Iraqi Journal of Market Research and Consumer Protection
(2024) 16(2): 172-187
DOI: http://dx.doi.org/10.28936/jmracpc16.2.2024.(15)
Al-Hashemi & Al-Janabi

المجلة العراقية لبحوث السوق
وحماية المستهلك

BIOCHEMICAL AND GENETIC IDENTIFICATION OF TWO LOCAL DIACETYL PRODUCER BACTERIAL ISOLATES

Abrar N. Al-Hashemi[1], Nidhal M. S. Al-Janabi[2]*
[1]Department of Food Science, College of Agricultural Engineering Sciences, University of Baghdad, Baghdad, Iraq. E-mail: abrar.abd2102m@coagri.uobaghdad.edu.iq
[2]Department of Food Science, College of Agricultural Engineering Sciences, University of Baghdad, Baghdad, Iraq, E-mail: nidhal.m@coagri.uobaghdad.edu.iq

Received 14/ 6/ 2023, Accepted 23/ 8/ 2023, Published 31/ 12/ 2024

This work is licensed under a CCBY 4.0 https://creativecommons.org/licenses/by/4.0

ABSTRACT

Four local bacterial isolates (Q3, K7, K2 and K4) were selected that were isolated from Qargoli cheese with the ability to produce diacetyl , all isolates showed a negative result in the catalase test, they fermented glucose when grown at 20 and 37 °C but all of them were unable to grow at 45 °C , the isolates K2, K4 and K7 in MRS broth and Q3 in M17 broth tolerated 0.5% and 2% NaCl excepted K7 , isolates K2 and K4 were able to grow at 5% NaCl ,the tested isolates were not tolerated 10% NaCl , isolates have acidic activity in skim milk and all of them gave a white clot that ranged from soft to very soft, isolate K4 ranked first in diacetyl production 59.59, 68.36 and 179.09 µg/ml at of 0.05, 0.1 and 0.15% sodium citrate concentration. Genetic examination proved that isolate Q3 is *Lactococcus lactis strain HANM Diacetyl* and K7 is *Pediococcus pentosaceus strain Bro DiAc*.

Keywords: Diacetyl , Genetic examination, PCR , biochemical test , *Lactococcus lactis* , *pediococcu pentosaceus*.

الفحوصات الكيموحيوية والتشخيص الجيني لعزلتين بكتيريتين محليتين منتجتين لثنائي الاسيتيل

ابرار نافع عبدالاله الهاشمي [1]، نضال محمد صالح الجنابي [2]
[1] قسم علوم الاغذية ، كلية علوم الهندسة الزراعية ، جامعة بغداد ،بغداد، العراق abrar.abd2102m@coagri.uobaghdad.edu.iq
[2] الاستاذ الدكتور، قسم علوم الاغذية، كلية علوم الهندسة الزراعية ، جامعة بغداد،بغداد، العراق، nidhal.m@coagri.uobaghdad.edu.iq

الخلاصة

اختيرت أربع عزلات بكتيرية محلية (Q3, K7 , K2 و K4) تم عزلها من جبن القرغولي تميزت بقدرتها على إنتاج ثنائي الأسيتيل ، أظهرت جميع العزلات نتيجة سلبية في اختبار الكاتليز ، و استطاعت العزلات تخمير الجلوكوز عند النمو بدرجة 20 و 37 م° وغير قادرة على النمو في 45 م ، تحملت العزلات K2 و K4 و K7 في الوسط MRS السائل و Q3 في الوسط M17 السائل التركيز 0.5٪ و 2٪ كلوريد الصوديوم فيما عدا K7 ، وكانت العزلات K2 و K4 قادرة على النمو عند 5٪ كلوريد الصوديوم. وجميعها لم تتحمل 10٪ كلوريد الصوديوم ، وكان للعزلات نشاط حامضي في الحليب الفرز وجميعها أعطت خثرة ناعمة بيضاء تراوحت من طرية إلى شديدة الطراوة ، واحتلت العزلة K4 المرتبة الأولى في إنتاج ثنائي الأسيتيل 59.59 و 68.36 و 179.09 ميكروغرام / مل عند تركيز 0.05 و 0.1 و 0.15٪ سترات الصوديوم . أثبت الفحص الجيني أن العزلة Q3 هي *Lactococcus lactis strain HANM Diacetyl* والعزلة K7 *Pediococcus pentosaceus strain Bro DiAc*.

الكلمات المفتاحية: ثنائي الاسيتيل ،التشخيص الجيني ، PCR ، الاختبارات الكيموحيوية ، *Lactococcus lactis , pediococcu pentosaceus*.

*The article is taken from a master's thesis by the first researcher.

INTRODUCTION

LAB metabolism creates other end-products beside lactic acid with organoleptic properties like improved flavor texture, and/or extended shelf-life (**Kleerebezemab *et al.*, 2000**). Several LAB under different physiological conditions are able to produce acetate, acetaldehyde, acetoin, diacetyl and 2,3-butanediol (**Hugenholtz & Starrenburg, 1992; Bintsis, 2018**). The LAB distribute products are relevant in fermentations. In minutely, diacetyl and acetaldehyde are required flavor compounds in dairy products. Diacetyl is an aromatic compound known for its buttery aroma and taste. It is found in several dairy products, fundamentally butter, margarine, sour cream and some cheeses (**Rincon-Delgadillo *et al.*, 2012; Clark & Winter, 2015**), this compound is synthesized originally by leuconostoc and some lactococci that can metabolize citrate, once a subject of controversy, the biosynthetic pathway of diacetyl is now well known, citrate is transported inside cells by a citrate permease (**Harvey, 1962**), and is then cleaved to acetate and oxaloacetate by citrate lyase. Oxaloacetate is decarboxylated by oxaloacetate decarboxylase, (**Suhad, 2001**) yielding pyruvate.a-Acetolactate synthase transforms pyruvate to acetaldehyde-thiamine pyrophosphate and condenses it with a second molecule of pyruvate to form α-acetolactate. Diacetyl originates from the chemical oxidative decarboxylation of a-acetolactate (**Hugenholtz & Starrenburg, 1992; Verhue, 1991**) and acetoin originates from the decarboxylation of α-acetolactate by a-acetolactate decarboxylase, by reduction of diacetyl by diacetyl reductase, or by nonoxidative chemical decarboxylation (**Hugenholtz, 1993**) ever after lactic acid bacteria produce relatively small quantities of diacetyl (generally less than 5 mg/liter), considerable work has been devoted to increasing its production. Factors such as pH, temperature (**Bassit *et al.,* 1995; Pack, *et al.,* 1967**) citrate concentration (**Petit *et al.,* 1989; Libudzisz &Galewska, 1991**) and oxygen (**Bassit *et al.,* 1993**) have been studied for their effects on diacetyl production. Metabolic engineering strategies have also been proposed to obtain strains producing larger quantities of diacetyl. So It has an antibacterial effect (**Al-Khafaji *et al.,* 2005; Al-Azawi & Al-Khafaji, 1988**).

Besides its natural appearance in dairy products, diacetyl has a high commercial value and it is manufactured for use as a food additive. Starter distillates are also relevant in the formulation of many food products such as cottage cheese and sour cream. The amount of diacetyl in SD ranges from 1.2 to 22,000 µg/g (0.00001–0.22 M). (**Hernandez-Valdes, *et al.*, 2020**)

MATERIALS AND METHODS

Biochemical tests:

The four highly productive isolates of the flavoring compound diacetyl(Q3, K7, K2 and K4) were selected based on the qualitative detection and quantitative estimation of it in a previous study of ours, with the four (Q3,K7,K2 and K4) isolates varying in their productivity and inferred by the culture characteristics of the colonies on the culture media (MRS and M17 at 37 ºC/ 48 hr) anaerobic conditions and the microscopic characteristics, biochemical test was conducted by using inoculum(1.5 x 10^8 cfu/ml MacFarland method) (Jawan, *et al.*, 2020) that included:

Catalase test:

Add a few drops of hydrogen peroxide (H_2O_2 3%) to the separated colony growing on the (MRS, M17) solid culture medium, the test is considered positive when bubbles are formed. According to (**Ramalingam& Karara, 2011**).

Glucose fermentation test:

Test medium was used (N.B, 1% glucose and 0.004% bromo cresol purple), then inoculated with the bacterial isolate (1.5 x 10^8) cfu/ml at (37 ºC/ 24 hr /anaerobic conditions). The change in color of the culture medium from violet to yellow in the medium is evidence of a positive test (**Jawan *et al.*, 2020; Ahmaed, 2014**).

Growth test at different temperatures:

The three isolates (K7,K2 and K4) were inoculated on tubes of MRS broth and Q3 isolate on M17 broth incubated at (20, 37, and 45) ºC for 24-48 hr , the appearance of growth with the change of color from violet to yellow and compared with the control tube without inoculum the medium is evidence of a positive test.

Test of growth in different concentrations of NaCl:

The tubes containing MRS and M17 broth were inoculated with 3 isolates and 1 isolate (1.5 x 10^8) cfu/ml respectively using different concentrations of NaCl (0.5, 2.0, 5.0, and 10%) w/v in the presence of bromo cresol purple (0.004%), the appearance of turbidity and a change in the color of the indicator from violet of yellow compared to the control tube (without inoculum) is the positive result (Jawan *et al.*, 2020).

Acidity activity:

Tubes containing skim milk 10% (w/v) were inoculated with (1.5 x 10^8) cfu/ml of culture. It was incubated at 37 C^O, with a change in the incubation period for each isolate (24, 48 and 72) hr. Acidity is tested by changing the pH using a pH-meter, clot, change in color, flavor, and aroma.

Standard curve of diacetyl

By adding different volumes of a standard diacetyl compound in test tubes, and completing the volume to 10 ml by distilled water, the absorbance of all tubes was measured using a spectrophotometer at 540 nm(table 1). Only Blank's solution of distilled water was used to zero the device at the same wavelength.

Table (1): Absorbance readings and corresponding concentrations for the diacetyl compound

No. tube	Conc. of standard diacetyl (µg/mL)	Absorbance (540 nm)
1	2.5	0.003
2	5	0.012
3	10	0.030
4	20	0.150
5	50	0.370
6	100	0.804
7	150	1.198
8	200	1.602

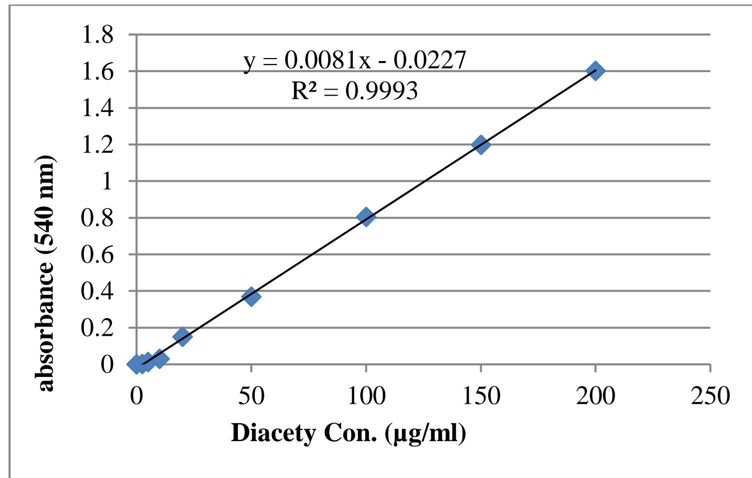

Figure (1): The standard curve of diacetyl.

THE ABILITY TO CONSUME CITRATE:

Addition of citrate at different concentrations to skim milk:

Different concentrations of sodium citrate (0.05%, 0.1%, 0.15%, 0.2% and 0.25%) were added to 100 ml of sterilized skim milk in test tubes. Each tube was inoculated with (1.5 x 10^8 cfu/ml) of the isolate. It was incubated at 37C°/24-48 hr , and its ability to consume citrate was tested. Then centrifugation was conducted and the absorbance was read at 540 nm (**Asmaa *et al.,* 2016**).

Addition of citrate at different concentrations to the broth medium:

Different concentrations of Sodium Citrate (0.05%, 0.1%, 0.15%, 0.2%, 0.25%) were added to 100 ml of the two broth media (M17 and MRS) according to the medium in which the isolate was grown, each tube was inoculated with (1.5 x 10^8) cfu/ml of culture was incubated at 37C°/24-48 hr . Then centrifugation was conducted and the absorbance was read at 540 nm.

Genetic identification:

The genetic identification of the two isolates Q3 and k7, characterized by higher productivity of diacetyl and distinct appearance in the biochemical test was carried out as follow:

DNA extraction:

Genomic DNA was isolated from bacterial growth according to the protocol of ABIOpure Extraction as the following steps:

- For pellet cells, 1ml of overnight culture for 2min at 13000 rpm. The supernatant was then discarded.
- For protein digestion and cell lysis, 20µl of Proteinase K solution (20 mg/ml) was added to 200µl of Buffer BL and cell pellet, then the tube was mixed vigorously using vortex and incubated at 56˚C for 30 min.

- From absolute ethanol 200µl was added to the sample, pulse-vortex to mix the sample thoroughly.
- All of the mixtures were transferred to the mini- column carefully, then centrifuged for 1 min at 6,000 x g above (>8,000 rpm), and the collection tube was replaced with a new one.
- From Buffer BW 600µl was Added to the mini- column, then centrifuged for 1 min at 6,000 x g above (>8,000 rpm) and the collection tube was replaced with a new one.
- From Buffer TW 700µl was applied. Centrifuged for 1 min at 6,000 x g above (>8,000 rpm). The pass-through was discarded and the mini- column was reinserted back into the collection tube.
- The mini-column was Centrifuged at full speed (>13,000 x g) for 1 min to remove residual wash buffer, then the mini-column was placed into a fresh 1.5 ml tube.
- From Buffer AE 100µl was added and incubated for 1 min at room temperature, then centrifuged at 5,000 rpm for 5min.

Quantitation of DNA:

Quantus Fluorometer was used to detect the concentration of extracted DNA to detect the goodness of samples for downstream applications. For 1 µl of DNA, 200µl of diluted Quantifluor Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected for both isolates Q3 and K7. The equation below was adopted to determine the purity:

DNA purity = (photo absorption at 260 nm) / (photo absorption at 280 nm)

Primer preparation:

Table (2): Preparation of primer.

Primer Name	Vol. of nuclease free water (µl)	Concentration(pmol/µl)
27F	300	100
1492R	300	100

These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 ºC) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

Reaction setup and thermal cycling protocol:

Table (3): PCR Component Calculation.

No. of Reaction	2	Rxn	Annealing temperature of primers	60
Reaction Volume /run	25	µl	No. of PCR Cycles	30

Amplification 16S RRNA:

Polymerase chain reaction (PCR) technology was used to amplify the *16S rRNA* gene of bacteria in order to confirm the type of the selected isolate using the following universal primer (4) according to what was stated in (**Sacchi *et al*., 2002**).

Table (4): Primers used to amplify the *16S rRNA* gene by PCR.

Primer Name	Sequence	Annealing Temp. (°C)	Product Size (bp)
27F	5`-AGAGTTTGATCCTGGCTCAG-3`	60	1500 bp
1492R	5`-TACGGTTACCTTGTTACGACTT-3`		

The amplification was 25 µl, and the Master Mix prepared by the American company Promega was added according to table (5) to the Eppendorf tube as follows:

Table (5): Materials added and their quantities to the reaction tube to amplify the 16SrRNA gene in PCR technology.

Master mix Components	Stock	Unit	Final	Unit	Volume 1 Sample
Master Mix	2	X	1	X	12.5
Forward primer	10	µM	0.5	µM	1
Reverse primer	10	µM	0.5	µM	1
Nuclease Free Water					8.5
DNA		ng/µl		ng/µl	2
Total volume					25
Aliquot per single rxn	23µl of Master mix per tube and add 2µl of Template				

The tube was transferred to a PCR device (thermal cycler) and the program shown in Table (6) was used for the purpose of amplifying the gene (*16S rRNA*) according to what was stated in (**Shripama *et al.*, 2013**) with some minor modifications.

Table (6): Phases of the PCR process After the end of the time allotted for the reaction, 5 µl of the amplification products of the *16S rRNA* gene were withdrawn to be used for electrophoresis.

Steps	Temperature	m: s	Cycles
Initial Denaturation	95°C	05:00	1
Denaturation	95°C	00:30	30
Annealing	60°C	00:30	
Extension	72°C	01:00	
Final extension	72°C	07:00	1
Hold	10°C	10:00	

Agarose gel electrophoresis:

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria. Solutions :1 X TAE buffer, DNA ladder marker, Ethidium bromide (10mg / ml)).

Preparation of agarose:

100 ml of 1X TAE was taken in a flask. 1.5 gm (for 1.5%) agarose was added to the buffer. The solution was heated to boiling (using Microwave) until all the gel particles were dissolved. 1µl of Ethidium Bromide (10mg/ml) was added to the agarose. The agarose was stirred in order to get mixed and to avoid bubbles. The solution was left to cool down at 50-60°C.

Casting of the horizontal agarose gel:

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was left to solidify at room temperature for 30 minutes. The comb was carefully removed, and the gel was placed in the gel tray. The tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel, as stated in (**Michael & Sambrook, 2012**).

DNA loading:

PCR products were loaded directly. For the PCR product, 5µl was directly loaded into the well. Electrical power was turned on at 100v/mAmp for 60min. DNA moves from Cathode to plus Anode poles. The Ethidium bromide-stained bands in gel were visualized using Gel imaging system.

Standard sequencing:

PCR products were sent for Sanger sequencing using ABI3730XL, an automated DNA sequencer, by Macrogen Corporation – Korea. in order to determine the sequences of nitrogenous bases, and these sequences were adopted in comparison with the available information about the amplified gene in the NCBI Gen Bank (National Center for

Biotechnology Information) and in light of the website according to the BLAST Nucleotide program for the purpose of identifying the type of the selected isolate.

RESULTS AND DISCUSSION

Biochemical tests: catalase test:

The test results (Table 7) showed that the catalase-negative isolates did not form bubbles. Therefore, we infer the absence of the catalase enzyme in these isolates because are unable to decompose H_2O_2 to water and oxygen as figure (2), agree with **(Khalil, 2020)**.

Table (7): Catalase test.

Isolates	Result
Q3	-
K7	-
K2	-
K4	-

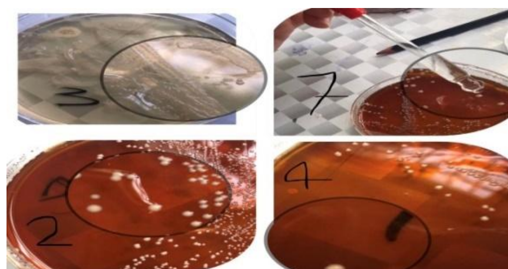

Figure (2): Add H_2O_2 (3%) to the bacterial isolate's colonies on MRS and M17 medium.

Glucose fermentation test:

The results of table (8) shows the ability of four bacterial isolates (Q3,K7,K2 and K4) glucose fermentation by changing the color of the culture medium in the presence of an indicator that gives a yellow color at the acidic pH , so the color of control did not change due to the absence of inoculum, this agree with (**Al-dosari, 2002**).

Table (8): Glucose fermentation test result

Isolates	Result
Q3	+
K7	+
K2	+
K4	+
Control	-

Growth test at different temperatures:

The results of table (9) show the ability growth of four bacterial isolates (Q3,K7,K2 and K4) at 20,37 and 45 ºC with some difference between them (20 ºC), it was found that the isolates have the ability to grow at t 20 ºC and 37 ºC , and they could not grow at 45 ºC , the best result we got at 37 ºC .

Table (9): Growth of isolates at different temperatures.

Isolates	20 ºC	37 ºC	45 ºCº
Q3	+	+	-
K7	+-	+	-
K2	+	+	-
K4	+	+	-
Control	–	–	-

(+-) poor growth , (+) growth ,(-) no growth , Control (C) without inoculum

Test of growth in different concentrations of Nacl:

That all isolates cannot grow in concentration 10% NaCl , but they can grow in 0.5% NaCl while isolate K7 grew only in 0.5%NaCl , isolates K7 and K4 were able to grow in 5% NaCl.

Table (10): The ability of bacteria to grow and tolerate different concentrations of NaCl.

Isolates	0.5% NaCl	2% NaCl	5% NaCl	10% NaCl
Q3	+	+	-	-
K7	+	-	-	-
K4	+	+	+	-
K2	+	+	+	-

Acidity activity:

The color of the clot formed (white) by the acidity caused by bacteria did not change through 24,48 and 72 hr at the same temperature (37 ºC). The control treatment was the highest in the pH during the three time periods (24, 48 and 72) hr (6.88, 6.75 and 6.60) respectively, noting that pH of all treatments decreased with the progression of the time period. As for the curd it was soft and tender, except clot of isolate K2 which was distinguished by its stuffiness at 37 ºC /72hr, with acidic flavor also clot of isolate K4 it was an acidic.

Table (11): The ability of the isolates to produce acid on the skim milk medium.

	24 hr/37 Cº				48 hr/37 Cº				72 hr/37 Cº			
Iso.	color	pH	Clot	Flavor	color	pH	Clot	Flavor	color	pH	Clot	Flavor
Q3	White	4.97	Soft	Mild acidity	White	4.64	Soft	Mild acidity	White	4.16	Soft	Acidic
K7	White	6.35	-	-	White	6.00	-	-	White	5.61	Soft	Mild acidity
K2	White	5.40	Very soft	Very mild acidity	White	4.92	Soft	Mild acidity	White	4.35	Solid clot	Acidic
K4	White	5.29	Very soft	Very mild acidity	White	4.57	Soft	Mild acidity	White	4.09	Soft	Acidic
Control	White	6.88	-	-	white	6.75	-	-	White	6.60	-	-

(-): no clot and not change flavor.

The ability to consume citrate:

Addition of citrate at different concentrations to skim milk:

The isolate K4 ranked first among the tested isolates in the production of diacetyl at first and third concentrations reached 59.59, 179.09 µg/ml, with superiority of isolate K2 at conc. 0.1% and conc. 0.2 % 98.48 and 184.03 µg/ml while the productivity of the four isolates decreased at the fifth conc. 0.25%.

Table (12): The ability of isolates to consume citrate added to skim milk.

	0.05%		0.1%		0.15%		0.2 %		0.25%	
Iso.	540 nm	Conc. DA. µg/ml	540 nm	Conc. DA. µg/ml	540 nm	Conc. DA. µg/ml	540 nm	Conc. DA. µg/ml	540 nm	Conc. DA. µg/ml
Q3	0.245	33.05	0.145	20.70	0.165	23.17	0.311	41.20	0.126	18.36
K7	0.212	28.98	-	-	0.956	120.83	1.302	163.54	-	-
K2	0.279	37.25	0.775	98.48	0.317	41.94	1.468	184.03	0.403	52.56
K4	0.460	59.59	0.531	68.36	1.428	179.09	1.011	127.62	0.323	42.68

(DA.): Diacetyl ,(-) : can't produce diacetyl

Addition of citrate at different concentrations to the broth medium:

It is clear from the results of table (13) that the addition of certain concentrations of sodium citrate to different media, it was found that the ability of bacteria to consume citrate is better when the culture medium is skim milk.

Table (13): The ability of isolates to consume citrate added to (MRS and M17) medium at different concentrations.

		0.05%	0.1%		0.15%	
Isolates	Abs. 540nm	Conc. DA. µg/ml	Abs. 540nm	Conc. DA. µg/ml	Abso. 540nm	Conc. DA. µg/ml.
Q3	0.151	21.44	0.216	29.47	0.157	22.19
K7	0.163	22.93	-	-	-	-
K2	-	-	-	-	-	-
K4	0.156	22.06	-	-	-	-

	0.2%		0.25%	
Isolates	Abs. 540 nm	Conc. DA. µg/ml.	Abs. 540 nm	Conc. DA. µg/ml.
Q3	0.387	50.58	0.336	44.28
K7	0.237	32.06	-	-
K2	0.150	21.32	-	-
K4	0.206	28.23	-	-

(DA.): Diacetyl ,(-) : can't produce diacetyl ,(Q3): on M17 broth,(K7,K2 and K4): on MRS broth.

Genetic identification:

The results obtained for the two isolates after electrophoresis of the amplified gene showed that one bundle representing the *16S rRNA* gene appeared in both isolates (Q3, K7) Figure (3), this indicates the success of the binding process between the primers and the target gene, which is the *16S rRNA* gene, without being bound to parts. The rest of the DNA extracted from the isolates to be diagnosed. The molecular size of the amplification products was estimated, and the gene size was 1500 base pairs from both isolates compared to the size of the

guide (ladder) used in electrophoresis along with the test samples. (**Jebur & Auda 2020; Abbas *et al.*, 2020**) also used the genetic diagnosis of a local isolate.

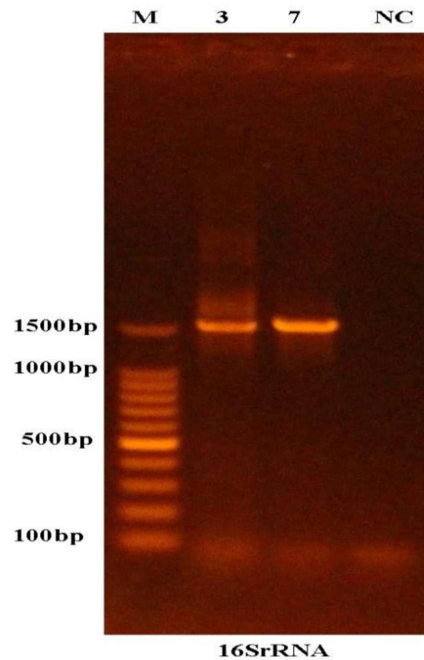

Figure (3): Results of the amplification of *16s RNA* gene of Unknown bacterial species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 3-7 resemble 1500bp PCR products.

The *16S rRNA* gene gives crucial results in diagnosis and is used to distinguish between different types of bacteria (**Naser *et al.*, 2013; Balcázar *et al.*, 2007**).

Analysis of amplification product sequences:

Table (14): FASTA of the *16S rRNA* amplified gene of isolate Q3.

Gene	The nitrogen base sequence of the 16S rRNA gene	The total number of nitrogenous bases
1_27F *16S rRNA*	AAAGTCTGACCGAGCACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTG GTAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCATCCAGTGACGGTAACTACCCAGAAA GGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGA TTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCT CAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCC ATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCT GGCCTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG GTAGTCCACGCCGTAAACGATGAGTGCTAGATGTAGGGAGCTATAAGTTCTCTGTATCGCA GCTAACGCAATAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAAT TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC CTTACCAGGTCTTGACATACTCGTGCTATTCCTAGAGATAGGAAGTTCCTTCGGGACACGG GATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC	1057

	AACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAACGAGACTGCC GGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGG CTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACAGTGATGTTTAGCTAATCT CTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGC TAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG TCACACCACGGGAGTTGGGAGTACCCGAAGTAGG	

Table (15): FASTA of the *16S rRNA* amplified gene of isolate K7.

Gene	The nitrogen base sequence of the 16S rRNA gene	The total number of nitrogenous bases
1_27F 16S rRNA	TGATTATGACGTACTTGTACTGATTGAGATTTTAACACGAAGTGAGTGGCGAACGGGTGA GTAACACGTGGGTAACCTGCCCAGAAGTAGGGGATAACACCTGGAAACAGATGCTAATA CCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGG ATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACG TAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACG GGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGT GAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAG TAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC CGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAG GCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATTGGAAACT GGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAG ATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGGC TCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGA TTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGTAATCCGC CTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATTGACGGGGGCCCGCACAAGCG GTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCT GACAGTCTAAGAGATTAGAGGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTG TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACT AGTTGCCAGCATTAAGTTGGGCAGTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGG TGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG ATGGTACAACGAGTCGCGAAACCGCGAGGTTAAGCTAATCTCTTAAAACCATTCTCAGTTC GGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCA TGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGT AACACCCAAAGCCGGT	1393

The results were analyzed by BLAST program to find similarity or genetic match with the NCBI Gen Bank, as the results showed that there was a match in the sequences of the nitrogenous bases of the amplified *16S rRNA* gene of the local isolate S2 with the sequences of the nitrogenous bases of the same gene for several strains of *Lactococcus lactis* and *pediococcu pentosaceus* registered in the NCBI bank. NCBI genes and *Lactococcus lactis* match rate was 99.91% with a strain belonging to this bacteria. The match rate was 99.81% with more than 80 other strains from the same isolate (Table 16), so the local isolate Q3 was considered to belong to the bacterium *Lactococcus lactis*, while the match was for the results of the nitrogen base sequences of the amplified *16S rRNA* gene of the local isolate K7 with the nitrogen base sequences of the same gene and other strains Also numerous of *pediococcu pentosaceus*

bacteria isolated from other sources and also registered in NCBI and the match rate was 99.93% (Table 17).

Table (16): Matching percentage of the sequences of the nitrogenous bases of isolate Q3 for several strains of *Lactococcus lactis* registered at the National Center for Biotechnology Information NCBI.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Lactococcus lactis strain HANM Diacetyl 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1953	1953	100%	0.0	100.00%	1057	OQ712009.1
Lactococcus lactis partial 16S rRNA gene, strain L-4	Lactococcus lactis	1947	1947	100%	0.0	99.91%	1462	LT853603.1
Lactococcus lactis subsp. lactis strain LM 0404 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	1941	1941	100%	0.0	99.81%	1453	OQ569384.1
Lactococcus lactis subsp. lactis strain LM 0403 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	1941	1941	100%	0.0	99.81%	1458	OQ569383.1
Lactococcus lactis subsp. lactis strain LM 0306 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	1941	1941	100%	0.0	99.81%	1456	OQ569380.1
Lactococcus sp. strain L1-2CT-A-OTU36MT(U71) 16S ribosomal RNA gene, partial sequence	Lactococcus sp.	1941	1941	100%	0.0	99.81%	1412	OM164537.1
Lactococcus sp. strain P1-5CT-J-OTU36MT(U07AKAV70) 16S ribosomal RNA gene, partial ...	Lactococcus sp.	1941	1941	100%	0.0	99.81%	1412	OM164513.1
Lactococcus sp. strain P1-19CT-B-OTU36MT(R28) 16S ribosomal RNA gene, partial seque...	Lactococcus sp.	1941	1941	100%	0.0	99.81%	1413	OM164429.1
Lactococcus lactis strain M34 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1941	1941	100%	0.0	99.81%	1431	OQ225229.1
Lactococcus lactis strain M31 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1941	1941	100%	0.0	99.81%	1433	OQ225199.1
Lactococcus lactis strain M33 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1941	1941	100%	0.0	99.81%	1422	OQ225197.1
Lactococcus lactis strain M3 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1941	1941	100%	0.0	99.81%	1429	OQ224981.1
Lactococcus lactis strain M2 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1941	1941	100%	0.0	99.81%	1432	OQ224980.1
Lactococcus lactis strain M1 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1941	1941	100%	0.0	99.81%	1430	OQ216739.1
Lactococcus lactis strain VHProbi V60 chromosome, complete genome	Lactococcus lactis	1941	11640	100%	0.0	99.81%	2416269	CP110849.1
Lactococcus lactis strain LL15 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1941	1941	100%	0.0	99.81%	1454	OQ002429.1
Lactococcus lactis strain LL14 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1941	1941	100%	0.0	99.81%	1447	OQ002428.1

Activate Windows

Table (17): Matching ratio of the sequences of nitrogenous bases of isolate K7 from strains of *pediococcu pentosaceus* registered at the National Center for Biotechnology Information NCBI

		Score	Score	Cover	value	Ident		
Pediococcus pentosaceus strain Bro DiAc 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2573	2573	100%	0.0	100.00%	1393	OQ714402.1
Pediococcus pentosaceus strain 4942 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1488	MT512069.1
Pediococcus pentosaceus strain HBUAS1-3 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1499	MT000126.1
Pediococcus pentosaceus strain MG5316 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1447	MN704679.1
Pediococcus pentosaceus strain L25 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1452	MN638797.1
Pediococcus pentosaceus strain L18 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1446	MN638790.1
Pediococcus pentosaceus strain L1 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1457	MN638773.1
Pediococcus pentosaceus strain IAH_35 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1458	MK990078.1
Pediococcus pentosaceus strain OCPP3 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1492	MK605953.1
Pediococcus pentosaceus strain 24 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1423	MH229959.1
Pediococcus pentosaceus strain HBUAS56024 16S ribosomal RNA gene, partial seque...	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1499	MK396642.1
Pediococcus pentosaceus strain S3c 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1433	MH169740.1
Pediococcus pentosaceus strain ACD43-3 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1454	MH111691.1
Pediococcus pentosaceus strain ACD43-1 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1447	MH111689.1
Pediococcus sp. strain CAU504 16S ribosomal RNA gene, partial sequence	Pediococcus sp.	2567	2567	100%	0.0	99.93%	1438	MF424971.1
Pediococcus pentosaceus strain CAU7108 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1439	MF423947.1
Pediococcus pentosaceus strain CAU:229 16S ribosomal RNA gene, partial sequence	Pediococcus pentosaceus	2567	2567	100%	0.0	99.93%	1481	MF369881.1
Pediococcus sp. strain CAU:1799 16S ribosomal RNA gene, partial sequence	Pediococcus sp.	2567	2567	100%	0.0	99.93%	1416	MF354845.1

Activate Windows

Recording local isolates globally:

Based on the results of the analysis and matching of the aforementioned nitrogenous base sequences ,the local isolates under study were registered in the NCBI Gen Bank and named the isolate Q3 isolated from Qargoli cheese and given the symbol *Lactococcus lactis strain HANM Diacetyl*, in the National Center for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov/nuccore/OQ712009 .
while the isolate K7 isolated from cheese was named Al-Qargoli K was also given the symbol *Pediococcus pentosaceus strain Bro DiAc,* the National Center for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov/nuccore/OQ714402 . (**Khalil, 2020**) also, two local isolates of lactic acid bacteria were recorded in the GenBank.

CONCLUSION

The isolates isolated from Qarghouli cheese Q3and K7 new isolates that produce a diacetyl compound and are safe for their application in food and medicine,it was showed good productivity of the diacetyl compound used in the food industry as a flavoring agent and a preservatives agent because it has antimicrobial properties, It is also known that lactic acid bacteria produce compounds with many benefits of *lactobacillus* bacteria and applied them in the production of therapeutic milk products. The PCR technique was used to identify the isolates, and it is considered the accurate method in the identification of genes.

REFERENCE

1. Abbas *et al.*, (2020). Identification local isolates of *Trichophyton mentagrophytes* and detection of keratinase Gene using PCR technique. *Iraqi Journal of Agricultural Sciences*. Baghdad University. *51*(6): 1534–1542.
2. Ahmaed, A. S. (2014). Dextran production from locally isolated bacteria *Leuconostoc mesenteroides* and utilization of the produced dextran in some food applications. Ph.D. Thesis. Department of Food Sciences. College of Agricultural Engineering. University of Baghdad.
3. Al-Azawi, Sh. Al-Khafaji, Z, M. (1988). Effect of diacetyl on the growth of Lipolytic Bacteria. *Journal of Agricultural Sciences.* University of Baghdad_ . 19.1.
4. Al-dosari, A, Y. (2002). Isolation and identification of *lactobacillus reuteri* bacteria from the stool of breastfed children, study of some of their developmental and therapeutic properties, and their use in the production of therapeutic milk products. Ph.D. thesis. Department of Food Sciences. College of Agricultural Engineering. University of Baghdad.
5. Al-Khafaji, Z, M, *et al.,* (2005). Effect of a flavor compound diacetyl on bacteria isolated from hospitals. *Iraqi Journal of Agricultural Sciences . Baghdad University* . 36, 179-184.
6. Asmaa, S., Akram, AL. rawi and Tariq, N., M. (2016). Production of dextran from a local isolate (*Leuconostoc mesenteroides*) and its its utilization in some food applications. *Iraqi Journal of Market Research and Consumer Protection*, *8*(1):367–377.
7. Balcázar. José Luis, Ignacio de Blas, Imanol Ruiz-Zarzuela, Daniel Vendrell, Olivia Gironés and José Luis Muzquiz. (2007). Sequencing of variable regions of the *16S rRNA* gene for

identification of lactic acid bacteria isolated from the intestinal microbiota of healthy salmonids. Comp Immunol Microbiol Infect Dis. 30(2):111-118.

8. Bassit, N., C. Y. Boquien, D. Picque, and G. Corrieu. (1993). Effect of oxygen concentration on diacetyl and acetoin production by *Lactococcus lactis* subsp. *lactis biovar diacetylactis*. Appl. Environ. Microbiol. 59:1893–1897.
9. Bassit, N., C. Y. Boquien, D. Picque, and G. Corrieu. (1995). Effect of temperature on diacetyl and acetoin production by *Lactococcus lactis* subsp. *lactis biovar diacetylactis* CNRZ 483. J. Dairy Res. 62:123–129.
10. Bintsis, T. (2018). Lactic acid bacteria as starter cultures: an update in their metabolism and genetics. AIMS Microbiol. 4: 665–684. doi: 10.3934/microbiol. 2018.4.665.
11. Clark, S., and Winter, C. K. (2015). Diacetyl in foods: a review of safety and sensory characteristics. Compr. Rev. Food Sci. Food Saf. 14: 634–643. doi: 10.1111/1541-4337.12150
12. Harvey, R. J., and E. B. Collins. (1962). Citrate transport system of *Streptococcus diacetylactis.* J. Bacteriol. 83:1005–1009.
13. Hernandez-Valdes, J. A., Solopova, A., & Kuipers, O. P. (2020). Development of *Lactococcus lactis* biosensors for detection of diacetyl. *Frontiers in Microbiology*, *11*, 1032.
14. Hugenholtz, J. (1993). Citrate metabolism in lactic bacteria. FEMS Microbiol. Rev. 12:165–178.
15. Hugenholtz, J., and M. J. C. Starrenburg.(1992). Diacetyl production by different strains of *Lactococcus lactis* subsp. *lactis var. diacetylactis* and *Leuconostoc spp*. Appl. Microbiol. Biotechnol. 38:17–22.
16. Hugenholtz, J., and Starrenburg, M. J. C. (1992). Diacetyl production by different strains of *Lactococcus lactis subsp. lactis var. diacetylactis* and *Leuconostoc spp.* Appl. Microbiol. Biotechnol. 38: 17–22
17. Jawan, R., Kasimin, M. E., Jalal, S. N., Faik, A. M., Abbasiliasi, S., & Ariff, A. (2020). Isolation, characterization and in vitro evaluation of bacteriocins-producing lactic acid bacteria from fermented products of Northern Borneo for their beneficial roles in food industry. In *Journal of Physics: Conference Series* 1358: 1.
18. Jebur, H. A. and Auda, J. M. (2020). Evalution of antimicrobial activity of partial purified bacteriocin from local isolate of *bacillus licheniforims* HJ2020 MT192715.1. *Iraqi Journal of Agricultural Sciences*. Baghdad University: 51 (6): 1644-1652.
19. Khalil, N. (2020). Isolation and identification of local *Limosilactobacillus fermentum* and studying its effects on reducing acrylamide in potato Chips. Master's thesis. Department of Food Sciences. College of Agricultural Engineering. University of Baghdad.
20. Kleerebezemab, M., Hols, P., and Hugenholtz, J. (2000). Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering. Enzyme Microb. Technol. 26: 840–848. doi: 10.1016/ S0141-0229(00)00180-0
21. Libudzisz, Z., and E. Galewska. (1991). Citrate metabolism in *Lactococcus lactis subsp. lactis var. diacetylactis* strains. Die Nahrung. 35:611–618.
22. Michael R. G., & Sambrook, J. (2012). Molecular Cloning A laboratory Manual.4th.ed. 1CSH Laboratory Press Cold Spring Harbor, New York, USA.

23. Naser Tajabadi, Makhdzir Mardan, Nazamid Saari, Shuhaimi Mustafa, Rasoul Bahreini. Mohd Yazid Abdul Manap. (2013). Identification of *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus fermentum* from honey stomach of honeybee. Brazilian Journal of Microbiology 44(3): 717-722.
24. Pack, M. Y., E. R. Vedamuthu, W. E. Sandine, P. R. Elliker, and H. Leesment. (1967). Effect of temperature on growth and diacetyl production by aroma bacteria single- and mixed-strain lactic cultures. J. Dairy Sci. 51:339–344.
25. Petit, C., F. Vilchez, and R. Marczak. (1989). Influence of citrate on the diacetyl and acetoin production by fully grown cells of *Streptococcus lactis subsp. diacetylactis.* Curr. Microbiol. 19:319–323.
26. Ramalingam, C., Bedi, J., & Karara, A. (2011). Isolation of heavy metal resistant marine fungi and Bacteria and their antibacterial activity. *Elixir Bio-Tech.*, 40:5618-5622.
27. Rincon-Delgadillo, M. I, Lopez-Hernandez, A., Wijaya, I., and Rankin, S. A. (2012). Diacetyl levels and volatile profiles of commercial starter distillates and selected dairy foods. J. Dairy Sci. 95:1128–1139. doi: 10.3168/jds.2011-4834
28. Sacchi, C. T.; Whiteney, A. M.; Mayer, L. W.; Morey, R.; Steigerwalt, A.; Boras, A.; Weyant, Robin, S. and Tanjja, P. (2002). sequencing of *16s rRNA* Gene A Rapid Tool for Identification of *Bacilluc anthracis*. *Emerging Infectious Disease journal.* 8(10): 1117 - 1123.
29. Shriparna M.; Dhananjay K.; Ashis K.N. and Ranadhir C. (2013). *16S rRNA* gene sequence analysis of the metagenome derived from water of river Mahananda at Siliguri: An approach to understand bacterial diversity. Indian journal of Biotechnology. 12: 80 – 87.
30. Suhad, K. K. (2001). Isolation and identification of some types of bacteria responsible for the flavor of Iraqi countryside cheese and using them as starters. Master's thesis. Department of Food Sciences. College of Agricultural Engineering. University of Baghdad.
31. Verhue, W. M., and F. S. B. Tjan. (1991). Study of citrate metabolism of *Lactococcus lactis subsp. lactis biovar diacetylactis* by means of 13C nuclear magnetic resonance. Appl. Environ. Microbiol.57:3371–3377.