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Purification of gelatinase from the bacteria contaminating gelatin production process.

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ABSTRACT

Gelatin contamination is of great concern from economic and public health point of view. In this study, bacterial contamination during different stages of gelatin manufacturing process was examined. One hundred and fifty four isolates were obtained from different stages of gelatin production process. Wet noodles samples acquired the highest percentage of bacterial isolates (14.93%). Majority of the bacterial isolates were classified as Gram-positive bacilli (79.8%). 51.3% of isolates showed moderate gelatinase activity. The most potent gelatinase producing isolate was identified as *Bacillus cereus* based on biochemical and 16S rRNA sequence analyses. Fluoroquinolones were the most effective antibiotics against bacterial isolates with the highest sensitivity rate (94.78%). Cinnamon oil exhibits the highest sensitivity rate (97.6%) and low MIC values (0.195 µl/ml). GC/MC analyses revealed that monoterpenes were the predominant components of the essential oils. The main constituent of cinnamon oil was cinnamaldehyde (63.69%). Gelatinase was purified by ion exchange and size exclusion chromatography. Zymography and SDS-PAGE analysis estimated that the purified enzyme is ~100 KDa molecular size.

Keywords: Gelatin, Gelatinase, *Bacillus cereus,* Zymography.

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INTRODUCTION

Gelatin is a proteinaceous colloid of animal origin derived by thermal denaturation of collagen **[1]**. Gelatin is applied in food industry, pharmaceutical industry and in the production of photographic films, matches and glues **[2].**

Gelatin acts as an excellent medium for the growth of many microorganisms **[3]**. Microbial contamination of industrial plants is a widespread problem that affects the quality and safety of gelatin **[4]**. *Bacillus* and related genera as an example of such contaminants constitute a hazard to the quality of gelatin. These contaminants can survive extreme manufacturing conditions, besides they have been mentioned in food poisoning incidents **[5].**

Certain plant-derivative essential oils were found to have antibacterial effects upon growth of microorganisms **[6]**. Therefore, may provide an alternative method for contamination prevention **[7]**. As they are almost safe, environmentally friendly and lack most side effects upon the products **[8]**.

Proteases represent one of the largest groups of industrial enzymes **[9]**. Microbes serve as a preferred source of these enzymes because of their rapid growth and limited space required for their cultivation **[10]**. *Bacillus sp*. prevalently used in such applications, produce a wide variety of extra-cellular enzymes, including proteases **[11]**.

Gelatinase is an important metalloprotease, it is widely used in chemical and medical industries and also in food and basic biological science **[12]**. The potential uses of gelatinase and their high demand requires the discovery of new strains of bacteria that produce enzymes with novel properties and the development of low cost industrial medium and extraction formulations **[13]**.

The present study aimed to isolate and identify bacterial contaminants during different gelatin processing stages along with studying the effect of antibiotics and essential oils on the growth of these contaminants. In addition, purifying and characterizing gelatinase from the most potent contaminants for its future applications in different industries.

MATERIALS AND METHODS

Bacterial samples collection

Samples were collected from the different stages of gelatin manufacturing process. Water used during processing, weak liquor stage, strong liquor stage, solid samples and swabs of drying zones **(Table 2)**.

Bacterial strains isolation

For liquid samples, 1ml was directly inoculated into nutrient agar plates and incubated at 37°C for 3 days and observed in each day. For the final dry product 10g of dry gelatin was soaked in 100 ml of autoclaved saline solution (10% w/v) then put in water bath at 45°C until completely dissolved. 1ml of this solution was inoculated in nutrient agar plates, then incubated at 37°C for 3 days and observed in each day. For the strong liquor and wet noodles they were processed like the dry product. Swab samples were taken by rubbing the surfaces of the conveyer belt and different zones of band dryer with sterilized loop and then directly streaked on nutrient agar plates and observed in each day.

Morphologically different single colonies were isolated from the plates that have bacterial growth and maintained on nutrient agar slants.

Identification of bacterial isolates

Identification of the selected bacterial isolates was carried out according to Bergey's Manual of Systematic Bacteriology **(1994)** and confirmed with 16s rRNA analyses in which DNA was extracted using GeneJET™ Genomic DNA Purification Kit (Thermo Scientific, USA) according to manufacturer's protocol and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3').

Screening for gelatinase

The bacteria were screened for gelatinase production in a semi-quantitative manner by streaking on 4% gelatin supplemented nutrient agar **[14]**. Plates were incubated for 24 hours at 37°C. Then enzyme activity was measured in terms of clearing zone diameter in cm that appeared after flooding the plates with Frazier reagent (12.5% acidified mercuric chloride).

Estimation of growth

The growth of the selected bacterial isolates was determined in protease production medium, by measuring the growth activity at 660 nm against un-inoculated medium as blank. Readings were taken on alternate days up to the 11th day of incubation at 37°C and 150 rpm in shaking incubator **[15].**

Estimation of Extracellular Protease activity

Extracellular protease activity was determined by casein digestion method described by **[16].** In brief, bacterial isolates were grown on soyabean casein digest medium and incubated in shaker incubator at 37°C and 150 rpm. After incubation period, 1.5ml of culture media was centrifuged at 10000 rpm, the filtrate was used to measure protease activity. The unknown concentration of tyrosine liberated after enzymatic reaction in a test tube was measured at 660 nm against a reagent blank using tyrosine as standard. Standard curve of tyrosine was prepared by taking tyrosine in following range: 27.5µM/ml to 275µM/ml. One protease unit was defined as the amount of enzyme that releases one µM of tyrosine per minute at 37°C and pH 7.5. All the experiments were done in triplicates and mean values were recorded.

Antibiotic sensitivity test

Test was performed by the disk agar diffusion method on Müller-Hinton(MH) agar medium **[17].** Selected bacterial isolates were inoculated on nutrient agar medium and incubated overnight at 37°C. Single colony was taken and suspended in saline solution to obtain bacterial suspension 1x10^{8} CFU/ml (0.5 McFarland standard). Sterilized swaps were soaked in this bacterial suspension and streaked thoroughly on MH agar plates. Disks containing antibiotics were placed onto the surface of the medium. The culture was incubated overnight at 37°C. After incubation, the diameters of the inhibition zones were recorded.

Essential oils susceptibility test

Plants used in this study were collected from Giza, Egypt **(Table 1)**. Identification of plants was verified by Herbarium, Botany and Microbiology Department, Faculty of Science, Cairo University, Giza, Egypt. Essential oils were obtained by hydrodistillation. Test was performed by the diffusion method on MH agar medium, bacterial suspension was prepared as described before. Sterilized swaps were soaked in this bacterial suspension and streaked thoroughly on MH agar plates **[18]**. 15µl of essential oils were applied into wells. Culture was incubated overnight at 37°C. After incubation, the diameters of inhibition zones were recorded.

Table (1): Common name, scientific name and family of the plants used.

Common name	Scientific name	Family
Cinnamon	*Cinnamomum verum* J.Presl	Lauraceae
Basil	*Ocimum basilicum* L.	Lamiaceae
Frankincense	*Boswellia sacra* Flueck.	Burseraceae
Fennel	*Foeniculum vulgare* Mill.	Apiaceae
Bergamot	*Citrus bergamia* Risso	Rutaceae
Mustard	*Sinapis alba* L.	Brassicaceae
Clove	*Syzygium aromaticum* (L.) Merrill & Perry	Myrtaceae
Ginger	*Zingiber officinale* Roscoc.	Zingiberaceae
Lavender	*Lavandula angustifolia* Mill.	Lamiaceae
Jojoba	*Simmondsia chinensis* (Link.) C.K. Schneid.	Simmondsiaceae
Lemon grass	*Cymbopogon citratus (*DC.*)* Stapf	Poaceae
Bitter almond	*Prunus dulcis* (Mill.) D. A. Webb	Rosaceae

Gas chromatography mass spectrometry (GC/MS)

The analysis of essential oils was carried out using a GC (Agilent Technologies 7890A) interfaced with a mass-selective detector (MSD, Agilent 7000 Triple Quad) equipped with an apolar Agilent HP-5ms (5%-phenyl methyl poly siloxane) capillary column (30m × 0.25mm and 0.25µm film thickness). The carrier gas was helium with the linear velocity of 1 ml/min. The injector and detector temperatures were 200°C and 250°C, respectively. Injection mode, split ratio 1:10, volume injected 1µl of the sample. The MS operating parameters were as follows: ionization potential 70 eV, interface temperature 250°C, and acquisition mass range 50–600. Identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds from NIST and WILEY library **[19].**

Determination of minimum inhibitory concentration (MIC)

Determination of minimum inhibitory concentration was done according to the criteria of the Clinical and Laboratory Standards Institute **[20].** By using broth micro-dilution technique that was conducted in 96 v-shaped multi wells micro-dilution plates. 50 µl of MH broth were added to each well. Fifty µl of the oil was mixed with10 µl Tween 20. This mixture was two-fold serially diluted by broth. Highest oil concentration was put in row 1 and lowest concentration at row 10. Row 11 used as negative control, the broth and oils without microorganism and row 12 was used as positive control for the microorganisms, culture in the broth without oils. An equal volume of 50 µl of the bacterial suspension $1x10^8$ CFU/ml was added in each well that would reduce the concentration of the oil by half and total volume in each of the 96 wells was 100 µl. Then incubate all at 37°C for 24 hours **[21]**.

After incubation, the MIC was recorded visually by adding 10 µl resazurin solution to all the wells and incubate for 2 hours at 37°C. Wells were assessed visually for color change; change from blue color into pink color is an indication of microbial growth. The growth in each well was compared with that of the growth control (oil free) wells. The MIC was recorded as the lowest concentration in each row which completely inhibited bacterial growth.

Enzyme purification

Gelatin nutrient culture medium was centrifuged at 12,000 rpm 4°C for 15 minutes, precipitate was discarded and supernatant (crude extract) was salted out by adding ammonium sulphate by 40% (fraction 1), 60% (fraction 2) and 80% (fraction 3) (w/v) at 4°C and with continuous slow stirring, until complete dissolving of the salt, the solution was again centrifuged at 12,000 rpm for 20 minutes. The precipitate was collected and desalted using a 25 mm cellulose dialysis membrane in a large volume of buffer (0.1 M phosphate buffer, pH 7.4) and stirred for 4 hours with changing the buffer at least twice in between then left overnight in the fresh buffer. The protein solution in the bag was then centrifuged at 12,000 rpm and 4°C for 20 minutes and the supernatant was collected and stored at -20°C for the next purification step **[12].**

The dialyzed protein was loaded on Sephadex G-150 column (1.5X30 cm), equilibrated with (0.1 M phosphate buffer, pH 7.4) and eluted with the same buffer at 4°C with observation of the filtrate activity and the column was washed with elution buffer, fractions of (2 ml) were collected and monitored for protein (280 nm) and gelatinase activity using spectrophotometer, the partially purified protein was loaded on DEAE-Cellulose for further purification. The purified fractions were collected and analyzed using SDS-PAGE for evaluating the activity. The SDS-PAGE was carried using 8 ml 7.5% acrylamide (w/v) separating gel and 2 ml 4% acrylamide (w/v) stalking gel, after applying samples with 4X sample buffer gel run with 1X running buffer at 150V at 4°C, then the gel was stained with Coomassie Brilliant blue R-250. The enzyme was sized with the help of the protein molecular weight marker (GeneDirex, Las Vegas, NV, USA) **[10].**

Zymography of gelatinase enzyme

Zymography of the enzyme extracted and the purified fractions of the selected bacterial isolates were performed using 1% (w/v) substrate gel **[22]**. The substrate used was food grade gelatin in 8 ml 7.5% acrylamide (w/v) separating gel and 2 ml 4% acrylamide (w/v) stalking gel. The samples were applied with 4X sample buffer and run with 1X running buffer at 150V for 1 to 2 hours at 4°C, then washing buffer was added

to the gel and incubated at room temperature with gentle agitation for 40 minutes. After the incubation period, the zymogram washing buffer was disposed and replaced with 1X zymogram developing buffer (Koma Biotech, Seoul, Korea) for 30 minutes, then replaced with fresh developing buffer and incubated overnight at 37°C. After the incubation period zymogram was stained with Coomassie Brilliant blue R-250 for 30 minutes then de-stained (45% ethanol and 10% acetic acid v/v).

Enzyme characterization

Effect of temperature on the enzyme activity was assayed by incubating the enzyme in gelatin agar medium plates in different temperatures for 24 hours. Effect of pH was assayed by incubating the enzyme in different pH values for 24 hours at 37°C.

The effect of EDTA with concentration of 16.8, 27.9 and 37.2 mg/ml was tested. The purified enzyme was inoculated on gelatin agar medium, and incubated at 37°C for 24 hours. The enzyme activity was assayed and compared to that of the non-inhibited enzyme activities.

RESULTS AND DISCUSSION

A total of one hundred and fifty four bacterial organisms were isolated from different stages of gelatin production process **(Table 2).** Forty four bacterial strains were isolated from the samples collected from the weak liquor main stage represented 28.57% of the total bacterial isolates. Following with 40 bacterial isolates (25.9%) from the solid samples and swaps stage, 36 isolates were collected from strong liquor stage (23.37%) and 34 bacterial isolates were isolated from water used in processing stages that 22.07% of the total bacterial isolates. Wet noodles samples were noticed to acquire the highest percentage of bacterial isolates (14.93%) amongst other sub-stages. Contamination in this stage may be explained by the high watery content of the wet noodles and the moderate temperature of the votator that produce these noodles, allowing bacterial growth.

Majority of the bacterial isolates were classified as Gram-positive bacilli (79.8%) while 14.93% of the isolates were classified as Gram-positive cocci. Gram-negative isolates percentage was less significant, that 3.24% of the isolates were Gram-negative bacilli and only 1.94% of the isolates were Gram-negative cocci, similar results were previously reported **[2].**

Identification of bacterial isolates

Based on results of gelatinase screening, the 15 most active isolates were selected to be identified from the results of the biochemical tests. The isolate (I E1 S1 1) was identified as *Bacillus megaterium,* (I F1 S1 2), (I E2 S2), (I B1 S3 5), (II D1 S3 1) and (III C2 S2 2) were identified as *Bacillus firmus,* (I B2 S2), (II C2 S1), (II D2 S1) and (III A1 S1 1) were identified as *Bacillus badius.* Isolate (II D1 S2) was identified as *Bacillus circulans.* Isolates (III A2 S2 1) and (III A2 S3 2) were identified as *Bacillus carboniphillus.* Isolate (IV C2 S1 2) was identified as *Bacillus acidiceler* and isolate (IV B2 S2) was identified as *Bacillus cereus.* Based on the results of the 16S rRNA identification and by comparing the obtained sequence against the BLAST server **(http://blast.ncbi.nlm.nih.gov/Blast.cgi)**, the best bacterial isolate giving the code (IV B2 S2) was confirmed as *Bacillus cereus.*

From the results of the identification and characterization of the selected isolates, *Bacillus* sp. were the dominant bacterial species over other bacterial isolates. Earlier studies reported dominance of these bacteria over other types of bacteria **[2] [3]**. This dominance may be due to the high tolerance and resistance of this species that endure in extreme environments. *Bacillus* species can be obligate aerobes or facultative anaerobes.

Under stressful environmental conditions, the bacteria can produce endospores and remain in a dormant state for very long periods **[23]**. In addition, *Bacillus* can produce copious amounts of enzymes which have the ability to hydrolyze different substances which may be vital for the survival of the bacteria **[4]**.

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Table (2): Count and percentage of bacteria isolated during the different stages of gelatin manufacturing

Stages	Gram positive		Gram negative		Total bacterial isolates count
	Bacilli No. (%)	Cocci No. (%)	Bacilli No. (%)	Cocci No. (%)	
I. Water used in processing	**30 (24.39)**	**3 (13.04)**	**0 (0)**	**1 (33.33)**	**34 (22.07)**
Chilled water used in liming pit	6 (4.87)	0 (0)	0 (0)	0 (0)	6 (3.89)
Chlorinated water at washer sink	8 (6.50)	2 (8.69)	0 (0)	1 (33.33)	11 (7.14)
Liquor extraction hot water	1 (0.81)	0 (0)	0 (0)	0 (0)	1 (0.64)
Water for flushing votator	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Water for cleaning conveyer belt	7 (5.69)	0 (0)	0 (0)	0 (0)	7 (4.54)
General washing water	8 (6.50)	1 (4.34)	0 (0)	0 (0)	9 (5.84)
II. Weak liquor	**38 (30.89)**	**5 (21.73)**	**1 (20)**	**0 (0)**	**44 (28.57)**
Before filtration	1 (0.81)	0 (0)	1 (20)	0 (0)	2 (1.29)
After filtration	6 (4.87)	0 (0)	0 (0)	0 (0)	6 (3.89)
Softener inlet	5 (4.06)	1 (4.34)	0 (0)	0 (0)	6 (3.89)
Softener tank	4 (3.25)	3 (13.04)	0 (0)	0 (0)	7 (4.54)
Softener outlet	7 (5.69)	1 (4.34)	0 (0)	0 (0)	8 (5.19)
Header tank	6 (4.87)	0 (0)	0 (0)	0 (0)	6 (3.89)
Before evaporator	9 (7.31)	0 (0)	0 (0)	0 (0)	9 (5.84)
III. Strong liquor	**25 (20.32)**	**9 (39.13)**	**1 (20)**	**1 (33.33)**	**36 (23.37)**
After evaporator	9 (7.31)	5 (21.73)	1 (20)	0 (0)	15 (9.74)
Before sterilizer	9 (7.31)	1 (4.34)	0 (0)	1 (33.33)	11 (7.14)
After sterilizer	7 (5.69)	3 (13.04)	0 (0)	0 (0)	10 (6.49)
IV. Solid samples & Swaps	**30 (24.39)**	**6 (26.08)**	**3 (60)**	**1 (33.33)**	**40 (25.97)**
Wet noodles	19 (15.44)	1 (4.34)	3 (60)	0 (0)	23 (14.93)
Final dry product	7 (5.69)	5 (21.73)	0 (0)	1 (33.33)	13 (8.44)
Swaps from the Drying rooms	4 (3.25)	0 (0)	0 (0)	0 (0)	4 (2.59)
Total	**123 (79.87)**	**23 (14.93)**	**5 (3.24)**	**3 (1.94)**	**154 (100)**

Screening for gelatinase

The gelatinase activity of the bacterial isolates was ranging from 0.2 cm to 3.0 cm. Thirty seven bacterial isolates (24%) have gelatinase activity ranging from 0.2 cm to 0.9 cm that was considered as low gelatinase activity. Seventy nine of the bacterial isolates (51.3%) have enzyme activity ranges from 1.0 cm to 1.9 cm which is a moderate enzyme activity and 27 isolates (17.5%) were found to have strong enzyme activity (more than 2cm). Eleven isolates (7%) found to have no gelatinase activity **(Tables 3a, 3b, 3c and 3d)**. Similar studies reported that the majority of the bacterial contaminations during gelatin production have moderate gelatinase activity **[2] [3].**

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Table (3a): Gelatinase activity of bacterial isolates from the water used in processing

I. Water used in processing

(A) Chilled water used in liming pit			(B) Chlorinated water at washer sink			(C) Liquor extraction hot water			(D) Water for flushing votator			(E) Water for cleaning conveyer belt			(F) General washing water		
Code	GA (cm)	Gm stain	Code	GA (cm)	Gm stain	Code	GA (cm)	Gm stain	Code	GA (cm)	Gm stain	Code	GA (cm)	Gm stain	Code	GA (cm)	Gm stain
I A1 S1	0.4	(+)	I B1 S1	1.0	(+)	I C2 S1	1.8	(+)	No bacterial growth detected in this sub-stage			I E1 S1 1	2.0	(+)	I F1 S1 1	0.4	(+)
I A2 S1	0.8	(+)	I B2 S1	1.6	(+)							I E1 S1 2	0.8	(+)	I F1 S1 2	2.2	(+)
I A1 S2	0.6	(+)	I B1 S2	1.2	(+)							I E2 S1	1.2	(+)	I F2 S1 1	1.4	(+)
I A2 S2	0.8	(+)	I B2 S2	2.1	(+)							I E1 S2	0.6	(+)	I F2 S1 2	1.6	(+)
I A1 S3	0.8	(+)	I B1 S3 1	1.2	(+)							I E2 S2	2.2	(+)	I F1 S2	1.2	(+)
I A2 S3	0.2	(+)	**I B1 S3 2**	0.4	(+)							I E1 S3	1.2	(+)	I F2 S2	1.0	(+)
			I B1 S3 3	0.8	(+)							I E2 S3	1.2	(+)	I F1 S3 1	1.4	(+)
			I B1 S3 4	1.0	(+)										**I F1 S3 2**	1.2	(+)
			I B1 S3 5	2.3	(+)										I F2 S3	ND	(+)
			I B2 S3 1	1.4	(+)												
			I B2 S3 2	ND	(-)												

Table (3b): Gelatinase activity of bacterial isolates from the weak liquor

II. Weak liquor

(A) Before filtration			(B) After filtration			(C) Softener inlet			(D) Softener tank			(E) Softener outlet			(F) Header tank			(G) Before evaporation		
Code	**GA (cm)**	**Gm stain**	**Code**	**GA (cm)**	**Gm stain**	**Code**	**GA (cm)**	**Gm stain**	**Code**	**GA (cm)**	**Gm stain**	**Code**	**GA (cm)**	**Gm stain**	**Code**	**GA (cm)**	**Gm stain**	**Code**	**GA (cm)**	**Gm stain**
II A2 S2	1.6	(+)	II B1 S1	1.4	(+)	II C1 S1	1.6	(+)	II D1 S1	1.4	(+)	II E1 S1 1	1.4	(+)	II F1 S1	1.2	(+)	II G1 S1 1	1.2	(+)
II A2 S3	0.2	(-)	II B2 S1	1.4	(+)	II C2 S1	2.2	(+)	II D2 S1	2.4	(+)	II E1 S1 2	1.2	(+)	II F2 S1	1.6	(+)	II G1 S1 2	1.2	(+)
			II B1 S2	1.0	(+)	II C1 S2	1.8	(+)	**II D1 S2**	2.2	(+)	II E1 S1 3	1.3	(+)	II F1 S2	1.8	(+)	II G1 S1 3	1.3	(+)
			II B2 S2	0.8	(+)	II C2 S2	1.0	(+)	**II D2 S2**	1.2	(+)	II E1 S2	1.6	(+)	II F2 S2	1.6	(+)	II G2 S1	1.5	(+)
			II B1 S3	1.2	(+)	II C1 S3	1.0	(+)	II D1 S3 1	2.3	(+)	II E2 S2	1.6	(+)	II F1 S3	0.2	(+)	II G1 S2	1.2	(+)
			II B2 S3	0.2	(+)	**II C2 S3**	ND	(+)	II D1 S3 2	1.4	(+)	**II E1 S3 1**	0.5	(+)	II F2 S3	ND	(+)	II G2 S2	1.0	(+)
									II D2 S3	1.8	(+)	II E1 S3 2	1.2	(+)				II G1 S3 1	1.7	(+)
												II E2 S3	2.2	(+)				II G1 S3 2	2.5	(+)
																		II G2 S3	1.4	(+)

GA; Gelatinase activity in cm, Gm stain; Gram stain, (+); Gram-positive, (-); Gram-negative, Bold text; cocci, Non-Bold text; bacilli, ND; no activity detected

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Table (3c): Gelatinase activity of bacterial isolates from the strong liquor

III. Strong liquor								
(A) After evaporation			(B) Before sterilization			(C) After sterilization		
Code	GA (cm)	Gm stain	Code	GA (cm)	Gm stain	Code	GA (cm)	Gm stain
III A1 S1 1	3.0	(+)	**III B1 S1 1**	0.6	(+)	**III C1 S1**	1.2	(+)
III A1 S1 2	2.0	(+)	III B1 S1 2	1.6	(+)	**III C2 S1**	1.6	(+)
III A2 S1 1	0.6	(+)	III B2 S1 1	2.0	(+)	III C1 S2 1	1.0	(+)
III A2 S1 2	0.2	(-)	**III B2 S1 2**	ND	(-)	III C1 S2 2	1.9	(+)
III A1 S2 1	1.8	(+)	III B1 S2 1	0.8	(+)	III C2 S2 1	0.9	(+)
III A1 S2 2	2.1	(+)	III B1 S2 2	1.3	(+)	III C2 S2 2	2.2	(+)
III A2 S2 1	2.3	(+)	III B2 S2 1	2.1	(+)	III C1 S3 1	ND	(+)
III A2 S2 2	1.4	(+)	III B2 S2 2	1.0	(+)	III C1 S3 2	1.4	(+)
III A1 S3 1	0.4	(+)	III B1 S3 1	0.2	(+)	III C2 S3 1	0.8	(+)
III A1 S3 2	ND	(+)	III B1 S3 2	0.4	(+)	**III C2 S3 2**	1.1	(+)
III A1 S3 3	ND	(+)	III B2 S3	0.6	(+)			
III A2 S3 1	0.6	(+)						
III A2 S3 2	2.2	(+)						
III A2 S3 3	ND	(+)						
III A2 S3 4	ND	(+)						

Table (3d): Gelatinase activity of bacterial isolates from solid samples and swabs

IV. Solid samples and swabs								
(A) Swabs from drying rooms			(B) wet noodles			(C) Final dry product		
Code	GA (cm)	Gm stain	Code	GA (cm)	Gm stain	Code	GA (cm)	Gm stain
IV A2 S2	1.4	(+)	IV B1 S1 1	1.0	(+)	**IV C1 S1 1**	1.0	(+)
IV A1 S3	1.0	(+)	IV B1 S1 2	1.6	(+)	IV C1 S1 2	2.2	(+)
IV A2 S3 1	1.5	(+)	IV B2 S1 1	0.6	(+)	**IV C1 S1 3**	0.6	(+)
IV A2 S3 2	0.8	(+)	IV B2 S1 2	1.2	(+)	**IV C2 S1 1**	0.2	(+)
			IV B3 S1	0.4	(-)	IV C2 S1 2	2.2	(+)
			IV B4 S1	ND	(-)	**IV C2 S1 3**	1.6	(+)
			IV B1 S2 1	1.5	(+)	**IV C1 S2 1**	2.0	(-)
			IV B1 S2 2	1.8	(+)	IV C1 S2 2	2.2	(+)
			IV B2 S2	2.5	(+)	IV C2 S2 1	2.0	(+)
			IV B3 S2	0.7	(-)	IV C2 S2 2	1.2	(+)
			IV B4 S2	1.3	(+)	**IV C1 S3**	1.8	(+)
			IV B4 S2 1	1.5	(+)	IV C2 S3 1	2.4	(+)
			IV B1 S3 1	1.0	(+)	IV C2 S3 2	1.0	(+)
			IV B1 S3 2	0.9	(+)			
			IV B1 S3 3	1.0	(+)			
			IV B1 S3 4	1.6	(+)			
			IV B2 S3 1	0.7	(+)			
			IV B2 S3 2	2.0	(+)			
			IV B3 S3 1	1.1	(+)			
			IV B3 S3 2	1.0	(+)			
			IV B4 S3 1	1.4	(+)			
			IV B4 S3 2	1.1	(+)			
			IV B4 S3 3	0.7	(+)			

GA;Gelatinase activity in cm, Gm stain; Gram stain, (+); Gram-positive, (-); Gram-negative, Bold text; cocci, Non-Bold text; bacilli, ND; no activity detected

Estimation of growth and extracellular protease activity

The growth activity of the most potent 15 bacterial isolates revealed that the majority of the bacterial strains (66.6%) reached their maximum growth activity 8-9 days after the incubation. Whereas, 13.3% of the selected bacterial isolates reached the maximum growth activity after 6 days of incubation **(Table 4)**, which was in consistent with previous observations **[3].**

Growth and extracellular protease activity correlation suggests a relation between the bacterial growth and protease secretion **[3] [10]**.

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Antibiotic sensitivity

From results illustrated in **(Figure 1),** gatifloxacin was found to have the highest susceptibility rate (94.78%) against the selected bacterial isolates followed by ofloxacin (93.2%) that means great tendency of the fluoroquinolones to inhibit the bacterial growth of the isolates **(Figure 2)**, which was in consistent with previous studies **[24] [25]**. On the other hand, ampicillin from aminopenicillins class has the lowest susceptibility rate (3.01%) as it faced a strong resistance from the bacterial isolates which was substantially approved formerly **[26] [27] [28].**

Table (4): Extracellular protease activity (EU/ml) of the most active bacterial isolates

Code	Incubation days										
	1	2	3	4	5	6	7	8	9	10	11
I B2 S2	16.59±0.01	16.56±0.01	16.62±0.01	16.59±0.01	16.65±0.01	16.65±0.01	16.62±0.01	16.65±0.01	16.74±0.04	16.62±0.01	16.65±0.01
II D1 S2	12.5±0.01	12.68±0.01	12.74±0.01	12.81±0.01	12.87±0.01	12.84±0.01	12.77±0.01	12.77±0.01	12.74±0.01	12.71±0.01	12.71±0.01
III A2 S3 2	15.79±0.08	15.82±0.01	15.95±0.04	16.04±0.01	16.1±0.01	16.07±0.01	16.07±0.08	16.07±0.01	16.04±0.04	16.01±0.01	16.01±0.01
IV C2 S1 2	18.08±0.08	18.26±0.01	18.42±0.01	18.66±0.01	18.63±0.01	18.6±0.01	18.6±0.01	18.57±0.01	18.6±0.08	18.54±0.01	18.57±0.01

EU/ml±Standard deviation, each reading is the mean of triplicates.

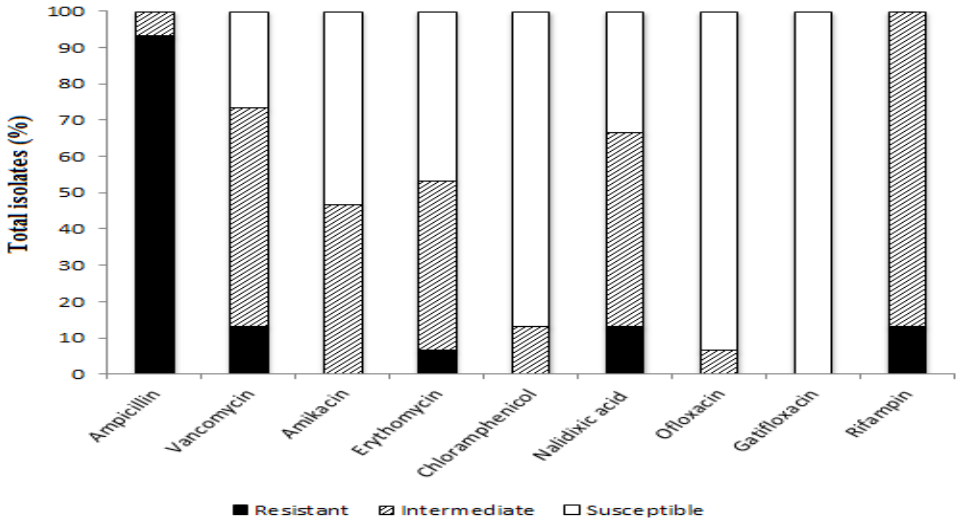

Figure (1): Antibiotic susceptibility percentage of the bacterial isolates

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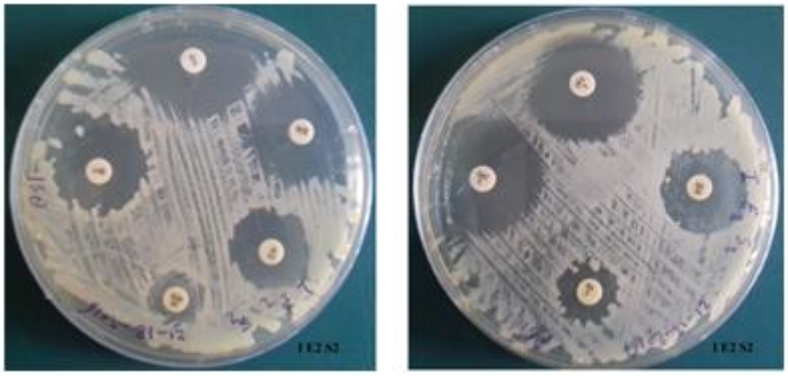

Figure (2): Antibacterial sensitivity of the I E2 S2 bacterial isolate against tested antibiotics after 24 hours incubation at 37°C. 1; erythomycin, 2; chloramphenicole, 3; amikacin, 4; vancomycin, 5; ampicillin, 6; gatifloxacin, 7; ofloxacin, 8;nalidixic acid, 9; rifampin.

Essential oils susceptibility

The secondary metabolites extracted from plant have wide potential as antimicrobial agents against many microorganisms **[29]**. Six essential oils showed antibacterial activity with varying degrees of activity **(Figures 3)**. Rest of the oils have no activity on the isolates, which might be a result of climatic factors of plant cultivation area and genetic factors of the plants and bacterial isolates as well **[30]**. Cinnamon oil was the most effective oil tested with the highest sensitivity rate (97.6%) and 2.14 cm mean inhibition zone diameter. Cinnamon oil showed antibacterial activity at low concentration (0.195µl/ml) which was lower than **[31]** who recorded MIC of cinnamon oil ranges between 0.391 µl/ml and 1.56 µl/ml. However, lower MIC ranges between 0.075µl/ml and 0.6µl/ml has been recorded for cinnamon oil **[32]**. In this context, 2.0 µl/ml of cinnamon oil was enough to inactivate tested microorganisms **[33]**. Difference of MIC may be explained by the genetic difference of plants, cultivation condition and the difference of the tested bacteria.

Clove oil and bergamot oil come next with (74.55%) and (67.03%) sensitivity rates respectively. Frankincense essential oil with sensitivity rate of (11.34%), only affects the growth of 5 isolates with 0.852 cm mean inhibition zone diameter. Limon grass oil was effective only on 2 isolates with 1.08 cm mean inhibition zone diameter and basil oil found to be effective only on one isolate with 1.4 mean inhibition zone diameter.

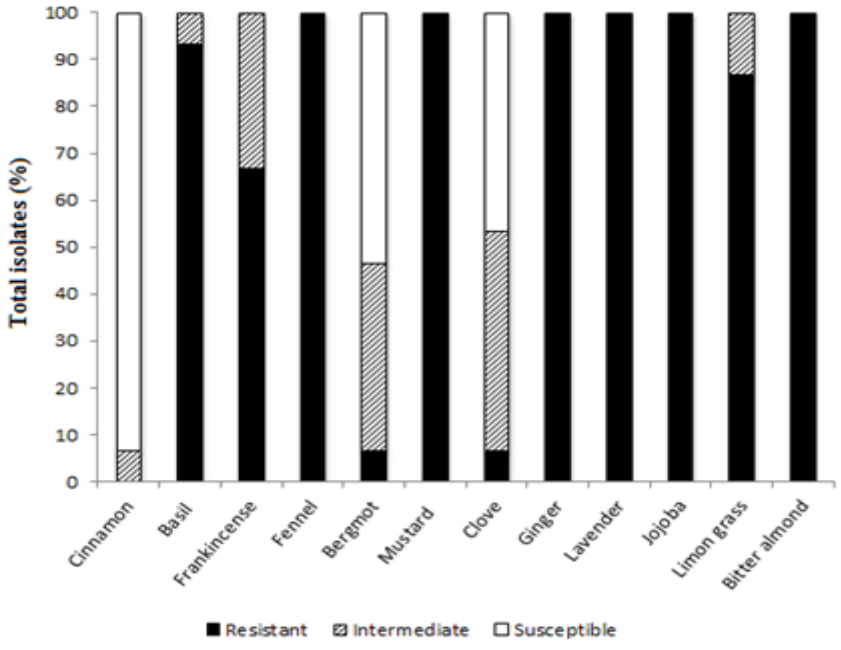

Figure (3): Antibacterial susceptibility percentage of the bacterial isolates against essential oils.

Chemical composition of the effective essential oils

GC/MS analysis revealed that cinnamaldehyde was the major constitute of cinnamon oil with 63.69% peak area **(Table 5)**. It is known that cinnamaldehyde interacts with the bacterial cell wall constituents by the rapid depletion of the cellular ATP of the energized cells **[34]**. This may cause disruption to the cell wall which leads to the death of the bacterial cells. Eugenol and isoeugenol were the major components of clove oil constituting 24.91% and 19.35% peak area respectively, these phenolic compounds exhibit broad antimicrobial activities. 2-(Hydroxymethyl)-5-methylphenol and 1-terpinenol were the major components of the bergamot oil constituting 14.41% and 10.46% peak area respectively, they both exhibit antibacterial activity by attacking lipids of the bacterial cell membrane **[35].** Frankincense essential oil contains cedrol, ledol, (-)-phyllocladene and cis-11-eicosenoic acid with 17.28%, 16.57%, 13.1% and 12.32% peak area respectively, these compounds are terpenes that were reported by **[6]** to have antibacterial effect on the bacterial growth. Limon grass oil contains limonene and longiverbenone as major components with 28.5% and 8.24% peak area respectively. Limonene has been used for the prevention of bacterial contamination and infection as it was found to have broad antibacterial effect on different bacterial strains **[36].** Basil was the least effective oil amongst the effective essential oils, depending on the GC/MS analysis, β-ocimene and anethole were the major constitutes of basil oil with 17.39% and 15.56% peak area respectively. β-ocimene has been reported in previous studies for having antibacterial and antifungal effects. Anethole has potent antimicrobial properties against wide variety of microorganisms-**[37][38].**

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Table (5): Common chemical components of the tested essential oils by GC-MS analysis.

No.	RT	RI	Compound	Chemical class	Area sum (%)					
					Cinnamon	Clove	Basil	Bergamot	Limon grass	Frankincense
1	6.84	1038	β-Ocimene	Monoterpenes	-	-	**17.39**	2.59	2.44	-
2	7.32	973.1	β-Pinene	Bicyclic monoterpene	-	-	2.44	2.45	-	-
3	7.39	1079	α-Terpinolen	Monoterpene alcohol	-	-	2.24	4.74	3.49	-
4	7.54	934	α-Pinene	Monoterpenes	3.12	-	0.65	2.54	3.39	-
5	7.8	1100	p-Mentha- 1,3,8-triene	Monoterpenes	-	-	0.67	2.19	1.25	-
6	7.88	ND	Eucalyptol	Monoterpene alcohol	-	-	4.32	-	-	-
7	8	1133	1-Terpinenol	Monoterpene alcohol	-	-	**12.14**	**10.46**	-	2.5
8	8.08	1023	Limonene	Cyclic terpene	-	-	-	-	**28.5**	-
9	8.27	997	2-Carene	Bicyclic monoterpen	-	-	1.16	1.13	5.31	-
10	8.7	1086	β-Linalool	Monoterpene alcohol	-	-	2.54	-	4.24	-
11	8.78	1332	4-Terpinenyl acetate	Hydrocarbon terpenes	-	-	-	3.34	-	0.57
12	8.88	1157	Isopregol	Phenol	-	-	0.41	3.65	1.12	-
13	9.6	1249	Anethole	Monoterpene alcohol	-	-	**15.56**	-	-	-
14	9.72	ND	Estragole	Monoterpene alcohol	-	-	**14.62**	-	-	-
15	9.75	1146	Isopulegol	Phenol	-	-	-	1.59	2.28	0.89
16	10.23	1480	β-Selinene	Hydrocarbon terpenes	-	-	-	4.82	0.83	-
17	10.28	1270	Bornyl acetate	Esters	-	-	-	**7.55**	-	-
18	10.7	1879	2-(Hydroxymethyl)-5-methylphenol	Phenol	-	-	-	**14.41**	-	-
19	11.42	1376	Methyleugenol	Monoterpene alcohol	-	-	4.92	0.17	-	-
20	11.9	1419	Caryophyllene	Hydrocarbon terpenes	-	-	2.24	0.48	0.36	-
21	11.98	1370	Ylangene	Hydrocarbon terpenes	-	-	1.29	0.69	0.41	-
22	12.18	1444	α-Himachalene	Hydrocarbon terpenes	-	-	0.84	0.16	0.42	-

Figures in bold indicate the major components of each oil, RT=retention time, RI=retention index, ND= not detected

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Continue table (5): Common chemical components of the tested essential oils by GC-MS analysis.

No.	RT	RI	Compound	Chemical class	Area sum (%)					
					Cinnamon	Clove	Basil	Bergamot	Limon grass	Frankincense
23	12.77	1500	β-Humulene	Hydrocarbon terpenes	-	-	2.5	0.94	0.38	-
24	13.13	ND	Phenol, 2-ethoxy-4-(2-propenyl)	Phenol	-	-	-	8.03	-	-
25	14.45	1022	1,8-Cineole	Monoterpene alcohol	8.75	-	-	-	-	-
26	15.63	1250	(-)-Phyllocladene	Hydrocarbon terpenes	-	-	-	-	-	**13.1**
27	15.82	1490	germacrene A	Hydrocarbon terpenes	-	-	-	-	-	6.14
28	16.1	1271	Thunbergol	Monoterpene alcohol	-	-	-	-	-	2.33
29	16.84	2105	Linoleic acid	Acids	-	-	1.75	0.22	2.69	8.97
30	16.86	ND	cis-11-Eicosenoic acid	Acids	-	-	-	-	4.53	**12.32**
31	17.06	2133	Oleic acid	Acids	-	-	1.38	0.2	-	2.79
32	17.18	1582	Ledol	Sesquiterpene	-	-	-	-	-	**16.57**
33	17.37	1597	Cedrol	Sesquiterpene	-	-	-	-	0.41	**17.28**
34	21.8	ND	3.4-2H-Coumarin, 4,4,5,6,8-pentamethyl-	Hydrocarbon terpenes	-	-	-	-	3.95	-
35	22.76	1404	Longiverbenone	Ketone	-	-	-	-	**8.24**	-
36	24.21	1178	Cinnamaldehyde	Hydrocarbon terpenes	**63.69**	-	-	-	-	-
37	26.61	1339	Isoeugenol	Monoterpene alcohol	-	**19.35**	-	-	-	-
38	28.12	ND	N-(o-Aminophenyl)-N-methyl-isobutyramide	Hydrocarbon terpenes	-	10.65	-	-	-	-
39	28.24	ND	Dimethyl aminopurine	Hydrocarbon terpenes	-	5.38	-	-	-	-
40	28.44	1339	Eugenol	Monoterpene alcohol	-	**24.91**	-	-	-	-
41	28.92	1419	Caryophyllene	Hydrocarbon terpenes	-	14.37	-	-	-	-
42	29.23	1335	Cinnamyl acetate	Hydrocarbon terpenes	9.93	-	-	-	-	-
43	32.18	ND	Acetic Acid 2- Methox-5-Prophenyl-Phenyl Ester	Esters	-	11.57	-	-	-	-

Figures in bold indicate the major components of each oil, RT=retention time, RI=retention index, ND= not detected

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Gelatinase enzyme purification

Purification of isolate (IV B2 S2) gelatinase enzyme was done first using ammonium sulphate fractionation (60%) followed by gel filtration chromatography on Sephadex G-150 column then DEAE-Cellulose column. 60% ammonium sulphate saturation was the most suitable concentration for the protein precipitation process based on the results of the gelatinase activity and SDS-PAGE (**Figures 4 and 5**) and (**Table 6)**, similar results reported previously **[39]**. SDS-PAGE results showed that the purified fraction of gelatinase enzyme from selected isolates was ~100 KDa molecular mass **(Figure 6).** Zymographic analysis of the selected isolates revealed the presence of gelatinases activity in gel **(Figure 7)**. Analysis of purified enzyme demonstrated a single band at ~100 KDa, this result suggest a success in purifying a single monomeric enzyme from a mixture of enzymes in the 60% dialyzed sample, also a homogeneity was confirmed as the clear colorless zone was at almost the same size as the single band from the SDS-PAGE. A 105 KDa enzyme was previously isolated and purified from *Bacillus cereus* strain that have gelatinolytic activity **[40]**.

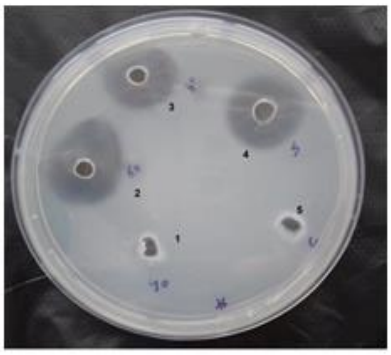

Figure (4): Activity of gelatinase fractions using 40%, 60% and 80% ammonium sulphate salt cut off technique 1; 40% saturation, 2; 60% saturation, 3; 80% saturation, 4; crude extract, 5; Control (no enzyme)**.**

Table (6): Activity of gelatinase fractions

Fraction no.	Salt concentration (%)	Diameter (cm)
Crude extract	0	1.9
1	40	0.2
2	60	1.8
3	80	1.4

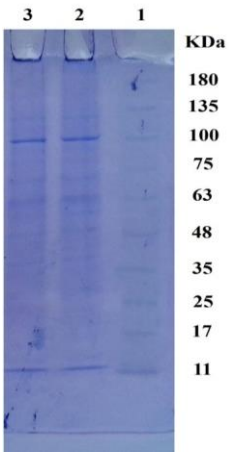

Figure (5): SDS-PAGE analysis of crude protein secreted by isolate (IV B2 S2) at 60% ammonium sulphate concentration. Lane 1: molecular weight marker indicated in KDa, Lane 2: protein with 60% ammonium sulphate, Lane 3: protein after dialysis

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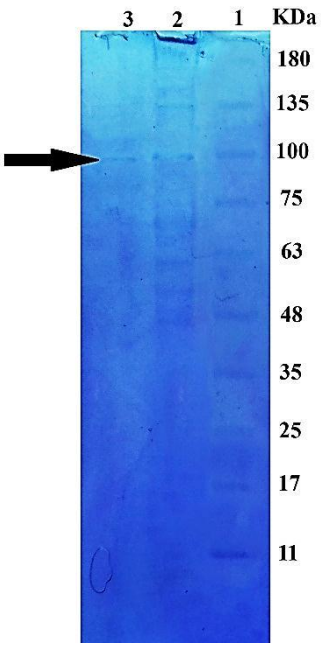

Figure (6): SDS-PAGE of purified enzyme fraction. Lane 1; protein marker (KDa), lane 2; enzyme solution after 60% ammonium sulphate fractionation, lane 3; purified enzyme**.**

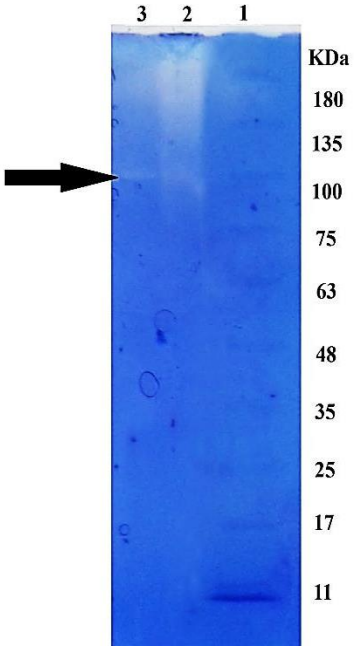

Figure (7): Zymography of purified enzyme fraction. Lane 1; protein marker (KDa), lane 2; enzyme solution after 60% ammonium sulphate fractionation, lane 3; purified enzyme.

Enzyme characterization

Characterization of enzyme activity showed that maximum enzyme activity recorded at 35°C and pH 6 while lowest activity was at 15°C and pH 5 **(Figures 8 and 9)**. In a previous study it was found that 35°C and pH 7.5 were optimum for maximum production of gelatinase **[12].** Another study recorded maximum enzyme activity at pH 7.5 and 50°C **[23]**. This difference may be due to the difference in processing stages and in the tested bacteria. The enzyme inhibitor, EDTA at different concentrations has a complete inhibitory effect upon the activity of the purified gelatinase.

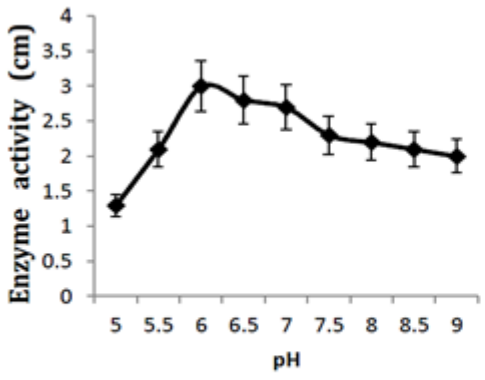

Figure (8): pH effect on the activity of gelatinase.

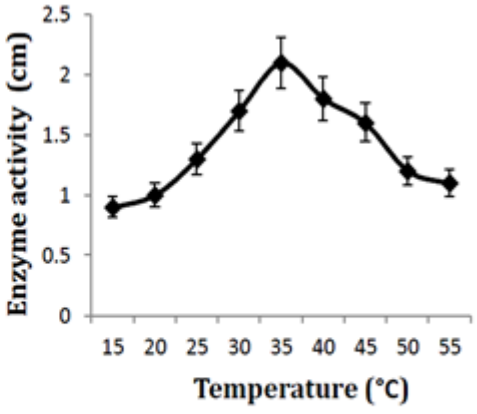

Figure (9): Temperature effect on the activity of gelatinase.

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CONCLUSION

It can be concluded that, *Bacillus* sp. were found to be dominant strains contaminating gelatin production process. Cinnamon oil had the highest sensitivity rate with low MIC and could be used as an alternative way to prevent industrial microbial contamination. In addition, the possibility of purifying gelatinase from bacterial contaminants for its numerous applications.

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