soluble fractions was measured at 280 nm. One unit of enzyme activity was equal to the amount of enzyme that liberates 1 µmol of tyrosine from casein/30 min at 37°C. A standard curve using the amino acid tyrosine was established.

Determination of soluble protein content

The content of soluble proteins was estimated using the bicinchoninic acid (BCA) assay kit (Sigma-Aldrich BCA-1, B9643), with bovine serum albumin as a standard.[18]

Antimicrobial activity test

The sensitivity of three different pathogenic bacteria against the extracellular crude protease enzyme produced from the three isolated bacteria was done, according to the previous studies.[19,20] The three pathogens strains were isolated and identified previously, Biology Department, Faculty of Science, Jazan University.[21] These pathogenic isolates were one Gram-positive bacterial strain, *Staphylococcus aureus*. In addition, Gram-negative bacterial strains, *Escherichia coli,* and *Klebsiella pneumoniae*.

The isolated bacterial cells were activated by growing them overnight on Nutrient agar supplemented with skim milk at

37°C. Ten milliliter of Casein Soyabean Digest Broth were inoculated with several recently growing colonies (fresh colonies) after which cells were allowed to grow for 3 h at 37°C with shaking at 150 rpm. A 100 ml of fresh Casein Soyabean Digest Broth were inoculated with 1 ml of the above culture. The new culture was allowed to grow overnight at 37°C with shaking. At the indicated time, CFU/ml was determined. In addition, 1.5 ml of the growing culture was taken and centrifuged in a microcentrifuge at 7000 rpm for 2 min. The supernatants were then used as crude enzyme to determine the activity of protease, protein content as well as to test as antimicrobial agents. Ten microliters of activated pathogen cells were inoculated to 10 ml of Casein Soyabean Digest Broth, supplemented with 5 or 10 µl crude enzyme extract. All cultures were incubated O/N at 37°C. At the indicated time, CFU/ml and measurement of turbidity at 600 nm were determined to detect the antimicrobial activity of the tested proteolytic isolated strains.

RESULTS

Isolation of proteolytic bacterial strains

Three samples were collected from different extreme environment, Jazan, Kingdom of Saudi Arabia as mentioned before. Isolation of bacterial colonies was done on M.A. with incubation at 30°C [Figure 2a]. The total bacterial count for salty soil, Hot spring, and Sea water samples was 1.2×10^7, 6×10^3, and 2×10^5 CFU/ml, respectively. Among the different isolates, three isolates (one bacterial isolate from each resource) with the maximum zone of clearance on skim M.A. and high capacity for the liquefaction of gelatin were selected [Figures 2b and c]. Bacterial isolates were purified using streaking technique on skim M.A. The three strains were coded as follow: S: Strain isolated from Salty soil, H: Strain isolated from Al-Harth Hot spring, and W: Strain isolated from Seawater.

Characterization of bacterial strains

After purification of selected maximum proteolytic strains, they were observed using the light microscope. The morphology graph of isolated bacteria showed that all isolates were Gram-positive and rod-shaped bacteria.

Catalase test was performed for all isolates. All of them were catalase-positive bacteria. Therefore, they were strict aerobes as well as facultative anaerobes.

Using API biochemical kit, the three of the Gram-positive rods isolated from extreme environment, Jazan, KSA, were characterized using some selective media [Table 1].

Antibiotic susceptibility test

Susceptibility test is used when microbe is unable to grow in the presence of one or more antimicrobial drugs. Testing is used to determine the potential effectiveness of specific antibiotics on the bacteria and/or to determine if the bacteria have developed resistance to certain antibiotics. The obtained results [Table 2] show the antibiotic susceptibility pattern of the bacterial isolates in this study. The bacterial isolate (W) recorded high sensitivity (71%) with different degrees against most antibiotics, but also showed resistance to the following antibiotics: PG, AX, ATM, TM, CXM, MUP, CPM, and CAZ. Furthermore, the bacterial strain (S) showed resistance to ATM only, and it was highly sensitive to the rest of the antibiotics (96%). In the meantime, the bacterial strain (H) showed sensitivity against most antibiotics (71%) and showed resistance to the following antibiotics: ATM, T, TEC, MUP 5, CD, AX, PG, and FC.

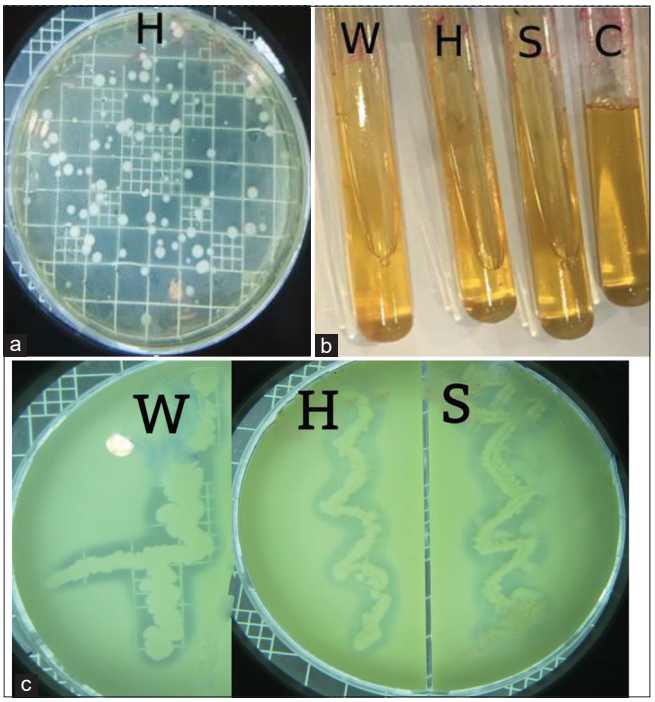

Figure 2: (a) Isolation on milk agar with incubation O/N at 30°C (Hot spring sample), (b) Protease qualitative determination of the bacterial isolates by gelatin liquefaction, where C: control (gelatin without inoculum), S: Isolated from Salty soil, H: Isolated from Hot spring and W: Isolated from Seawater, (c) Protease qualitative determination of the bacterial isolates on skim milk nutrient agar, Where, S: Isolated from Salty soil, H: Isolated from Hot spring and W: Isolated from Seawater.

16S rRNA identification

Three Gram-positive *Bacilli* bacteria with maximum zone of clearance on skim milk, and high capacity for the liquefaction of gelatin due to proteolytic activity were selected to identify. After DNA extraction, followed with PCR, the molecular identification of the bacterial isolates was performed using 16S rRNA gene sequencing. The obtained DNA sequences were aligned at the NCBI database. [Table 3] shows the

Table 1: Biochemical characterization of Gram-positive rods bacterial isolates using API tests.

No.	Test	Reaction	Isolate S	Isolate H	Isolate W
1	ONPG	β-galactosidase	−	−	−
2	ADH	Arginine dihydrolase	+	+	+
3	LDC	Lysine decarboxylase	+	+	−
4	ODC	Ornithine decarboxylase	+	+	−
5	CIT	Citrate utilization	−	−	−
6	H_2S	H_2S production	−	−	−
7	URE	Urea hydrolysis	+	−	−
8	TDA	Tryptophan deamination	+	+	+
9	IND	Indole production	+	+	−
10	VP	Acetoin production	+	+	+
11	GEL	Gelatin hydrolysis	+	+	+
12	GLU	Glucose fermentation	−	−	−
13	MAN	Mannitol	−	−	−
14	INO	Inositol	−	−	−
15	SOR	Sorbitol	−	−	−
16	RHA	Rhamnose	+	+	+
17	SAC	Sucrose	−	−	−
18	MEL	Melibiose	+	+	+
19	AMY	Amygdalin	−	−	−
20	ARA	Arabinose fermentation	+	+	−
21	ESC	Aesculin hydrolysis	+	+	+
22	PYRA	Pyrrolidonylaryl-amidase	−	+	−
23	αGAL	α-galactosidase	−	+	−
24	βGUR	β-glucuronidase	−	+	−
25	βGAL	β-galactosidase	−	+	−
26	PAL	Alkaline phosphatase	−	+	−
27	LAP	Leucine arylamidase	−	+	−
28	RIB	Ribose fermentation	+	+	+
29	MAN	Mannitol fermentation	−	−	−
30	SOR	Sorbitol fermentation	−	−	−
31	LAC	Lactose fermentation	−	+	−
32	TRE	Trehalose fermentation	−	−	+
33	INU	Inulin fermentation	+	−	−
34	RAF	Raffinose fermentation	−	−	−
35	AMD	Starch fermentation	+	−	+
36	GLYG	Glycogen fermentation	+	−	+

+: Positive, −: Negative

accession no, identity percentage and identification of the isolates. The sequences and phylogenetic tree of the selected strains identified 16S rRNA is shown at [Figures 3-5]. The *Bacilli* species were identified as *Bacillus subtilis* (isolated from salty soil.), *Bacillus licheniformis* (isolated from Al-Harth hot spring), and *Bacillus cereus* (isolated from seawater).

Factors affecting on bacterial growth and protease production

Some factors affecting on bacterial growth and protease production were studied as mentioned above. The bacterial growth was monitored by calculating (CFU/ml). The protease activity was monitored using casein method as mentioned before regarding to tyrosine standard curve. One unit of enzyme activity can defined as the amount of enzyme that liberates 1 μmol tyrosine/30 min at 37°C using casein as a substrate.

[Figures 6a and b] illustrate that there is a considerable increase in the level of both of protease activity (Units/ml) and bacterial growth (CFU/ml) of the culture that was directed by *B. subtilis* at 37°C. The maximum protease activity was 64.12 units/ml, while the maximum bacterial count was 1.9×10^7 CFU/ml. The same was observed with *B. licheniformis* where the optimum bacterial growth was 1.7×10^8 CFU/ml and protease activity 82 units/ml to 37°C then decreased at 45°C. On the contrary, the growth of *B. cereus* and its activity gradually increased up to 45°C where reached to 5×10^7 CFU/ml and 116 units/ml.

Table 2: Antimicrobial effect of different antibiotics on bacterial isolates.

No.	Antibiotic	Conc.(µg/disc)	Isolate W		Isolate S		Isolate H	
			Status	Halo-zone mm	Status	Halo-zone mm	Status	Halo-zone mm
1	Cefoxitin	30	S	12	S	31	S	26
2	Aztreonam	30	R	0	R	0	R	0
3	Cefotaxime	30	S	9	S	23	S	30
4	Ceftazidime	30	R	0	S	18	S	20
5	Amoxicillin/Clavulanate	30	S	10	S	30	S	14
6	Meropenem	10	S	28	S	40	S	36
7	Amikacin	30	S	30	S	30	S	20
8	Cefepime	30	R	0	S	30	S	33
9	Piperacillin/Tazobactam	110	S	27	S	30	S	27
10	Imipenem	10	S	37	S	46	S	39
11	Tetracycline	30	S	18	S	22	R	0
12	Teicoplanin	30	S	18	S	18	R	0
13	Neomycin	30	S	25	S	25	S	17
14	Mupirocin	200	S	10	S	39	S	25
15	Mupirocin	5	R	0	S	30	R	0
16	Rifampicin	5	S	14	S	20	S	13
17	Erythromycin	15	S	29	S	35	S	13
18	Vancomycin	30	S	18	S	25	S	9
19	Clindamycin	2	S	22	S	20	R	0
20	Trimethoprim/Sulfamethoxaz	25	S	18	S	35	S	22
21	Cefuroxime	30	R	0	S	21	S	25
22	Trimethoprim	5	R	0	S	35	S	23
23	Ciprofloxacin	5	S	22	S	35	S	31
24	Amoxicillin	10	R	0	S	31	R	0
25	Cefazolin	30	S	8	S	37	S	13
26	Penicillin	10	R	0	S	40	R	0
27	Fucidin acid	10	S	13	S	28	R	0
28	Nitrofurantoin	300	S	22	S	25	S	25

R: Resistance, S: Sensitive

Table 3: The bacterial isolates identification after alignment at the national center for biotechnology information database.

Isolation resource	Sequence length	Accession number	Identity (%)	Identification
Isolated S	1483 bp	KU229984	96.75	*Bacillus subtilis*
Isolated H	1095 bp	MK622385	93.49	*Bacillus licheniformis*
Isolated W	1434 bp	KJ534410	96.43	*Bacillus cereus*

It was noticed that the enzyme activity and bacterial growth of *B. subtilis and B. cereus* strains were increased at alkaline medium. The maximum growth was 2×10^7 and 7.5×10^7 CFU/ml for both strains, respectively. However, the maximum activity was 67.5 and 56 units/ml, respectively. However, *B. licheniformis* gave the highest growth and activity at neutral pH with values 7×10^7 CFU/ml and 33.9 units/ml as demonstrated at [Figures 7a and b].

In a trial to study the effect of salinity on the enzyme activity, it was found that the bacterial growth and enzyme activity of *B. subtilis* were reached to the maximum at 5% NaCl with values 2×10^7 CFU/ml and 67 units/ml, respectively. However, the maximum bacterial growth for *B. licheniformis* and *B. cereus* was at 2% NaCl. The bacterial count was 3.1×10^7 and 4.6×10^7 CFU/ml. The enzyme activity was maximum for *B. lichinoformis* at control culture with 33.9 units/ml and the maximum enzyme activity for *B. cereus* was at 2% NaCl with 106 units/ml. It was noticed that the growth of all strains was inhibited at 10% NaCl [Figures 8a and b].

Use of the extracted crude protease as antimicrobial agent

The sensitivity of three pathogenic bacteria against the extracellular crude protease enzyme produced from the three isolated bacteria was done. These pathogenic isolates were

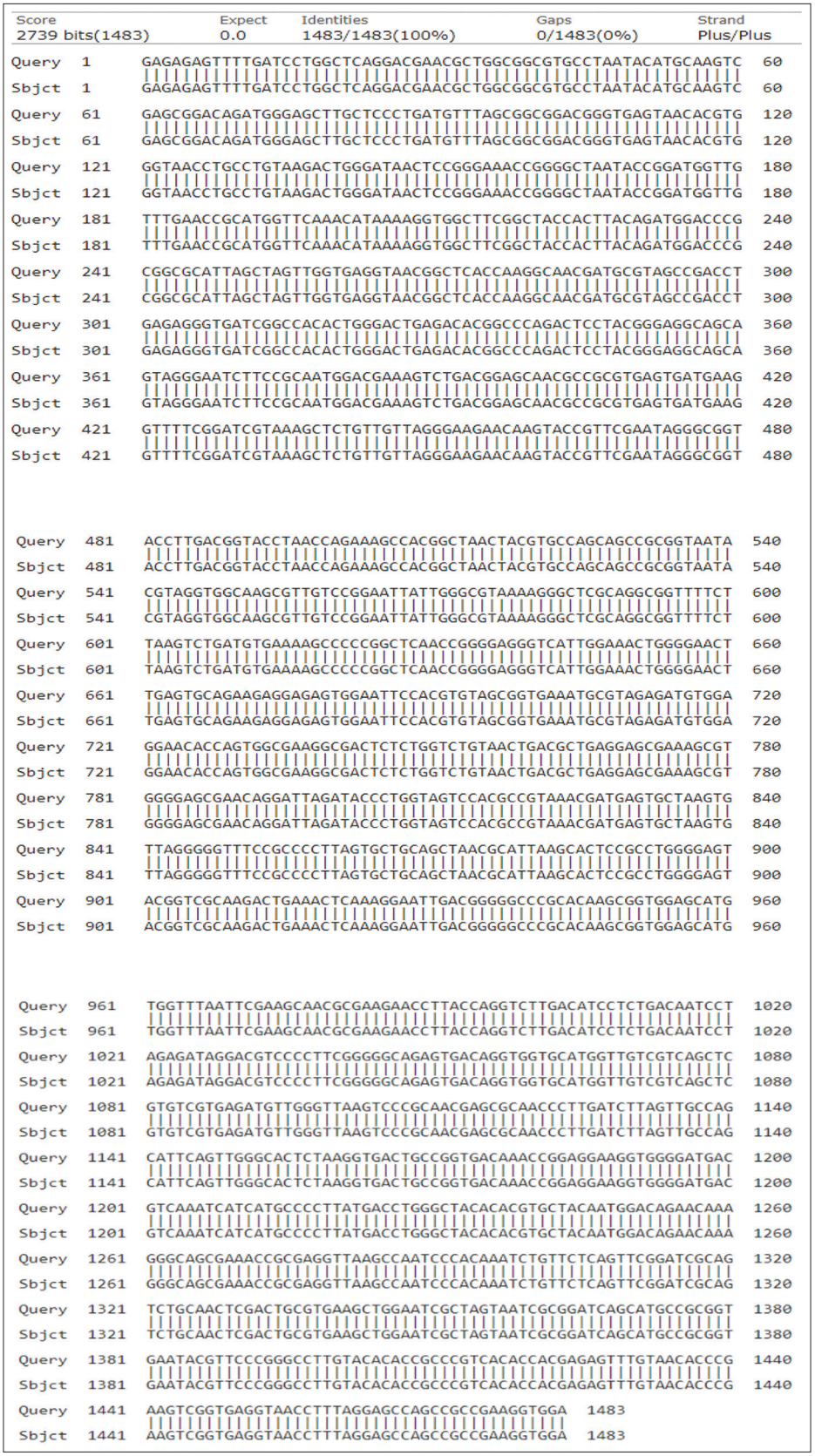

Figure 3a: The homology of partial DNA sequences of the 16S rRNA gene of the bacterial strain isolated from Salty water, Jazan, KSA (code S) and the corresponding gene of *Bacillus subtilis* strain (accession number KU229984).

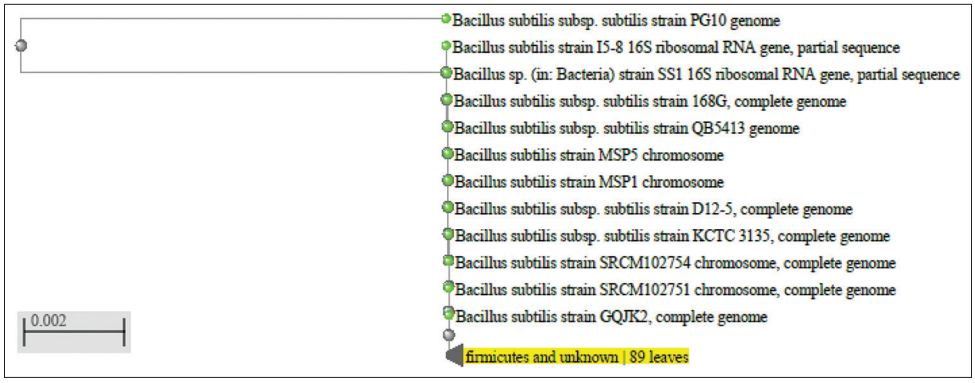

Figure 3b: Phylogenetic tree of the 16S rRNA sequence results of *Bacillus subtilis*.

Gram-positive bacterial strain, and *S. aureus*. In addition, Gram-negative bacterial strains, *E. coli,* and *K. pneumoniae*.

In an attempt trial to test the susceptibility of the pathogenic bacteria against antibiotics, it was found that *E. coli* recorded resistance (40%) to the following antibiotics: AX, TM, E, CD, PG, VA, T, RP, Colistin, Fosfomycin, CTX, and AK, in addition, showed high sensitivity against the rest of antibiotics. *S. aureus* pathogen recorded high resistance (63%) to the following antibiotics: AX, TM, Cephalexin, CZ, CXM, Ceftriaxone, CTX, CAZ, CPM, ATM, Gentamicin, Tazobactam, IMI, MEM, NI, Colistin, CIP, CTX, and AK, in addition, showed sensitivity to the rest of antibiotics. Furthermore, *K. pneumoniae* recorded resistance (43%) to the following antibiotics: AX, TM, CAZ, NI, E, CD, PG, VA, T, RP, Colistin, and Fosfomycin, moreover, showed sensitivity to the rest of the tested antibiotics [Table 4].

As mentioned above, the activated isolated bacterial cells were inoculated to fresh Casein Soyabean Digest Broth. The new culture was allowed to grow overnight at 37°C with shaking. At the indicated time, CFU/ml was determined. In addition, 1.5 ml of the growing culture was taken. The supernatants were then used as crude enzyme to determine the protease activity, protein content as well as to test as antimicrobial agents. [Table 5] shows the bacterial growth (CFU/ml), protease activity (Units/ml), soluble protein content (µg/ml), and specific activity (Units/ml/mg/ml) for *B. subtilis, B. licheniformis,* and *B. cereus* which their extract were used as antimicrobial agents. [Table 5] shows that 1 µl crude enzyme extracted from *B. subtilis*, *B. licheniformis,* and *B. cereus* contain 0.073, 0.076, and 0.092 specific activity (Units/µl/µg/ml).

Ten microliters of activated tested pathogen cells were inoculated to 10 ml of Casein Soyabean Digest Broth, supplemented with 0, 5, or 10 µl crude enzyme extract. All cultures were incubated O/N at 37°C. At the indicated time, measurement of O.D. of the turbidity at 600 nm was determined to detect the antimicrobial activity of the tested crude protease enzyme on the growth of pathogenic bacteria. [Figures 9-11] show a high effect on inhibiting the growth of pathogenic bacteria using 5 µl of crude enzyme with specific enzyme activity 73, 76, and 92 (Units/ml/mg/ml) for *B. subtilis, B. licheniformis,* and *B. cereus,* respectively. All pathogenic bacteria were totally inhibited with 10 µl more than 5 µl of crude enzyme.

DISCUSSION

Bacteria are ubiquitous and highly diverse prokaryotes that can survive in adverse habitats. Thermophilic bacteria are microbes that mostly inhabit hot springs, live, and survive in temperatures between 45°C and 122°C. Thermophiles including bacterial and archaeal species are found in various geothermally heated regions of the earth such as hot springs and deep sea hydrothermal vents.[22] In the Kingdom of Saudi Arabia, there are ten geothermal springs with different deep temperatures of and different flow rates. They are distributed in Al-Lith and Gizan areas. Of these, Ain Khulab Quwa, Ain Khulab, Ain ad Damad, Ain Mijara Quwa, Ain al Wagrah, and Ain al Wagrah Dam are located in Gizan meanwhile Ain Jumah, Ain al Harra, Ain ad Darakah, and Ain Markub are located in Al-Lith area.[23]

Microbial produced enzymes that are secreted into the media are highly reliable for industrial processes and applications.[24] Protease enzymes are at the forefront of commercial enzymes as they have an industrial importance that makes up a large percentage of other enzymes. Commercially, it is used in various industrial food fields as softening of meat, bread, cheese, and beer and textiles industry as well as use in the field of pharmaceuticals, medical, and cosmetics production.[25]

In the current study, on the basis of maximum production of protease and gelatinase, three bacterial isolates were identified using 16S rRNA gene sequence analysis as *B. cereus,* and *B. subtilis* isolated from halophilic habitat, while *B. licheniformis* was isolated from thermophilic habitat (Al-Harth Hot spring). The selection of strains was depended on the choice Gram-positive and catalase-positive isolates. Therefore, they

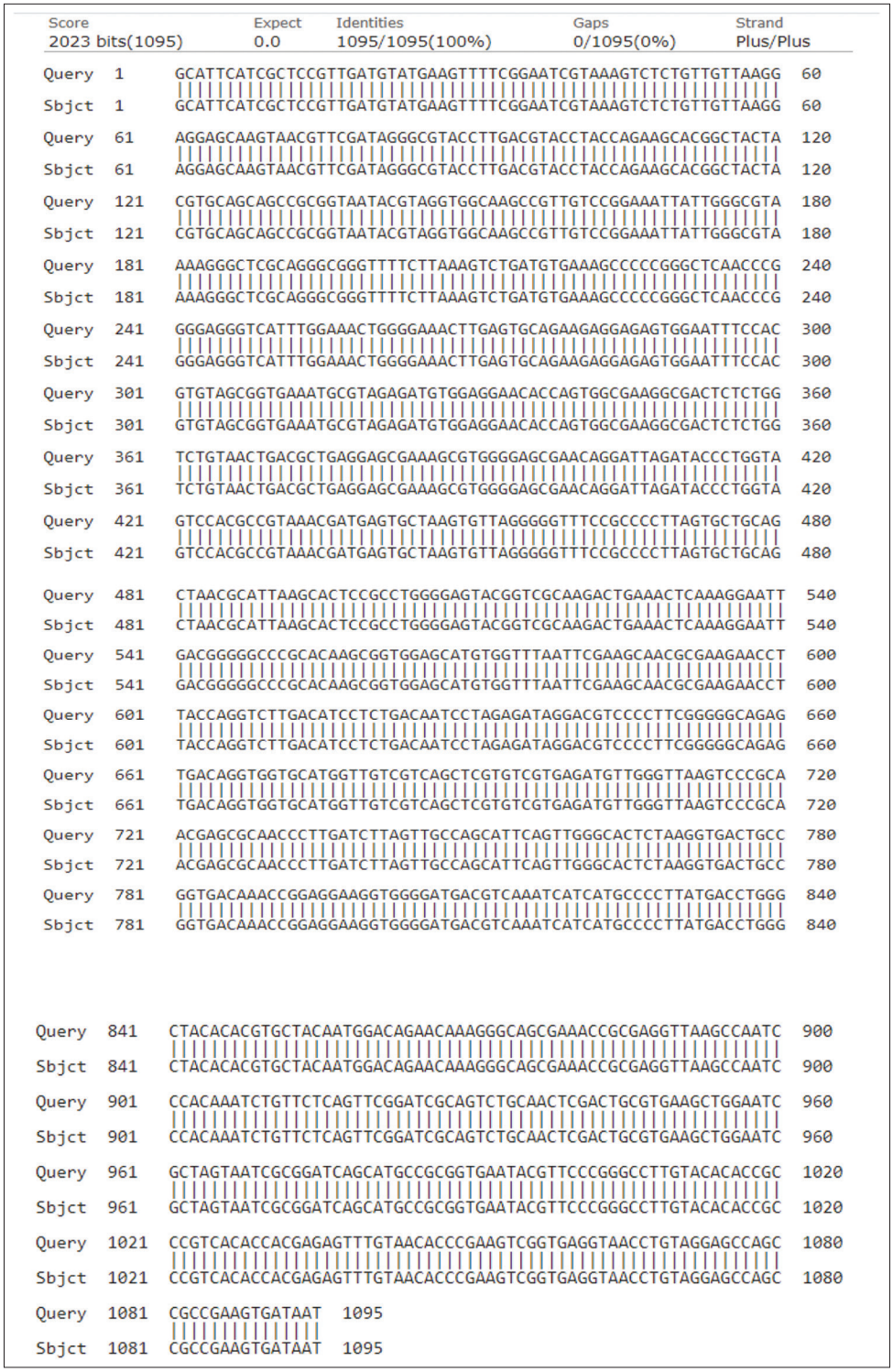

Figure 4a: The homology of partial DNA sequences of the 16S rRNA gene of the bacterial strain isolated from Al-Harth hot spring, Jazan, KSA (code H) and the corresponding gene of *Bacillus licheniformis* strain (accession number MK622385).

are strict aerobes or facultative anaerobes. The importance of *Bacillus* species in the industries was illustrated. Where, the genus *Bacillus* is probably the most important bacterial source of extracellular enzymes and is capable of producing high yields of alkaline and neutral proteolytic enzymes with remarkable properties, such as high stability toward extreme

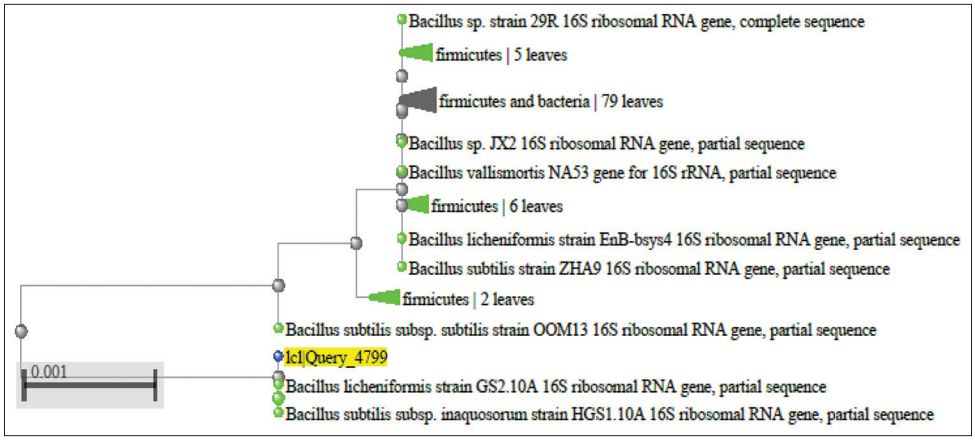

Figure 4b: Phylogenetic tree of the 16 S rRNA sequence results of *Bacillus licheniformis*.

temperatures, pH, organic solvents, detergents, and oxidizing compounds.[26] In addition, *Bacillus sp.* can form endospore allowing it to tolerate harsh environmental conditions. It can secrete proteins into the medium, which is considered as a major advantage. This allows the accumulation of the native product with high yield in a pure form. Moreover, they have no known pathogenic interaction with animals or human.[27]

B. subtilis is a Gram-positive and catalase-positive bacteria found in the soil. *Bacillus* microflora has the ability to form a solid, protective microbial that allows it to withstand harsh environmental conditions.[28] In addition, *B. licheniformis* is a Gram-positive bacterium, often found in soil. These bacteria have the ability to live at high temperatures and are considered one of the most important types of bacteria producing industrial enzymes and that for their ability to produce alkali serine proteases, examples of industrial products are detergents.[29] Meanwhile, *B. cereus* is an endemic bacterium that lives in the soil. This is a human-causing strain that is transmitted through contaminated food. Harmless strains of wax *Bacilli* are used as feed additives in bio-nutrition to reduce *salmonella* in the intestines of animals. This improves the growth of animals and makes their meat safe and suitable for human consumption.[30]

Biochemical test reactions that are not universally positive or negative within a species may define biotypes of the species. Since bacteria will react differently to these tests, it is much like the bacterial "fingerprint." To characterize the selected strains, the biochemical tests were done for all strains. They appeared utilization of some carbohydrates and other substrates as shown in the results. These results were compatible with the previous studies.[1,31]

The differences among strains may also be detected by variations in sensitivity to fixed concentrations of chemicals such as the antibiotic susceptibility test, where the antibiotics resistance in bacteria is a major health problem in many countries.[32] Resistance to antibiotics is acquired by a change in the gene makeup of bacterium, which can occur either by a gene mutation or by transfer of antibiotic resistance genes between bacteria in the environment.[33] The susceptibility of *Bacilli* to different antibiotics has been studied, and it has been demonstrated that in principle it should be possible to identify species based on the results of susceptibility tests. A large number of *Bacillus* strains assigned to different species were tested to determine their susceptibilities to antibiotics. Some clear differences between species were observed.[34] The antibiotic susceptibility test is performed to detect possible drug resistance and to assure susceptibility to drugs of choice for particular contamination.[1]

In the current study, bacterial isolates recorded high sensitivity with different degrees against tested antibiotics. In the previous studies, *B. subtilis* isolated from Al-khoba hot spring, Jazan, another *B. subtilis* isolated from tap water and *B. thuringiensis* were sensitive against most of tested antibiotics.[1,22,35] In a similar study, as part of a clinical-microbiological study, 89 strains of *Bacillus* spp. isolated from clinical blood cultures were tested for antimicrobial agent susceptibility to 18 antibiotics. MIC susceptibility tests revealed all *B. cereus* strains to be susceptible to IMI, VA, chloramphenicol, gentamicin, and CIP. Disk susceptibility testing suggested that *B. cereus* was rarely susceptible to PGs, semisynthetic PGs, or cephalosporins with the exception of mezlocillin.[36] In addition, the *B. cereus* isolates from cereals were susceptible to most of the antibiotics tested, but they were highly resistant to ampicillin, CPM, oxacillin, and PG.[37] In addition, the MIC for eight antibiotics was determined for 85 *Bacillus* species strains, *B. subtilis* subsp. *subtilis* ($n = 29$), *B. licheniformis* ($n = 38$), and *B. sonorensis* ($n = 18$). The antibiotic susceptibilities and characteristics of 94 *B. licheniformis* strains isolated from traditional Korean fermented soybean foods were assessed. The minimum inhibitory concentration (MIC) tests revealed that all strains were susceptible to gentamicin, kanamycin, T, and VA and that antibiotic resistances were expressed in a strain-specific manner. The resistances of *B.*

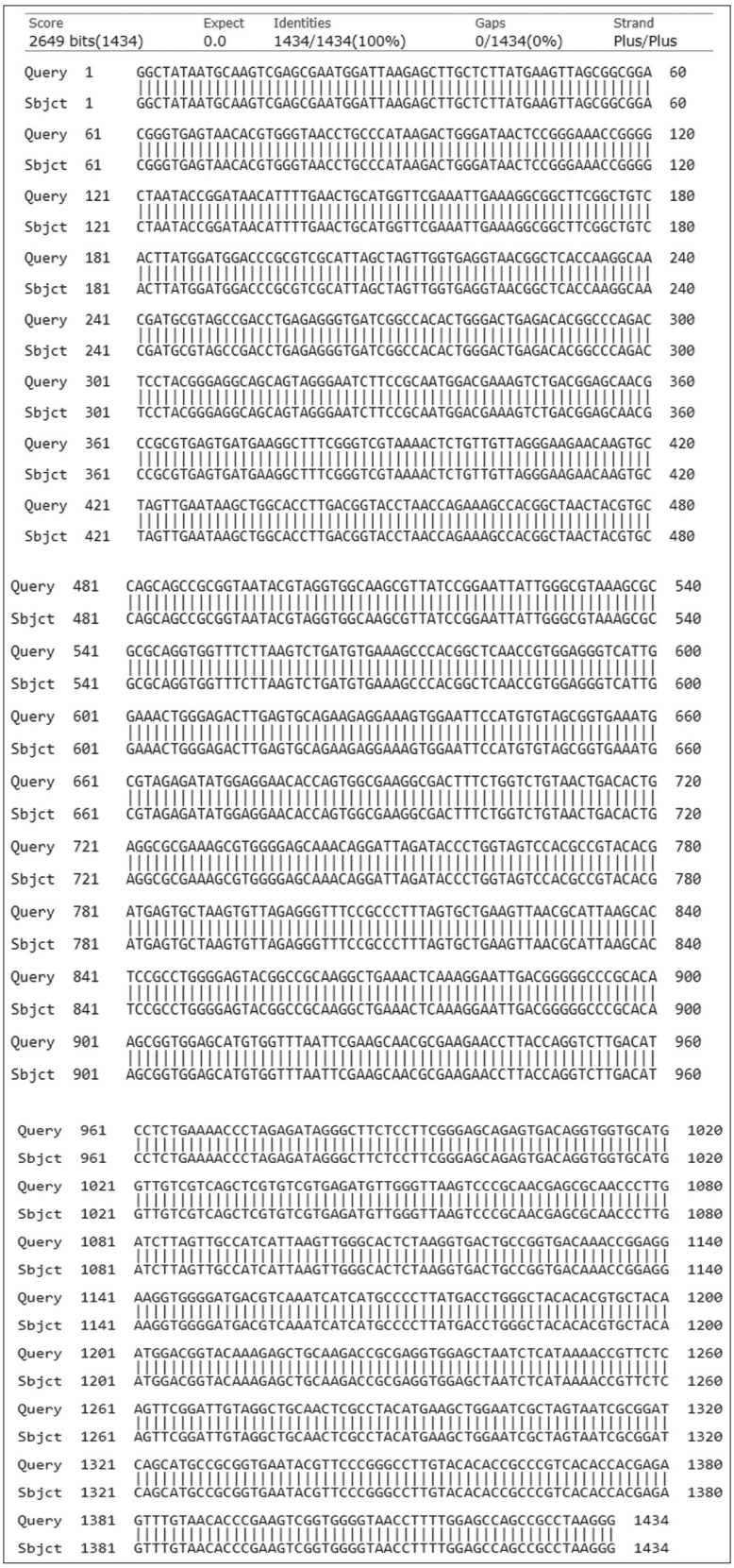

Figure 5a: The homology of partial DNA sequences of the 16S rRNA gene of the bacterial strain isolated from Farasan Island sea water, Jazan, KSA (code W) and the corresponding gene of *Bacillus cereus* strain (accession number KJ534410).

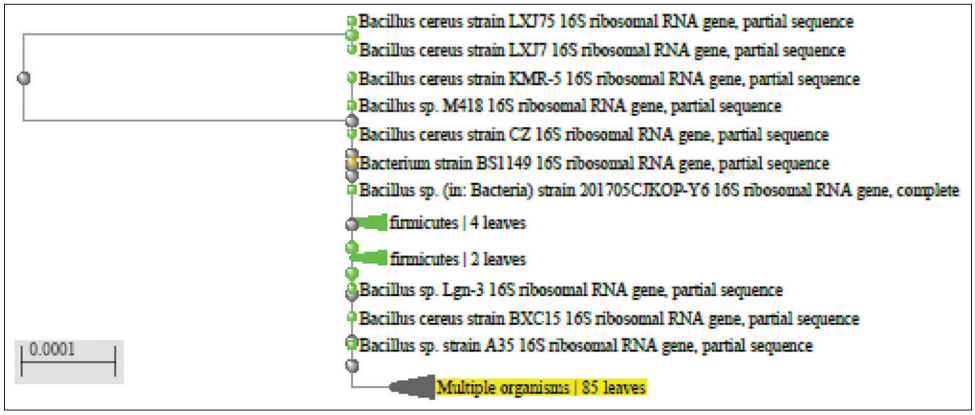

Figure 5b: Phylogenetic tree of the 16 S rRNA sequence results of *Bacillus cereus*.

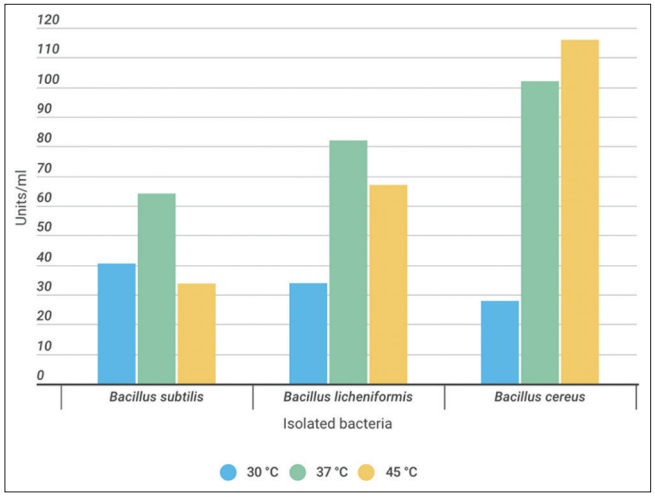

Figure 6a: Monitoring protease activity (Units/ml) throughout the utilization process of casein soyabean broth using *Bacillus subtilis*, *Bacillus licheniformis,* and *Bacillus cereus* at 30, 37, and 45°C for 24 h.

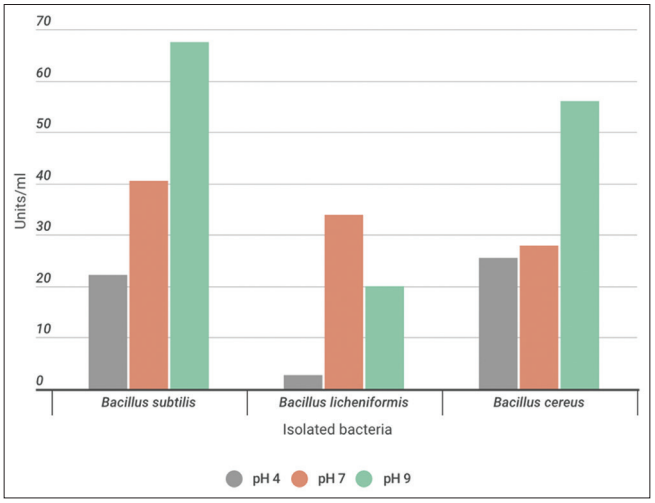

Figure 7a: Monitoring protease activity (Units/ml) throughout the utilization process of casein soyabean broth using *Bacillus subtilis*, *Bacillus licheniformis,* and *Bacillus cereus* at pH 4, 7, and 9 for 24 h at 30°C.

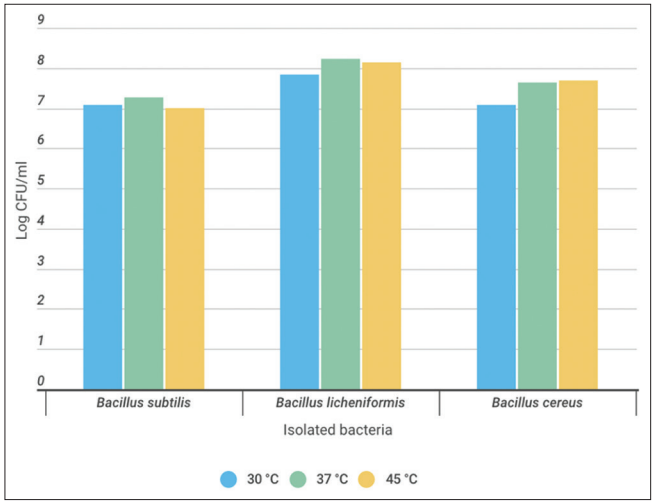

Figure 6b: Monitoring the total bacterial count (log CFU/ml) throughout the utilization process of casein soyabean broth using *Bacillus subtilis*, *Bacillus licheniformis,* and *Bacillus cereus* at 30, 37, and 45°C for 24 h.

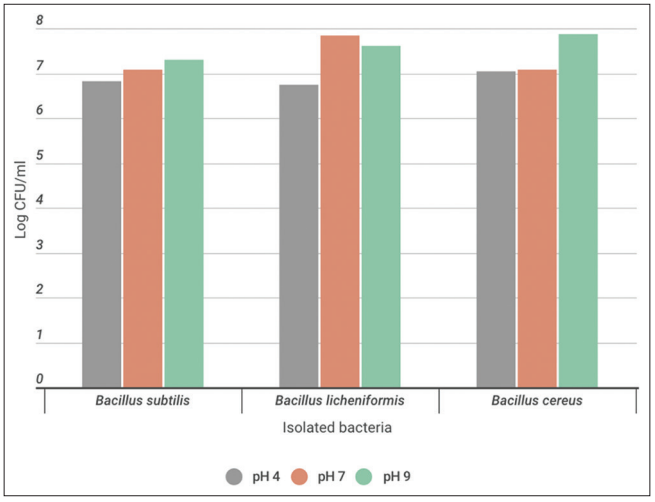

Figure 7b: Monitoring the total bacterial count (log CFU/ml) throughout the utilization process of casein soyabean broth using *Bacillus subtilis*, *Bacillus licheniformis,* and *Bacillus cereus* at pH 4, 7, and 9 for 24 h at 30°C.

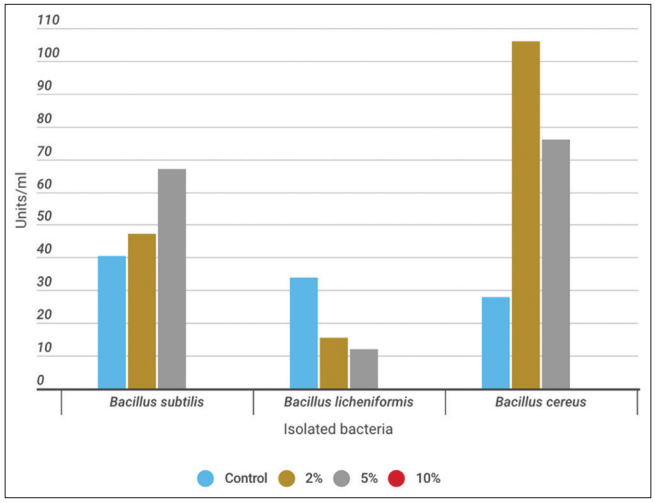

Figure 8a: Monitoring protease activity (Units/ml) throughout the utilization process of casein soyabean broth supplemented with 2, 5, and 10% NaCl using *Bacillus subtilis*, *Bacillus licheniformis,* and *Bacillus cereus* for 24 h at 30°C.

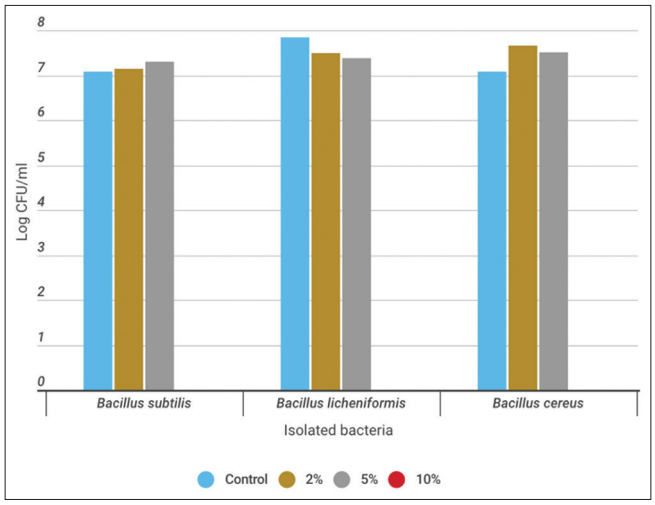

Figure 8b: Monitoring the total bacterial count (log CFU/ml) throughout the utilization process of casein soyabean broth supplemented with 2, 5, and 10% NaCl using *Bacillus subtilis*, *Bacillus licheniformis,* and *Bacillus cereus* for 24 h at 30°C.

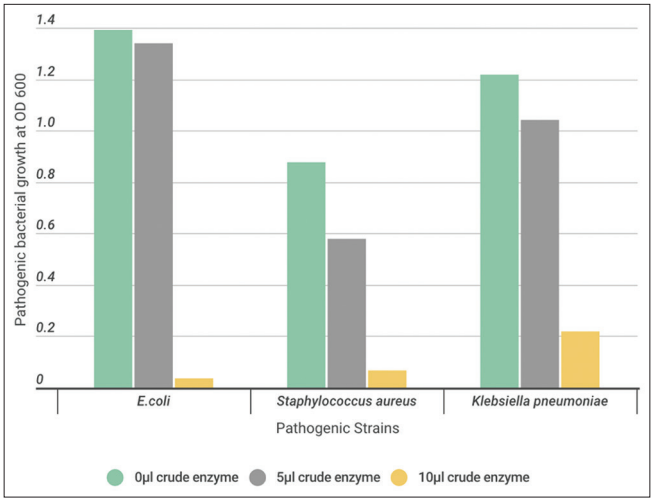

Figure 9: The effect of different volumes (0, 5, and 10 µl) of crude protease enzyme extracted from *Bacillus subtilis* on the pathogenic strains.

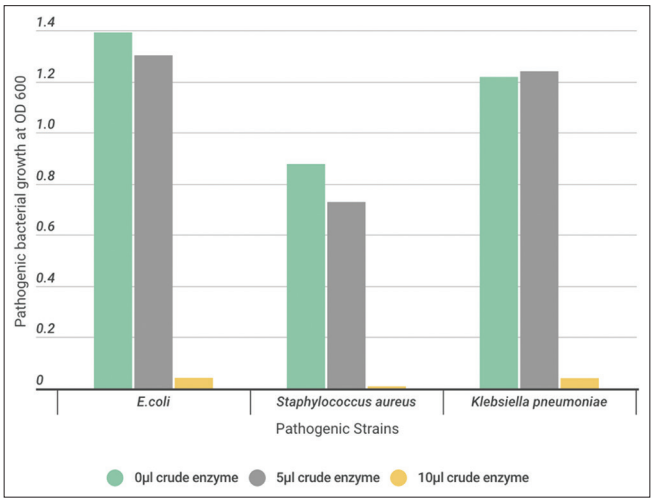

Figure 10: The effect of different volumes (0, 5, and 10 µl) of crude protease enzyme extracted from *Bacillus licheniformis* on the pathogenic strains.

licheniformis to chloramphenicol and streptomycin were established as intrinsic characteristics.[38]

In the current study, due to the importance of proteases in the industries, the extracellular protease production from *B. cereus, B. licheniformis,* and *B. subtilis* was chosen for further work. Due to the ever-increasing economic relevance of proteases, optimization of some fermentation parameters for *Bacillus sp*. was done. The effect of temperature, pH, and salinity on the bacterial growth and enzyme production was studied. Proteases production by microorganisms is affected by the medium compositions in addition to the environmental conditions. Thus, it is important to explore the influencing factors to attain the maximum protease production.[1] Cultivation temperature affects protein synthesis by influencing rate of biochemical reactions within the cell and consequently inducing or repressing enzyme production.[39]

The optimum temperature of the growth of *B. subtilis* and *B. licheniformis* was at 37°C. However, *B. cereus* showed high growth with protease activity at the optimum temperature of 45°C. In addition, it was found that the maximum growth with protease production for *B. subtilis* at 37°C.[40] Nevertheless, in another study of two isolates *Bacilli* that showed growth at high temperature 45°C.[41] In a previous study, *B. licheniformis* has been isolated and produced extracellular proteases and was able to grow at temperatures up to 60°C and at pH values up to 9.0 when casein was used as carbon source.[42]

Table 4: Effect of antibiotics on the tested pathogenic bacterial strains.

No.	Generic name	Conc.	*Escherichia coli*		*Staphylococcus aureus*		*Klebsiella pneumoniae*	
			Status	Halo-zone mm	Status	Halo-zone mm	Status	Halo-zone mm
1	Amoxicillin	10	R	0	R	0	R	0
2	Trimethoprim	25	R	0	R	0	R	0
3	Amoxicillin/Clavulanate	30	S	26	S	28	S	28
4	Cephalexin	30	S	25	R	0	S	28
5	Cefazolin	30	S	25	R	0	S	28
6	Cefuroxime	30	S	25	R	0	S	28
7	Ceftriaxone	30	S	26	R	0	S	26
8	Cefotaxime	30	S	26	R	0	S	26
9	Ceftazidime	30	S	26	R	0	R	--
10	Cefepime	30	S	26	R	0	S	28
11	Aztreonam	30	S	26	R	0	S	26
12	Gentamicin	10	S	22	R	0	S	24
13	Amoxicillin	10	S	24	S	35	R	0
14	Tazobactam	110	S	28	R	0	S	28
15	Imipenem	10	S	28	R	0	S	28
16	Meropenem	10	S	28	R	0	S	28
17	Nitrofurantoin	300	S	24	R	0	R	--
18	Colistin	10	S	14	R	0	S	26
19	Cefoxitin	30	S	25	S	26	S	26
20	Ciprofloxacin	5	S	26	R	0	S	28
21	Erythromycin	15	R	0	S	26	R	0
22	Clindamycin	2	R	0	S	25	R	0
23	Penicillin	10	R	0	S	35	R	0
24	Vancomycin	30	R	0	S	20	R	0
25	Tetracycline	30	R	0	S	28	R	0
26	Rifampicin	5	R	0	S	28	R	0
27	Cotrimoxazole/Colistin	25	R	0	S	28	R	0
28	Fosfomycin	200	R	0	S	27	R	0
29	Cefotaxime	30	R	0	R	0	S	26
30	Amikacin	30	R	0	R	0	S	26

R: Resistance, S: Sensitive

pH affects the growth of microorganisms by particularly affecting the enzymes involved in the biosynthesis and growth process. For each microorganism, the growth rate is determined for a specific pH value. There is also a maximum value of maximum pH, which is the maximum value in which growth can be observed, and any increase in this value leads to preventing growth. Minimum pH is the lowest value in which growth can be observed.[43] Most natural environments have pH values between 5 and 9 and most organisms have pH optimal in this range. The protease enzyme showed activity in the wide range of pH from 6 to 11 with maximum protease activity at pH10.[44] In the current study, the protease enzyme

Table 5: The bacterial count, protease activity, soluble protein content, and specific activity for *B. subtilis, B. licheniformis,* and *B. cereus* grown on casein soybean digest broth at 37°C for 24 h.

Bacterial strain	Bacteria count	Protease activity	Soluble protein content (mg/ml) 1	Specific activity
	CFU/ml	Units/ml		(Units/ml/mg/ml)
B. subtilis	2.7×10^7	40.2	0.546	73
B. licheniformis	3.2×10^7	46.6	0.608	76
B. cereus	6.8×10^6	49.7	0.54	92

1-Specific activity is dividing Enzyme activity (Units/ml)/Soluble protein (µg/ml). *B. cereus*: *Bacillus cereus*, *B. licheniformis*: *Bacillus licheniformis*, *B. subtilis*: *Bacillus subtilis*

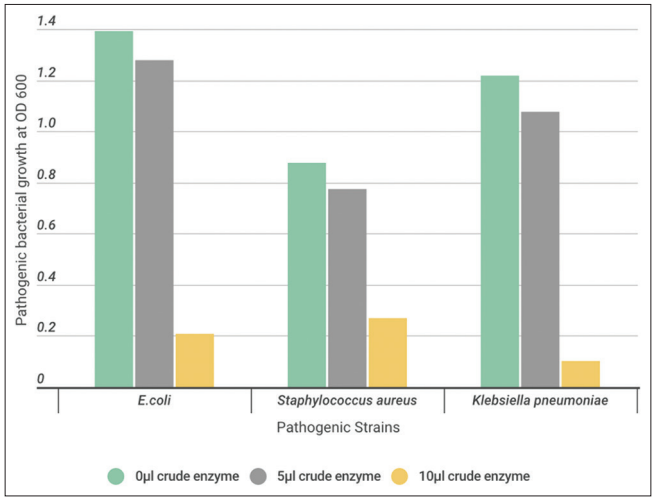

Figure 11: The effect of different volumes (0, 5, and 10 µl) of crude protease enzyme extracted from *Bacillus cereus* on the pathogenic strains.

activity and bacterial growth of *B. subtilis* and *B. cereus* strains were increased at alkaline medium. On the other hand, *B. licheniformis* gave the highest growth and activity at neutral pH. These results were agreeing with a previous study, where *B. subtilis* fermentation gave high level of protease production at 45°C after 36 h at pH-10.[45] In another study, the optimal pH and optimal temperature of enzyme production from *Bacillus* sp. strain were 8.0 and 45°C, respectively. Studies on enzymatic characterization revealed that crude protease showed maximum activity at pH 9.0 and 60°C, which is indicating the enzyme to be thermoalkaline protease.[2]

Proteases from halophilic microorganisms possess the advantage of being stable under high salinity levels. To maintain osmolality in saline environments, the microorganisms adopt mainly two strategies: One followed by most moderately halophilic bacteria, accumulating organic compatible solutes in the cytoplasm, and the second followed by the halobacteria, accumulating inorganic salts in the cytoplasm. In the current study, it showed that *B. subtilis* was halotolerant within concentrations at 5% NaCl, while *B. licheniformis* and *B. cereus* showed maximum growth at 2% NaCl. However, in a previous study, where the influence of NaCl concentrations on the fermentation process of *Bacillus thuringensis* isolated from mangrove rhizosphere to treat whey waste was investigated. Among the salinity supplemented media, the highest bacterial growth (log CFU/ml 9.04) and maximum protease production (125.03 U/ml) were recorded in the presence of 10% NaCl.[1] In another study similar to the current study, *Bacillus luteus* H11 isolated from an alkaline soda lime was remarkably stable in the presence of Na Cl up to 5 M. The enzyme was active in a broad range of pH values and temperatures, with an optimum pH of 10.5 and a temperature of 45°C. The halo-alkaline protease produced by *B. luteus* H11 seems to be significant from an industrial perspective because of its tolerance towards high salinity and alkalinity as well as its stability against some organic solvents, surfactants, and oxidants.[46] Meanwhile, Protease producing halotolerant bacterium was isolated from saltern pond sediment and identified as *B. licheniformis*. The maximum protease production (141.46 U/mg) was obtained after addition of 1M NaCl.[47]

Antibiotic resistance appears because of evolution by natural selection. The effect of the antibiotic creates environmental pressure on the bacteria, but the mutations that appear in some bacterial cells cause them to escape the effect of the antibiotic. After that, this feature is transferred to the next offspring, which is characterized as a generation with full antibiotic resistance. Several studies have shown that the way antibiotics are used generally affects the evolution of the number of microorganisms resistant. The overuse of broad-spectrum antibiotics, such as second and third-generation cephalosporins, accelerates the development of methylene resistance. Other factors include inaccurate medical diagnosis, doctor prescribing unnecessary medications, inappropriate use of antibiotics by the patient, as well as the use of antibiotics as additional materials to feed and grow livestock.[40] Hence, bacterial proteases are an extensive collection of enzymes that have vital roles in cell viability, stress response, and pathogenicity. Their perturbation clearly offers the potential for antimicrobial drug development, both as traditional antibiotics and anti-virulence drugs.[48]

In this study, the susceptibility of three pathogenic bacteria against the extracellular crude protease enzyme produced

from the three isolated bacteria was done. These pathogenic isolates were *S. aureus*, *E. coli,* and *K. pneumoniae*. A high effect on inhibiting the growth of pathogenic bacteria was registered. These results suggest that protease enzyme, could represent a promising antimicrobial drug strategy. In a similar study, 29 soil-isolated *Bacillus* species were tested against to some bacteria in terms of their general inhibition effects. Isolates have been found to be effective against Gram-positive and Gram-negative bacteria, while their extensive inhibition effect is particularly effective against gram-positive bacteria. In comparison, *B. cereus* strain has an inhibitory effect on Gram-positive as well as Gram-negative bacteria. In addition, certain isolates are more effective against test bacteria compared to other antibiotics.[49] Meanwhile, extracellular protease was isolated and purified from *Xylaria psidii* KT30 and used as antibacterial agent against Gram-positive bacteria; *B. subtilis* and *S. aureus*.[50] In another study, the effect of *Bacillus pumilus* proteolytic enzymes on the structure of 7-day-old *S. marcescens* biofilms was examined. A high efficacy of subtilisin-like protease and glutamyl endopeptidase in biofilm removal was noticed.[51] Furthermore, it was revealed the capacity of *B. cereus* to generate antimicrobial peptides from casein, through the production of extracellular enzymes, presents a new model of antagonistic competition.[52] *B. subtilis* VCDA that had proven antibacterial activity was screened for its production of bioactive extracellular proteases. In another study, the antibiofilm activity of the *B. licheniformis* protease enzymes was evaluated toward four pathogenic bacterial strains, *S. aureus*, *Pseudomonas aeruginosa*, *E. coli*, and *B. subtilis,* and the results showed that proteases from *B. licheniformis* might be useful for controlling biofilm formation by some pathogenic bacteria.[53]

CONCLUSION

In general, the extracellular crude protease enzyme of the isolated bacteria can able to use as antimicrobial agents against pathogenic bacterial strains. Further studies are recommended in future prospective. The recommended studies are the study of further factors affecting extracellular protease enzyme activity, scaling up of enzyme production, enzyme purification, studying the kinetics of enzyme, the effect of inhibitors, and immobilization of enzyme. Focusing on the application as biology control on a large number of pathogenic bacteria and fungi with different concentrations to determine MICs with a comparison with the effect of antibiotics is recommended.

Declaration of patient consent

Patient's consent not required, as there are no patients in this study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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How to cite this article: Al-Thobaiti BM, Abada E, El-Gayar K. Biochemical and molecular characterization of proteolytic bacterial strains isolated from Jazan region, KSA with the application as an antibacterial agent. Mod J Microb Biol 2021;3(1):1-17.