J. Agric. Sci. Mansoura Univ., 32 (10): 8273 - 8286, 2007

GENETIC IMPROVEMENT OF *Lactobacillus casei* SUBSP *Casei* via PROTOPLAST FUSION

Sharaf, O.M.; Nivien A. Absoereh*; Azzat B. Abd-El-Khalak and Kawther El-Shafei

Dairy Department, * Microbial Genetic Department, Genetic Engineering and Biotechnology Division, National Research Centre, Cairo, Egypt.

ABSTRACT

Protoplast fusion has been used to combine genes from different organisms to develop strains with desired properties. This is a powerful technique in engineering microbial strains for desirable industrial properties. This study describe the successful use of protoplast fusion, using two auxotrophic strains of *Lactobacillus casei subsp. Casei & Entreococcus faecium*, Protrophic strains were obtained as a result of recombination in fused protoplasts at frequencies of 0.06-0.6% based on the number of protoplasts subjected fusion. The use of this technique resulted in novel strain of *Lactobacillus casei spp. Casei* that tolerate 8% NaCl. The effect of novel *L.casei subsp. Casei* on quality and properties of Domiati cheese during storage at room temperature for 45 days were studied. Moisture, (59.78, 56.12, 54.93, 53.30 and 52.60) and total nitrogen content was decreased, while soluble nitrogen (8.4, 10.72, 14.34, 20.21 and 23.60) contents increased during storage. total count of new *Lb.casei spp. Casei* was more than 10^9 cfu/g through storage period. Cheese manufacture from milk with adding new *Lb. casei spp*. casei as a starter to enhance flavor intensity of the resultant cheese (after 45 days) more pronounced if compared with that of the control cheese in all pickling periods.

Keywords: Protoplast fusion, *Lactobacilus*. *casei*, *Enterococcus faecium*, Domiati cheese

INTRODUCTION

Domiati cheese is the most popular soft cheese in Egypt. It is consumed after pickling in its salted whey for a period of 4 months. The fresh cheese is mild but at 12 months or order the cheese has a pungent flavor due to the proteolytic and lipolytic activities. Domiati cheese is traditionally made from milk salted with 5-15% Nacl (ElAbed et al,,2003 and Abo Donia 1986).

Sodium chloride is important ingredient of cheese which exerts a major influence on its composition, micro flora, ripening, texture, flavor and quality (Salem and Abied 1997). Therefore, enterococci genus was predominated, 95% of the cocci isolated from Egyptian Domiati cheese. The dominance or persistence of enterococcus in some cheeses during ripening can be attributed to their wide range of growth temperature, their high tolerance to heat, salt and acid (Wessels *et al* 1990 and Bahy El-Din *et al* 2002). Enterococci play an important role in ripening of traditional cheese, probably through proteolysis, lipolysis, and citrate breakdown, hence contributing to their typical taste and flavour (Manolopoulou *et al* 2003 and Moreno *et al* 2002)

For many years, scientists in the dairy field have tried to shorten the maturation period and reduce the inherent costs, as well as to enhance

flavour intensity, for various cheeses. So far, lipolytic and proteolytic enzymes have been used extensively in the dairy industries, for hydrolysis of milk fat with the purpose of flavor enhancement or acceleration of ripening processes in cheeses and cheese-related products, but bitterness and rancidity have precluded their use on a large scale.(Nelson *et al* 1997).

Ripening of cheese is a series of complex biochemical processes necessary for the development of cheese flavor and texture (Madkor et al 2000).During cheese manufacture, the initiation of the fermentation process begins at the addition of starter cultures to milk. However, the presence of starter cultures is not sufficient to explain flavor formation in raw milk cheese (Mday and Casey,2004).In addition to starter bacteria a number of other proteolytic and lipolytic agents in cheese contribute to cheese ripening including milk enzymes, and adjuncts non-starter lactic acid bacteria consisting mainly of *Lactobacillus* (Madkor *et al* 2000).After 4 to 6 week. with the concomitant increase of non-starter LAB such as *Lactobacills casei* reaching levels of 106to 107 cfu/g cheese. (Fenster *et a1*2003, Kieronczyk *et al* 2003 and Madey and casey 2004).

Lb.casei was predominated in good quality of mutant cheddar cheese and play a fundamental role in the development of organoleptic characteristic of fresh white cheese. (Shin *et al* 2004 and Dpesic and Novonovic 2005). Many others reported that *Lb.casei* has the peptidolytic, esterolytic, and proteolytic activity (Chio *et a1* 2004,Roy *et a1* 1997, and Shin et al 2004).

Most of the genetic discoveries in bacteria were done on only two microorganisms, *Escherichia coli* and *Bacillus subtilis.* A new start in the genetics of lactic acid bacteria, especially those who are beneficial for human, came from the possibility to produce and regenerate protoplasts and finally" transform protoplast(Steidler, 2003).

Methods in improving microbial strains have relied upon either mutagenesis followed by selection to improve properties, or manipulation of specific gene known to play an important role in the desired phenotype. Other reports have described the successful use of protoplast fusion (Without the initial mutagenesis step) to combine metabolic capabilities of two different organisms, for example, protoplast fusion of *Acietobacter* sp.A3 and *Pseudomonas putida* OP gg have been fused to generate strains with enhanced abilities to degrade hydrocarbons (Hanson and Desai, 1996), and protoplasts of *Kluyveromyces* SR Y -85 and *Saccharomyces cerevisiae* E-15 have been fused to generate strains with enhanced ability to produce sorbitol under fermentation conditions (Wei *et al*. 2001).

Genome shuffling is a recently introduced method that is much more efficient for evolution strains with desirable phenotypes. It involves generation of a heterogeneous population of mutants by treatment with biochemical mutagen, followed by recursive fusion of protoplass to allow recombination. Recent reports have described the use of genome shuffling to improve the production of tyrosine by *Streptomyces fradiae* (Zhang *et al* 2002) and acid tolerance of Lactobacillus (Patraik *et al*. 2002) Dai and Copley 2004).

This study recently reported that experiments designed to identify optimal conditions for protoplast regeneration and determined the degree of

heat which is good to see regeneration at 37°C. After protoplast fusion between two strains; *Lb.casei, En.faecium*, the new strain which grow in (ard,leu) could tolerate 9% of Na Cl and recorded 70-80 the survival percentage.

This investigation aimed to induce new lactobacillus strain carrying important genes related to tolerate high concentration of NaCl via genetic techniques to be using it for shorten the pickling period and improve the flavor and quality of Domiati cheese.

MATERIALS AND METHODS

Bacterial strains

Enterococcus feacium was isolated in Dairy Microbiology Lab., Dairy Dept., National Research Center, Egypt (El-Shafei *et al.,* 2002). *Lactobacillus casei* spp. *casei, Lactococcus lactis*, *Streptococcus thermophilus* and *lactobacillus bulgaricus* were kindly obtained from Chr. Hansen, S Lab, Denmark. All strains were subcultured and prepared in Elliker broth and sterilized skim milk, *En. Feacium* and *L. casei* spp. *casei* were used as a wild type.

Mutagensis

In this study MRS medium supplemented with either arginine or leucine (100g/ml) and a DV lamp was used as a mutagenic agent in both strains of *Lb. casei* spp. casei & *E. feacium* Auxotrophic mutant were isolated mutant and nutrition requirements using minimal media supplemented with different requirements were used.

Protoplast Formation

Protoplasts were formed by modification of a previously reported method (Weiss,1976) the buffer which used in thise method (EDTA,0.IM,Ph8.0), SMM buffer (0.5 sucrose, 20m M sodium maleate, 20m M Mgc12 pH6.5) and lysozyme (1mg 1ml) .

Protoplast Regeneration

Several conditions for regeneration of protoplasts were explored. The MRS containing 0.5 M sucrose and either arg (100mg/ml) or Leu (100mg) ml) autoclaved plastic spreaders. The plates were subsequently incubated at either 2.5 or 37°C.

Protoplast Fusion

Protoplasts were fused using a modification of Previously reported methods (Dai and Capley, 2004). 0-5 ml of protoplast formed of each auxotroph 1 ml of DNase of (promega was added + 0 digest DNA released from cells that had lysed during protoplast formation and then SMM buffer containing (40% v/v PEG 6000, 10m M CaCl2 and 5% v/v DMSo) was added.

PHYSIOLOGICAL AND BIOCHEMICAL REACTION

The determination of new gene's was performed according to morphological, physiological and biochemical characteristics as described by Mund (1986) &Kandler and Weiss (1986). The two procedure, wild type and new strains were tested for cell morphology, gram reaction and catalase formation.

Growth at 15 ,37 ,and 45 °C in sterilized skim milk (11 %) were observed from 24 -48 hr at 37 C. Growth at the presence of 4,6.5 and 9% NaCl and gas production from glucose were performed using modified Elliker broth (Guessas and Kihal 2004) . The fermentation of arabinose, cellobise manitol and raffinose (1 %w/v) was described by Nair and Surendran (2005) . The resistance of 3% Bile salt and pH 9.6 were tested according to El-Shafei *et al* 2002).

CHEESE PREPARATION

Fresh Buffaloes milk was heated to 72°C for ~30 sec. then rapidly cooled to 37C, calcium chloride and sodium chloride were added at the rate of 0.02% and 6% respectively. Milk was divided into three equal parts, the first was control (C) which inoculated with 1% of yoghurt culture *(Lb. bulgaricus* and *Str.thermophilus)*, the second part inoculated with 0.5% of Lb. casei spp. Casei + 0.5% *Laclactis* .
(A), and the third part was also inoculated by O. 5
Lac./aclis and O. 5 from new strain (B).
Domiate cheese was manufacture from each Partition by conventional method of Fahmi and Sharara . (1950)

The obtained cheeses were pickled in their respective whey, and kept at room temp. (20°C). Samples were CHEMICALLY, microbiologically and sensory evaluation analyzed when fresh and after 7, 15, 30,and 45 days. All treatments were canied out in triplicates.

CHESSE ANALYSIS

Chemical analysis

Cheese samples were analyzed for total Nitrogen (T.N) and soluble Nitrogen(S.N), according to Ling (1963). Total volatile fatty acid (TVFA) were determined as described by Kosikowski (1982).

Microbiological analysis

The total Lactic acid bacteria was counted on Elliker agar. The behaviour of new strain and *Lb. casei* during cheese storage was determined in L.C. agar(Shah 2000)

Organaleptic evaluation

Domiati cheese samples were organoleptically assessed by 15 panel Members of the experts at Dairy Science Dept., National Research Center, for flavors (60). Body and texture (30), appearance (10).

RESULT AND DISCUSSION

Initial experiments were done to optimize conditions for formation and regeneration of protoplasts with the two strains (*Lactobacillus casei* spp. *casei (arg) and Enterococcus faecium*)(leu-) to be used in protoplast fusion experiments. Two protocol for formation of protoplasts has used. One protocol was a modification of the method done by Weiss (1979).

Table (1) summarizes the results of experiments designed to identify optimal- condations for protoplast regeneration, it has been found that

protoplast regeneration is slightly more efficient at 25°C than at 37°C. However, exposure to melten top agar at 45°C, a commonly used procedure for spreading protoplasts on plates.

Table (1): Effect of protoplast generation and recovery conditions on protoplast regeneration frequency

Protoplast Generation method	Regeneration medium	Plating method	Regeneration Teamperature(˚C)	Regeneration Frequency L.c	Regeneration Frequency En.f
(1)Lysozyme (2)EDTA	MRS+0.5M sucrose	Spreading	25	45	75
(1)Lysozyme (2)EDTA	MRS+0.5M sucrose	Spreading	37	50	75
(1)Lysozyme (2)EDTA	MRS+0.5M sucrose	Dilution in soft agar	25	5	16
(1)Lysozyme (2)EDTA	MRS+ arg or leu, as required	Dilution in soft agar	25	0.5	0.3

The best results were achieved by spreading protoplasts on soft agar plates containing MRS and 0.5 M sucrose and incubating at 25°C.
This protocol gave consistently high regeneration frequencies averaging 70% in three separate experiments (Table 2) and was used for subsequent protoplast fusion experiments

Protoplast fusion experiments were done between *Lb.casei* spp. casei (arg-) and *En. Faecium* (L/eu-). Recombination between genomes of these two strains can lead to strains that grow on MRS medium at the absence of both arginine and leucine and in the precence of 6% NaCl and tolerate 3% bil salt. Protoplasts were generated from each strain by treatment with EDTA and lysozyme as described above and subjected to fusion with PEG6000 for various times.

Recoveries of viable cells after fusion were in the range 5-55% (Table 3), somewhat lower than those obtained from protoplasts that had not been subjected fusion. But still in an acceptable range. However, 5-30% were able to grow on media+ NaCl after examined this in media without Leu and arg. and it became prototrophic.

The generation of wild type obtained in these experiments can be attributed to recombination between parental genomes in fused diploid or multiploid protoplasts rather than spontaneous reversion of mutations,

transfer of genetic material by conjugation or transfer motion. No prototrophic (tolerate colonies) were obtained if the protoplasts were mixed without PEG 6000 or if the individual auxotrophs were subjected to protoplasting and fusion. The experiments were carried out with F. strains that are deficient in conjugation. Furthermore the inclusion of DNAse in the fusion medium minimizes the possibility that genetic material was transferred by uptake of DNA rleased from lysed cells. Protoplasts of two strains *(L.casei* and *Enfaecium) typically,* protoplast formation efficiencies of 90-95 % could be achieved.

Table (2): Frequencies of protoplast formation and regeneration achieved in several experiment.

Expt.	Strains	(a) Cells/ml Before protoplast regeneration	(b) Viable cells/ml after protoplast regeneration	(c) Viable cells/ml after lysis of protoplast	Protoplast formation frequency	Regeneration frequency
1	*Lb.casei spp.casei*	$1.9x10^8$	$1.5x10^8$	$1.4x10^7$	93	70
	En.faecium	$6.3x10^7$	$4.9x10^7$	$1.6x10^6$	97	75
2	*Lb.casei spp.casei*	$1.3x10^8$	$1.0x10^8$	$6.3x10^6$	94	75
	En.faecium	$4.1x10^8$	$1.9x10^8$	$5.4x10^6$	99	45
٣	*Lb.casei spp.casei*	$1.2x10^8$	$7.5x10^7$	$3.0x10^6$	98	59
	En.faecium	$2.8x10^8$	$1.9x10^6$	$8.8x10^6$	97	66

(a)Assessed by counting colonies on MRS plate before addition of lysozyme and EDTA
(b)Assessed by counting colonies on MRS+0.5 μ SACROSE
(c)Assessed by counting on MRS after dilution of protoplasts with distilled water.

Table (3) : Generation of Protorophs by fusion of protoplasts from two auxotrophic Parental strains: Lb.casei

Expt.	Fusion time (min)	Recovery after fusion%	Colonies tested	% that grow on MRS 3% Nacl	% that grow on MRS+6% Nacl	% that grow on MRS on first patch	% that grow on MRS after second patch	% that grow on MRS after third patch	Over all freq. of st. formation %[a]
1a[b]	5	20	300	62	30	9	1.5	0.5	0.12
1b[b]	15	50	300	50	31	17	7.4	1.3	0.60
1c[c]	5	52	300	60	31	15	3.4	1.3	0.60
1d[d]	5	25	300	61	35	14	3.2	0.5	0.18
2a[b]	5	18	500	55	40	8	1.6	0.4	0.8
2b[b]	15	9	400	45	52	8	1.5	0.9	0.06
3[b]	15	22	450	54	43	20	1.4	0.4	0.05

a-Calculated by multiplying the % of colonies obtained after regeneration on MRS sucrose that were capable o
growth on Nacl after the thied patch by the fraction of protoplasts capable of regeneration after fusion.
b-Cells plated on MRS immediately after fusion.

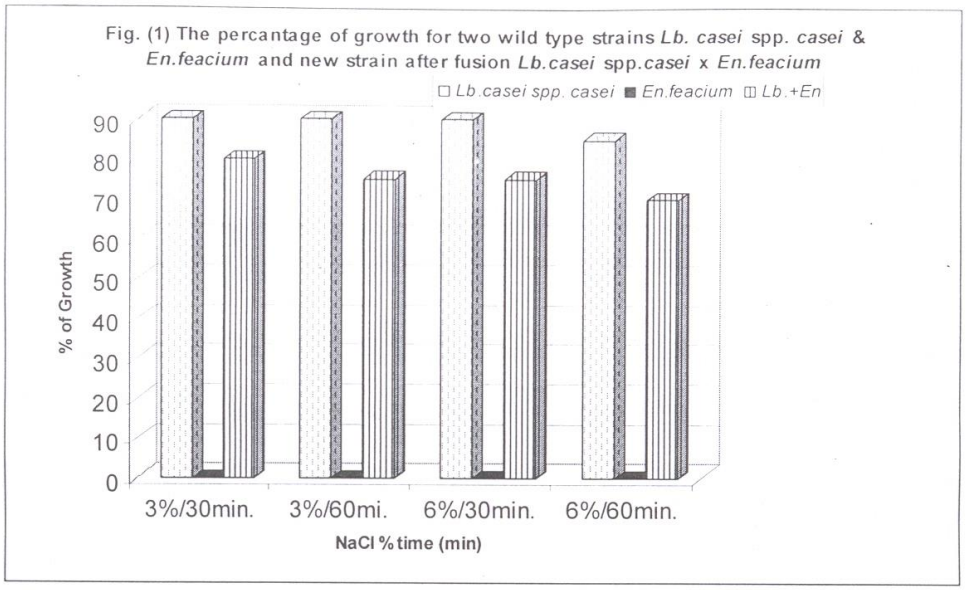
c-Cells plated on MRS after 1h of shaking at 37 °C.

d-Cells plated on MRS after 1h of standing at 37°C.

Tables 1 and 2 summarize the efficiencies of formation and regeneration of protoplasts of two strains *(L.casei* and *En.faecium),* typically, protoplast formation efficiencies of 90-95 % could be achieved.

The success of protoplast formation and regeneration is likely to be organism specific, so the comparison with previous work using *E.coli* is most relevant. Reported work with E.coli (Tsenin et al. 1986) may have been due to a combination of a less gentile method for generating protoplasts. These findings raise an important issue with respect to evaluate previous reports for the efficiency of protoplast fusion, since the number of wild type strains is often assessed by the phenotype of colonies immediately after fusion, and thus the actual number of prototroph may be overestimated.

The percentage of growth for two wild type strains *Lb. casei* and *Enfaecium* and fusion between *Lb. casei* & *Enfaecium* (Fig. I) could tolerate 3% NaCl and having a survival percentage ranged between 75- 80% When the percentage of NaCl reached to 6% the survival reached to 70, 75 % at 30,60 min continually.



This model predicats that the colonies obtained after regeneration on LB sucrose and many cases will be hereto us such heterogeneity has been well documented in previous studies of protoplast fusion (Coetzee *et al* 1979).

Based upon both technical and organize factors. It is important to generate protoplasts and to allow regeneration after fusion under gentle conditions so that riverside damage to the cells does not occur. Thus, the efficiency of recombination during protoplast fusion in grame positive is higher than that obtained in the most reports using grame negative .Given the improvement in cells recovery cells after fusion of protoplasts obtained using the methods described here were believe that genome shuffling will be useful for efforts to evolve improved strains

Characteristic properties of the wild type strains and new strain:

Table (4) shows that the new strain of *Lb. casei spp.casei* was short round rods, failed to ferment raffinose. It could grow at NaCl 6 and 9 %(w/v) and bile salt 3%. These results confirm that new *Lb.casei* can survive at high concentrations of sodium chloride.

Changes in counts of the Lactic acid bacteria and new *Lb.casei* spp. *casei* counts in Domiati cheese during pickling period at room temperature was achieved.

Data in Table (5) show that counts of the lactic acid bacteria counts in cheeses manufactured with *Lb.casei spp casei* increased during pickling, reaching a maximum after two weeks and after one week in control Thereafter, the counts decreased along pickling period reaching the minimum after 45 days of storage.

There results coincide with those obtained by Effat (2000) and Mehanna *et al* (2002). On the other hand the counts of wild *Lb.casei* spp *casei* and new *Lb. casei* gradually increased during the first two weeks of pickling, after viable cells were still at 109 cfu/gm for new *Lb.casei* spp *casei,* but the count of wild *Lb.casei spp casei* decreased 1.710g cycle after 45 "days of pickling, salt concentration increase during cheese pickling. This is an important selection factor for growth of salt-tolerant bacteria (new *Lb.casei* Spp *case I*) during the late stage of cheese pickling

Table(4): Morphological, cultural physiological characteristics and biochemical characteristics of the wild taupe strains and new strain

Characteristics	*En.feacieum*	*L.casei* spp. *casei*	*New*
Cell morphology	Cocci	Rods	Short round rods
Gram strain reaction	G +	G +	G +
Catalase activity	--	--	--
Growth at			
5C	-	-	-
10C	-	+	-
45C	+	-	+
Growth in medium with Nacl			
2%	+	+	+
4%	+	+	+
6%	+	-	+
9%	+	-	+
Growth in medium with pH 9.6	++	-	+
Growth in 3% bile salt	++	-	+
Fermentation 0f sugars			
Arbinose	-	-	-
Cellobise	+	+	+
Raffinose	+	-	-
Manitole	-	+	+

Table (5) Count of lactic acid bacteria strains used in cheese during pickling at room temp.

Pickling period days	Log count of Lactic acid bacteria			Log count of *Lb.casei* spp. *casei*		
	A	B	C	A	B	C
Zero	7.5	7.47	8.9	7.7	8.0	-
7	8.5	8.9	9.2	8.7	9.2	-
15	9.0	9.6	8.2	8.8	9.47	-
30	6.6	6.8	6.2	7.6	9.0	-
45	6.1	6.5	6.0	7.0	9.0	-

A: Treatment (1) with wild *Lb.casei* spp *casei*
B: Treatment (2) with new *Lb.casei* spp *casei*
C: Cheese control.

Chemical properties

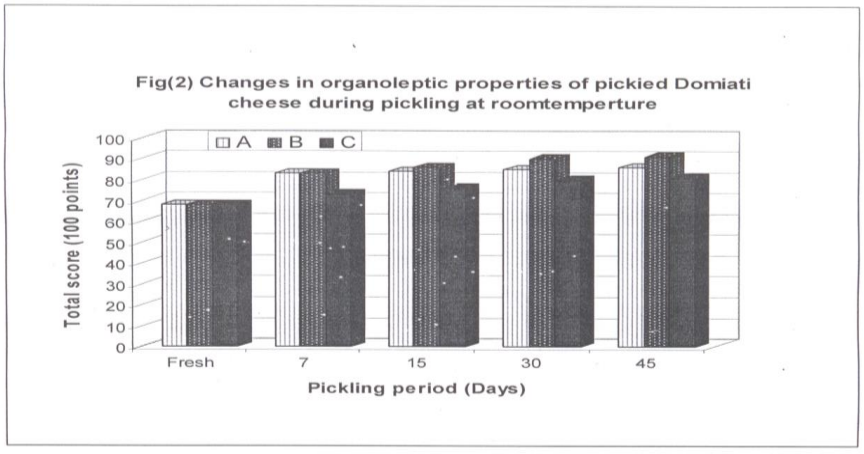
It is shows from Table (6) that moisture content in Domiate cheese (control and all treatment) decreased gradually as pickling progressed until 45) days. Also, the obtained results indicated that in all treatments, the rate of accumulation of (W.S.N) increased as pickling period increased. This was attributed to the proteolysis through pickling. Similar results were findings by Degheidi *et al.,* (1998) and Mehanna et al (2002). The increase in W.S.N. in cheese manufactured with new *Lb. casei* spp *casei* was higher as compared with control cheese. The effect of adding *Lb.casei* spp *casei* on the development of total volatile fatty acids (T.V.F.A.) in Domiate cheese during pickling was shown in table (6). From these results it could be noted that T.V.F.A. gradually increased in cheese manufactured with *Lb. casei* spp *casei* than the corresponding in control cheese.

Treated cheese contained high T.V.F.A

through all pickling intervals as compared with colitrol one. These 'findings could be attributed to the lipolytic effect of the add *Lb.casei* spp *casei,* which led to the increase in T.V.F.A in cheese during pickling and/or addition, this could be on the basis that the explained increasing rate of accumulation of free amino acids which act as precursors for free fatty acids through domination
reaction Degheidi *et al* (1998).

Sensory evaluation of cheese

The quality of Domiati cheese was organoleptically examined through the pickling period and scoring results are given in Fig (2) .It is obvious that the quality of all treated cheeses gradually improved as the progression of pickling period than that of control cheese which reached the highest levels at the end of pickling (45days). It appears that cheese made from milk with adding *Lb.casei* spp *casei* to starter even gained the highest scores as compared with other treatments . It was characterized by good mature flavaur, typical smooth body and texture with out off flavours. These improvements in cheese flavor during pickling period may be due to the rate of proteolysis and lipolsis in cheese.



Fig(2) Changes in organoleptic properties of pickied Domiati cheese during pickling at roomtemperture

Table (6) Chemical properties of Domiati cheese during pickling storag

Treatment	Pickling Period	Moisture	WSN/TN	pH	T.V.F.A as ml of o.1N Naoh
A	Fresh	59.81	8.4	5.8	10
B		59.78	8.4	5.8	10
C		60.10	8.4	5.8	10
A	7	55.95	10.81	5.8	17
B		56.12	10.72	5.8	17
C		56.52	8.80	5.8	12
A	15	54.22	13.21	5.7	20
B		54.93	14.34	5.7	20
C		54.82	9.10	5.7	15
A	30	53.24	19.99	4.6	24
B		53.30	20.21	4.6	26
C		53.90	17.50	4.7	16
A	45	52.44	21.82	4.1	25
B		52.60	23.60	4.0	28
C		52.82	17.99	4.1	17

REFERENCES

Abo-Donia S.A.(1986): Egyptian. Domiati soft white pickled cheese.Review Neuozealand J.Dairy Sci. Technol. 21:167-177

Bahy El-Din B., El-soda M. and.ezzat N (2002).Protealytic, lipollytic and autolytic activeties *of* enterococci strains isolated from Egyptian Dairy products. Lait 82 . 289-304 .

Chio Y. J., Miguez c.B., and Lee Y.J.(2004*).* Characterization *of* heterolagous gene Expression *of* Novel Esterase *from lactobacillus casie*CL96.J.Applied and Environment Micro.*70:* 3213-3221

Coetzee, J.N., Sirggel, F.A., Lecatsas, G.(1979). Genetic recombination in fused speroplasts *of* Providence Alcaliiens J.Gen.Microbiol.l14, 313-322.CTA 1998

Dai, M,H.and Capley D.(2004). Genome shuffling improves degradation *of* anthropogenic pesticide pentachlorophenol *by* sphingobium Worophenlpcuns ATCC 39723 Appl.Environ.Microbiol. *70,23991-2397*

Degheidi,M.A.; Abd Rabou,N.S. and Ismail, A.A. (1998). Improvement *of* Domiati cheese quality during pickling using Jack Fruit Lipase. Egypt.J.Dairy Sci.26: *103-110.*

Dpesic M.and Navonovic L. (*2005).* Microbiology study *of* fresh white cheese (Aserbion caraftvariety). Applied Ecology and Environmental Research 4:129-134

Effat, RA. (2000). Antifungal substance from some lactic acid bacteria and propioni bacteria for use as food preservatives .J.Agric.Sci.Mansoara Univ25:6291-6295.

EI-Abed, M., Abd-EI-Fattah, M. Osman, S.G. (2003).Egyptian J.Dairy Sci. 31: 125-138.

EI-Shafei,K.,Ibrahim G.A. and Tawfik N.F (2002). Beneficial uses *of* locally isolated Lactic acid bacteria. Egyptian J.Dairy Sci.30: 15-25.

Foulquie Moreno M.R., Sarantino polllos.P., Tsakalidoll E. and Devis L. (2006). The role and application of Enterococci in food and health. International J. of Food microbiology 106 : 1-24

Fenster K., L., Parkin K. L., and Steele J.,L.(2003) .Nucleotide sequencing, purification and biochemical properties of an arylesterase from *Lactobacillus casei* J.Dairy Science86:2547-2557.

Guessas ,S. and Kihal, M. (2004). Characterization of lactic acid bacteria isolated from Algerian arid zone raw goats milk. Afr.J.Biotechnol. 3: 339-342.

Hanson"K.G. and Desai,A.J.(1996). Acinetobacter sp A3 and hydrocarbon degradation Intergeneric protoplast fusion between *Pseudomonos putida* Dp99 for enhanced Biotechnol.Lett 19:1369-1374

Kandler,O.and Weiss,N. (1986).Genus Lactobacillus. In Berge)"s Manual of Systematic Bacteriology, vo1.2 (Eds.P.H.A. Smeath,N.S. Mair and M.E.Sharpe). Baltimore, MD:Williams and Wilkins,pp.1209-1215

Kosikowski,F.(1982).Cheese and fermented milk foods.2th ed.573.F.V.Kosikowski and Associates,Ithaca,N.Y.,U.S.A.

Kieronczyk,A., Skeie S., Langstrud T., and Yvan M,(2003). Cooperation between lactococcus lactis and Non starter Lactobacillus in the formation of cheese. J. Dairy Sci. 88-89

Ling,E.R. (1963). A text book of dairy chemistry, vol-2 practica1.3rd ed. Chapman andHall Ltd.,London.

Madkor S., A., Tong, P.S., El-soda M. (2000) Ripening of cheddar cheese with AddedAttenuated Adjunct culture of lactobacilli.J.Dairy Sci.83:1684-1691

Manolopulou,E.,Sarantinnopoulas,P.,Zoidou,E.,Aktypis,A.,MoschopaulouE.,Ka ndara kis,IG.,Anifantakis E.M.' (2003) Evaluation of microbial population during traditional feta cheese manufacture and ripening. Inter.J of Food Microbiology 82:153-161.

Madey L., and Casey M.G (2004). Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains International J of Food Micro. 90:139-159.Egyptian J. of Dairy Sci.30:217-229

Moreno,M.R. F .,Leisnd,J.J., Tee,L.K.;Radu,S.,Rusul,G., Vancanneyt,M.,De Vuyst,L.,(2002) Microbial analysis of Malaysian tempe, and characterization of two bacteriocins produced by isolates of *EnterococcusfaeGium.J.* of Applied Microbiology 92:147-157.

Mrngffua Dai, Ziesman,S, Ratcllliffe,T, Gill"T.R. and Copley,S.D.(2004). Visualization of protoplast and utilization of recombination in fused protoplasts of auxotrophhic strain of *E.coli* Metabolic Engineering 1-8 Nauk. SSSR243, 1066-1068.

Mund,J.O.(1986) Genus *Enterococci.* In Bergey,s Manual of Systematic Bacteriology.Vo1.2 (Eds.P.H.A. Smeath,N.S.Mair and M.E.Sharpe). Baltimore,MD: Williams and Wilkins,pp.l 063-1 078.

Nair P.S. and Surendran, P.K. (2005). Biochemical characterization of lactic acid bacteria isolated from fish and prawn. J. of Culture Collections. 4:48-52.

Nelson J.H.,R.G. Jensen and R.E.Pihos (1997). Pregastric esterases and other oral lipases- a review J.Dairy Sci. 60:327-362.

Patraik,R., Loouuie,S., Gavrilovic", V.,Perry,K., ,Ryan,C.M., dell cardayre.,S. (2002). Genome shuffling of Lactobacillus for improve acid tolerance. Nature. Biotecnol. 20;707-712

Roy D, Petre M., Blanchelle, L., Sovoie L.,Belanger, G., Ward, P. and J.L.Manbois (1997). Monitoring proteolysis and cheese juice composition during ripening of cheddar cheese made from microfiltered milk .Lait 77:521-541.

Salem A.S.,and Abeid A.M. (1979). Low sodium and cholesterol domiaticheese. Egyptian 1. Dairy Sci.25:123-134.

Shah, N.P. (2000). Probiotic bacteria selective enumeration and survival in dairyfood.J.Dairy Sci.83:894-898.

Shin J.G.W.M.Jean, G.RKin and RH. Leen (2004) Purification and characterization of intracellular proteinase from *Lactobacillus casei* ssp. *casei* LLG. J.Dairy Sci. 87: 4097-410

Steider,L. (2003). Genetically engineered probiotics. Best Practice and Research Clin.Gastro.17:861-876.bactirophage resistance and impact on product quality summary reports of European commission supported STD- 3 projects (1992-1995) published by CTA 1998. .

Tsenin, A.N., Karimov, G.A., Rybchin ,V.N.(1986). Recomination during fusion of protoplasts of *E.coli* K 12. DokI.Akad.Naak.SSSR 243:1066-1068.

Wei,w,wu,K., Xie, Z.,and Zhu"X.(2001). Intergeneric protoplast fusion between *Klyvuyeromyces* and *Sacchromyces cervisiae* to produce sorbitol from Jerusalem artichokes Biotechnol. Lett 23: 799-803.

Weiss"R.L.(1979). Protoplast formation in *E.coli*. *J* .Bacteriol. 128:668-675.

Wessels,D.,Jooste,P.J ,Mostert ,J.F. (1990). Technologically important characteristics of Enterococcus isolates from milk and dairy products. International Journal of Food Micro. 10:349-352.

Zhang, Y., X., Perry, K., Vinci , V. A., P)owell, K., Stemmer, W.P.C. delcardayre, S.B. (2002). Genome Shuffling leads to rapid phenotypic improvement in bacteria. Nature. 6: 415-425.

التحسين الوراثي لسلالات اللاكتوباسيلاسي **عن طريق الدمج** البروتوبلاستي

أسامة شرف ، نيفين أبو سريع*، عزات عبدالخالق وكوثر الشافعي
قسم الألبان و* قسم الوراثة الميكروبية – قطاع الهندسة الوراثية والتكنولوجية – المركز القومى للبحوث – القاهرة - مصر

وقد استخدم دمج البروتوبلاسيت في هذه الدراسة للحصول على سلالات ذات صفات وراثية جديدة مرغوبة، يعتبر الدمج البروتوبلاستي أحد طرق الهندسة الوراثية لتحسين صفات الكائنات المختلفة.

وفي هذا البحث أمكن الحصول على :

1- طافرنين الأولى Leu (ليوسين) من الإنتيروكوكاس فاثيم والثانية arg أرجنين من اللاكتوباسيلاسي كاس
2- استخدمت درجات حرارة مختلفة ومعاملات مختلفة (EDTA) وبعض الإنزيمات مثل الليوزيم (Laysozyme) وذلك للوصول إلى التكشف البروتوبلاستي
3- أمكن عمل الدمج البروتوبلاستي باستخدام البولي اثيلين جليكول 6000 6000 (PEG) وأمكن الحصول على سلالات تنمو على MRS بدون الاضافات الغذائية.
4- تم اختبار تحمل السلالات الجديدة لكلوريد الصوديوم للـ Nacl ووجد أنها تتحمل حتى تركيز 6% باختلاف الوقت وكانت نسبة الحيوية من 70-80%
5- بالنسبة لتأثير السلالات الجديدة من اللاكتوباسيلاسي على جودة الجبن الدمياطي أثناء التخزين على درجة حرارة الغرفة لمدة 45 يوم وجد تحسين ملحوظ في المحتوى النيتروجيني لها.
6- وجد تحسن ملحوظ في الطعم والملمس بعد التخزين لمدة 45 يوم بعد إضافة السلالة المحسنة بالمقارنة بالجبن المصنع السلالة الأصلية الـ . Wild Type