Int.J.Curr.Microbiol.App.Sci (2016) *5*(1): 546-552

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 1(2016) pp. 546-552**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.501.055

Obtaining of *Lactobacillus delbrueckii* Cold Sensitive *Rif* Mutants for Shelf Life Prolongation of Dairy Products

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Keywords

Lactobacillus delbrueckii, Milk fermentation, Rifampicin-resistant mutants, Shelf life

Article Info

Accepted:
25 December 2015
Available Online:
10 January 2016

A B S T R A C T

The present study was aimed at using cold-sensitive (CS) starter as powerful alternative for shelf life prolongation of fermented dairy products. Utilized replica plating technique to select six cold-sensitive mutants of *Lactobacillus delbrueckii* MDC 9617 from about 400 rifampicin-resistant (Rif) mutants which losing ability to grow on LAPTg and ferment milk at minimal temperature. Whole CS mutants during growth at optimal temperaturebasically hold milk clotting rate, as well as the organoleptic properties of fermented dairy product. There are two CS Rif mutants CS-Rif3 and CS-Rif 8also containing highgrowth and milk fermentation rates property. Dairy Products which fermented by CS starters maintain the viable cells count, titratable acidity, texture and flavor at last for one month of refrigerator condition inmutuality to the native starter.Thereby, cold sensitive *rif* mutations can be used as an alternative solution for shelf life prolongation of dairy products at refrigerator temperatures also for technological characteristics ameliorate of starter cultures.

Introduction

The shelf life of dairy product reducing cause of metabolic activity of dairy starters at low temperatures during cooling and storage (Mitropoulou *et al.*, 2013). Nowadays for dairy products shelf life prolongation are used pasteurization or chemical preservatives, which affect the viability of useful probiotic microorganisms and refused from consumers (Tamime and Robinson, 2011). Growth of microorganisms in rich media at extreme temperatures limited by cold or heat sensitivity of one or more key protein(s) involved in global cellular processes such as DNA replication, transcription, translation or cell division (Lengeler *et al.*, 1999). There are fundamental connections between rifampicin resistance, RNA polymerase structure and function and global gene expression (Hua *et al.*, 2011). Rifampicin can specifically block transcription initiation but not elongation (Ingham and Furneaux, 2000). Resistance to rifampicin is associated with mutations in the gene coding for the beta subunit of RNA polymerase (*rpoB*) (Garibyan *et al.*, 2003; Lai *et al.*, 2002). *Rif* mutations of RNA polymerase have been found involved in a variety of physiological processes and possessing pleiotropic effects, including:cell growth (Jin and Gros, 1989; Kogoma, 1994; Maughan *et al*., 2004); the ability of mutants to support the growth of various bacteriophages; the ability to maintain the F' episome; interaction with other genes mutant alleles; uracil sensitivity; exopolysaccharides over synthesis and thermosensitivity of LABs (Hovhannisyan *et al*., 2010; Hovhannisyan and Barseghyan, 2015).

A spontaneous *rif* mutation of *Saccharopolyspora erythraea* caused slow-growth and stimulated erythromycin production by global changing of transcriptional profile (Elisabetta *et al*., 2009).

So webelieve that metabolic activity of LAB starters at low temperatures will be limited byCS mutations of RNA polymerase byshift up of T_{min}. The objective of this work is assess the shelf life of fermented dairy products and select rifampicin resistance CS starters.

Materials and Methods

The strain of *Lactobacillus delbrueckii* MDC9617obtained from the Microbial Depository Center of NAS RA (Armenia).

N-methyl-N′-nitro-N-nitrosoguanidine (NTG) induced mutagenesis was performed as described earlier (Hovhannisyan *et al*., 2010). Rifampicin and NTG purchased from Serva (Germany).

Skimmed milk, LAPTg medium (0.5% bacto tryptone, 0.3% yeast extract, 0.1% glucose, Tween 80), for softening 1.5% agar was added (Huys *et al*., 2002).

NTG Mutagenesis and Selection of Rifampicin-Resistant CS Mutants

Overnight incubated L. *delbrueckii* MDC 9617 (10^9 CFU/ml) was used for obtaining Rifampicin-resistant mutants, treated by 300 μ/ml dos of NTG by plating on LAPTg agar containing 100 µg/ml of rifampicin and incubated at 37°C.

Used replica plating method to screening CS mutants of Rif colonies, then cultivated at 27°C. Colonies, which were not growing at minimal temperature, were isolated for farther investigation.

Rate of Growth

Diluted 20 times overnight incubated cultures in fresh LAPTg broth and growth at 37˚□ with water bath shaker at 200 rpm.

Optical density (OD_{600}nm) measured every half an hour.

Milk Clotting Rate

1.8 ml of sterile skim milk was inoculation by 0.2 ml of overnight broth cultures and incubated at 37°C. milk coagulation checked every 30 min intervals.

Titratable Acidity

Was executed titratable acidity of the fermented milk according toThorner (^{o}T) (Tamimeand Robinson, 2011).

Shelf Life Test

The 50 ml aliquots of pasteurized skimmed milk inoculated by overnight *L. delbrueckii* CS *rif* mutant starter were poured in 12 properly covering cans, then fermented for 6 hours at 37°C, and stored in refrigerator at 6°C. Three cans of milk fermented by each starters tested on physical, microbiological and chemical characteristics at a week intervals.

Statistical Analysis

Statistical analysis of the data was done using Student's t test computer, taking the criterion of P value <0.05 sufficient for significant differences in the results.

Results and Discussion

Phenotypic Character of *L. delbrueckii* MDC 9617

The strain *L.delbrueckii* MDC 9617 isolated from human origin has temperature growth optimum (T_{opt}) 36-40 °C, minimum (T_{min}) 28°C and maximum (T_{max}) 46°C, cells are H_2O_2 producing, non-motile rods, catalase negative, gram-positive, oxidase positive.In contrast to *Lactobacillus delbrueckii* strains, which cleanly grow in the milk, this strain to acidify milk by high rate and forms a solid clot.

Obtaining of Rifampicin Resistant Mutants

Its known that more isolated rifampicin-resistant mutations coming to very narrow bunch 81-bp region in *rpoB* gene (Telenti *et al.*, 1993), for this reason their spontaneous frequency is low (3.5×10^{-8})(Hua *et al.*, 2010). Earlier we have manifested that use of Nitrosoguanidine induced mutagenesis increases the yield of *rif* mutations in LAB up to 2.2×10^{-5}(Hovhannisyan *et al.*, 2010).
In this study, using of NTG mutagenesis allowed increasing the frequency yield of rifampicin resistant mutants of *L. delbrueckii* MDC 9617from $4.5 \times 10^{-8}$to 4.1 $\times$ 10^{-5}. About 400 colonies formed on LAPTg agar with 100µg/ml of rifampicin were replica plated on the same medium and incubated at 29°C.For further investigation picked up The colonies, which are not growing on replica plates from mother plates. The among the Rif mutants the frequency of obtaining CS strains was appraised about 1.0%.Six of Rif mutants, which permanently have lost the capability to grow at minimal temperature suggested as Cold-Sensitive.

The ranges of strain *L. delbrueckii* MDC 9617CS mutants growth temperature in LAPTg broth was illustrated (Table 1).

How table 1 shows, due to *rif* mutations the optimal temperatures of wild strain and CS mutants were not changed (36-40°C). But minimal temperatures of growth of CS mutants raising to 5 degrees.

The growth rate of CS mutants in LAPTg medium at 37°C was studied (Figure 1).

The figure1curves shows, except of CS-Rif 3 mutant, which had high growth rate and CS-Rif 9, which growth rate was lower, than wild strain, the growth rate of other CS mutants at optimal temperature are same as compared with parental strain. Was determined the milk coagulation rate by CS starters at 37°C comparatively (Figure 2).

Figure 2 illustrated that, mutant CS-Rif 3 has higher milk coagulation rate (5 h), the CS-Rif 4 and CS-Rif 8 mutants have same milk clotting rate comparative with parental strain. The mutants CS-Rif1, CS-Rif 7 and CS-Rif 9 coagulated milk slowly for6.5 and 7 hours respectively.

The profile of milk fermentation by CS mutant starters in temperature range from 28 to 46 °C were studied (Table 2).

How table 2 shows, CS-Rif 1 and CS-Rif 8 mutants incapable to ferment milk below 32°C. Also CS-Rif 3, CS-Rif 9 unable clotting milk below 34°C. Other mutants minimum temperature of milk curdling are30°C.There are 3 mutants did not ferment milk at maximum temperature (46°C).So, the minimum temperature of milk fermentation of CS mutants increased from 1 to 5°C.

Table.1 Description of Growth Temperatures Range of CS *Rif* Mutants in LAPTg Broth

Growth Temperatures	Cold-Sensitive Mutants Strains						
	MDC 9617	CS-Rif 1	CS-Rif 3	CS-Rif 4	CS-Rif 7	CS-Rif 8	CS-Rif 9
T_{min}	28	32	33	30	29	31	32
T_{opt}	36-40	36-40	36-40	36-40	36-40	36-40	36-40
T_{max}	46	45	46	46	43	43	46

Table.2 Temperature Profile of Milk Fermentation by CS Mutant Starters

Strains	Temperature, °C						
	28	30	32	34	37	40	46
MDC 9617	+	+	+	+	+	+	+
CS-Rif 1	-	-	+	+	+	+	+
CS-Rif 3	-	-	-	+	+	+	-
CS-Rif 4	-	+	+	+	+	+	+
CS-Rif 7	-	+	+	+	+	+	-
CS-Rif 8	-	-	+	+	+	+	-
CS-Rif 9	-	-	-	+	+	+	+

"+" – ferment, "- "- not ferment

Table.3 Quantity of Titratable Acidity and Viable Cells of Fermented Milk used *Lactobacillus delbrueckii*MDC 9617, CS Mutant Starters, Stored at 6 °C

Strains	**Time of storage, days**							
	1		7		14		28	
	CFU/ml	˚T	CFU/ml	˚T	CFU/ml	˚T	CFU/ml	˚T
MDC 9617	$3.4x10^9$	110	$2.9x10^9$	115	$6.4 x 10^8$	130	$9.8x10^7$	140
CS-Rif 1	$2.6x10^9$	95	$2.5x10^9$	95	$5.8x10^8$	120	$8.1x10^7$	130
CS-Rif 3	$2.7x10^9$	75	$2.3x10^9$	78	$5.9x10^8$	90	$7.6x10^7$	105
CS-Rif 4	$2.8x10^9$	90	$2.4x10^9$	92	$6.1x10^8$	119	$8x10^7$	132
CS-Rif 7	$3.1x10^9$	100	$2.6x10^9$	101	$5x10^8$	115	$7.7x10^7$	125
CS-Rif 8	$3.2x10^9$	95	$2.1x10^9$	97	$5.2x10^8$	110	$8.5x10^7$	120
CS-Rif 9	$2.9x10^9$	70	$2.5x10^9$	70	$6.1x10^8$	80	$6.4x10^7$	127

Figure.1 CS Mutants Growth Rate in LAPTg at 37°C

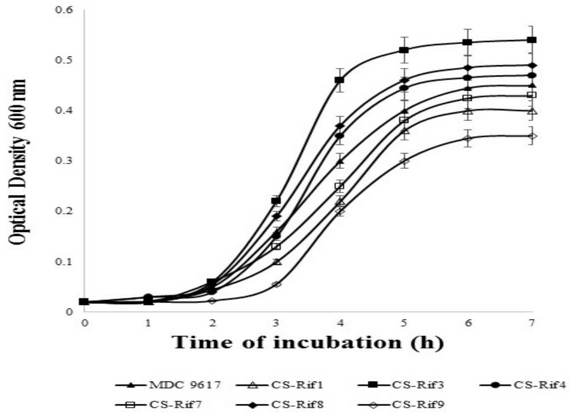
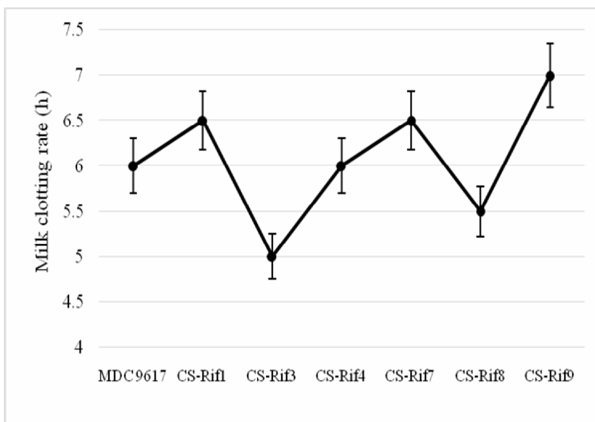


Figure.2 Rate of Curdling Milk by CS Mutant Starters at 37°C



The quality of dairy probiotic products assessed by the number of viable microorganisms and organoleptic properties (Shafiee *et al.,* 2010, Beal *et al.,* 1999). In connection with this has been studied the microbial titer and titratable acidity of yogurts during storage at 6°C. Used titer of viable cells (CFU/ml) and organic acids amount as criterion for calculation of product quality fermented by CS starters (titratable acidity, ˚T) (Table 3).

According to data, titer of cells and titratable acidity of fermented by *L. delbrueckii* MDC 9617milk were higher in initial day of storage compared with products where used CS mutants starters, cause of cessation time of CS mutants growth the parental strain continue to grow at lower temperature. Considering at further storage the viability of CS mutants stayhigher and productsacidity are lower than for *L. delbrueckii*MDC 9617. Study of data offers use these CS mutantsforlong preservation of dairy products quality, fermented.

Thereby, utilization of*rif* mutations can be significant for shelf life prolongation of dairy products,and also for obtaining starter cultures strains with improved technological characteristics and organoleptic properties.

As mentioned, is it is possible to isolate CS mutants possessing of high milk fermentation and specific growth rate simultaneously, because rifampicin-resistant mutants of LAB by frequency of about, 1.0 %. Totally cold sensitive *rif* mutations can be powerful alternative for shelf life prolongation of dairy products at refrigerator temperatures as well as for improving technological characteristics of starter cultures.

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How to cite this article:

Alireza G. Goodarzi. 2016. Obtaining of Lactobacillus delbrueckii Cold Sensitive Rif Mutants for Shelf Life Prolongation of Dairy Products. *Int.J.Curr.Microbiol.App.Sci.* 5(1): 546-552
http://dx.doi.org/10.20546/ijcmas.2016.501.055