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Effect of N_2 Laser Fluence on Lactic Acid Production by *Streptococcus thermophilus*

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Abstract: The aim of this work was to improve the lactic acid production by subjecting lactic acid isolates to N_2 laser radiation. Fifteen samples of fermented dairy products were collected from local markets. Two others were brought from the dairy product manufacture of Abu Ghraib and other dairy product factories in Baghdad. Eleven isolates were identified as Cocci lactic acid bacteria. All isolates had been subjected to the biochemical tests (catalase production, gelatin liquefaction, growth in litmus milk, NH_3 from arginine, CO_2 production, growth at 10 and 45 ^oC, growth at 4% NaCl, growth at 0.1% of methylen blue and fermentation of nine carbon compounds. For selecting efficient isolates in production of lactic acid, all isolates were propagated in whey medium. After propagation one isolate S9 of *Streptococcus thermophilus* produced highst total titrable acid (TA%) when it reached 1.28% therefore this isolate was selected for subjection to N_2 laser with wavelength of 337.1 nm, with different fluences. After treatment with N_2 laser at fluence of 18.95 J/cm2, production of lactic acid by this isolate reached 17 mg/ml.

Introduction

Scheele was first who discovered lactic acid in yogurt in 1780. In 1857 Pasteur studies proved the presence of microorganisms producing lactic acid in yogurt (Foster *et al*., 1958). Both *Streptococcus* and *lactobacillus* are related with faculatatively anaerobic taxa evolved as individual lines of descent about (1.5 –2) billion years ago when the earth passed from an aerobic to an anaerobic environment (Teuber and Erko, 1988).

According to the modern development in bacterial taxonomy, lactic acid bacteria comprise several genera; *Streptococcus, Lactobacillus, Lactococcus, Pedococcus, Leuonostoic, Enterococcus, Aerococcus*. Depending on their metabolisms, similarities in the physical, and nutritional needs; these genera are grouped together as lactic acid bacteria. One of the common major properties of these genera is the production of lactic acid as a main or virtually sole end product of the fermentation of sugar (Lndquest, 1998: Teuber, 1999). These bacteria were classified at first time after isolation it from sour milk in Sweden in the year 1857 (Teuber, 1995). In 1873 and by chance Joseph Lister obtained this bacteria pure culture for first time, he described it as *Bacterium lactis*. In 1909 Lohnis renamed this species as *Streptococcus lactis* and recently its accepted name is *Lactococcus lactis*.

Taxonomic changes have taken place in this group of bacteria, particularly in the genus (*Streptococcus*). Traditionally the Sherman criteria along with the Lancifield antigen have been used in the characterization of grouping of species within *Streptococcus* two decades during the past depending on nucleic acid studies, the genus *Enterococcus* and *Lactococcus* have been spun off from *Streptococcus spp*. One may still note the use of out dated terminology such as *Streptococcus faecalis* and *Streptococcus lactis*. These species are now called *Enterococcus faecalis* and *Lactococcus lactis* respectively (Lndquest, 1998). Due to the

important role of *Streptococcus spp* in production of lactic, this study aimed to investigate the effect of N_2 laser on production of lactic and by *Streptococcus thermophilus*.

Materials and Methods

Samples Collection and Isolation of Lactic Acid Bacteria

Samples were collected (in sterilized plastic packages) from dairy factories and local markets in Baghdad, Regarding lyophilized starters (*Lactobacillus bulgaricus* and *Streptococcus thermophilus)* samples were brought to the laboratory in their sealed packages. MRS liquid medium is inoculated by 1% of milk and yoghurt samples, and incubated at 37 ^{o}C for 24-hr. Pour plate method was used for isolation of lactic acid bacteria according to Harrigan and MacCance (1976). After incubation, colonies surrounded by zones were selected. Isolated colonies were, separately, streaked on MRS agar for purification, then incubated at 37 ^{o}C for 24 hr. under anaerobic conditions.

Identification Tests of Lactic Acid Bacteria

Cocci Isolates of LAB were identified depending on cultural, microscopical and physiological characteristics (biochemical tests), which include ammonia production from arginin, growth at 4% NaCl, growth in 0.1% of methylen blue, carbohydrate sources fermentation and CO_2 production from glucose also growth in 10 and 45 ^{o}C.

Selection of Efficient Isolates for Lactic Acid Production

Equal percents from the suspension of the isolates which had been perviously grown in MRS medium into flasks containing 100 ml sterilized whey with pH= 6.0. All flasks were incubated with 37 ^{o}C. Amounts of acid production were estimated. The pH value was daily determined by a pH- meter to keep it equal to 6 during fermentation period by adding calcium hydroxide whenever needed.

Mutation Methods

Preparation of Isolates

Ten ml of MRS broth was inculcated with (1%) culture (3.8- 4.6x10^9 cell/ml) of each (*Streptococcus thermophilus,*) isolate,and incubated at 37 ^{o}C for 24 hr. After incubation, the fermentation medium was centrifuged 3000 rpm for 15 min. at 10 ^{o}C. Cells pellets were washed twice with physiological saline (pH= 7), then mixed by vortex and suspended in Tris-HCl, pH= 6.0 (Shaba, 2000).

N_2-Laser Device

Pulsed N_2 laser (type Molectron UV 24) was used in this study. Its wavelength is 337.1 nm with 1.5 mJ per pulse and 10 ns pulse duration.

Laser Treatment

One milliliter of each prepared isolate (as mentioned in bacterial preparation) was suspended in Tris–HCl. They were transferred to sterile ependroff tubes, having top diameter of 1 cm and bottom of 3 mm. Nitrogen laser treated the transferred bacteria. All samples were treated at room temperature in a dark place. After treatment, samples were put in ice to avoid DNA replication (Bauer *et al.* 1998). Samples were then exposed to laser irradiation for the following periods (0.25, 0.5, 1, 3, 5, 7, and 10) min with (8, 16, 24 and 32) Hz repetition rates for each exposure. Table (1) shows the N_2 laser parameters, which were used in this work.

Table (1) The experimental laser parameters

No. of pulses per sec.	Exposure time (sec)	Energy density J/cm^2
8	15	0.47
	30	0.94
	60	1.80
	180	5.68
	300	9.47
	420	13.26
	600	18.94
16	15	0.47
	30	1.89
	60	3.78
	180	11.36
	300	18.94
	420	26.52
	600	37.89
24	15	1.42
	30	2.84
	60	5.68
	180	17.05
	300	28.45
	420	39.78
	600	56.84
32	15	1.89
	30	3.78
	60	7.57
	180	22.73
	300	37.89
	420	53.05
	600	75.78

The laser fluence was calculated from the following steps:

1. Energy Density (ED): Energy per unit area, i.e., ED (in J /cm^2) = E / A , where E is a single photon energy and A is the area.

2. Fluence (F) = ED. N , where N is the number of pulses per second and N = n.t. Here t is the exposure time in seconds and n is the number of pulses.

The ependroff tube was located on a fixed base, while a mirror was used to adjust the reflected laser beam on the required area, and lens were used with focal length 5 cm to concentrate the laser beam on the bottom of the ependroff tube.

Detection and Determination of Lactic Acid

The following procedure (Barker and Summerson, 1954) was employed for the detection and determination of lactic acid:

a. Portions of 10 ml from the fermentation medium was put in sterilized test tubes, separately, then centrifuged by 4000 rpm, for 20 min. Supernatant solution were taken and put in another sterilized test tubes.
b. Two milliliters of each of the supernatant solution of samples were added to 8 ml of trichloroacidic acid (10%) in sterilized test tubes, separately, mixed thoroughly by vortex. Turbid suspensions were appeared in all samples due to the protein denaturation. They were centrifuged by 3000 rpm for 10 min to get rid of proteins. Supernatant solutions were also put in sterilized test tubes.
c. Five standard solutions of lactic acid were prepared with different concentrations are (2, 4, 6, 8, and 10) mg/l (Fig. 1).
d. Two milliliters of supernatant from the centrifuged fermentation medium of each sample were taken and put in sterilized test tubes separately. Another 2 ml of five standard solutions were taken and put separately in another test tubes.
e. To each tube, one ml of 20% copper sulfate solution was added and diluted to 10 ml, then mixed thoroughly by vortex.
f. One gram of CaO powder was added to each tube stoppers. They were shacked vigorously until the solution was uniformly dispersed. They were allowed to stand for half an hour then centrifuged with 3000 rpm for 10 min.
g. One milliliter from each supernatant solution was taken and put into sterilized test tubes, 0.05-ml of 4% copper sulfate solution was added to each tube.
h. To each tube, 6 ml of concentrated sulfuric acid were added. The sulfuric acid should be added drop by drop at first. Contents of the tube were mixed well during the addition of acid. All tubes were placed upright in boiling water bath for 5 min, then transferred to cold water and cooled to 10 ^{o}C.
i. Tenth milliliter of para-hydroxydiphenyl reagent, was added drop by drop to each tube. All tubes were placed in beaker continuum water at 30 ^{o}C for half an hour. Finally all tubes were placed in boiling water for exactly 90 second, then in cooled water at 10 ^{o}C or less.
j. The colored solution was transferred to the suitable container and optical density was determined by spectrophotometer model 20 at 560 nm.

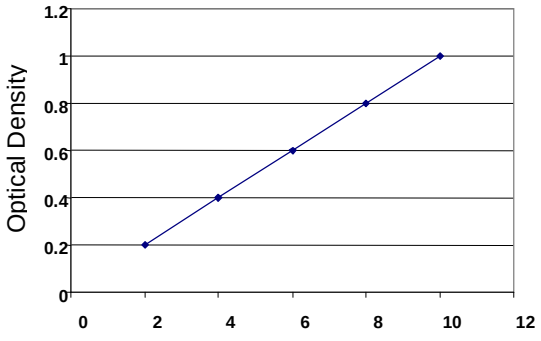

Fig. (1) Standard curve of lactic acid

Results and Discussion

Identification of LAB (Cocci)

From milk and milk product samples locally collected, 11 isolates were primary detected as

Cocci LAB according to clear zone around their colonies on MRS agar containing 1% of $CaCO_3$. Colonies grown on the MRS agar were circular, convex, slimy and white color. Such characteristics are similar to the colonies formed by the coccal genera of LAB. Microscopical examination of the LAB isolates, showed that most of the isolates are coccal cells, arranged in long chains (7-10 cells), with presence of single and pairs, they were G(+) nonsporeforming. The medium MRS was very suitable for growth of LAB cocci due to its contents of essential mineral salts that encourage the growth of this bacteria (De Man, 1960: Tiwari *et al.*,1979). Biochemical tests were used for the differentiation between the species of LAB (Cocci). Table (2) shows these tests.

Table (2) Biochemical characteristics of the eleven isolates of lactic acid bacteria (LAB)

A- Fermentation of carbohydrates (CHO) sources

Isolate no.	Glucose	Lactose	Mannsose	Mannitol	Arabinose	Xylose	Galectose	Maltose	Raffinose
St.1	(1)+	(1)+	--	(1)+	--	--	(4)+w	(2)+w	--
St.2	(1)+	(1)+	--	(1)+	--	--	(3)+w	(3)+w	--
St.3	(1)+	(2)+	(2)+	(1)+	--	(1)+	(1)+	(1)+	--
St.4	(1)+	(1)+	--	(1)+	--	--	(3)+	(3)+w	--
St.5	(1)+	(2)+	(1)+	(1)+	--	(1)+	(1)+	(1)+	--
St.6	(1)+	(2)+	(3)+	(1)+	--	(3)+	(3)+	(1)+	--
St.7	(1)+	(1)+	--	(1)+	--	--	(3)+w	(2)+w	--
St.8	(1)+	(1)+	--	(1)+	--	--	(3)+w	(1)+w	--
St.9	(1)+	(3)+	(4)+	(1)+	--	--	(2)+	(1)+	--
St.10	(1)+	(1)+	--	(1)+	--	--	(2)+w	(3)+w	--
St.11	(1)+	(1)+	--	--	--	--	(w)	(2)+w	--

(+)=Positive test, (--) = Negative test, w = weak change with color, ( ) = Days of incubation

B- Other biochemical characteristics

Isolates	Catalase	Gelatin	NH_3 from Argnine	Growth in litmus milk	Growth at 45 °C	Growth at 10 °C	Growth in 4% NaCl	Growth in 0.1% methyln blue
St.1	-	-	-	+	+	-	-	-
St.2	-	-	-	+	+	-	-	-
St.3	-	-	+	+	-	+	+	+
St.4	-	-	-	+	+	-	-	-
St.5	-	-	+	+	-	+	+	+
St.6	-	-	+	+	-	+	+	+
St.7	-	-	-	+	+	-	-	-
St.8	-	-	-	+	+	-	-	-
St.9	-	-	+	+	-	+	+	+
St.10	-	-	-	+	-	-	-	-
St.11	-	-	-	+	-	-	-	-

(+)=Positive test, (--) = Negative test

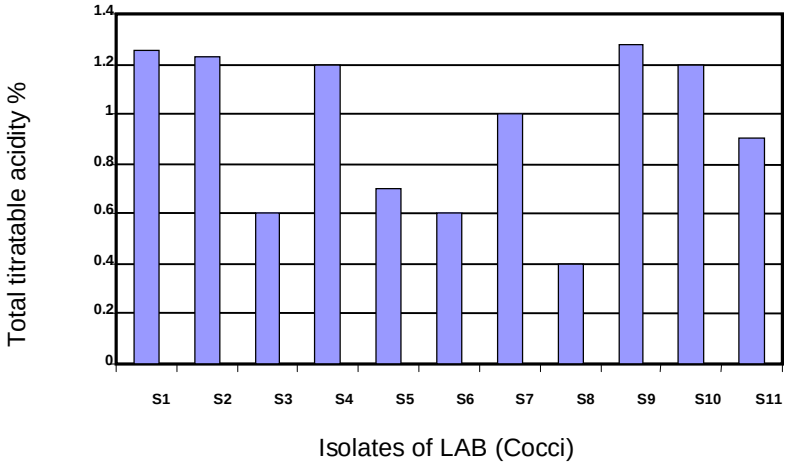

Fig. (2) Total acidity of lactics production Cocci

Efficient Isolates for Lactic Acid Production

Figure (2) shows the production of lactic acid by the isolates grown in fermentation medium (whey) expressed in percentages of total acidity. After 5 days of fermentation, isolates which belonging to species produced more lactic acid higher than the other isolates of lactic acid bacteria. Depending on the most efficient isolates in acid production, one isolate has been selected for further study; they are ***Sterpt. therophilus*** (St. 9) that gave 1.28 of total acidity. This isolate was subjected to treatment with N_2 laser. Some investigators (Rodes, 1975: Casida, 1968: Teuber, 1993: Singh, 1977) used ***Strept.therophilus*** to produce lactic acid after propagation in various by –production media (e.g. whey and molasses).

Isolates Mutation

Inoculum Size and Treatments

One selected lactics isolate (St. 9) was irradiated with N_2 laser. Nitrogen (N_2) laser was used to treat (4.6 $x10^9$) cells/ml of St. 9. The following parameters of N_2 laser have been applied; (8, 16, 24 and 32) pulse/s. 0.25, 0.5, 1, 3, 5, 7 and 10 min. exposure time. Above inoculum size is usually regarded as suitable for similar kinds of studies. Singh and Ranganathan (1977) exposed 2 $x10^9$ cells/ml of lactics cultures to gamma radiation (as a physical mutagen) and obtained high efficiency isolates in production of lactic acid (Hopwood, 1970).

Production of Lactic Acid

Since the active mutants may occur when the percentage of killing reaches 99.9% (Milganiveva, 1971), fluence that lead to obtain maximum percentage of killing for the test bacteria (***Strept. theromphilus***) was selected for production of lactic acid. Twelve colonies belonged to ***Strept. theromphilus*** growing on MRS agar were tested for production of lactic acid using Barker and Summerson (1954) method procedure that depends on the standard curve, as shown in Fig. (1).

Despite that most of the isolates produced lactic acid were belonging to the parent bacteria, two isolates demonstrated high production of acid in comparison to the wild type isolate. These isolates were (S11) that produced 19 mg/ml. and isolate (S9) produced 17 mg/ml of lactic acid after treatment with N_2 laser fluences 22.5 and 18.95 J/cm^2 respectively. Fig. (3) illustrates the amount of lactic acid produced by the colonies of ***Strept.therophilus***. Few studies are available in this field of investigation. Milganieva (1971) found that production of lactic acid in whey medium increased by ***Lb. bulgaricus*** and its isolates after treatment with x- ray under temperature 46 ^oC, pH= 5.5- 6.0 with absence of oxygen (Milganiveva, 1971). While Al-Azzawi (1995) used ultraviolet light for improving ***Lb.bulgaricus*** and ***Strept. thermophilus*** production of lactic acid in whey medium and found that one of the mutated isolate highly efficient in producing remarkable concentrations of lactic acid (Mahdi, 1982).

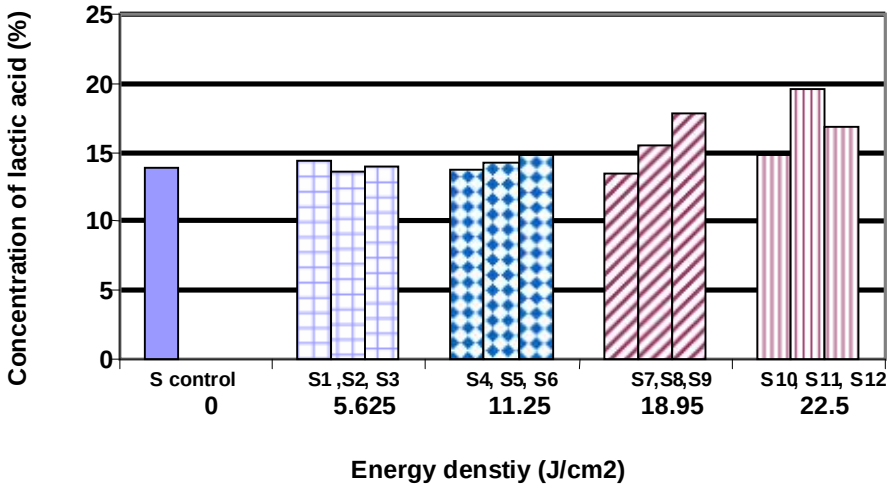

Fig. (3) Concentration of lactic acid produced by *strept. Thermophilus* isolates.

Effect of pH on Production of Lactic Acid

Figure (4) indicates that values of pH decreased during propagating of *Strept. thermophilus* and their mutants in the fermentation medium (whey) after subjection to various pulses of N_2 laser. In general, pH values decreased for *Strept. thermophilus* and its mutants. Maximum decrease (3.55) was achieved for the isolate (S11) after 120 hr of fermentation and 22.5 J/cm^2 fluence. It was appeared that the pH decrease was recorded after 96 hr of fermentation, and that the decrease in the pH values for the parent isolate was less than that of its isolates.

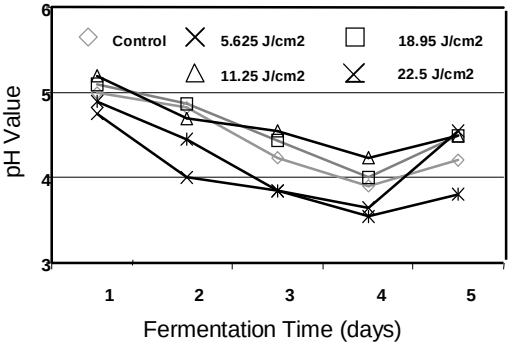

Fig. (4) Changes in pH values during propagating *Streptococcus thermophilus* isolates (S9) in whey medium contain maniral salts at different fluences.

Mahdi (1982) mentioned that greater amount of production of lactic acid was achieved when pH value for whey medium was fixed at 6 (Kempe *et al.*, 1950). Similar findings were obtained by Kempe *et al.* (1950), Marshall, (1970) and Tiwari, (1979). They found that production of greater amount of lactic acid is achieved at pH ranging between 5.5 -6.0, and any increase or decrease from that range, will lead to a decrease in the production of the acid amount (Kempe *et al*, 1950: De-Man, 1960: Marshall, 1970). For this purpose, Ca (OH) $_2$ was used in this study for controlling pH changes during fermentation**.**

Effect of N_2 Laser Fluence on Acid Production

The effect of N_2 laser fluence on the concentration of lactic acid is illustrated in Fig. (3). Highest concentration of acid was obtained for *Strept. thermophilus* isolate at fluence of 22.5 J/cm^2.

The effect of N_2 laser of 337.1 nm wavelength is most likely photochemical due to the absorption of laser light at this certain wavelength and certain energy densities by certain chromophores and lead to either biostimulation or bond breaking as follows:

If the photon is absorbed by the molecule, and its energy is less than that of the bond, the molecule should be affected. The excitation mechanisms lead to activate the molecule as:

$$(A\text{-}B) + h\nu = (A\text{-}B)^*$$

where (A-B) is a molecule consists of two atoms, hν is the photon energy and (A-B)* is the excited molecule. If the photon energy is equal or higher than the dissociation energy of the bond, it may be broken and produce energy as:

$$(A\text{-}B)^* + h\nu = A + B + E_{KIN}$$

Table (3) illustrates the dissociation energy of the bonds, while Table (4) shows the photon energy for some lasers.

Table (3) Energy dissociation of some bonds

Type of bond	Dissociation Energy (eV)
C=O	7.1
C=C	6.4
O-H	4.8
N-H	4.1
C-O	3.6
C-C	3.6
C-N	3.0

Table (4) The wavelength and photon energy of some types of lasers.

Laser type	Wavelength nm	Photon energy eV
ArF	193	6.4
KrF	248, 268	5.0, 4.9
Nd:YLF 4W	263	4.7
XeCl	308	4.0
N_2	337	3.6
Argon Ion	488, 514	2.4
Nd:YLF 2W	526.5	2.3
He - Ne	633	2.0
Diode	800	1.6
Nd:YAG	1064	1.2
HO:YAG	2120	0.6
Er:YAG	2940	0.4

The effect of laser radiation on the cells depends on its wavelength. This effect is either direct when the wavelength is located in the UV region 190 to 280 nm where the chromophore or molecule that absorbs radiation is DNA, or it is indirect when the wavelength locate in UVA-region (320- 400 nm). there is no effect on DNA at this wavelength of laser beam ,because of DNA don't absorbed laser light in this wavelength, so addition of specific chromophor to these wavelengths between (320-400) nm, will induce the effect on DNA and considering as exogenous chromophores. (DeWith and Greulich, 1995). The most probable chromophore which absorb the light of N_2 laser (337.1 nm) represents the reduced form Nicotinamide Adenine Dinuclatide Phosphate NADP (H) (Fig. 5), where NADP (H) acts as a donor for H atoms or acceptor electrons in reductive biosynthesis reactions also enters in krebs cycle and pentose phosphate pathway for producing Adenosine Tri- Phosphate (ATP) (Simeth, 1985). It is clear that the above description is coincidence with the conclusions of Pukhoua (1995) which concluded that the NADP (H) of the macrophage cells absorbs N_2 laser light with 337.1 nm (Pukhoval, 1995).

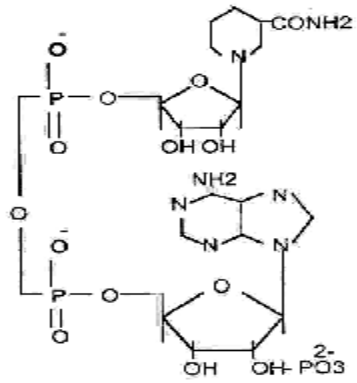

Fig. (5) Compound of Nicotinamide Adenine Dinucleotide Phosphate (Reduced form).

Table (4) illustrates that the photon energy of the N_2 laser is 3.6 eV, so the probable bonds may be affected during the interaction are the bonds between (C-C, C-O, C-N) and as mentioned above either biostimulation or bond breaking occur according to the type of interaction.

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Streptococcus thermophilus *Ù Ķ Ķ й Ǖ ɥ Ǖ γ Ķй ĸǑç*

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الخلاصة

هدفت الدراسة الى تحسين أنتاج حامض اللاكتيك بتعريض عزلات بكتريا اللاكتيك الى أشعة ليزر النتروجين . وجمع لهذا الغرض 15 نموذج من منتجات الألبان المتخمرة من الأسواق المحلية وبعص مصانع الألبان في بغداد وضواحيها . أمكن تشخيص 11 عزلة تعود لبكتريا اللاكتيك الكروية عبر أخضاعها الى كافة الفحوصات الزراعية والمجهرية والكيموحياتية . ولأختبار قابلية العزلات على أنتاج الحامض فقد نميت في وسط الشرش . وتم أختيار عزلة تعود لبكتريا Streptococcus thermophilus لمعاملتها بليزر النتروجين ذا الطول الموجي1ر337 نانومتر. ثم قورنت العزلات المعرضة لليزر مع العزلات الأبوية من حيث تركيز حامض اللاكتيك المنتج والأس الهيدروجيني .