

Lactic Acid and Diacetyl Production of Nitrosoguanidine Mutants Derived from *Lactobacillus casei* 34143 in Soymilk

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Production of acid and diacetyl by nitrosoguanidine mutants of *Lactobacillus casei* 34143 was evaluated in soymilk. Optimum temperature for growth of five cultures varied between 34.5 and 35.9°C. However, the temperature required for maximum production of diacetyl in soymilk in the parent culture was 32.7°C and mutants such as N-14, N-15 and N-25 required a temperature of 29.6°C, while another mutant, L-7 required 26.4°C for production of diacetyl. Parent and mutant cultures were deficient in citrate permease and citritase synthetic mechanisms, although lactose-utilising mutants (N-25 and S-3-1) possessed both β -galactosidase and phospho- β -galactosidase.

Key words : *Lactobacillus casei*, nitrosoguanidine mutants, soymilk, lactic acid, diacetyl

Introduction

Investigations in our laboratory have shown that a few nitrosoguanidine (NTG) mutants of *Lactobacillus casei* subsp. *alactosus* 34143 acquired a lactose-fermenting mechanism, while a small number of mutants became either high acid producers or high diacetyl producers¹⁾. This indicates a possible genetic variations among mutants and differences from the parent culture in their genetic makeup. The change in the DNA base sequence must have resulted in differing synthetic patterns of certain enzymes involved in lactose fermentation and diacetyl production. During recent years several investigators have established the role of plasmids in lactose metabolism and diacetyl production²⁻⁵⁾.

This study was made to determine the optimum incubation temperature and time for growth and production of diacetyl and acid by selected mutants and parent culture in tryptone yeast extract broth (TY broth) and soymilk. An investigation was also made to determine their

ability to synthesize citrate permease, citritase, β -galactosidase and phospho- β -galactosidase.

Materials and Methods

Source and maintenance of cultures. *Lactobacillus casei* subsp. *alactosus* 34143 and its NTG mutants, namely L-7, N-14, N-15 and lactose-positive (*lac*⁺) mutants N-25 and S-3-1 had been maintained in this laboratory. They were grown in TY broth routinely.

Media. The following media were used in this study.

a) Tryptone yeast extract broth (TY broth)¹⁾.

b) Soymilk was prepared from defatted soy bean flour as described earlier¹⁾.

Effect of incubation temperature.

a) Cultures were inoculated at 1% level in TY broth and incubated in temperature gradient incubator Model TN-3 (Advantec) maintained at

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5–60°C. After 24 hours of incubation, the growth was measured at 450 nm with Hitachi Model 101 spectrophotometer and expressed as Optical Density (O. D.).

b) Cultures were inoculated at 1% level in soymilk and incubated in temperature gradient incubator. After 24 hours of incubation, the samples were withdrawn and assessed for acid production.

c) Cultures were inoculated at 1% level in soymilk and incubated in temperature gradient incubator for up to 4, 8, 12, 16, 20 and 24 hours and the fermented soymilk was tested for diacetyl production.

Effect of incubation time. Mutants and parent culture were inoculated at 1% level in soymilk and incubated at 35°C. The fermentation was terminated at 4, 8, 12, 14, 16, 18, 20 and 24 hours and the samples were assessed for acid and diacetyl contents.

Analysis. Titratable acidity in soymilk was determined by titration with 0.1N NaOH. The Owades and Jakovac method as modified by Pack *et al.*⁶⁾ was used for diacetyl estimation. A standard curve was prepared by using dimethylglyoxime⁷⁾.

Isolation of plasmids. Plasmid profile of parent and mutant cultures was determined as described by Anderson and McKay⁸⁾.

Enzymes.

a) Citrate permease production by the parent culture 34143 and its mutants was determined as described previously by Harvey and Collins⁹⁾.

b) Citritase synthesis by the cultures was studied by following the method of Harvey and Collins¹⁰⁾.

c) β -Galactosidase synthesis by lac⁺ mutants, N-25 and S-3-1 was determined by following the method of Citti *et al.*¹¹⁾.

d) The method of McKay *et al.*¹²⁾ was used for determining the synthesis of phospho- β -galactosidase by mutant cultures N-25 and S-3-1.

Results and Discussions

Effect of incubation temperature on growth of parent and its mutants is illustrated in Fig. 1. It was found that the optimum growth temperature of five cultures varied between 34.5 and 35.9°C. These observations indicate that the mutants do not differ much in their requirement of optimum growth temperature from the parent culture 34143. It was also observed that growth of all cultures occurred at 13°C and also at 48.5°C.

Data presented in Table 1 reveal that the parent culture produced the highest amount of titratable acidity in soymilk at 34.5°C, and the mutants N-14 and N-15 exhibited the same trend as their parent in the production of acid in soymilk. However, the other mutant, namely N-25, produced more acid in soymilk at 32.8°C, although the growth in TY broth of this culture was maximum at 34.5°C. These findings indicate that the growth medium has some influence over the optimum growth temperature of this culture.

Preliminary work in our laboratory revealed that parent culture 34143 produces more diacetyl at 12 hours of incubation, as opposed to 18 hours incubation for mutant cultures N-14 and L-7 and 24 hours for N-25. The results of this study indicate that the parent culture 34143 produces the highest amount of diacetyl at 32.7°C and all other mutants except L-7 require an optimum temperature of 29.6°C, while mutant culture L-7 requires 26.4°C for maximum production of diacetyl in soymilk (Fig. 2).

Data presented in Table 2 indicate the effect of incubation time on acid production by the cultures in soymilk. As the incubation period increased, the amount of acid produced by the parent culture 34143 also increased up to 24 hours of incubation. However, mutant culture L-7 failed to produce any considerable amount of acid at 35°C.

Effect of incubation time on diacetyl production by the cultures is presented in Table 2. The

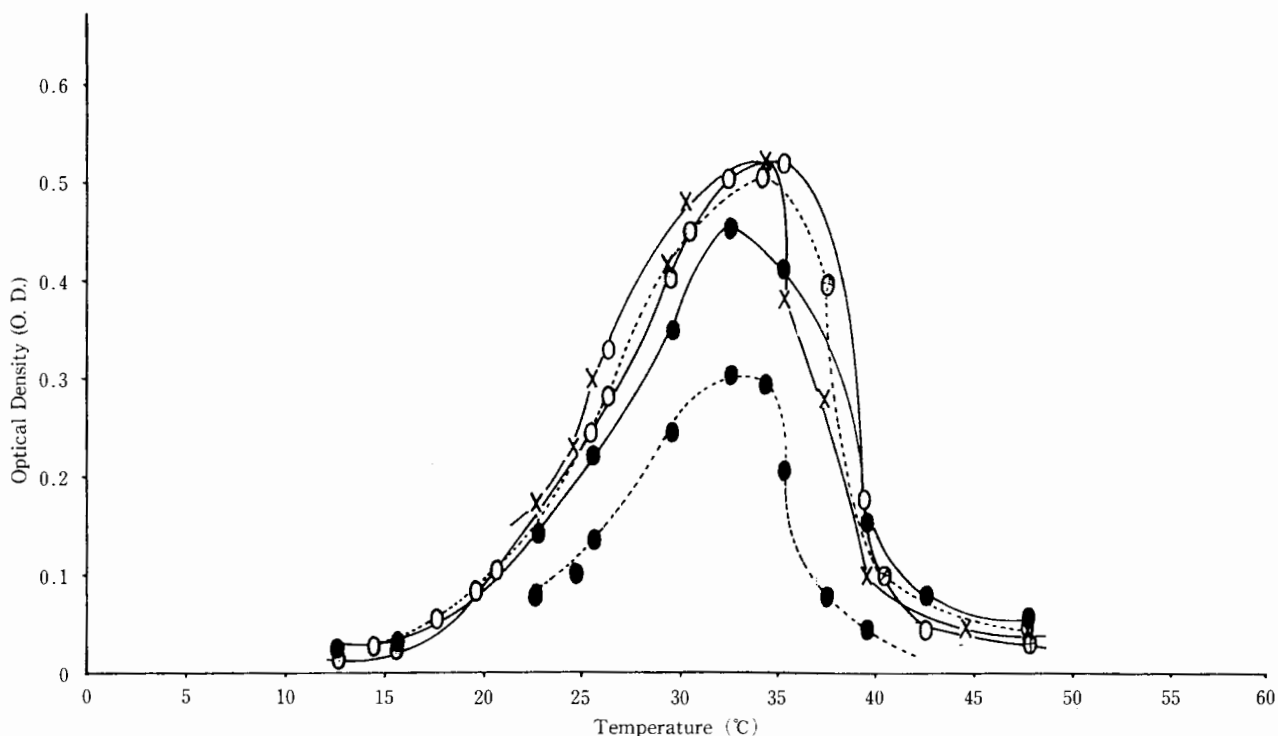


Fig. 1 Effect of temperature on the growth of *Lactobacillus casei* subsp. *alactosus* 34143 and its mutants in TY broth. The growth was measured at 450 nm with spectrophotometer and expressed as optical density. 34143 (O---O), N-25 (O—O), N-15 (●---●), L-7 (●—●), N-14 (x---x).

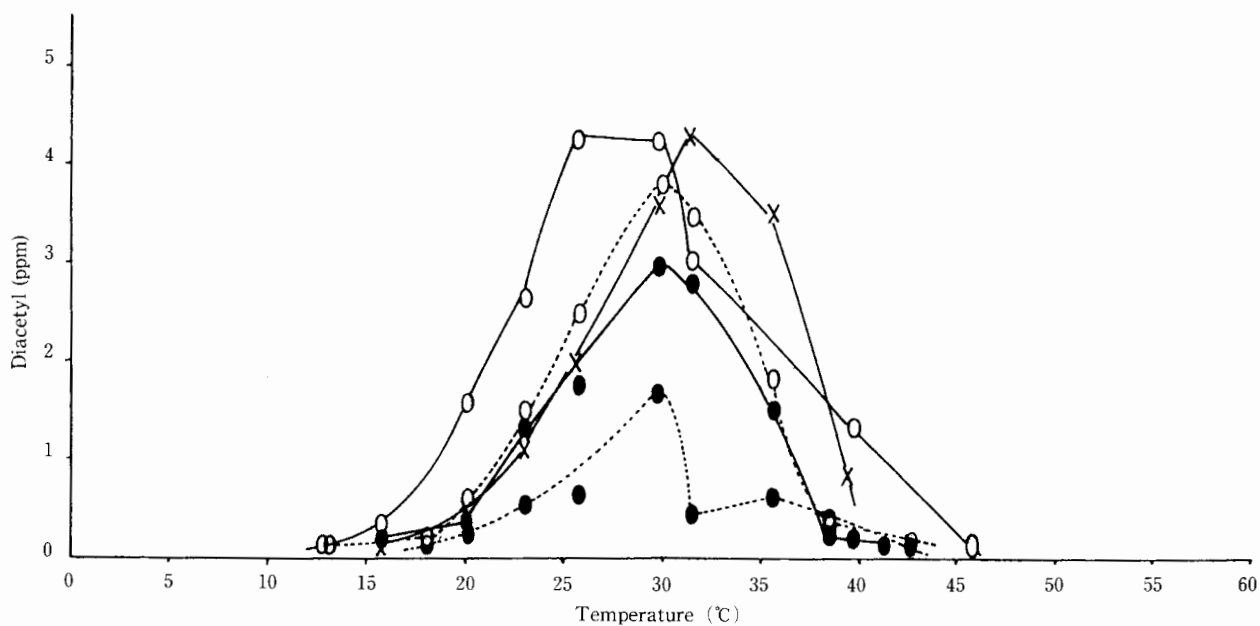


Fig. 2 Effect of temperature on the production of diacetyl by *Lactobacillus casei* subsp. *alactosus* 34143 and its mutants in soymilk. The diacetyl content was estimated after 18 hours of incubation in case of N-14 and L-7, 14 hours of incubation in case of 34143 and N-25, and 12 hours of incubation in case of N-15. 34143 (x---x), L-7 (O—O), N-14 (O---O), N-15 (●---●), N-25 (●—●).

data indicate that 34143 culture produced the highest amount (1.18 ppm) of diacetyl at 12 hours of incubation. Like parent culture, mutant culture N-15 also produced the highest amount of diacetyl at 12 hours of incubation. The diacetyl production by mutant culture L-7 progressively increased with the increase in the period of incubation up to 16 hours, thereafter it showed a decreasing trend. A *lac*⁺ mutant of the parent culture, N-25 failed to produce significant amounts of diacetyl when compared to the other mutants.

Parent and its mutants were deficient in citrate permease and citritase enzyme synthetic mecha-

Table 1 Effect of incubation temperature on acid production by parent and mutant cultures of *Lactobacillus casei* subsp. *alactosus* 34143 in soymilk^{a)}

Incubation temperature (°C)	Titratable acidity (%) ^{b)}			
	34143	N-14	N-15	N-25
15.2	0.01	0.01	0.00	0.00
18.5	0.01	0.01	0.00	0.00
21.7	0.01	0.01	0.02	0.06
25.0	0.04	0.04	0.06	0.11
28.0	0.07	0.05	0.07	0.29
31.3	0.14	0.13	0.22	0.62
34.5	0.27	0.28	0.28	0.61
37.6	0.10	0.16	0.12	0.50
40.8	0.05	0.00	0.02	0.02
44.8	0.00	0.00	0.00	0.00
48.5	0.00	0.00	0.00	0.00

a) Titratable acidity of fermented soymilk was assessed after 24 hours of incubation.

b) Expressed as percent lactic acid.

nisms. Earlier studies in our laboratory also indicated that although the parent failed to produce diacetyl from sodium citrate, it did produce enhanced amounts of diacetyl and acetoin when peptone-yeast extract-glucose broth medium was supplemented with 0.5% of sodium pyruvate¹³⁾. These observations reveal that both parent and mutant cultures do not produce diacetyl from citrate but convert pyruvate into diacetyl.

The *lac*⁺ mutants, namely N-25 and S-3-1, were able to synthesize both β -galactosidase and phospho- β -galactosidase. Therefore, the initial steps of lactose utilization by these two mutants may involve a phosphoenol-pyruvate dependent phosphotransferase system coupled with phospho- β -galactosidase as known to group N streptococci¹⁴⁾.

The plasmid profile of *lac*⁺ and *lac*⁺ mutants of 34143 culture revealed that neither these mutants nor their parent culture possessed different sizes of plasmids. These observations indicate that the *lac*⁺ mutants may possess the genes for lactose utilization in the chromosome. The nitrosoguanidine affected the chromosomal DNA base sequence in such a way that a few of the mutants of *Lactobacillus casei* subsp. *alactosus* 34143 became *lac*⁺ by acquiring β -galactosidase and phospho- β -galactosidase synthetic mechanisms.

Table 2 Effect of incubation time on acid and diacetyl production by parent and cultures of *Lactobacillus casei* subsp. *alactosus* 34143 in soymilk

Incubation time (hours)	34143		L-7		N-15		N-25	
	Diacetyl (ppm)	TA ^{a)} (%)	Diacetyl (ppm)	TA ^{a)} (%)	Diacetyl (ppm)	TA ^{a)} (%)	Diacetyl (ppm)	TA ^{a)} (%)
4	0.21	0.02	0.19	0.01	0.17	0.01	0.01	0.17
8	0.63	0.03	0.65	0.04	1.28	0.02	0.01	0.14
12	1.18	0.63	2.14	0.02	3.02	0.04	0.37	0.25
16	0.70	0.56	3.30	0.00	1.85	0.22	0.59	0.30
20	0.71	0.64	2.04	0.03	0.99	0.25	0.72	0.16
24	0.93	0.66	0.72	0.00	1.02	0.48	0.73	0.04

a) Titratable acidity was expressed as percent lactic acid.

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Lactobacillus casei 34143 の ニトロソグアニジン処理変異株による 大豆乳での乳酸およびジアセチル生成

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大豆乳の発酵に有効な乳酸菌株を検索する目的で、ラクトース非発酵性 *Lactobacillus casei* 34143 のニトロソグアニジン (NTG) 処理変異株について、液体培地での生育性ならびに大豆乳培地での乳酸とジアセチル生成能を温度勾配培養法によって検討した。また、NTG 処理変異株におけるクエン酸およびラクトースの取込みと分解に関与する酵素の活性についても調べた。その結果、親株ならびに変異株の液体培地での生育至適温度はほぼ同じ値を示し、いずれも34.5℃から35.9℃の範囲であった。しかしながら、大豆乳培地での乳酸とジアセチル生成能には違いを示す菌株があった。とくにジアセチル生成の至適温度は親株が32.7℃であるのに対して、変異株 (N-14株, N-15株および N-25株) では29.6℃であり、そして変異株の L-7 株では26.4℃であった。一方、供試した親株と変異株はすべてクエン酸透過酵素ならびにクエン酸分解酵素活性を示さなかったが、ラクトース代謝能を獲得した変異株 (N-25株と S-3-1 株) は β -ガラクトシダーゼとフォスフォ- β -ガラクトシダーゼの活性を示した。