RAPD AND SCAR ANALYSIS OF PROTOPLAST FUSANTS AND PLASMID TRANSFORMANTS AS NEW CONSTRUCTED LACTIC ACID PRODUCERS

EL-FADLY, G. *; KARIMA A. MOHAMED; A. ISMAIL**;**

A. ABOU-SHOSHA* AND A. KHATTAB**

*** GENETICS DEPT., FAC. OF AGRIC., KAFR EL-SHEIKH, TANTA UNIV. EGYPT**

**** APPLIED MICROBIAL GENETICS LAB. , N.R.C., DOKKI, GIZA; EGYPT**

ABSTRACT

Different intraspecific, interspecific and intergeneric protoplast fusion crosses were carried out using antibiotic response as additional genetic markers for fusants detection . Enhancement of lactic acid productivity on intraspecific level reached up to 35-89% higher than the orginal strain. Interspecific protoplast fusion between *Lb. casei* subsp . *rhamnosus* NRRL B-445 (lactose fermentative and not ferment starch) and *Lb. amylovorus* NRRL B-4540 (starch fermentative and non lactose fermentative) yielded nine fusants most of them were able to ferment lactose and starch and exhibited lactic acid productivity . Intergeneric protoplast fusion was carried out between the high lactic acid producer UV- isolate D_{33} (could not produce bacteriocin) and *Streptococcus lactis* subsp. *cremoris* No. 66 (produce bacteriocin) yielded 20 fusants and most of them exhibited acid and bacteriocin productivities.Six transformation experiments were conducted using pGIT032 plasmid containing lactate dehydrogenase gene. The excellent transformant was T_{2-3} which produced about 7% more lactic acid than the original strain . Plasmid profile analysis showed that all of the tested transformants exhibited an extra plasmid (pGIT032) more than the plasmid content of recipient strains. Molecular variations between four fusants and four transformants compared to their original strain showed that many differences in RAPD banding patterns profile were detected. The application of four specific primers using SCAR analysis confirmed the occurrence of gene transfer and recombination in association with intergeneric protoplast fusion.

INTRODUCTION

Lactobacilli have been of recent interest for genetic improvement due to its potential applications and its Generally Regarded As Safe (GRAS) status. Many strains are transformable and therefore amenable to genetic manipulation . However , the fate of recombinant molecules in strains of bacteria designed for release into the environment, particularly food chain , has been of some public concern .

Improvement of industrial strains, including selection for desirable characteristics such as acid production is an ongoing requirement.

Many researchers have investigated methods for improvement of strains through genetic modifications and molecular biological studies including fusion of protoplasts (**Ward *et al.*, 1993; Yeeh *et al.*, 1996**) as a tool for genetic manipulation . The exchange of genetic information has been monitoed by following the acquisition of an indicator plasmid (**Simon *et al.*, 1985**) and or the recombination of chromosomal markers (**Gasson, 1980**) .

Various molecular typing methods such as restriction fragment length polymorphism, pulsed field electrophoresis, ribotyping and the random amplified polymorphic DNA (RAPD) technique have been used for finer discrimination of *Lactobacillus* strains (**Tilsala- Timisjarvi and Alatossava, 1998**).

The present study aimed to construct superior lactic acid producing bacteria for improvement lactic acid fermentation. Molecular analysis were conducted to study the genetic variabilities of the genetically constructed fusants and transformants.

MATERIALS AND METHODS

1- Microorganisms

a- **Lactic Acid Bacterial Strains:** Three original strains, four UV treated isolates and five MNNG treated isolates used in the present study are listed in Table (1).

b. ***Micrococcus luteus*** **(NRRL B-287):** it was as a tester for the detection and bioassay of the bacteriocin production (**Kim *et al.*, 1997**).

2- Media:

a- *Lactobacillus* carrying medium (LCM) **Efthymiou and Hansen (1962).** This medium was used for pre-growing for protoplasting.

b- Regeneration medium (RM), **Lee-Wickner and Chassy (1984).** This medium was used for regeneration of fused protoplasts

c- Luria medium (LB), **Sambrook *et al.*, (1989).** It was used for culturing and maintaining *E.coli.*

d- Fermentation medium, **Rincon *et al.*, (1993).**

3- Reagents and Buffers

a- Protoplast formation and fusion buffers, **Lee Wickner and Chassy (1984)and Cocconcelli *et al.*, (1986).**

b- Buffers for plasmid isolation form *E.coli*, **Sambrook *et al.*, (1989).**

c- Buffers for isolation of total and plasmid DNA from *Lactobacilli*, **Anderson and Mckay (1983).**

Table (1): Code number and sources of the original and isolate lactic acid bacterial strains.

Code No.	Original Strain/ Isolate	Source
40 45	*Lactobacillus amylovorus* B-4540 *Lactobacillus casei* subsp. *rhamnosus* B-445	Northern Regional Research Laboratory (NRRL), 1815 North University St., Peoria, USA.
66	*.Streptococcus lactis* subsp. *cremoris*	National Research Center, Cairo, Egypt.
D_{1},,D_{32},D_{33},D_{34}	UV treatment for 2 min	**Khattab (2002)**
30 / 32 40/4 , 40/6 50/6 , 50/31	100ug MNNG/ml for 30 min 100ug MNNG/ml for 40 min 100ug MNNG/ml for 50 min	**Khattab (2002)**

d- Reagent and primers for molecular analysis

i- Polymerase chain reaction (PCR) reagents: For PCR technique,

Ready-To-Go PCR Beads (Amersham, Pharmacia Biotech. No. 27-9555-01) were used. Each bead contains all of the necessary reagents, except primer and DNA templet, for performing 25 µl PCR amplification reaction.

ii- Primers: Table (2) presents the two different primer sets which

were used in the present study. The first set consists of six random 15-mer primers. The second set consists of four specific primers for amplification of some regions of lactose operon encode, the tagatose-6-phosphate kinase, tagatose 1,6-diphosphate aldolase, enzyme II^{lac} and part of phospho-β-galactosidase according to **McCormick *et al.* (1995)**. All primers supplied by Gulf-Biotech Laboratory.

4- Enzymes: Sigma products, i.e, lysozyme and mutamolysin were used through this study for preparing protoplasts and DNA.

5- Antibiotics: the following antibiotics were used according to **Curragh and Collins (1992) and Fortina *et al.*, (1990).** 5 µg/ml of Ampicillin (Ap), Erythromycim (Em), Lincomycin (Lm), Mitomyicn (Mm); 10 µg/ml of Novobiocin (Nb); 40 µg/ml of chloramphencol; 100 µg/ml of Cephalosporin C (Cp), Tetracycline (Tc), Vancomycin (Vm) and 200 µg/ml of Kanamycin (Km), Neomycin (Nm) and Streptomycin (Sm).

6- Plasmid: Plasmid used in this study was pGIT032. It was kindly provided by Prof. Dr. **Ferain. T.** (Molecular Genetic Lab., Genetics Unit, University of Louvan, Belgium). The size of this plasmid is 6.7 kb and contains lactate dehydrogenase gene, erythromycin resistance gene and ampicillin resistance gene. It also contains two origins of replication, ori I for replication in *E. coli* and ori II for replication in *Lactobacillus* strains.

Table (2): The nucleotide sequences of the applied primers.

Serial No.	Type of primer	Cat. No	Nucleotides sequence
1	Random	9050320	5'-GGG GTT TGC CAC TGG-3'
2	Random	9050321	5'-CAT ACC CCC GCC GTT-3'
3	Random	9050310	5'-GTG TTG TGG TCC ACT-3'
4	Random	9050322	5'-AAC CTC CCC CTG ACC-3'
5	Random	9050303	5'-TGA GTG GTC TAC GTG-3'
6	Random	9050314	5'-CCC CAA CGC CGA CTC-3'
7	Specific	9030837	5'-ACA CGT TGA AAC ACA TAA TGC-3'
8	Specific	9030838	5'-TTA CTA TAC TTT ATC AGT CC-3'
9	Specific	9030839	5'-AGT AGG ATC CAG AGG AGC AAA GTA ATT AAT G-3'
10	Specific	9030840	5'-CCA TGC ATG CTC AGC TAA TTC TAA ATC AAC-3'

Methods:

1. Isolation of antibiotic resistant isolates: Many of the original strains in addition to the higher and lower lactic acid producing isolates were streaked on the surface of MRS plates each supplemented with specific antibiotics. The plates were incubated at 37°C for 3 days. Colonies which exhibited resistance to specific antibiotic(s) were retested to be sure of its stability.

2- Protoplast formation, fusion and fusant (s) detection were carried according to **Lee-Wickner and Chassy (1984).**

3- Fermentation and determination of lactic acid production was carried out according to the methods of **Khattab (2002).**

4- Starch hydrolysis assay: The parental strains and the obtained fusants after interspecific protoplast fusion were inoculated onto the surface of LCM medium containing 2% starch instead of glucose. The plates were incubated at 37°C overnight and then overlayed with 2% iodine solution. Clear zones around the bacterial growth area were measured.

5- Bacteriocin assay: Bacteriocin was measured according to **kim *et al.*, (1997).**

6- Plasmid transformation:

a. Isolation of plasmid DNA from *E. coli,* was conducted as reported by **Sambrook *et al.* (1989).**

b. Transformation of *Lactobacilli* and selection of transformants, **Jane and Savage (1986).**

c. Isolation of plasmid DNA from *Lactobacilli* the procedure steps of plasmid isolation was carried out according to **Anderson and Mckay (1983).**

7- Randome Amplified Polymorphic DNA (RAPD) analysis: To each ready To-Go- PCR bead, 12 ng of the used random primer and 40 ng of the purified DNA template were added. The total volume of the amplification reaction was completed to 25 µl using sterile distilled

water. The amplification protocol was carried out according to **Williams *et al.*, (1990)** with some modifications of cycling programme using Techne (Genius) Thermal Cycler. The cycling programme used was thirty – five cycles each consifs of 94 C (1 min), 51-57 C (2 min) according to GC ratio of each primer **(Qiagen, 1997)** and72 C (2 min) for DNA polymerization .

8- Sequences Characterized Amplified Region (SCAR) analysis :The SCAR analysis was carried out using the same method of RAPD analysis with exception of using two specific primers, forward and reverse primers, at the same time to amplify specific sequence or DNA region. Two SCAR amplification experiments were achieved. The first one was carried out using primers Nos. 7 and 8 (Table 2) and the primer annealing temperature was 50°C for 2 min. The second experiment was repeated two times using primers No. 9 and 10 with two primer annealing temperatures, i.e., 60°C and 50°C.

9- PCR products analysis: The amplified DNA products from RAPD analysis were electrophorated (using Hoefer HE 99 X Max Submarine Electrophoresis Unit) on 1.5% agarose gel and 1 X TBE buffer at consistent 100 volt for about 3 hrs. The DNA products from SCAR analysis were also electrophorated as same as of RAPD but the concentration of agarose was 1% only. The different bands sizes were determined against 100 bp ladder from Amersham-Pharmacia Biotech. Company and the separated bands were stained with 0.5 µg/ml ethidium bromide and photographed using both Polaroid Instant Camera and UV Transeliminator.

RESULTS AND DISCUSSION

Iintraspecific Protoplast Fusion Crosses:

Induction of protoplasts was carried out from some different isolates which varied in both lactic acid productivity and antibiotic response. Four intraspecific, one interspecific and one intergeneric protoplasts crosses were conducted as presented in Table (3). The productivity of the obtained intraspecific fusants and its relation to either the wild type or the higher producer parent were presented in Table (4).

Data in Table (4) showed that intraspecific protoplast fusion proved to be on effective tool to improve lactic acid production. In addition, protoplast fusion between the two higher lactic acid producing isolates (crosses 1 and 2) proved to be the most effective one since the enhancement of lactic acid productivity reached up to 35.09 and 35.89 percents higher than the original strain No. 45 for the two isolated fusants Nos. 1-9 and 2-8. Furthermore, in spite of the fact that protoplast fusion between D_{23} and 40/6 included the two lower lactic acid producing parents, but on the other hand, with one exceptional case (No.

3-5) all of the resulted fusants were higher producers than the original culture No. 45.

Table (3): Antibiotic response for the parental strains and numbers of the obtained fusants from the different protoplast fusion crosses.

Serial No.	Type of protoplast fusion	Genotype of selected original strains and isolates No.	No. of obtained fusants
1	Intraspecific	D_{33} (*Sm^r, Tc^r, Nb^r*) x D_{34} (*Cp^r, Nm^r, Cm^r, Vm^r*)	9
2	Intraspecific	D_{33} x D_1 (*Cp^r, Nm^r, Cm^r*)	11
3	Intraspecific	D_{32} (*Sm^r, Km^r, Nb^r*) x 40/6 (*Cm^r*)	10
4	Intraspecific	45* (*Nm^r*) x 50/31 (*Sm^r*)	13
5	Interspecific	45 x 40** (*Cm^r*)	9
6	Intergeneric	D_{33} x 66*** (*Cp^r, Nm^r, Lm^r, Cm^r, Vm^r*)	20

* 45 = *Lb. casei* subsp. *rhomnosus* No. 45

** 40 = *Lb. amylovorus* No. 40

*** 66 = *Streptococcus lactis* subsp. *cremoris* No. 66

The above results were in agreement with those obtained by **Iwata *et al.* (1986)** who isolated new fusants which appeared to involve a rearrangement in genetic material after intraspecific protoplast fusion between *Lactobacillus fermentum* 604 possesses *Tet^r* and *Lb. fermentum* 604 possesses *Ery^r*. Moreover, **Kanatani *et al.* (1990)** isolated fusants which exhibited recombination between a range of chromosomal marker after intraspecific protoplast fusion between isogenic *Lactobacillus plantarum* strains. Furthermore, **Chassy (1987)** and **Ward *et al.* (1993)**and **Yeeh *et al.*(1996)** reported that protoplast fusion could be used for the genetic manipulation and improvement of *Lactobacilli.* In addition, **Ward *et al.* (1993)** studied the molecular analysis of the obtained fusants after intraspecific protoplast fusion between *Lactococcus lactis* subsp. *cremoris* strains and showed that, out of the four protoplast fusions examined; three appeared to involve a rearrangement in genetic material while in the fourth, the fusants appeared similar to one of the parental strains. These results were in agreement with the results in this study.

Lactic Acid Productivity of Fusants after Interspecific Protoplast Fusion:

The interspecific protoplast fusion was carried out between *Lb. casei* subsp. *rhamnosus* No. 45 and *Lb. amylovorus* No. 40. The first parent, as mentioned before, was high lactic acid producer and lactose fermentative but could not hydrolyze starch. On the other hand, the second parent was low lactic acid producer and starch fermentative but could not ferment lactose.

Table (4): Lactic acid productivity of the parental strains and the created fusants out of four different *Lb. casei* subsp. *rhamnosus* protoplasts crosses.

Crosses	Parents and fusants No.	Lactic acid concentration (g/L)	Lactic acid % to higher parent	Lactic acid % to W.T.	Crosses	Parents and fusants No.	Lactic acid concentration (g/L)	Lactic acid % to higher parent	Lactic acid % to W.T.
	W.T.	87.5	-	100.00	Cross 3	$P_1 D_{32}$	48.7	100.00	55.66
Cross 1	$P_1 D_{33}$	110.0	100.00	125.71		P_2 40/6	27.2	55.85	31.09
	$P_2 D_{34}$	109.6	99.64	125.26		3-1	104.5	214.58	119.43
	1-1	111.9	101.73	127.89		3-2	103.9	213.35	118.74
	1-2	102.6	93.27	117.26		3-3	101.9	209.24	116.46
	1-3	115.7	105.18	132.23		3-4	103.5	212.53	118.29
	1-4	112.5	102.27	128.57		3-5	72.9	149.69	83.31
	1-5	116.4	105.82	133.03		3-6	89.0	182.75	101.71
	1-6	93.0	84.55	106.29		3-7	89.4	183.57	102.17
	1-7	112.2	102.00	128.23		3-8	90.8	186.45	103.77
	1-8	112.6	102.36	128.69		3-9	90.0	184.80	102.86
	1-9	118.2	107.45	135.09		3-10	94.7	164.46	108.23
Cross 2	$P_1 D_{33}$	110.0	100.00	125.71	Cross 4	P_1 W.T	87.5	100.00	100.00
	$P_2 D_1$	109.8	99.82	125.49		P_2 50/31	79.2	90.51	90.51
	2-1	116.4	105.82	133.03		4-1	92.7	105.94	105.94
	2-2	107.8	98.0	123.20		4-2	95.6	109.26	109.26
	2-3	99.3	90.27	113.49		4-3	95.0	108.57	108.57
	2-4	114.7	104.27	131.09		4-4	94.4	107.89	107.89
	2-5	111.5	101.36	127.43		4-5	94.1	107.54	107.54
	2-6	107.6	97.82	122.97		4-6	73.7	84.23	84.23
	2-7	114.3	103.91	130.63		4-7	87.0	99.43	99.43
	2-8	118.9	108.09	135.89		4-8	96.4	110.17	110.17
	2-9	115.0	104.55	131.43		4-9	95.7	109.37	109.37
	2-10	117.3	106.64	134.06		4-10	95.4	109.03	109.03
	2-11	114.7	104.27	131.09		4-11	98.2	112.23	112.23
						4-12	96.3	110.06	110.06
						4-13	96.3	110.06	110.06

Nine fusants were obtained from this cross and all of them exhibited resistance to both parental opposite antibiotics confirming the recombination evidence between the two genomes. The second evidence confirming the genetic recombination between the two genomes was detected from the results in Table (5) and Figure (1).

Data in Table (5) showed that only three fusants (Nos. 5-8, 5-3 and 5-9) proved to be higher lactic acid producer than the parental strain No. 45. The increasing percents were 0.57 (fusant No. 5-8); 2.86 (fusant No. 5-3) and 5.94 (fusant No. 5-9). All of the rest fusants exhibited lactic acid productivity lower than the parental strain No. 45 and so higher than the other parent (No. 40).

On the other hand, in Figure (1) the lytic zone of starch (indication of amylase production) was not observed by fusants Nos. 5-1 and 5-6. The rest of fusants produced amylase enzyme with variable degrees as recorded from the clear lytic zone around the bacterial growth.

Table (5): Lactic acid productivity and starch hydrolysis of regenerated fusants after interspecific protoplast fusion between *Lb. casei* subsp. *rhamnosus* No. 45 and *Lb. amylovorus* No. 40.

Parents and fusants No.	Lactic acid concentration (g/L)	Lactic acid % to higher parent (W.T.)	Starch lytic zone (mm)
P_1 original st. No. 45	87.5	100.00	00
P_2 original st. No. 40	00.4	00.46	17
5-1	77.8	88.91	00
5-2	79.4	90.74	16
5-3	90.0	102.86	15
5-4	86.8	99.20	14
5-5	77.8	88.91	17
5-6	83.2	95.09	00
5-7	81.4	93.03	16
5-8	88.0	100.57	13
5-9	92.7	105.94	12

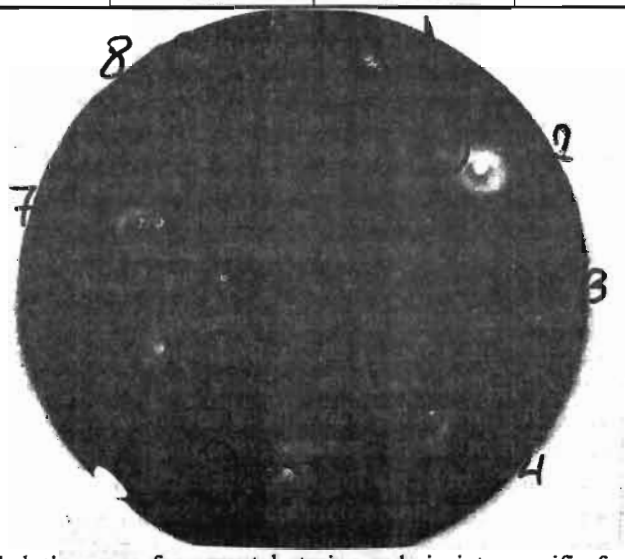

Fig. (1):Starch lytic zones for parental strains and six interspecific fusants (1) original strain No. 45, (2) original strain No. 40, (3) 5-1, (4) 5-2, (5) 5-3, (6) 5-4, (7) 5-5 and (8) 5-6.

Furthermore, data in Table (5) and Figure (1) showed also that the clear lytic zone of *Lb. amylovorus* No. 40 was 17 mm and all regenerated fusants showed either less than or approximately (5-2, 5-5 and 5-7 fusants) the same amylase activities to the *Lb. amylovorus* No. 40.

This study is the first in the application of interspecific protoplast fusion between *Lactobacillus casei* subsp. *rhamnosus* NRRL-445 and *Lb. amylovorus* NRRL-4540 but some authors used different species in interspecific protplast fusion. **Yeeh *et al.* (1996)** obtained eight fusants from the interspecific protoplast fusion between *Lb. acidophilus* (maltose and galactose fermentative but could not ferment lactose) and *Lb. casei* (lactose and galactose fermentative but could not be maltose fermentative). They found that, four fusants were able to ferment maltose, lactose and galactose, six fusants were generally greater in the abilities of lactic acid production than their parents and two fusants exhibited an increase in β-galactosidase activity up to 7.6-8% when compared to *Lb. acidophilus*. Whereas, other two fusants had increased phospho-β-galactosidase activity up to 137-200% when compared to *Lb. casei.*

Lactic acid productivity of fusants after intergeneric protoplast fusion:

According to **Khattab (2000)**, isolate No. D_{33} proved to be the most efficient isolate for lactic acid production out of all UV and MNNG treated isolates. In addition, the original strain No. 66 (*Streptococcus lactis* subsp. *cremoris*) proved to be the efficient one for bacteriocin and lactic acid production in comparison with the other original strain No. 55. Plotoplast suspensions from both genotypes were mixed and the fusants were isolated. Lactic acid and bacteriocine productivities of the obtained twenty fusants were determined and compared with the two parents under the same condition.

Data obtained from the intergeneric fusants of UV-isolate No. D33 and *Streptococcus lactis* subsp. *cremoris* No. 66 protoplasts were presented in Table (6).

Results presented in Table (6), showed that two fusants (Nos. 6-6 and 6-8) out of the twenty ones proved to be higher bacteriocin producers than the rest. They produced bacteriocin which induced inhibition zone (15 mm) approximately the same as the original strain No. 66 (16 mm).

On the other hand, no one of the obtained recombinant produced lactic acid more than the higher lactic acid producer parent (D_{33}), but all fusants produced lactic acid higher than the original strain No. 45 except two fusants (Nos. 6-8 and 6-12). The recombinant No. 6-7 proved to be the highest lactic acid producer one since it showed 23.43 percent production higher than the original strain (No. 45) and at the same time is considered as bacteriocin producer recombinant.

The above results showed that intergeneric protoplast fusion seemed to be also a valuable way to combine genes from different lactic acid bacteria genera since it resulted higher producer recombinants for both lactic acid and bacteriocin.

This study is the first in the application of intergeneric protoplast fusion between the isolate No. D_{33} which was descended from Lactobacillus *casei* subsp. *rhamnosus* NRRL-445 treated with UV-light and *Streptococcus lactis* subsp. *cremoris* No. 66 but some authors used other different genera in intergeneric protoplast fusion. **Cocconcelli *et al.* (1986)** suggested that, protoplast fusion can be used in intergeneric combination of different genes in lactic acid bacteria and as a method for the genetic manipulation and improvement of *Lactobacilli.* The isolated fusants hydrolyzed lactose and trehalose, also these fusants are resistant to erythromycin and grow at 42°C after intergeneric protoplast fusion between *Streptococcus lactis* SH4174 (lactose-negative, trehalose-positive, did not grow at 42°C and harboured erythromycin resistant plasmid pAMβ) and *Lactobacillus reuteri* DSM20016 (lactose-positive, trehalose-negative, grow at 42°C and erythromycin sensitive).

Moreover, **Chassy (1987)** reported that protoplast fusion has several potential advantages such as: obtaining the desired recombination without needing cloning vector. A cloned and specifically modified gene could be returned to that strain by fusion with the cloning host. Recombinational events would insert an altered copy of the gene in the target genome. The natural barriers to transformation such as nucleases and restriction system might be minimized during protoplast fusion.

The obtained results agreed with **Van der Lelie *et al.* (1988)** who demonstrated that the transfer of plasmids can be occurred between *Bacillus* sp. and *Lactococcus lactis* subsp. *cremoris* by protoplast fusion at frequencies ranging from 10^{-5} to 10^{-7} per recipient.

Plasmid Transformation and Lactic Acid Production:

-Transformation Detection

Six transformation experiments were conducted for this purpose. In each experiment, protoplasts from the recipients, i.e., the original strain 45 and five UV and MNNG treated isolates, i.e., D_{33}, 40/6, 30/32, 40/4 and 50/6 were prepared as mentioned in materials and methods. Furthermore, these recipient strains were tested for erythromycin sensitivity. The obtained results showed that all of these recipients were sensitive to 10 µg/ml erythromycin. Then 300 µg of plasmid DNA (pGIT032 *Ery*^{r}), which carried lactate dehydrogenase gene, and PEG were mixed with recipient protoplasts. After transformation, protoplasts were spread on the regeneration medium containing 10 µg/ml erythromycin as a selective marker. Results showed that a large number of transformants was obtained after each transformation experiment. These results confirmed the occurrence of transformation with the donor plasmid (pGIT032) which carried the erythromycin resistance gene. Only five transformants from each experiment (Table 7) were randomly selected to determine lactic acid productivity and plasmid isolation.

Table (6): Lactic acid and bacteriocin productivities of the intergeneric fusants of UV-isolate No. D_{33} and *Streptococcus lactis* subsp. *cremoris* No. 66 protoplasts.

Parents and fusants No.	Lactic acid concentration (g/L)	Lactic acid % to higher parent	Lactic acid % to W.T.	Inhibition Zone (mm)
W.T. (original st. No. 45)	87.5	79.55	100.00	00
P_1 D_{33}	110.0	100.00	125.71	00
P_2 (Original st. No. 66)	72.0	65.45	82.29	16
6-1	105.6	96.00	120.69	13
6-2	101.3	92.09	115.77	9
6-3	94.9	86.27	108.46	9
6-4	100.6	91.45	114.97	00
6-5	102.5	93.18	117.14	00
6-6	88.7	80.64	101.37	15
6-7	108.0	98.18	123.43	11
6-8	85.1	77.36	97.26	15
6-9	98.2	89.27	112.23	14
6-10	91.5	83.18	104.57	14
6-11	104.8	95.27	119.77	13
6-12	82.0	74.55	93.71	00
6-13	90.5	82.27	103.43	7
6-14	97.4	88.55	111.31	8
6-15	103.8	94.36	118.63	00
6-16	94.9	86.27	108.46	00
6-17	88.7	80.64	101.37	00
6-18	91.5	83.18	104.57	13
6-19	105.0	95.45	120.00	14
6-20	98.5	89.55	112.57	12

- Plasmid Profile of the Transformants and Their Parental Strains

Plasmid DNA was prepared from each of the donor, recipient and some transformant isolates. The plasmids number and size of transformants was examined against the plasmid profiles of both donor and recipient strains and λ DNA Hind III molecular weight marker. Two out of the six plasmid transformant groups could not produce any lactic acid as their recipient parents (40/4 and 50/6 isolates). Therefore, they were neglected from plasmid profile analysis.

Results in Figure (2) showed that four bands with approximately 23.1, 16.3, 9.4 and 6.7 kb were detected at the donor strain (lane 1) which containing only the plasmid pGIT032. Moreover, all of the *Lactobacilli* recipient strains exhibited six bands with approximately 23.1, 20.0, 6.9, 6.8, 4.0 and 3.8 Kb. On the other hand, all of the transformant isolates harbored 8 bands with approximately 23.1, 20.0, 16.3, 9.4, 6.9, 6.8, 4.0 and 3.8 kb. These results strongly confirmed the evidence of transformation by the donor plasmid pGIT032 where the

transformed isolates harbored two additional band with approximately 16.3 and 9.4 kb when compared was the recipient strains.

The above results were in agreement with those obtained by **Chassy and Flickinger (1987)**. They found that the electrophoretic mobility of the plasmid pNZ12 isolated from *Lb. casei* transformants was identical to that observed with plasmid preparations from *E. coli* when DNA plasmid was analyzed by agarose gel electrophoresis.

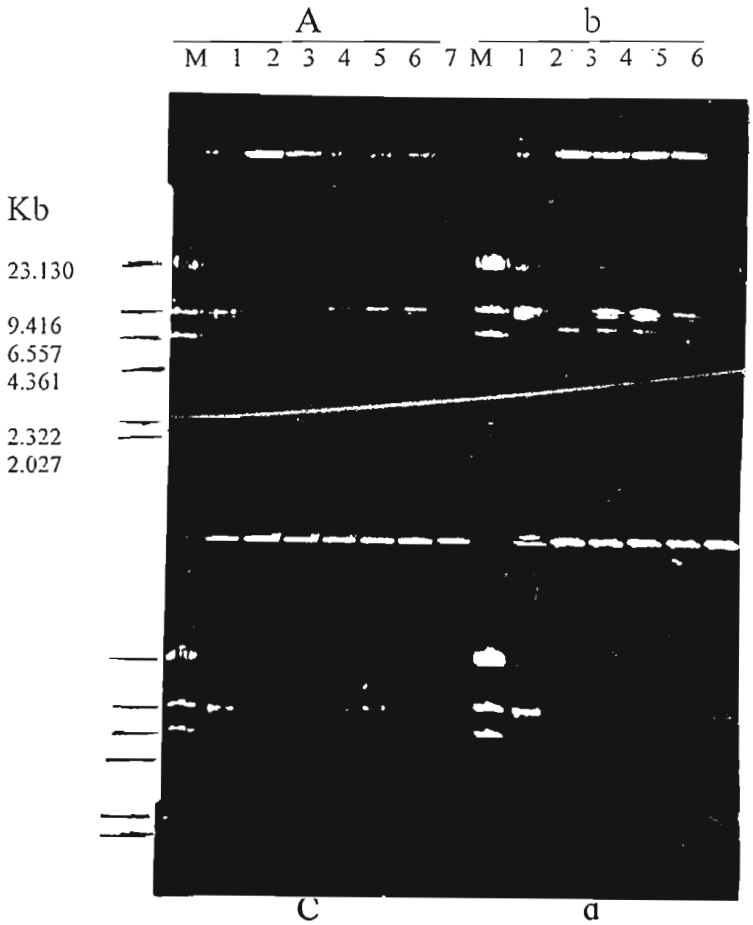

Fig. (2): Photograph of plasmid profiles of donor (lanes 1), recipients (lanes 2) and transformants: Lanes 3-6 (a) from recipient No. D_{33}, Lanes 3-5 (b) from recipient No. 30/32, Lanes 3-7 (c) from recipient (the original st. No. 45), and lanes 3-6 (d) from recipient No. 40/6 against 3. DNA Hind III marker (Lane M).'

- Lactic Acid Productivity of Transformants

Data in Table (7) presents the lactic acid productivity of the six recipient isolates and five created transformants derived from each as a result of plasmid acquiring.

Transformation experiment No. 1 was carried out using the high lactic acid producer isolate No. D_{33} as a recipient. Five transformants were selected from this experiment to determine their lactic acid efficiency. All transformants proved to be a little bit higher lactic acid producers than the recipient strain. The excellent transformant was T_{1-5} which produced 2.37 percent more lactic acid than the recipient strain and at the same time represents 28.34 percent more than the original strain No. 45.

The second transformation experiment involved the original strain No. 45; as a recipient and the donor plasmid pGlT032. All of the tested transformants produced lactic acid more than the recipient strain. The highest record of lactic acid production, i.e., 93.7 g/L was obtained from the transformant No. T_{2-3}which represents about 7% lactic acid more than the mother strain No. 45.

The third transformation experiment was conducted using the low lactic acid producer isolate No. 40/6 as a recipient. As shown in Table (7) the lactic acid productivity was improved. However, the production ranged from 90.2 g/L (transformant No. T_{3-5}) to 92.5 g/L (transformant No. T_{3-4}). Transformant No. T_{3-4} exceeded its parental recipient isolate in the production efficiency with more than three folds (3.40) and at the same time represents 5.71 percent more than the mother strain No. 45.

The fourth transformation experiment involved the transformation of isolate No. 30/32 which could not produce any amount of lactic acid. Results clearly showed that, the tested transformants produced higher lactic acid. Furthermore, the highest transformant, No. T_{4-3}, produced 4.23 percent lactic acid more than the original strain No. 45. Moreover, the lowest transformant, T_{4-5}, produced 83.2 g/L lactic acid which was only about 5% less than that of the original strain No. 45. Such results could be taken as an indication that the MNNG treated isolate No. 30/32 may be contain a suppression mechanism which blocked pathway of lactic acid production in a position where lactate dehydrogenase took place.

On the other hand, when each of the two isolates Nos. 40/4 and 50/6 were transformed with pGIT032, all of the tested transformants could not produce any amount of lactic acid. This result confirmed that the suppression of the biochemical lactic acid pathway of these two isolates occurred in any step before the biosynthesis of pyruvic acid.

Table (7): Lactic acid productivity of the recipient strains and the created transformants out of six different genetic transformation experiments of the original strain No. 45 and five isolates with pGIT032.

Transformation Experiment No.	Recipients and transformants No.	Lactic acid concentration (g/L)	Lactic acid % to recipient	Lactic acid % to W.T.
Original strain	W.T.	87.5	-	100.00
Transformation 1	D_{33}	109.7	100.00	125.37
	T_{1-1}	111.6	101.73	127.54
	T_{1-2}	111.4	101.55	127.31
	T_{1-3}	111.4	101.55	127.31
	T_{1-4}	111.8	101.91	127.77
	T_{1-5}	112.3	102.37	128.34
Transformation 2	45*	87.5	100.00	100.00
	T_{2-1}	92.5	105.71	105.71
	T_{2-2}	92.3	105.49	105.49
	T_{2-3}	93.7	107.09	107.09
	T_{2-4}	93.2	106.51	106.51
	T_{2-5}	93.6	106.97	106.97
Transformation 3	40/6	27.2	100.00	31.09
	T_{3-1}	90.3	331.99	103.20
	T_{3-2}	91.7	337.13	104.80
	T_{3-3}	92.0	338.24	105.14
	T_{3-4}	92.5	340.07	105.71
	T_{3-5}	90.2	331.62	103.09
Transformation 4	30/32	00.0	000.00	000.00
	T_{4-1}	85.9	∞	98.17
	T_{4-2}	89.5	∞	102.29
	T_{4-3}	91.2	∞	104.23
	T_{4-4}	84.3	∞	96.34
	T_{4-5}	83.2	∞	95.09
Transformation 5	40/4	0	0	0
	T5-1 to T5-5	0	0	0
Transformation 6	50/6	0	0	0
	T6-1 to T6-5	0	0	0

* Original strain No. 45

The obtained results also were in agreement with those obtained by **Harlander *et al.* (1984)** since they found that, after transformation of *Streptococcus sanguis* (*Lac*$^-$) with plasmid containing lactose metabolizing gene, the obtained transformants conferred the ability to ferment lactose into lactic acid and erythromycin resistant. Moreover, **Porro *et al.* (1999)** reported results with good harmony of the obtained results, since, they obtained a yield of lactic acid as high as 109 g/L after introducing lactate dehydrogenase gene into *Kluyveromyces lactis* yeast strain in which the unique pyruvate decrarboxylase gene had been deleted. On the other hand, the obtained results were in disagreement with those obtained by **Ferain *et al.* (1994)** since they used plasmid

pGIT032 for the transformation of *Lb. plantarum* DG301. They found that lactate dehydrogenase activity increased up to 13 folds through this gene dosage effect after transformation. These differences may be due to the different transformed genetic background. In addition, they did not use the specific medium for fermentation during lactic acid determination of transformant strains. They used MRS medium, so they could not observe any change in lactic acid production capacities.

Random Amplified Polymorphic DNA (RAPD) Analysis

The genetic effects of protoplast fusing on the DNA nucleotide sequence of the obtained fusants was evaluated in comparison with the original strain *Lb. casei* subsp. *rhomnosus* No. 45. The effect of the plasmid transformation by pGIT032 on the DNA nucleotide sequence was also considered. Table (2) presents the six random 15- mer primers used in this study. Lactic acid productivity of the selected fusants and transformants were presented in Table (8). Bands sizes were detected against 100 bp ladder marker Lane (M).

Table (8): Lactic acid productivity and pedigree of the selected fusants and transformants for molecular analysis.

Fusants			Transformants		
Fusant No. and parents	Lactic acid productivity (g/L)	Lactic acid % to W.T.	Transformant No. and Recipients	Lactic acid productivity (g/L)	Lactic acid % to W.T.
W.T	87.5	100	W.T.	87.5	100
1-9 (D_{33} x D_{34})	118.2	135.09	T1-4 (D_{33})	111.8	127.77
2-8 (D_{33} x D_1)	118.9	135.89	T1-5 (D_{33})	112.3	128.34
3-5 (D_{32} x 40/6)	72.9	83.31	T4-4 (30/32)	84.3	96.34
4-6 (W.T. x 50/31)	73.7	84.23	T4-5 (30/32)	83.2	95.09

Figure 3-I and II presents the random amplified banding patterns of the original strain No. 45 (lane 1), four selected fusants (lanes 2-5) and four selected plasmid transformants (lanes 6-9) as result of using primer No. 1 (upper-I) and primer No. 2 (lower-II).

With regard to use primer No. 1, all the four fusants DNA exhibited a common band with size of 1900 bp as in the original strain No. 45. Moreover, each fusant presents different polymorphic banding pattern since four, four, three and four bands with different sizes were detected for fusants Nos. 1-9, 2-8, 3-5 and 4-6, respectively. In spite of the fact that a common parent (the high producer D_{33} isolate) was used in the first two fusants (1-9 and 2-8), however, each of them exhibited completely different sizes of the amplified bands. Meanwhile both of these fusants exceeded the original strain with about 35% of lactic acid.

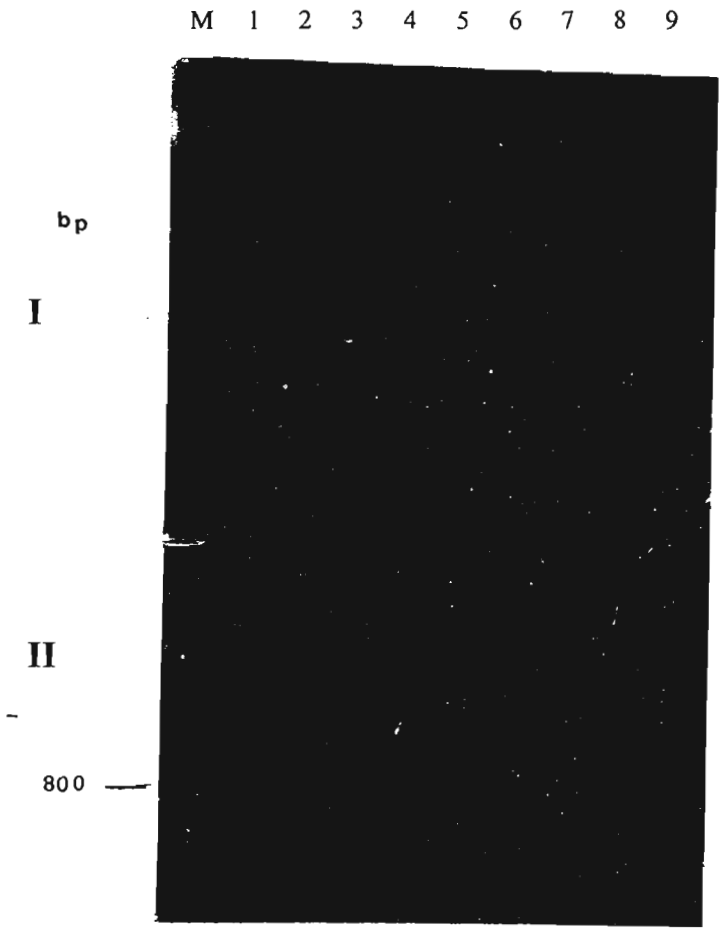

Fig. (3): Photograph of DNA amplified banding patterns based on RAPD eight recombinants against original strain No. 45 (Lane1) and 100 bp ladder marker(Lane M)using primer No.1(Upper) and No.2 (Lower). Recombinants sequence (Lanes 2 to 9), 1-9, 2-8, 3-5, 4-6, $T1_{-4}$, T_{1-5}, T_{4-4}, T_{4-5}.

On the other hand, the four plasmid transformants showed two polymorphic banding patterns. The transformants Nos. T_{1-4} and T_{1-5} can be grouped together since both of them exhibited identical four amplified bands. The other two transformants (T_{4-4} and T_{4-5}) showed identical three amplified bands. These results confirmed the plasmid transformation process since each constructed transformant group descended form the same recipient isolate. Moreover, each group produced nearly the same amount of lactic acid.

The application of primer No. 2 against the four fusants induced different polymorphic banding patterns for the first three ones, i.e., 1-9, 2-8 and 3-5. On contrary no amplified bands could be obtained when the DNA of the fusant No. 4-6 was used as a template (lane II-5); although its parents were the W.T. strain and 50/31 isolates. The plasmid transformants showed the same trend as following using primer No. 1 since transformants T_{1-4} and T_{1-5} exhibited six identical amplified bands and Nos. T_{4-4} and T_{4-5} DNA contained only three regions complement the primer No. 2 sequence. It was noticeable that the three bands of the second group were found with the exact sizes among the first group bands.

Using primers 3 and 4 against the selected fusants (Figure 4-I and II, lanes 2-5) exhibited extreme different random amplified banding patterns between all of the tested fusants from one side and their original parental strain from the other side. On contrary, although the same trend occurred between the tested transformants and the original strain, however, each two transformants descended from the same recipient showed almost identical bands for numbers, sizes and intensities.

As a result of primers Nos. 5 and 6 application (Fig 5-I and 5-II), the obtained banding patterns indicated that, three bands were detected following the application of each primer against the original strain with sizes of 1400, 1200 and 1000 bp for primer No. 5 and 1300, 1100 and 500 bp for primer No. 6.Figure 5-I and II exhibited different polymorphic banding patterns between all of the tested fusants and transformants in comparison with the original strain. Meanwhile both two groups of the transformants behaved identically with each primer either primer No. 5 or primer No. 6.

Generally, out of all previous random amplification polymorphic banding patterns of the four protoplast fusants on the level of intraspecific as well as the four transformants exhibited quite different polymorphism manner than the original strain against all of the applied 15-mer random primers. Moreover, out of these results no exact correlation could be pointed the lactic acid productivities from the other one between primers sequences and repeated complementary sequences on the templates from one side and the lactic acid productivities from the other side. The above differences in RAPD profile confirmed the evidence of genetic variation in genomes after the protoplast fusion and transformation with pGIT032. Furthermore, some of these differences, based on RAPD analyses, could be used as genetic markers for genetic diversity of lactic acid production characteristics.

The obtained results showed excellent harmony with those reported by **Ward *et al.* (1993)**. They demonstrated that intraspecific protoplast fusion induced rearrangements of the genetic material in

Lactococcus lactis subsp. *cremoris*. Moreover, **Thompson *et al.* (1997)** studied the molecular analysis of antibiotic mutation by RAPD-PCR analysis in *Lactobacillus plantarum* and they found some differences and similarities in RAPD profile between streptomycin and other mutants. On the other hand, **Urbach *et al.* (1998)** suggested that the genetic variation among closely related strains could be detected by RAPD-PCR technique.

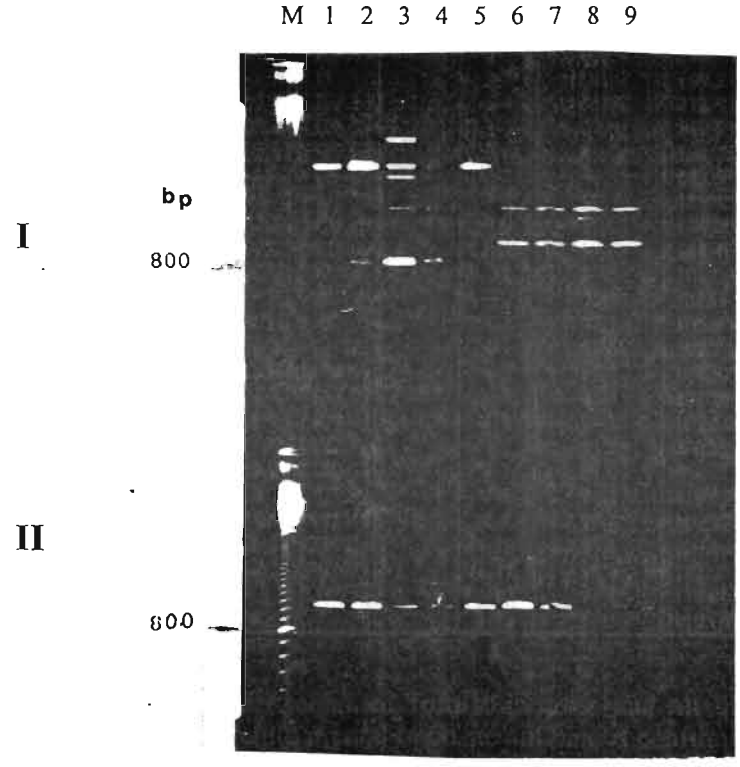

Fig. (4): Photographs of DNA amplified banding patterns based on RAPD eight recombinants against original strain (Lane 2) and 100 bp ladder marker (Lane M) using primers No. 3 (upper) and No. 4 (lower). Recombinants sequence (Lane 2 to 9), 1-9, 2-8, 3-5, 4-6, T_{1-4}, T_{1-5}, T_{4-4}, T_{4-5}.

Sequence Characterized Amplified Region (SCAR) analysis

Genetic determination of some regions of lactose operon encode, the tagatose-6-phosphate kinase, tagatose 1, 6-diphosphate aldolase, enzyme II^{lac} and part of phospho-β-galactosidase, was carried out by sequence characterized amplified region (SCAR)analysis of the parental strains and some fusants obtained after intergeneric protoplast fusion.

The first SCAR analysis was performed using specific primers Nos. 7 and 8 listed in Table (2) and the results were shown in Figure (6). This figure represented a photograph of amplified DNA band based on

SCAR analysis for parental strains [D_{33} (UV-treated isolate derived from *Lb. casei* subsp. *rhamnosus* NRRL-445) and original strain No. 66 (*Streptococcus lactis* subsp. *cremoris* 2)] and 12 fusants obtained after intergeneric protoplast fusion. The band size was detected against 100 bp ladder marker from Amersham Pharmacia Biotech. (Lan M).

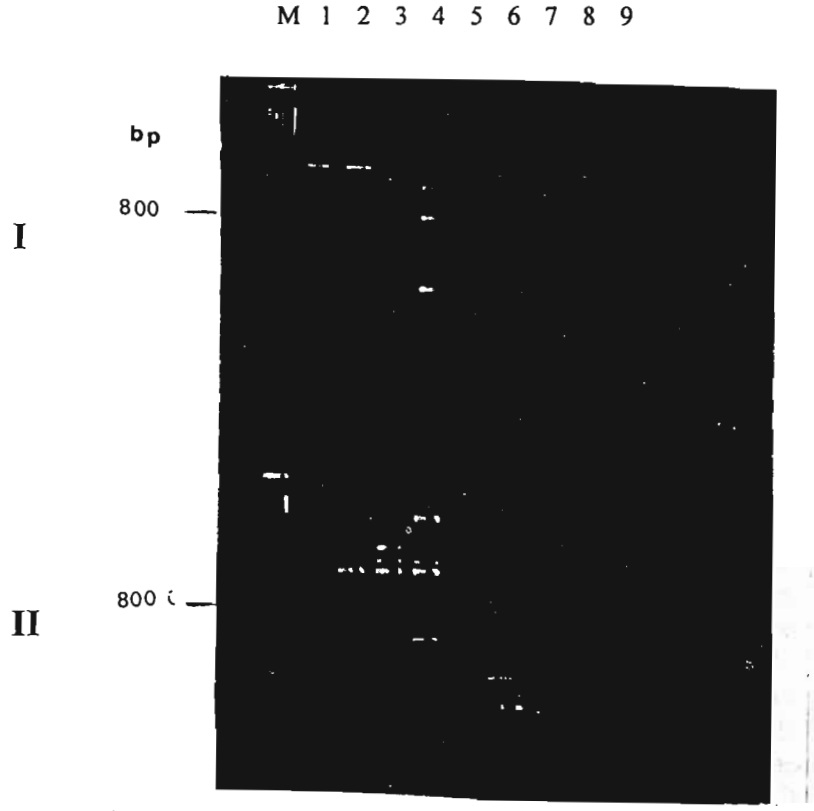

Fig. (5): Photographs of DNA amplified banding patterns based on RAPD eight recombinants against original strain No. 45 (Lane 2) and 100 bp ladder marker (Lane M) using 15-mer random primers No. 5 (upper) and No. 6 (lower). Recombinants sequence (Lane 2 to 9) 1-9, 2-8, 3-5, 4-6, T_{1-4}, T_{1-5}, T_{4-4}, T_{4-5}.

Results in Figure (6) showed that the amplified band with 2000 bp was occurred in the high lactic acid producer parent D_{33}. On the contrary, this band could not be detected in the second parent (the original strain No. 66). On the other hand, the amplified band with 2000 bp was also detected in only two fusants Nos. 6-6 and 6-11 out of 12

tested fusants. Fusants No. 6-6 produced 88.7 g/L lactic acid and the inhibition zone of bacteriocin was 15 mm. While fusant No. 6-11 produced 104.8 g/L lactic acid and the inhibition zone of bacteriocin was 13 mm.

The above results showed that, the high lactic acid producer parent No. D_{33} contained DNA sequences that could be amplified using specific primers Nos. 7 and 8 for tagatose 6-phosphate kinase and tagatose 1, 6 diphosphate aldolase genes. On the other hand, the original strain No. 66 did not contain the DNA sequence which can complement these two specific primers. The obtained results confirmed the occurrence of gene combination in association with intergeneric protoplast fusion since fusants Nos. 6-6 and 6-11 contained an amplified band with 2000 bp and at the same time produced bacteriocin. The above results were in agreement with **McCormick *et al.* (1995)**. They used the same primers as in the present study, i.e., Nos. 7 and 8 to amplify the tagatose-6-phosphate kinase and tagatose 1,6-diphosphate aldolase and they obtained an amplified band with 2000 bp.

On the other hand, no amplification could be detected using the specific primers Nos. 9 and 10 with either the parental strains or the tested fusants obtained after intergeneric fusion although the original strains Nos. 45 and 66 were strongly lactose fermentors. Moreover, in spite of the fact that all specific primers were designed for *Lactococcus lactis* genome according to **McCormick *et al.* (1995)**, the two specific primers Nos. 9 and 10 could not complement with the DNA sequences neither in the parental strains nor any other fusants. These may be due to any change (s) in the nucleotide sequence of their genomes that prevented the annealing with these primers.

In spite of the extensive examination of the literature about the genetic determination of the genes associated with tagatose pathway in *Lb. casei* subsp. *rhamnosus*; no published data could be detected to be deal with this point. So, this study may be considered as the first novel one to act on some of the tagatose pathway genes using isolate No. D_{33} derived from *Lb. casei* subsp. *rhamnosus* NRRL B-445

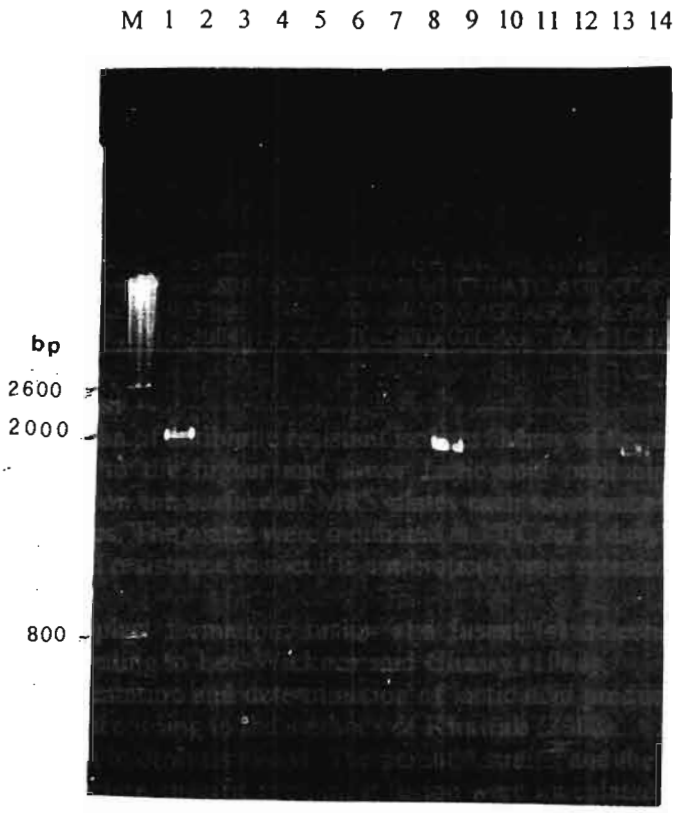

Fig. (6): Photograph of DNA amplifie d based on SCAR for parental strains and 12 fusants obtained after intergeneric protoplast fusion against 100 bp ladder marker (Lane M). Lane 1 (D_{33}), Lane 2 (Original strain No. 66), Lanes 3 to 14 (fusants 6-1 to 6-12)

REFERENCES

Anderson, D.G. and L.L. McKay (1983). Simple and rapid method for isolating large plasmid DNA from lactic *Streptococci*. Appl. Environ. Microbiol., 46: 549-552.

Chassy, M.B. (1987). Prospects for the genetic manipulation of *Lactobacilli*. FEMS Microbiol. Lett., 46: 297- 312.

Chassy, M.B. and J.L. Flickinger (1987). Transformation of *Lactobacillus casei* by electroporation. FEMS Microbiol. Lett., 44: 173-177.

Cocconcelli, P.S.; L. Morelli; M. Vescovo and V. Bottazzi (1986). Intergeneric protoplast fusion in lactic acid bacteria. FEMS Microbiol. Lett., 35: 211-214.

Curragh, H.J. and M.A. Collins (1992). High level of spontaneous drug resistance in *Lactobacillus*. J. Appl. Bacteriol., 73: 31-36.

Efthymiou, C. and C.A. Hansen (1962). An antigenic analysis of *Lactobacillus acidophilus*. J. Infectious Diseases, 110: 258-267.

Ferain, T.; D. Garmyn; N. Bernard; P. Hols and J. Delcour (1994). *Lactobacillus plantarum* Ldhl gene: Overexpression and deletion. J. Bacteriol., 176: 596-601.

Fortina, M.G.; C. Parini and P.L. Manachini (1990). Genotypic and phenotypic correlationships among some strains of *Lactobacillus helveticus*. Biotechnol. Lett., 12: 765-770.

Gasson, M.J. (1980). Production, regeneration and fusion of protoplasts in lactis *Streptococci*. FEMS Microbiol. Lett., 9: 99-102.

Harlander, S.K.; L.L. McKay and C.F. Schachtele (1984). Molecular cloning of the lactose-metabolizing genes from *Streptococcus lactis*. Appl. Environ. Microbiol., 48: 347-351.

Iwata, M.; M. Mada and H. Ishiwa (1986). Protoplast fusion of *Lactobacillus fermentum*. Appl. Envrion. Microbiol., 52: 392-393.

Jane, H.C. and D.C. savage (1986). Genetic transformation of rifampicin resistance in *Lactobacillus acidophilus*. J. Gen. Microbiol.,132:2107-2111.

Kanatani, K.; K. Yoshida; T. Tahara; M. Sakamoto and M. Oshimura (1990). Intraspecific protoplast fusion of *Lactobacillus plantarum*. Agric. Biol. Chem., 54: 225-227.

Khattab, Abd El-Nasser (2002). Molecular and biochemical studies of genetically constructed lactic acid bacteria. Ph. D., Genetic Dept., Faculty of Agriculture, Kafr El-Sheikh, Tanta Univ., Egypt.

Kim, W.S.; R.J. Hall and N.W. Dunn (1997). The effect of nisin concentration and nutrient depletion on nisin production of Lactococcus lactis. Appl. Microbiol. Biotechnol., 48: 449-453.

Lee-Wickner, L.J. and M.B. Chassy (1984). Production and regeneration of *Lactobacillus casei* protoplasts. Appl. Environ. Microbiol, 48: 995-1000.

McCormick, C.A.; H.G. Griffin and M.J. Gasson (1995). Construction of a food-grade host/vector system for *Lactococcus lactis* based on the lactose operon. FEMS Microbiol., Lett., 127: 105-109.

Porro, D.; M.M. Bionchi; L. Brambilla; R. Menghini; D. Bolzani; V. Carrera; J. Lievense; C.L. Liu; B.M. Ronzi; L. Frontali and L. Alberghina (1999). Replacement of a metabolic pathway for large-scale production of lactic acid from engineered yeasts. Appl. Environ. Microbiol., 65: 4211-4215.

Qiagen (1997). Taq PCR handbook for Taq DNA polymerase, Tag core kit and Taq PCR master Mix Kit p. 21.

Rincon, J.; J. Fuertes; A. Moya; J.M. Mondeagudo and L. Rodriguez (1993). Optimization of the fermentation of whey by *Lactobacillus casei*. Acta Biotechnol., 13: 323-331.

Sambrook, J.; E.F. Fritsch and Maniatis (1989). Molecular cloning; A laboratory Manual, Second Edition Cold Spring Harbor.

Simon, D.; A. Rouault and M.C.Chopin (1985). Protoplast transformation of group N *Streptococci* with cryptic plasmids. FEMS Microbiol. Lett., 26: 239-241.

Thompson, J.K.; K.J. Mc Conville; C. Mc Reynolds; S.D. Moorhouse and M.A. Collins (1997). Mutation to antibiotic resistance occur during stationary phase in *Lactobacillus plantarum* ATCC 8014. Microbiology, 143: 1941-1949.

Tilsala-Timisjarvi, A. and T. Alatossava (1998). Strain-specific identification of probiotic *Lactobacillus rhamnosus* with randomly amplified polymorphic DNA derived PCR primers. Appl. Environ. Microbiol., 64: 4816-4819.

Urbach, E.; C. Schindler and S. Giovannoni (1998). A PCR fingerprinting technique to distinguish isolates of *Lactococcus lactis*. FEMS Microbiol. Lett., 162: 111-115..

Van der Lelie, D.; J.M.B.M. Van der Vossen and G. Venema (1988). Effect of plasmid incompatibility on DNA transfer to *Streptococcus cremoris*. Appl. Environ. Microbiol., 54: 865-871.

Ward, L.J.H.; J.C.S. Brown and G.P. Davey (1993). Insertion sequence analysis of protoplast fused strains of *Lactococcus lactis* sp. *cremoris*. FEMS Microbiol. Lett., 113: 315-320.

Williams, J.G.K.; A.R. Kubelik; K.J. Livak; J.A. Rafalshi and S.V. Tingey (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic marker. Nucl. Acids Res., 18: 6531-6535.

Yeeh, Y.K; Y.B. Jo and O.C. Kwon (1996). Protoplast fusion between *Lactobacillus casei* and *Lactobacillus acidophilus*. Biotechnol. Lett., 18: 805-808.

إستخدام تفاعل البلمره المتسلسل العشوائى والمتخصص لتقييم بعض المندمجات والمتحولات البلازميدية المعدله وراثياً لإنتاج حمض اللاكتيك

جمعه الفاضلى*؛ كريمه محمد**؛ على عبده اسماعيل**؛
على أبوشوشه*؛ عبدالناصر خطاب**

* قسم الوراثة – كلية الزراعة – كفرالشيخ – جامعة طنطا – مصر
** معمل الوراثة الميكروبية التطبيقية – المركز القومى للبحوث – الدقى – جيزه – مصر

تهدف هذه الدراسة إلى الحصول على سلالات بكتيرية ذات كفاءة عالية فى إنتاج حمض اللاكتيك وإدخال صفات وراثية جديدة عن طريق الدمج الخلوى والتحول الوراثى بالإضافة إلى أجراء تقييم وراثى على المستوى الجزيئ لبعض هذه التراكيب الوراثية الجديدة
وتشير أهم النتائج المتحصل عليها إلى ما يلى :

١. تم إجراء أربعة تهجينات على مستوى الدمج الخلوى تحت النوع لعزلات مختلفة الإنتاجية (عالية ــ متوسطة ــ ومنخفضة) باستخدام المضادات الحيوية كعلامات وراثية لعزل المندمجات الخلوية حيث ارتفعت نسبة الزيادة فى حمض اللاكتيك إلى أكثر من ٣٥% مقارنة بالسلالة الأصلية .

٢. عند التهجين على مستوى الدمج الخلوى بين الأنواع بين السلالة الأصلية *Lb.casei* (قادرة على تخمير اللاكتوز وغير قادرة على تخمير النشا) والسلالة *Lb.amylovorus* (قادرة على تحليل النشا وغير قادرة على تخمير اللاكتوز) تم الحصول على تسع مندمجات خلوية معظمها له القدرة فى ذات الوقت على تخمير اللاكتوز وإنتاج الاميليز المسئول عن تحليل النشا والاستفادة منه كمصدر للكربون.

٣. عند إجراء التهجين على مستوى الدمج الخلوى بين الأجناس بين العزلة رقم D_{33} (مرتفعة الإنتاجية لحمض اللاكتيك وغير منتجة للبكتريوسين) والسلالة *S. lactis* subsp. *cremoris* (منخفضة الإنتاجية لحمض اللاكتيك ومنتجة للبكتريوسين) تم الحصول على ٢٠ من المندمجات الخلوية معظمها قادر على إنتاج حمض اللاكتيك والبكتريوسين فى ذات الوقت .

٤. تم إجراء ست تجارب مختلفة للتحول الوراثى باستخدام بلازميد pGIT032 والحامل لجين اللاكتات دهيدروجينيز حيث بلغت نسبة التحسين في إنتاجية حمض اللاكتيك ٧% فى المتحولة الوراثية T_{2-3} وقد تبين من عزل البلازميد للمتحولات الوراثية أنها تحتوى على البلازميد pGIT032 بالإضافة إلى المحتوى البلازميدى للسلالات المستقبلة .

٥. عند إجراء تفاعل البلمرة المتسلسل العشوائى لاربع مندمجات خلوية وأربع متحولات وراثية أوضحت النتائج وجود العديد من الاختلافات في الحزم المتضاعفة بين السلالات المعدلة وراثيا والسلالة الأصلية كما تم إجراء تفاعل البلمرة المتسلسل المتخصص باستخدام أربعة بوادئ متخصصة لبعض الجينات فى أوبرون اللاكتوز وذلك للآباء والسلالات المعدلة وراثيا الناتجة عن الدمج الخلوى بين الأجناس حيث أوضحت النتائج ان إثنين من المندمجات الخلوية أعطت حزمة متضاعفة وزنها ٢٠٠٠ زوج من القواعد باستخدام البوادئ المتخصصة ارقام ٧ &٨ مما يثبت حدوث نقل جينى متخصص بعد الدمج الخلوى.