ISOLATION AND IDENTIFICATION OF ALOCALSTRAIN OF PROBIOTIC BACTERIAL ACTOBACILLUS PLANTARUM AND STUDIED THE TOLERANCE ABILITY FOR DIFFERENT LEVELS OF pH

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ABSTRACT

Lactobacillus plantarum IRQ12 was isolated from local pickle producing from *Cucumis sativus* submerged in 5% salt solution for one week far from sun light, selective media was used for isolation and activation process which was MRS broth and MRS agar, biochemical tests like catalase, nitrate reducing, productive gas, citrate consumption, produced ammonia from arginine, produced indole from tryptophan, fluid gelatin, ability of growth in 15, 45 °C, carbohydrates fermentation test, were carried out. DNA of bacteria was extracted by DNA extraction kit, identification by *16S rDNA* gene. the local strain tolerance ability was investigated for different levels of pH 4, 3, 2 and 1.5 for 0, 1, 2 and 3 hours, log cfu/ml at pH 4 after 3 hours of treatment was 7.83 and at pH 3 the log cfu/ml decrease to 6.8 after 3 hours of treatment. The log cfu/ml was decreased to 5.76 and 5.05 at pH 2 and 1.5 respectively after 3 hours of treatment.

INTRODUCTION

The word probiotic derivative from Greek language which mean “for life” and it’s used from the old ages throw notes the positive affect from fermented food of human health, in spite of they didnot know how these products are used (35). Probiotic are microorganisms found in food Supplements which affect as a useful for human throw the improvement of digestion properties via status of microbial equilibrium(17). Lactic acid bacteria may produce a different

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compound which inhibit the growth of factors which causes the diseases, included organic acid like lactic, citric, bacitracin and reuterin which also effected on acid degree, so it affected the factors which causes the diseases, it also maybe toxic for microbes, and it also will be useful for digestive disorders such as diarrhea, typhoid and dysentery and others (43).

It is a group of Gram positive bacteria, non-spore formal, cocci or bacilli produce lactic acid as final production from carbohydrate (27). Lactic acid bacteria included *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Bifidobacterium*. It is existing in all gastrointestinal tract, lactobacillus bacteria is distinguished intestinal flora and it has a very important role of decrease (the lactose intolerance, diarrhea, cholesterol rate),Immune response and cancer prevention (38).

Lactobacillus plantarum is non-pathogenic bacterium naturally existing in human saliva and gastrointestinal tract. As a member of the lactic acidbacteria, it is commonly used in food fermentation, Being used as a probiotic, its biotherapeutic applications have been increasingly recognized, *L. plantarum*is rod-shaped, It's genome is the largest among all lactic acid bacteria andhas been fully sequenced, *L. plantarum* is a facultative anaerobic (36).It has a high capability of tolerance for a low pH always less than 4,its ability of food metabolism makes it important for industrial application, used as a starter or pharmacy probiotic bacteria (31,34,45). It is isolated for human saliva and it exists in some plant, human and animal gastrointestinal(18), row camel milk (4), raw cow milk (20) and human faeces(9).

The aim of this study is to diagnosis of *L.plantarum* isolated from local pickles, identification by traditional and molecular methods as well as studies the tolerance of the low level of pH.

MATERIALS AND METHODS

Traditional methods: The local method used to produce the Iraqi pickle as a source to isolate the bacteria under study, fresh *Cucumis sativus* purchased from the local market, the vegetable washed well to remove the dust and dirt exposed to the atmospheric air for two hourswith flipping each 30 minutes, salt solution (5%) was made and sterilized by autoclave at 121° Cfor 15 minutes. The cucumber was cut into small pieces 2 cm, and put in plastic container which is previously sterilized by UV light with a wave length 254 nm for 15 minutes, the container filled with saline solution fully and stored in the lab far from sun light for a week, selective media

used in isolation and activation process like MRS agar, MRS broth which is suitable for *L. plantarum* growth (24,15,41). It was grown at37°C in anaerobic condition with using some compounds as sodium carbonate and sodium azide, L.Cysteine.HCl, sodium acetate with pH 5.4 (16,40,39,23).

Biochemical characterization of the isolates

Catalase, nitrate reductase, and gas production was tested according to (5), citrate consumption, production of ammonia from arginine and indole production from tryptophan was tested according to (23),fluidity of gelatin according to (6),Ability to grow at a temperature of (15) and (45)°C was tested according to (5) carbohydrate fermentations according to (13).

Method DNA extraction: The DNA extractedusing Automated Nucleic Acid Extraction systemAccording to the method of the company, theelectrophoresis gel prepared for the detection of DNA molecules(1).The bacterium was identified using 16S ribosomal DNA (*16S rDNA*) by the polymerase chain reaction (PCR) technology to amplify *16SrDNA* according (33).

Table (1) Sequence of forward and reverse primers of *16S rDNA* gene of bacteria under study

Primers		Sequence	Length	Optimizing TA^^
27	Forward	5'-AGAGTTTGATCCTGGCTCA-3'	16	51.8°C
1492	Reverse	5'-GGTTACCTTGTTACGACTT-3'	19	51.8°C

Table (2) Materials and its quantity for amplify *16S rDNA* gene (50 mμ) in PCR

No	Reagent	Volume
1	DNA template	10 µl (30 ng)
2	Forward primer	2 µl (20 pmol)
3	Reverse primer	2 µl (20 pmol)
4	Go Taq Green Master Mix. 2x	25 µl
5	Nuclease-free water	11 µl
Total volumes		50 µl

Table (3) PCR program conditions for *16S rRNA* gene amplification

Steps	Temperature	Time	No. of cycles
Initial denaturation	°C59	2min	1
Denaturation	°C59	30 Sec	30
Annealing	51.8°C	45 Sec	
Extension	72°C	1.5 min	
Final extension	72°C	5 min	1

The method of **(7)**was used for preparing the sample for the sequence of nucleotides bases andidentification by Blast program. For acidtolerance the method of (19)was used.

RESULTS AND DISCUSSION

The microscopic Identification tests showed that the bacteria is bacilli shape, long or short, single or double which is short chains, Immobile and non-spores formal Figure (1).

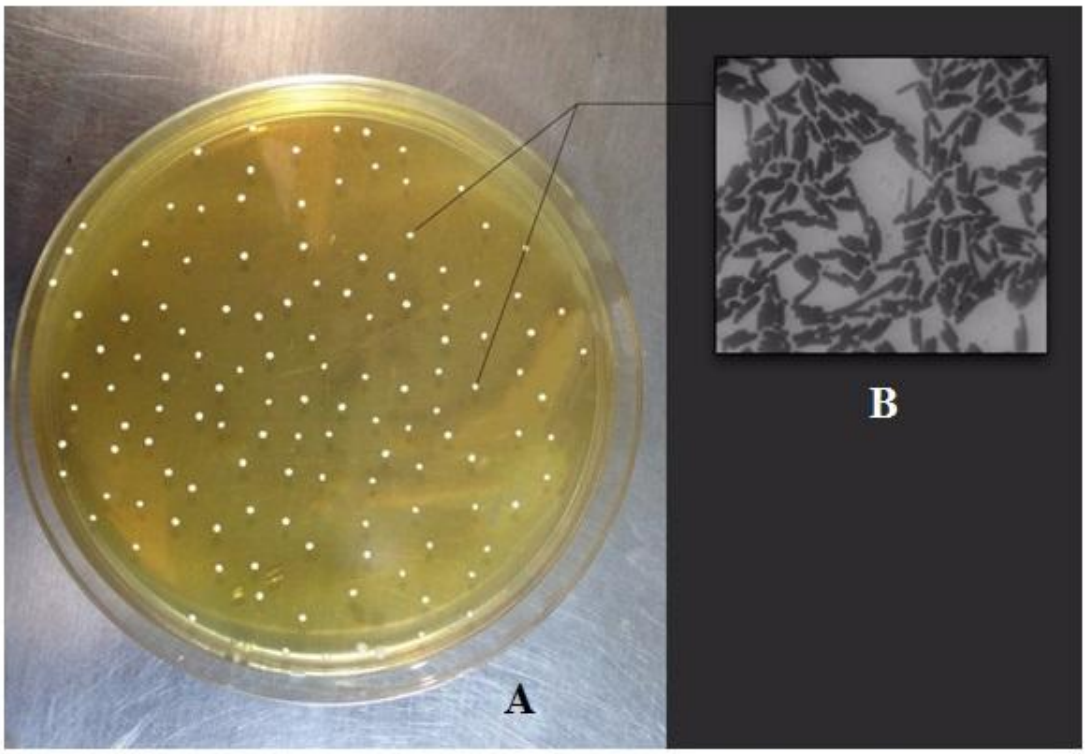

Figure (1) (A) shape of local bacterial isolates for *L.plantarum* on MRS Agar (B) gram stain under the microscope

Table (4) represents the biochemical tests for *L.plantarum*, the results indicate that bacteria is Gram positive, negative for catalase test because of its inability to produce peroxide whichanalyzes the hydrogen peroxide into oxygen and water (29).The test illustrated that the bacteria isNegative to reduce the nitrate because of the lack of Nitrate reductase enzyme which convert the nitrate to ammonia so the nitrate is unavailable to interact with sulfonic acid(2,16),the results showthat the bacteriaunable to consume the citrate, non-productive gas, does not produce ammonia from arginine, non-capable of producing indole from tryptophan, it's unable to liquefy gelatin because it could not able to produce the gelatinase, the results indicate the ability of bacteria to grow at 15°C and its non-ability to grow at 45°C because it is Mesophilic bacteria group(29)**,** This results agree with (25,44,2)

Table (4) Biochemical tests results for local strain *L.plantarum* IRQ12

L.plantarum IRQ12	Test	
+	Gram	1
–	Catalase	2
–	Nitrate reductase	3
–	Citrate consumption	4
–	Productive gas	5
–	Ammonia from arginine	6
–	Indole from tryptophan	7
–	Gelatin liquefy	8
+/–	Grow at 15/45°C	9

Table (5) illustrate carbohydrates fermentationtest in MRS broth for different kinds of sugar, the results indicate that the bacteria under study can ferment (glucose, sucrose, raffinose, Sorbitol, fructose, Salicine, Esculin, Ribose, glucose Monohydrate, Rhamnose, Melibiose, lactose, dextrose, Mannitol, Cellibiose, amichdolin, Trehalose), while it cannot ferment (Inositol, arabinose and Xylose).

Table (5) Carbohydrates fermentation tests for local strain *L.plantarum* IRQ12

L.plantarum IRQ12	Sugars	No.
+	Amygdaline	1
–	Arabinose	2
+	Cellibiose	3
+	Dextrose	4
+	Esculine	5
+	Fructose	6
+	Glucose	7
+	Gluconate	8
–	Inositol	9
+	Lactose	10
+	Mannitol	11
+	Melibiose	12
+	Raffinose	13
+	Rhmnose	14
+	Ribose	15
+	Salicine	16
+	Sorbitol	17
+	Sucrose	18
+	Trehalose	19
–	Xylose	20

This result is agree with (14,12,22,2).

Molecular Identification of *Lactobacillus plantarum* IRQ12 by 16S ribosomal DNA

Figure (2) show the DNA extracted from *L.plantarum* IRQ12strain, *16SrDNA* was amplified by PCR device Figure (3), gene bands appear at 1500 bp, compared with (1 KB) DNA standard Ladder.

Sequencing for *16S rDNA*gene and identification of *L.plantarum* IRQ12

Table (6) appears the sequences of the nucleotide bases for *L.plantarum* IRQ12 strain which is prepared from local pickle *Cucumis sativus* comparing with the standard global strains which is the similarity = 99%.

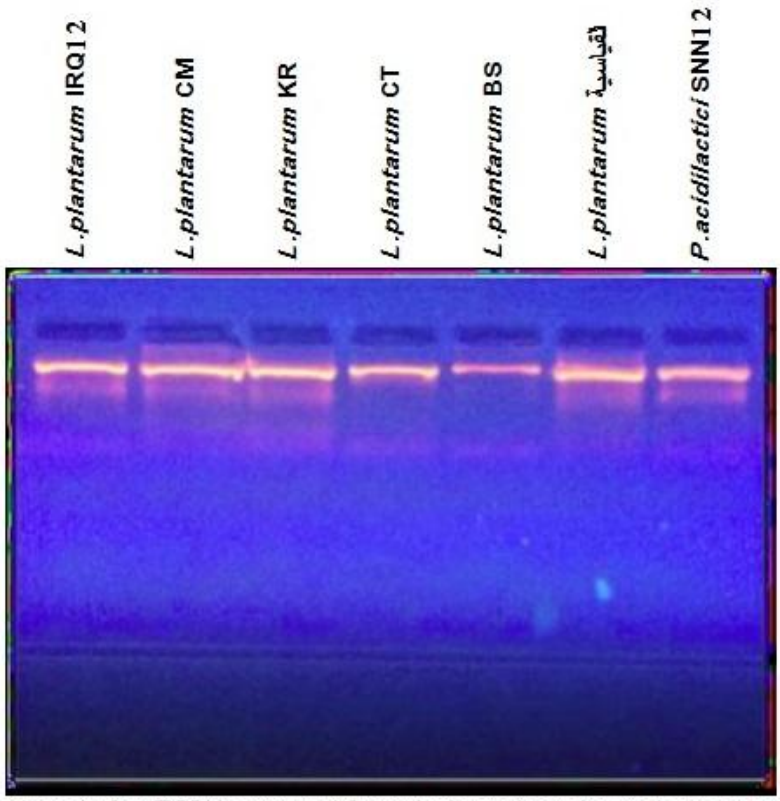

Figure (2) Electrophoresis for (DNA) extracted from the bacteria under study on agarose gel 0.8%, 70 volts for one hour

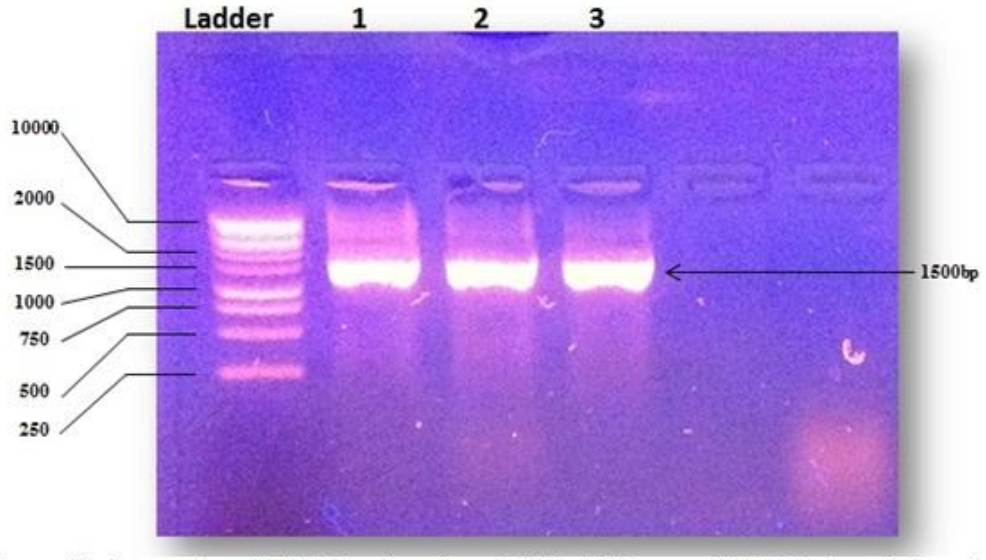

Figure (3) Conventional PCR for detection of *16Sr DNA* gene (1500 bp) Lan: 1, Lan: 2, Lan: 3 positive for *16Sr DNA* gene, compared with marker DNA ladder

Table (6) Sequences of *16S rDNA* gene, isolation source, similarity strain% for identified bacteria

Similarity %	DNA amplified	Source	Sequences	Strain	
99	1041	Cucumis sativus pickles	AAATGGCGGCAGACTATACATGCAGTCGAACGAACTCTGGTAT TGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAA CTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATA ACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCG CATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGAT GGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACC ATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACA TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGT AGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCC GCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAA AGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATT TAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA ATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC GAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTC AACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAA GAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATA TATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAA CTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGA TACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGG AGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTC	*Lactobacillus plantarum IRQ12*	1

Lactobacillus plantarum IRQ12 strain Tolerance for different pH values

The local strain was growninan acidic media four different pH 4, 3, 2 and 1.5 for different periods 0, 1, 2 and 3 hours, Figure (6) indicate the local strain(IRQ 12)tolerance for the acidic media, as the results illustrate it has high tolerance for the acidic conditions, the log cfu/mL is decrease to pH 4 from 10.02 at period 0 to 9.93 , 8.78 and 7.83 at the periods 1, 2, and 3hours respectively, the log cfu/mL was decreased from 9.91 at period 0 to 9.85, 7.88 and 6.8 at 1, 2, and 3hours respectively when the pH decreased to 3,when the pH decrease to 2 the log cfu/mL decreased from 9.81 at 0 time to 9.7, 7.7 and 5.76 at 1, 2, and 3hours respectively, decreasing appear clearly at pH 1.5 as the log cfu/mL decreased from 9.65 at 0 time to 8.91, 6.5 and 5.05 at 1, 2, and 3hours respectively. While the percentages of the log decreasing rates table (7) for pH4, 3, 2 and 1.5 for IRQ12 strain after three hours of treatment (21.85, 31.38, 41.28 and 47.66)% respectively.

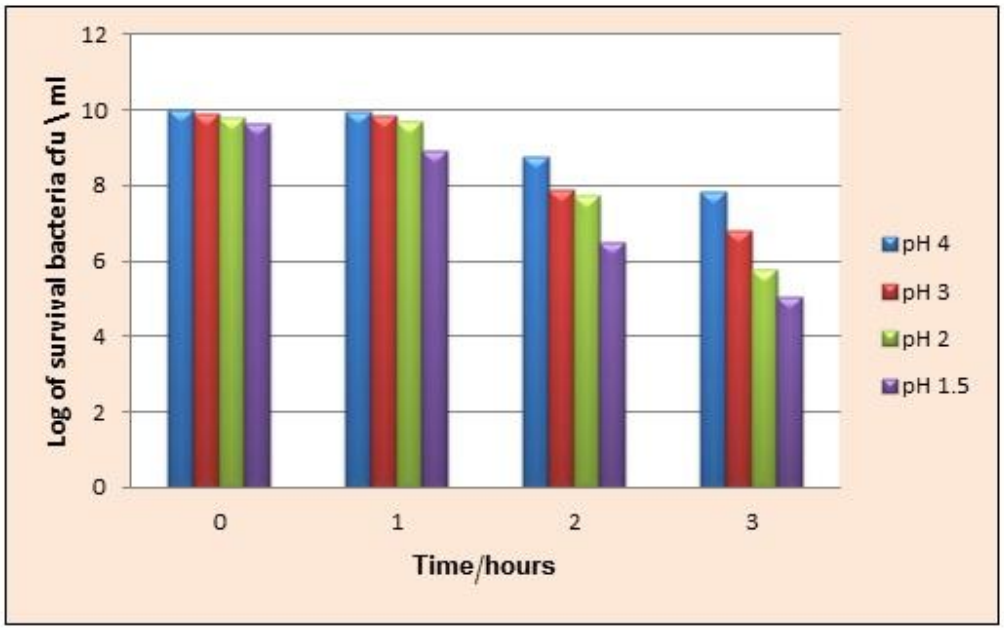

Figure (6) Tolerance of *L.plantarum* IRQ12 strain for different pH values

Table (7) The percentages of log of the survival bacteria / ml for *L.plantarum* IRQ12 strain when tolerance for different of pH values in different periods time

pH 1.5		pH 2		pH 3		pH 4		Time of treatment/hours
% of lowering of survival bacteria	Log of survival bacteria/ ml	% of lowering of survival bacteria	Log of survival bacteria/ ml	% of lowering of survival bacteria	Log of survival bacteria/ ml	% of lowering of survival bacteria	Log of survival bacteria/ ml	
0	9.65	0	9.81	0	9.91	0	10.02	0
7.66	8.91	1.12	9.7	0.60	9.85	0.89	9.93	1
32.64	6.5	21.50	7.7	20.48	7.88	12.37	8.78	2
47.66	5.05	41.28	5.76	31.38	6.8	21.85	7.83	3

As it was mentioned by (8 ,10,37,42),the lactic acid bacteria exposure to low values of pH2 and 1.5 for 3 hours is of the high stress – conditions the fact that these conditions are higher than the natural environment of stomach, the survival of the bacteria in stomach about 60-90 minute while the tolerance of bacteria for the conditions was an indicator for produce the fermented therapeutic products contain sufficient number of survival bacteria pass throw the stomach reaching to the minute and the large intense.Tolerance of pH it was indicate that the number of survival content was approximate exactly when treated for 3 hours in pH 2, 1.5 as the survival numbers was 95×10^3, 42×10^2 respectively (26).

Also this result was agree with (11) who studied two isolated of bacteria *L.plantarum* (DKL109, DKL119) at pH 2 for 1, 2 hours as the log cfu/ml for isolated DKL109 (5.96, 5.98) respectively, and (8.46, 8.26) respectively for the isolated DKL119.In addition(28)who studied the tolerance of 19 isolates of bacteria *L.plantarum* of pH 2.5, 2 for 0, 1, 2, 3 hours, all the isolates have a tolerance for acid condition and it is approximate for the results of (3) when he studied the tolerance of *L.plantarum* at pH 2, 3, 4 for 0, 1, 2, 3 hours which the log cfu/ml decrease from

10, 10.2, 10.01 to 5.38, 6.08, 6.31 respectively and there is no signal for intolerance of any isolates to acid conditions.

The tolerance of lactic acid bacteria for the low value of acidity may be due to the physiological state of the cytoplasm which is organize the rate of acidity between (pH outside) and (pH inside) the cell (32).(21) interpret that the (inside pH) when decreasing in some kinds of bacteria may lead to stop in the acidity of enzyme or could damage the proteins and nucleic acids, (30) explain that the variation of lactic acid bacteria for the harsh acidic condition due to (motive proton) which use as a power source in transfers throw cytoplasmic membrane.

عزل وتشخيص البكتريا العلاجية *Lactobacillus plantarum* IRQ12 من المخلل المحلي ودراسة قابلية تحملها لمستويات مختلفة من الرقم الهيدروجيني

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الخلاصة

عزلت بكتريا *L.plantarum* IRQ12 من المخلل المحلي لخيار القثاء والمغمور في محلول ملحي 5% لمدة أسبوع واحد بعيداً عن أشعة الشمس، استخدمت أوساط انتقائية لعملية العزل والتنشيط MRS الصلب و مرق MRS، أجريت الاختبارات الكيموحيوية كفحص الكاتليز والنترات وانتاج الغاز واستهلاك الستراتوانتاج الامونيا من الارجنين وانتاج الاندول من التربتوفان وسيولة الجيلاتين والقدرة على النمو في 15 و 45 °م، كما أجري اختبار تخمير الكربوهيدرات لمجموعة مختلفة من السكريات. أستخلص الحامض النووي للبكتريا باستخدام نظام آلي وتم التشخيص التأكيدي الجيني بوساطة جين *16S rDNA* وتم اجراء اختبار مدى مقاومة السلالة المحلية لمستويات مختلفة من الأس الهيدروجيني ولفترات مختلفة من المعاملة إذ بلغ لوغاريتم الأعداد الحية / مل عند 4 pH 7.83 بعد مرور 3 ساعات من المعاملة، أما عند 3 pH فقد كان لوغاريتم الأعداد الحية / مل 6.8 بعد 3 ساعات من المعاملة لتنخفض الأعداد الحية الى 5.76 و 5.05 عند 2 pH و 1.5 على التوالي بعد 3 ساعات من المعاملة.

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