J Ind Microbiol Biotechnol (2007) 34:729–737
DOI 10.1007/s10295-007-0249-x

ORIGINAL PAPER

A kinetic study on the plasmid stability of three *Lactococcus lactis* strains

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Received: 3 May 2007 / Accepted: 25 July 2007 / Published online: 28 August 2007

Absract The plasmid stability of three wild type *Lactococcus lactis* strains and their mutants was investigated at different incubation time and temperatures in two different media [M17 broth and reconstituted skim milk (RSM)]. The results showed that both incubation times and temperature are effective on plasmid loss. The plasmid profiles of wild type strains exhibited 8 to 9 distinct plasmid species with molecular weights from 2.1 to 24.0 kb. Lactose fermentation ability was found to be encoded by 22.2 (strain U70), 23.6 (strain U29) and 24.0 (strain U52) kb plasmids in the wild type strains, respectively. The stabilities of the plasmids were explained by applying a second-order polynomial modeling system. Reasonable fittings were obtained for the model and the adjusted regression coefficients (R^2_{adj}) were between 0.76 and 0.99 for the overall data. Overall, it was found that incubation time had the most profound effect on plasmid stability, with plasmid loss occurring after 72 h, while temperatures in the range of 15–40°C also induced plasmid instability.

Keywords *Lactococcus lactis* · Plasmid stability · Second-order polynomial modeling

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Introduction

Lactococcus lactis strains are widely used as starter cultures for the manufacture of cheese and other fermented dairy products on the account of their function of preservation and contribution to flavour and aroma [3, 5]. Since early 1970s, plasmid biology of *L. lactis* has gained more interest when their vitality for milk fermentations was understood. This is because that they are used primarily for lactic acid production as a result of breakdown of lactose, which was found to be plasmid encoded [20, 23]. Currently, it is known that, genetic determinants of industrially important traits including lactose utilization, proteolytic activity, phage resistance, citrate fermentation and bacteriocin production are generally encoded by plasmids which are omnipresent among *L. lactis* strains, with most isolates containing multiple plasmids ranging in size from 2 to 80 kilobases (kb) [8, 9, 24, 26]. A major challenge to the dairy industry is the provision of genetically stable strains having predictable properties under industrial fermentation conditions. Due to the potential unstable nature of plasmids, an extensive knowledge of plasmid stability functions is essential in order that plasmid encoded industrial traits can be maintained during extreme manufacturing conditions [14, 18]. Some environmental factors such as pH, specific growth rate, immobilization, medium composition and temperature exhibit a strong effect on segregational plasmid instability [2, 21, 22, 25, 27].

However, the effect of temperature on lactococcal plasmid stability has been less studied, despite its great importance for dairy fermentations. The aim of this study was thus to determine the effect of incubation temperature and time on the stability of lactococcal plasmids and to describe the effect of these factors on plasmid stability by use of a suitable mathematical model.

Materials and methods

Bacterial strains and culture conditions

Strains of *Lactococcus*, isolated from traditional milk fermentations in Türkiye, were obtained from the Ankara University Culture Service. They were grown routinely in M17 medium [29] at 30°C with lactose replaced by glucose when necessary (GM17). Bacterial stocks were stored in M17 broth containing 40% glycerol at –80°C.

Determination of plasmid stability

L. lactis strains were inoculated in M17 broth [29] and RSM (10%; Oxoid, UK) with an inoculum rate of 1% and incubated at 15, 20, 25, 30, 35 and 40°C for 24, 48 and 72 h. Plasmid stability was determined by the replica method [13] after 24, 48 and 72 h incubation periods for each of the incubation temperatures. One hundred colonies from M17 agar medium were plated onto lactose indicator agar medium [19] and were checked for their lactose fermentation abilities for each incubation temperature and time. The lysis procedure of Anderson and McKay [1] was used to isolate plasmid DNA from lactococcal plasmids. Plasmid sizes were estimated on 0.7% agarose gels by comparing their relative mobility to commercial covalently closed circular (ccc) DNA markers containing plasmid species of 16.2, 14.2, 12.1, 10.1, 8.1, 7.0, 6.0, 5.0, 4.0, 3.0 and 2.1 kb (Bathesda Research Laboratories, Product No: 56222SA, USA).

The model and model assessment

A second-order polynomial function (Eq. 1) was proposed to describe the percent of the plasmid stability (p) as a function of incubation temperature (T) in °C and duration (t) in h:

$$p(T,\ t)\ =\ a + bT + cT^2 + dt + et^2 + fTt \qquad (1)$$

where a, b, c, d, e and f are the parameters of Eq. 1.

A backward regression procedure was applied to determine the parameters of this equation. The backward regression procedure begins with all candidate variables in the function and then systematically removes variables that are not significantly associated with the target, until a model with only significant parameters is obtained.

For the model assessment study, regression coefficient (R^2) and adjusted regression coefficient (R^2_{adj}) values were used to investigate the goodness-of-fit of the model. The parameters of the second-order polynomial function were obtained by using the SigmaPlot 2000 Version 6.00 (Chicago, IL, USA).

Results

The plasmid profiles of three *L. lactis* strains and their mutants were analysed (Fig. 1). The tested strains contained 8 (*L. lactis* subsp. *cremoris* U70) or 9 (*L. lactis* subsp. *lactis* U29 and *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52) distinct plasmid species with molecular weights from 2.1 to 24.0 kb. Comparison of the plasmid profiles of wild type strains and their different plasmid cured mutants showed that it was sufficient to obtain lactose negative (Lac^-) phenotypes from all lactose positive (Lac^+) wild type *L. lactis* strains, curing by the highest molecular weight plasmids only. These results strongly suggested that 22.2, 23.6 and 24.0 kb plasmids were encoded lactose fermentation ability in *L. lactis* subsp. *cremoris* U70, *L. lactis* subsp. *lactis* U29, *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52, respectively.

Figure 2 shows the fitting of Eq. 1 to *L. lactis* subsp. *lactis* U29 (plasmid content: 12.3 kb) in RSM (Fig. 2a), *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52 (plasmid content: 24.0 kb) in M17 broth (Fig. 2b), *L. lactis* subsp. *cremoris* U70 (plasmid content: 20.2 kb) in RSM (Fig. 2c) and *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52 (plasmid content: 24.0 kb) in RSM (Fig. 2d), respectively. Visual inspection of Fig. 1 indicated that reasonable fittings were obtained and support for this statement comes from the corresponding R^2 and R^2_{adj} values listed in Tables 1, 2, 3, 4, 5, 6. R^2_{adj} for the overall data (all the plasmid content for each microorganisms in both M17 broth and RSM) is between 0.76 and 0.99. $R^2_{adj} = 0.76$ (or $R^2_{adj} = 0.99$) suggests that only 24% (or 1%) of the total variation were not explained by the model (Eq. 1). Starting from Eq. 1 and after eliminating non-significant parameters, the listed parameters (with their standard errors) in Tables 1–6 were obtained for each strain, each of plasmid content in M17 broth and RSM. The data in the Fig. 2b and d (24.0 kb plasmid in M17 broth and in RSM, respectively) indicates that plasmid stability is higher in RSM than in M17 broth. This was also characterized by the higher surface (fitting of Eq. 1) in Fig. 2d. A comparison between M17 broth and RSM can be made in terms of the prediction of plasmid stability percent by using the parameter values given in Tables 3 and 4, respectively. For example, plasmid stability percent of (plasmid content 24.0 kb) *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52 in M17 broth incubated at 32°C for 60 h can be calculated as about 21 while in RSM it is about 58.

It was observed that as the time of incubation increases plasmid stability decreases. Therefore, the partial derivative of the model (Eq. 1) with respect to t should always be

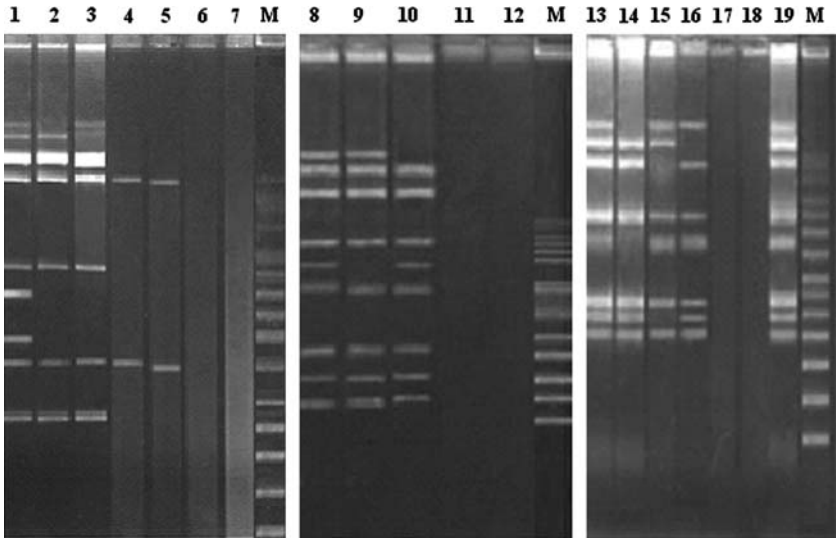

Fig. 1 Plasmid profiles of wild type *L. lactis* strains and their mutants. Lane 1 (wild type *L. lactis* subsp. *lactis* U29): 23.6, 22.1, 19.6, 16.7, 15.0, 12.3, 7.7, 7.1 and 5.7 kb; lanes 2, 4, 6 and 7: Lac^- mutants of U29; lane 3: Lac^+ mutant of U29; lanes 8 and 9 (wild type *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52): 24.0, 22.7, 20.5, 18.6, 8.7, 7.7, 5.9, 4.6 and 2.8 kb; lanes 10, 11 and 12: Lac^- mutants of U52; lanes 13 and 19 (wild type *L. lactis* subsp. *cremoris* U70): 22.2, 20.2, 18.2, 12.0, 9.5, 6.7, 6.0 and 5.2 kb; lanes 14, 17 and 18: Lac^- mutants of U70; lanes 15 and 16: Lac^+ mutants of U70 and M (ccc DNA markers): 16.2, 14.2, 12.1, 10.1, 8.1, 7.0, 6.0, 5.0, 4.0, 3.0 and 2.1 kb

negative. In other words, for a fixed level of temperature, the plasmid stability percent (p) should always be decreasing for increasing incubation times:

$$\frac{\partial p}{\partial t} = d + 2et + fT < 0 \tag{2}$$

This requirement should hold throughout the complete interpolation region.

Temperature effect on plasmid stability is similar as the effect of incubation time, however, when the temperature increases above 30°C plasmid stability decreases significantly ($P < 0.05$). Hence;

$$\frac{\partial p}{\partial T} = b + 2cT + ft < 0 \quad \text{if } T > 30^{\circ}\text{C} \tag{3}$$

Additionally, it was suggested that in the critical region where plasmid stability percent reaches its highest values the sign of the derivative should not be defined. Alternatively to this methodology, achieving maximum plasmid stability percent value in a certain region within the experimental domain can be translated in negative values of the second derivative of the plasmid stability percent with respect to the incubation temperature for any constant incubation time:

$$\frac{\partial^2 p}{\partial T^2} = 2c < 0 \tag{4}$$

The proposed model (Eq. 1), which was used to describe the plasmid stability of *L. lactis* strains and their mutants in M17 broth and RSM, is also obeying the mathematical constraints formulated in the above Eqs. 2, 3 and 4 by most of the plasmid contents of each strain in broth and RSM; however, there were some exceptions where model requirements were violated. For example, for *L. lactis* subsp. *lactis* U29 in M17 broth plasmid contents 15.0 and 5.7 and *L. lactis* subsp. *cremoris* U70 in RSM plasmid contents 22.2, 12.0 and 6.0 did not obey the constraint given in Eq. 2. This may be due to the unrealistic predictive capability of the polynomial function (Eq. 1) used [10]. It is known that polynomial function is not appropriate in certain environmental combinations. Nevertheless, since most of the plasmid contents in broth and RSM obeyed the constraint as mentioned above the model structure for these can be retained. For the ones whose model structure did not obey the constraints it is possible to obtain new parameter values (for the same model) by suitable optimization techniques as described in great detail by Geeraerd et al. [10]; however, this was not done in this study since the software, we used, can not perform such an operation (constrained linear least squares problem).

Discussion

The extreme commercial use of *L. lactis* strain has led to several fundamental and applied studies aimed at increasing our understanding of this lactic acid bacterium (LAB) to select better strains or to improve them through genetic

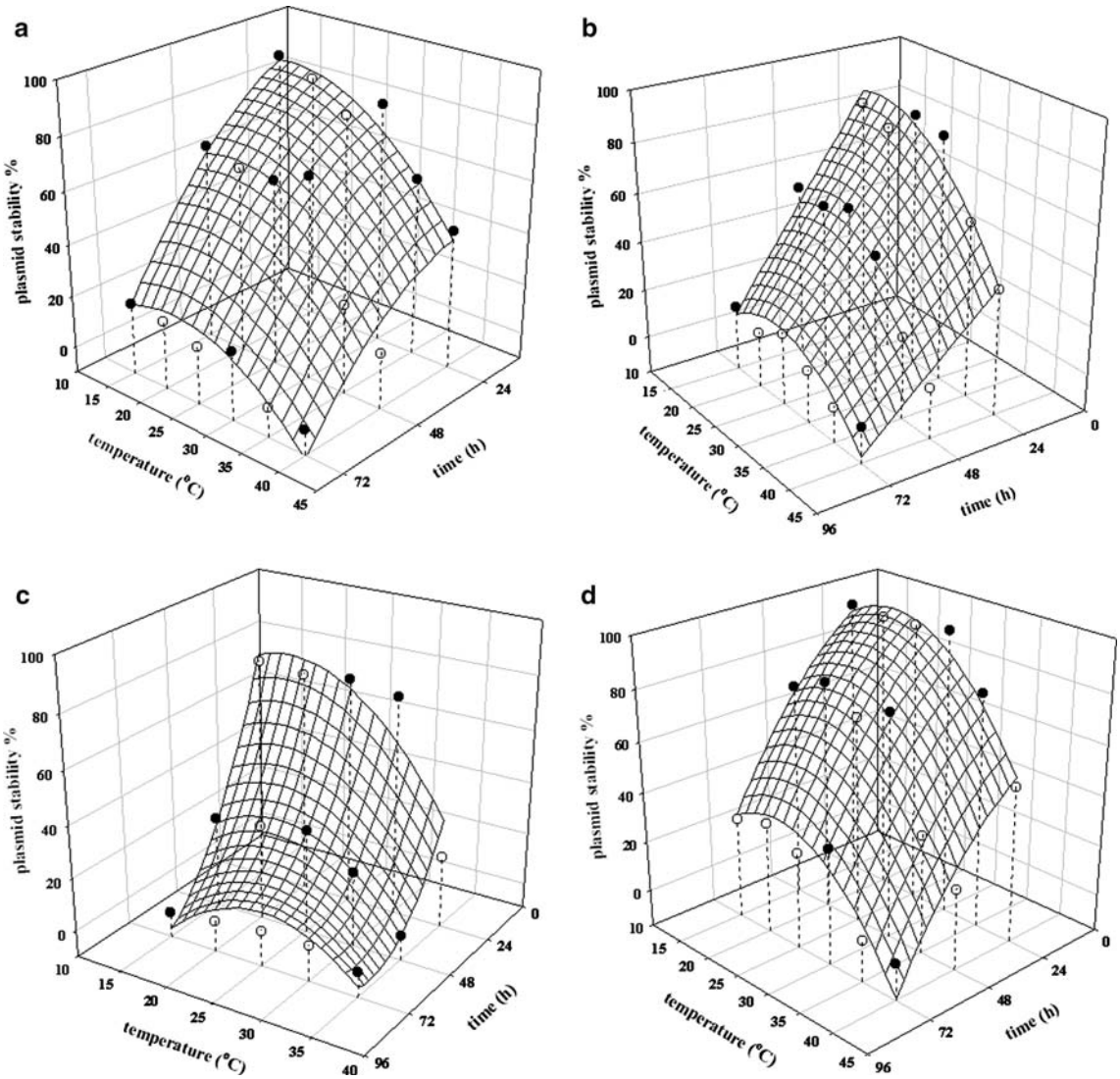

Fig. 2 **a** The three dimensional surfaces of the quadratic polynomial model (Eq. 1 with significant parameters) for *L. lactis* subsp. *lactis* U29 (12.3 kb) in RSM at different incubation temperatures and times. Experimental data points under the surface (*open circle*) and above the surface (*filled circle*). **b** The three dimensional surfaces of the quadratic polynomial model (Eq. 1 with significant parameters] for *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52 (24.0 kb) in M17 broth at different incubation temperatures and times. Experimental data points under the surface (*open circle*) and above the surface (*filled circle*). **c** The three dimensional surfaces of the quadratic polynomial model (Eq. 1 with significant parameters) for *L. lactis* subsp. *cremoris* U70 (20.2 kb) in RSM at different incubation temperatures and times. Experimental data points under the surface (*open circle*) and above the surface (*filled circle*). **d** The three dimensional surfaces of the quadratic polynomial model (Eq. 1 with significant parameters) for *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52 (24.0 kb) in RSM broth at different incubation temperatures and times. Experimental data points under the surface (*open circle*) and above the surface (*filled circle*)

modification [7, 17]. Plasmids are the most suitable genetic tools for such modifications and should be considered as a genetic obligation in milk fermentations [20, 23]. A substantial number of genes that are important for dairying were found to be plasmid encoded, and because of the structural or segregational instability, the traits specified by the genes are often lost [18, 31]. Thus, identification of the stabilities of industrially important traits in *L. lactis* has become a key process for the selection of starter culture strains. Furthermore, cryptic plasmids in lactococci have received considerable attention because the purpose of constructing cloning vectors to enhance the properties of LAB [4, 31]. High level plasmid instability characteristics of *L. lactis* strains, isolated from traditional fermented milk environments in Türkiye, showed similarities with lactose plasmid stability data in the literature [6, 11, 12, 15, 16, 28]. Results of this study indicate that the tested strains are more stable in plasmid content at lower incubation

Table 1 Parameters of Eq. 1 ± standard errors for *L. lactis* subsp. *lactis* U29 in M17 broth

Parameters	*L. lactis* subsp. *lactis* U29 plasmid content (kb) in M17 broth								
	23.6	22.1	19.6	16.7	15.0	12.3	7.7	7.1	5.7
A	80.8 ± 23.3	102.5 ± 25.2	96.2 ± 39.9	146.5 ± 11.9	179.7 ± 20.2	150.4 ± 12.6	148.6 ± 9.9	138.8 ± 25.9	183.4 ± 8.4
b	2.3 ± 1.8	2.5 ± 1.7	3.7 ± 2.6	–	–	–	–	3.1 ± 1.5	–
c	–0.09 ± 0.03	–0.09 ± 0.03	–0.1 ± 0.05	–0.06 ± 0.01	–0.04 ± 0.01	–0.06 ± 0.01	–0.04 ± 0.01	–0.09 ± 0.03	–0.04 ± 0.005
d	–	–2.2 ± 0.3	–2.1 ± 0.5	–2.4 ± 0.4	–4.7 ± 0.9	–2.4 ± 0.4	–2.2 ± 0.3	–4.3 ± 0.7	–5.3 ± 0.4
e	–0.02 ± 0.003	–	–	–	0.03 ± 0.009	–	–	0.02 ± 0.006	0.03 ± 0.004
f	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.006
R^2	0.95	0.95	0.91	0.93	0.93	0.92	0.95	0.97	0.99
R^2_{adj}	0.94	0.94	0.88	0.91	0.90	0.90	0.94	0.96	0.98

Table 2 Parameters of Eq. 1 ± standard errors for *L. lactis* subsp. *lactis* U29 in RSM

Parameters	*L. lactis* subsp. *lactis* U29 plasmid content (kb) in RSM								
	23.6	22.1	19.6	16.7	15.0	12.3	7.7	7.1	5.7
a	55.3 ± 23.5	96.0 ± 26.9	92.7 ± 33.4	84.7 ± 29.7	72.8 ± 40.0	74.2 ± 25.7	95.7 ± 23.7	113.0 ± 25.6	154.0 ± 16.2
b	5.4 ± 1.8	3.4 ± 2.1	3.7 ± 2.5	3.7 ± 2.3	5.9 ± 2.6	2.6 ± 2.0	3.5 ± 1.8	5.1 ± 1.7	–
c	–0.1 ± 0.03	–0.07 ± 0.04	–0.09 ± 0.05	–0.09 ± 0.04	–0.1 ± 0.05	–0.09 ± 0.04	–0.08 ± 0.03	–0.1 ± 0.03	–0.05 ± 0.01
d	–	–1.8 ± 0.1	–1.7 ± 0.2	–1.5 ± 0.1	–2.6 ± 1.9	–	–1.5 ± 0.1	–3.8 ± 0.7	–3.1 ± 0.7
e	–0.02 ± 0.001	–	–	–	0.01 ± 0.01	–0.02 ± 0.003	–	0.03 ± 0.007	0.009 ± 0.007
f	–	–	–	–	–	0.02 ± 0.01	–	–	0.03 ± 0.01
R^2	0.95	0.94	0.91	0.92	0.90	0.94	0.95	0.96	0.95
R^2_{adj}	0.94	0.93	0.90	0.90	0.86	0.92	0.94	0.94	0.94

Table 3 Parameters of Eq. 1 ± standard errors for *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52 in M17 broth

Parameters	*L. lactis* subsp. *lactis* biovar. *diacetylactis* U52 plasmid content (kb) in M17 broth								
	24.0	22.7	20.5	18.6	8.7	7.7	5.9	4.6	2.8
a	102.9 ± 26.4	132.8 ± 11.6	125.1 ± 15.1	35.3 ± 32.0	133.6 ± 8.6	105.3 ± 10.6	62.9 ± 25.1	72.9 ± 5.4	66.3 ± 4.8
b	3.2 ± 1.7	–	–	4.2 ± 2.4	–	–	2.6 ± 1.7	–	–
c	–0.1 ± 0.03	–0.06 ± 0.01	–0.07 ± 0.02	–0.09 ± 0.05	–0.07 ± 0.009	–0.06 ± 0.01	–0.1 ± 0.03	–0.06 ± 0.01	–0.06 ± 0.01
d	–2.0 ± 0.3	–2.4 ± 0.4	–2.3 ± 0.5	–2.8 ± 0.7	–2.5 ± 0.3	–2.0 ± 0.4	–1.8 ± 0.3	–	–
e	–	–	–	0.02 ± 0.007	–	–	–	–0.02 ± 0.003	–0.02 ± 0.003
f	0.02 ± 0.01	0.04 ± 0.01	0.045 ± 0.02	–	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
R^2	0.95	0.91	0.88	0.83	0.94	0.91	0.90	0.91	0.91
R^2_{adj}	0.94	0.89	0.85	0.76	0.93	0.88	0.86	0.89	0.89

Table 4 Parameters of Eq. 1 ± standard errors for *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52 in RSM

Parameters	*L. lactis* subsp. *lactis* biovar. *diacetylactis* U52 plasmid content (kb) in RSM								
	24.0	22.7	20.5	18.6	8.7	7.7	5.9	4.6	2.8
a	41.9 ± 23.2	157.1 ± 12.0	119.3 ± 38.2	–	88.8 ± 32.3	–	–	70.5 ± 39.2	–
b	6.1 ± 1.8	–	3.0 ± 2.5	6.8 ± 1.4	3.3 ± 1.9	7.0 ± 0.5	6.2 ± 0.4	3.9 ± 2.3	7.7 ± 0.6
C	–0.14 ± 0.03	–0.05 ± 0.01	–0.1 ± 0.04	–0.1 ± 0.03	–0.1 ± 0.03	–0.2 ± 0.02	–0.2 ± 0.02	–0.1 ± 0.04	–0.2 ± 0.02
d	–	–2.5 ± 0.4	–2.5 ± 0.5	–1.9 ± 0.8	–1.3 ± 0.8	–	–	–1.3 ± 1.0	–
e	–0.01 ± 0.001	–	–	0.01 ± 0.008	–0.02 ± 0.008	–0.02 ± 0.004	–0.02 ± 0.003	–0.01 ± 0.009	–0.02 ± 0.01
f	–	0.03 ± 0.01	0.05 ± 0.02	–	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.02	0.04 ± 0.02
R^2	0.95	0.94	0.91	0.81	0.95	0.93	0.90	0.91	0.88
R^2_{adj}	0.94	0.93	0.88	0.76	0.93	0.91	0.88	0.87	0.86

Table 5 Parameters of Eq. 1 ± standard errors for *L. lactis* subsp. *cremoris* U70 in M17 broth

Parameters	*L. lactis* subsp. *cremoris* U70 plasmid content (kb) in M17 broth							
	22.2	20.2	18.2	12.0	9.5	6.7	6.0	5.2
a	75.4 ± 49.5	161.3 ± 7.7	64.5 ± 28.7	130.7 ± 10.6	168.7 ± 25.7	74.7 ± 11.9	121.8 ± 8.2	116.0 ± 4.4
b	5.9 ± 3.7	–	2.8 ± 2.1	–	–	1.6 ± 0.4	–0.09 ± 0.2	–
c	–0.2 ± 0.07	–0.02 ± 0.008	–0.1 ± 0.04	–0.09 ± 0.01	–0.07 ± 0.02	–	–	0.03 ± 0.005
d	–2.6 ± 0.6	–4.9 ± 0.3	–1.8 ± 0.3	–2.6 ± 0.4	–4.9 ± 1.1	–3.1 ± 0.4	–3.6 ± 0.3	–3.6 ± 0.2
e	–	0.04 ± 0.003	–	–	0.03 ± 0.01	0.03 ± 0.003	0.03 ± 0.004	0.03 ± 0.002
f	0.05 ± 0.02	0.01 ± 0.007	0.04 ± 0.01	0.07 ± 0.01	0.06 ± 0.02	–0.03 ± 0.008	–	–0.02 ± 0.004
R^2	0.91	0.99	0.92	0.93	0.88	0.98	0.99	0.99
R^2_{adj}	0.87	0.99	0.89	0.91	0.83	0.97	0.98	0.99

Table 6 Parameters of Eq. 1 ± standard errors for *L. lactis* subsp. *cremoris* U70 in RSM

Parameters	*L. lactis* subsp. *cremoris* U70 plasmid content (kb) in RSM							
	22.2	20.2	18.2	12.0	9.5	6.7	6.0	5.2
a	–	120.5 ± 42.2	68.2 ± 40.1	–	132.4 ± 63.0	110.8 ± 15.4	140.8 ± 12.1	94.4 ± 8.0
b	9.7 ± 0.7	5.3 ± 2.9	5.5 ± 3.0	7.8 ± 0.6	4.8 ± 4.3	2.5 ± 1.3	–0.6 ± 0.4	1.4 ± 0.3
c	–0.3 ± 0.03	–0.2 ± 0.06	–0.2 ± 0.06	–0.2 ± 0.03	–0.2 ± 0.08	–0.06 ± 0.03	–	–
d	–	–4.7 ± 0.9	–2.4 ± 0.5	–	–5.0 ± 1.4	–4.0 ± 0.3	–3.3 ± 0.4	–2.5 ± 0.3
e	–0.02 ± 0.005	0.02 ± 0.009	–	–0.02 ± 0.005	0.02 ± 0.01	0.03 ± 0.003	0.02 ± 0.003	0.02 ± 0.002
f	0.03 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.03 ± 0.02	0.06 ± 0.03	–	0.01 ± 0.009	–0.02 ± 0.006
R^2	0.90	0.95	0.92	0.89	0.89	0.99	0.99	0.99
R^2_{adj}	0.87	0.92	0.89	0.86	0.83	0.99	0.99	0.99

temperatures than higher incubation temperatures. However, it should be further investigated to understand whether this is a common phenomenon among lactococci or not. Incubation time was found to have a more pronounced effect on plasmid stability. Hence, stable starter cultures must be selected by considering incubation times in manufacturing processes where *L. lactis* strains were used as starter cultures.

The criteria for selection of a model form for such data (combined incubation time-temperature data) should essentially include: (i) accurate description of the data, (ii) ease of synthesis and use, (iii) parsimony (i.e., simplicity), (iv) (at least partial) biological interpretability of parameter values.

When two or more factors (such as incubation time and temperature) affect the plasmid stability, response surface type models [quadratic (such as Eq. 1) and cubic equations] can describe the effects of all the factors and their interactions. In this study, we selected to use quadratic model because Eq. 1 is enough to describe the data accurately and it is also easy to use. Moreover, aiming at the most parsimonious model, i.e., a model with the minimum number of parameters as possible and at the highest parameter accuracy, the selection of the developed polynomial model (Eq. 1) is justified [30]. Since Eq. 1 is an empirical model its parameters has no biological meaning which can be a disadvantage.

Equation 1 can successfully be used to predict the plasmid stability of *L. lactis* subsp. *lactis* U29, *L. lactis* subsp. *lactis* biovar. *diacetylactis* U52 and *L. lactis* subsp. *cremoris* U70 in M17 broth and in RSM; however, predictions should be done within the experimental domain previously used to develop the model. For example, plasmid stability percent of (plasmid content 19.6 kb) *L. lactis* subsp. *lactis* U29 in RSM incubated at 37°C for 30 h can be calculated as 55 by using the values given in Table 2. It is also possible to calculate the plasmid stability percent other strains in M17 broth or RSM by using the corresponding values given in Tables 1–6; however, as mentioned above interpolation region should be used since any extrapolation (outside the range of experimental domain) may lead to erroneous predictions.

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