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Optimum Conditions for the Formation of Tetramethylpyrazine Flavor Compound by *Lactococcus lactis* ssp. *lactis* biovar. *diacetilactis* FC1

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To produce the tetramethylpyrazine (TMP) flavor compound, *Lactococcuss lactis* subsp. *lactis* biovar. *diacetilactis* (*L. diacetilactis*) FC1 was cultivated in the TMP medium containing 3% (w/v) of Na-citrate and 6% (w/v) arginine-HCl as substrates of acetoin and NH_3, respectively, which are the two precursors of the TMP. After 19-day fermentation at 34°C, 0.57 g/l or 4.19 mmole/l of the TMP was produced. This was the first result showing that the TMP could be produced by *L. diacetilactis*.

Tetramethylpyrazine (TMP) flavor compound can be produced microbiologically by condensation of two moles of acetoin and two moles of ammonia into one mole of the TMP (1, 2, 4). It has been known that *Lactococcus lactis* ssp. *lactis* biovar. *diacetilactis* (*L. diacetilactis*) FC1, a dairy starter used as a diacetyl producer, can produce both the acetoin and the ammonia (5). The authors reported in the previous studies the optimum conditions for the acetoin formation from citrate (5) and for the ammonia from arginine-HCl (6) by the *L. diacetilactis* FC1. In the present study, optimum fermentation conditions for simultaneous formation of the two precursors of the TMP, i.e., the acetoin and ammonia, and the formation of the TMP from the culture of *L. diacetilactis* FC1 were investigated.

MATERIALS AND METHODS

Fermentation Conditions

L. diacetilactis FC1 was the same strain as used in the previous studies (5, 6). Fermentation was performed using TMP production medium which was based on the Lactose-Citrate Medium (3) but modified to contain 3% (w/v) of Na-citrate, 6% (w/v) of arginine-HCl, 2 mg/l of thiamine-HCl, 1% (w/v) of galactose, and tryptone instead of petone, according to the results of previous studies (5, 6). Cultivation was done in a shaking cultivator at 34℃ and 150 rpm for up to 19 days.

Analysis of the Culture

Cell growth and concentration of the acetoin and ammonia were analyzed by the methods in the previous studies (5, 6). To isolate the TMP from the liquid culture, the culture was filtered, extracted with diethylether in a continuous liquid-liquid extracter for 12 h, eluted through a column packed with anhydrous Na_2SO_4 and Al_2O_3 to remove water and other impurities, and concentrated in a rotary evaporator to get oleoresin. The TMP content in the oleoresin was analyzed with a gas chromatograph (GC, Pye Unicam, PU 4500) with OV 101, stainless steel column and a flame ionization detector. The GC was operated at injector temperature of 220℃, detector temperature of 250℃, and temerature programing of 80~190℃ at 3℃/min. The TMP was identified by the retention time of the standard TMP and the concentration was determined by comparing peak area of the TMP with that of the qunoxaline which was added as a internal standard.

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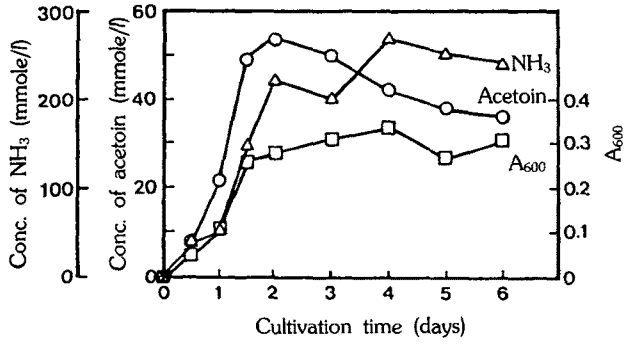

Fig. 1. Fermentation course of *L. diacetilactis* FC1 cultured in the TMP production medium at 34°C.

RESULTS AND DISCUSSION

Fermentation in the TMP Production Medium

Fig. 1 shows the characteristics of acetoin and ammonia formation and the cell growth when *L. diacetilactis* FC1 was cultivated in the TMP production medium containing both Na-citrate and arginine-HCl. The cell growth was slow and the absorbance of the culture liquid (A_{600}) reached about 0.25 in the stationary phase after 30 h of cultivation, while the A_{600} was about 0.80 after 20 h when media for production of acetoin and NH_3 were used (5, 6). This result suggested that coexistance of the citrate, arginine, and their metabolites in the TMP production medium influenced negatively on the growth of the cell because the optimum fermentation conditions for acetoin and NH_3 production were investigatied independently in the previous studies (5, 6) and the conditions were combined for the production of TMP in this study. Therefore further studies for the optimum conditions of acetoin and NH_3 production in the TMP medium would be necessary to increase the productivity of acetoin and NH_3.

The concentration of the acetoin and ammonia decreased after the stationary phase and this probably was due to condensation of the two precursors into the TMP. After 6-day fermentation, the culture was extracted with diethylether, analyzed by GC, and the TMP was identified in the gas chromatogram of the oleoresin as shown in Fig. 2. This result supported the Adachi's (1) and Rizzi's (8) postulation that biological TMP is synthesized by the reaction between acetoin and NH_3. Also, this is the first report that the TMP could be produced from acetoin and ammonia produced by *L. diacetilactis*. *Bacillus subtilis* (4, 7) and *Corynebaterium glutamicum* were only microorganisms known to produce the TMP but productivities were very low except for a mutant of *C. glutamicum* (2).

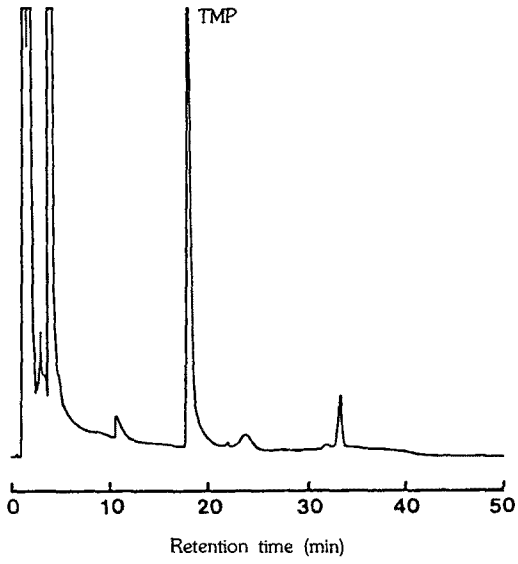

Fig. 2. Gas chromatogram of volatile compounds from 6-day culture of *L. diacetilactis* FC1 in the TMP production medium at 34°C.

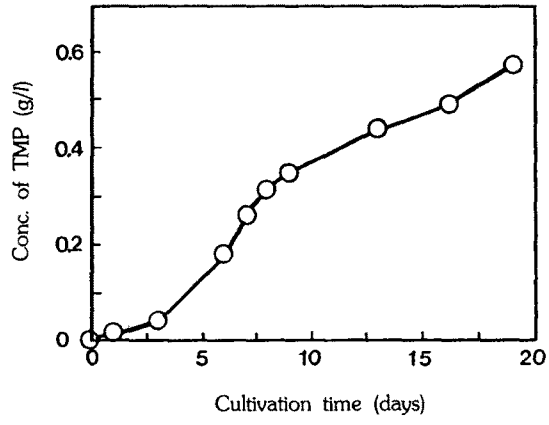

Fig. 3. TMP production course of *L. diacetilactis* FC1 cultured in the TMP production medium at 34 °C.

The TMP Production Course

Production course of the TMP when *L. diacetilacits* FC1 was cultivated in the TMP medium was shown in Fig. 3. The content of TMP increased rapidly for the first 10 days and then increased steadily to reach 0.57 g/l (4.19 mmole/l) after 19 days. It was shown in Fig. 1 that the cell began to die and the production of acetoin and NH_3 did not increase after two days of fermentation. However Fig. 3 illustrates that the TMP production continued until 19 days of culture suggesting that the increase of TMP was not directly related to the cell growth. This implies that the formation of TMP was resulted from

nonenzymatic condensation between acetoin and NH_3.

If we consider that two moles of acetoin and two moles of NH_3 are condenced into one mole of TMP and the concentration of acetoin was 35 mmole/*l* in the later period of the fermentation in Fig. 1, the 4.19 mmole of TMP after 19 days culture means that only 16% of acetoin was converted into TMP. The conversion rate was very low and this was probably due to that the condensation of acetoin and NH_3 into TMP is not a biochemical but a chemical reaction which is accelerated at much higher temperature than that of fermentation (8). It has been known that many factors such as concentration of acetoin and NH_3, pH, and temperature are important in the conversion of acetoin and NH_3 into TMP (8).

Therefore, further studies to find optimum conditions to increase the condensation of the TMP from acetoin and NH_3 produced in the citrate fermentation of *L diacetilactis* would be necessary.

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