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Effect of Volume Concentration Ratio of Cell-free Medium on Tetramethylpyrazine Production by *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis* FC1

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Acetoin and ammonia, the precursors of tetramethylpyrazine (TMP) having "nutty" or "roasted" flavors, were produced by cultivating *Lactococcus lactis* ssp. *lactis* biovar. *diacetilactis* FC1. The effects of the volume concentration ratio (VCR) of cell-free medium on the formation of TMP were investigated using a rotary evaporator at 70°C and 80°C. As the VCR increased, the formation of TMP and the conversion ratio of acetoin to TMP increased. More TMP were formed at 70°C than at 80°C. As the VCR increased, the concentration of acetoin decreased implying the formation of TMP from acetoin and ammonia.

Various microorganisms have been employed to produce many different flavors (2, 4, 14). These include esters, lactones, menthol, diacetyl, pyrazines, terpenes, and glutamic acid which are essential in achieving certain food flavors (4, 16). For example, pyrazines produce roasted, meaty, or smoky flavors (11, 12). Recently, more than one hundred different kinds of pyrazines have been identified in various food products. Among the various pyrazine derivatives, tetramethylpyrazine (TMP), an alkylpyrazine, was found to be responsible for the characteristic aroma of fermented soy bean or natto (1, 3, 5, 10). Its precursors are acetoin and ammonia; one mole of TMP is produced when two moles of each are condensed together (1). TMP, which was first isolated from the culture of *Bacillus subtilis* by Kamiya *et al.* (5), has also been produced by the culture of a mutant of *Corynebacterium glutamicum* (3).

TMP was produced through the production of the two precursors *i.e.*, acetoin and ammonia using *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis* FC1 (*L. diacetilactis*). It was Kim and Lee (6, 7, 8) who first reported that TMP could be produced using *L. diacetilactis*. The optimum conditions for the production of acetoin and ammonia by *L. diacetilactis* were: initial pH 5.5 of the culture medium, cultivation temperature of 34°C, 3% (w/v) of Na-citrate, 6% (w/v) of arginine-HCl, and 1% (w/v) of galactose as a carbon source. A Fed-batch culture of *L. diacetilactis* in lactose-citrate broth with aeration at 34°C for 98 hr had been previously carried out by Kim *et al.* (9). The optimum conditions for the production of acetoin and ammonia were achieved by adding citrate and arginine to 156 mM and 50 mM after 18 hr of fermentation, and citrate and galactose to 156 mM after 36 hr of fermentation, respectively. TMP formation was maximized by heating the cell free supernatant at 121°C for 4 hr with an initial pH of 8.3. The conversion ratio of acetoin to TMP ranged over 1.5 to 8.6% (9).

To enhance the utilization of acetoin, the concentration of cell-free medium was carried out. We investigated the effects of the volume concentration ratio (VCR) of the cell-free medium on the concentrations of acetoin and ammonia, the formation of TMP, and the conversion ratio of acetoin to TMP.

MATERIALS AND METHODS

Microorganism and Fermentation Medium

Pure stock cultures of *L. diacetilactis* was maintained at −20°C and cultivated on a lactose-citrate medium as previously described (6, 7, 8, 9). Galactose solution (1%, w/v) and thiamine-HCl (2 mg/*l*) were pre-filtered through a Whatman membrane filter (pore size: 0.45 μm) and added to the medium.

Cultivation Method

The culture (200 m*l*), contained in a 500 m*l* flask,

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was incubated in a shaking incubator (Vision Scientific Co., LTD., Korea) at 34°C, at 200 rpm for 20 hr. The inoculum (1%) was aseptically transferred to a 5 *l* jar fermentor (Korea Fermentor Co., Korea) containing 2.5 *l* of the medium. Free cells were cultivated at 34°C and at pH 5.5 with an aeration rate of 0.6 vvm and an agitation rate of 200 rpm. For the fed-batch cultivation, citrate and arginine solutions were added to the medium to reach 156 mM and 50 mM respectively after 18 hr, and citrate and galactose solutions were added to reach equally 156 mM after 36 hr.

Concentration of the Culture Supernatant and TMP Formation by Condensation

The culture medium was clarified at 693×g, at 4°C for 15 min. The supernatant was concentrated using a rotary vacuum evaporator (Tokyo Aikakikai Co., Japan) at 70°C between VCR 1.5 and 3.0, and at 80°C between VCR 2.0 and 4.0. The concentrated supernatant was heated and condensed at 121°C, at pH 8.3 for 1 hr following the previously reported procedure of optimum conditions for TMP formation (9).

Citrate, Acetoin, Arginine, and Ammonia Analysis

Citrate, acetoin, arginine and ammonia concentrations were determined by the methods of Marier and Boult (13), Westerfeld (17), Rosenberg *et al.* (15), and Wriston (18).

TMP Analysis

The pH of the samples were adjusted to 8.3 to make TMP insoluble in water which was then extracted with diethylether using a continuous liquid-liquid extractor for 12 hr. The concentration of TMP in the extract was analyzed using a gas chromatograph PU4500 (Pye Unicam, Philips, Netherlands) equipped with a flame ionization detector. The stainless steel column (phase: OV 101, support: diatomite CS 100~120 mesh size) was first run at 80°C with a temperature increase of 3°C per min till it reached 190°C. The temperatures of the injector and detector were 220°C and 250°C, respectively. Quinoxaline was added to samples as an internal standard. The TMP was identified by the retention time, and the concentration of the TMP was determined by comparing the peak area of TMP with that of quinoxaline. The conversion ratio of acetoin to TMP was calculated by the following equation,

$$\text{Conversion Ratio (\%)} = \frac{\text{TMP Concentration (mM)}}{1/2\ \text{Acetoin Concentration (mM)}} \times 100$$

where 1/2 in the denominator indicates the two moles of acetoin converted into one mole of TMP.

RESULTS AND DISCUSSION

Concentration of Acetoin, Ammonia, and TMP at Various VCR's

In the previous study on TMP production, the conversion ratio of acetoin to TMP ranged over 1.5 to 8.6% (9). To enhance the conversion of acetoin to TMP, concentration of the culture supernatant was carried out. Concentrations of two precursors (acetoin and ammonia) of TMP and that of TMP were monitored while concentrating the culture supernatant (Fig. 1, Fig. 2). As VCR was increased from 1.0 to 1.5, 2.0, 2.5, and 3.0

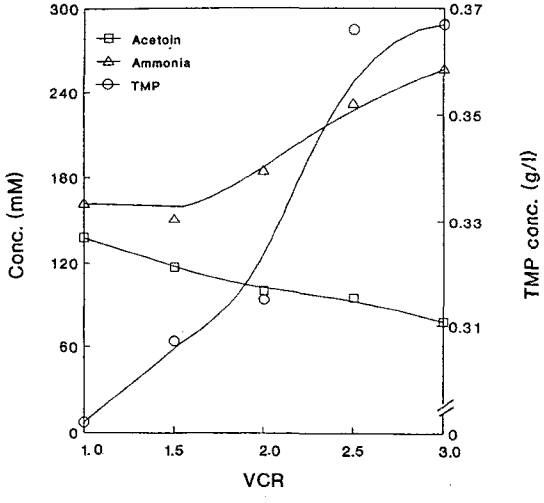

Fig. 1. The concentration of acetoin, ammonia, and tetramethylpyrazine (TMP) at various volume concentration ratios (VCR's) and concentration temperature of 70°C.
L. diacetilactis was cultivated at 34°C for 98 hr and then the culture supernatant was concentrated using a rotary evaporator at 70°C.

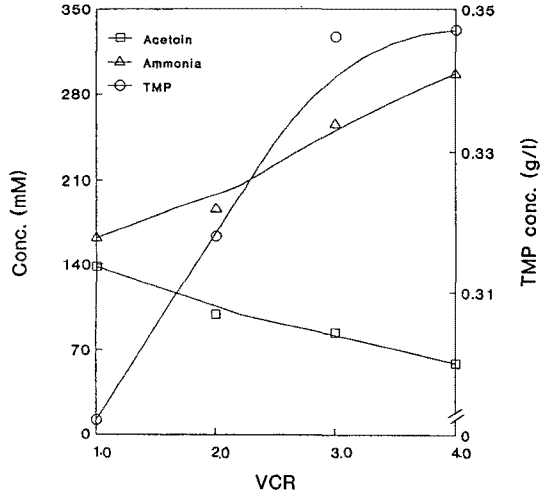

Fig. 2. The concentration of acetoin, ammonia, and TMP at various VCR's and concentration temperature of 80°C.
L. diacetilactis was cultivated at 34°C for 98 hr and then the culture supernatant was concentrated using an evaporator at 80°C.

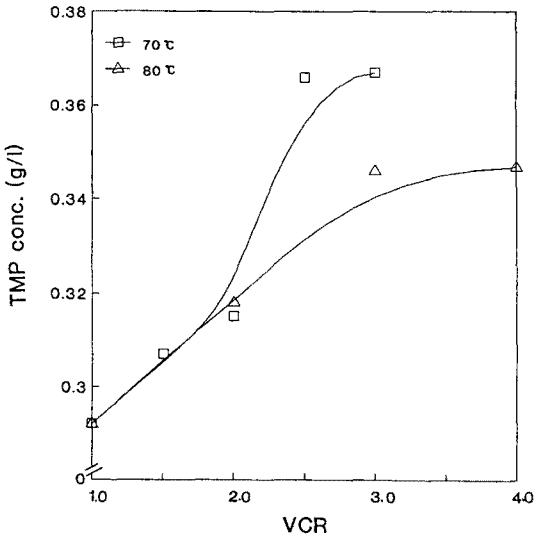

Fig. 3. The TMP productivity during concentration of the culture supernatant followed by heat treatment.
L. diacetilactis was cultivated at 34°C for 98 hr and the culture supernatant was concentrated using a rotary evaporator at 70°C and at 80°C, and then heated at 121°C for 1 hr.

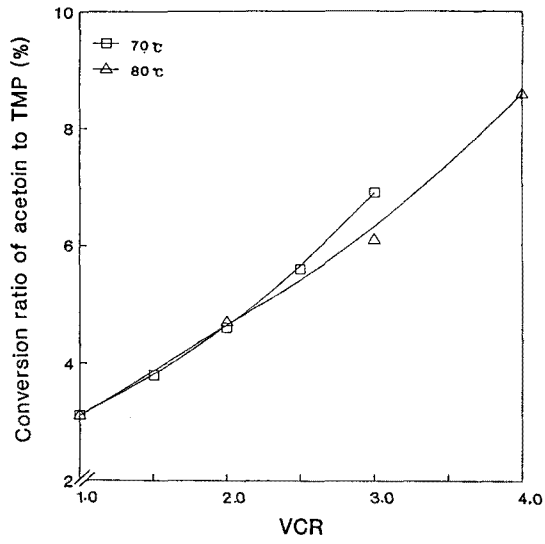

Fig. 4. The conversion ratio of acetoin to TMP during concentration of the culture supernatant followed by heat treatment.
L. diacetilactis was cultivated at 34°C for 98 hr and the culture supernatant was concentrated using an evaporator at 70°C and at 80°C, and then was heated at 121°C for 1 hr.

at 70°C, the concentration of acetoin was decreased from 138 to 117, 100, 95, and 78 mM, due to the increased conversion ratio of acetoin to TMP. When VCR was increased from 1.0 to 2.0, 3.0, and 4.0 at 80°C as depicted in Fig. 2, the concentration of acetoin was also decreased from 138 to 99, 84, and 59 mM showing a similar tendency as presented in Fig. 1.

The increase of VCR from 1.0 to 3.0 at 70°C contributed to the increase of ammonia concentration from 162 mM to 257 mM as shown in Fig. 1. As VCR increased from 1.0 to 4.0 at 80°C, the concentration of ammonia increased from 162 mM to 297 mM (Fig. 2). These results indicate that enough amount of ammonia still exists in the culture supernatant after TMP formation.

The concentration of TMP also increased when VCR increased at both 70°C and 80°C. This may be attributed to the increase of TMP formation stimulated by heat during evaporation because the culture supernatant was concentrated at relatively high temperatures over 70°C (11, 12). Before concentration, the concentration (162 mM) of ammonia was much higher than that (138 mM) of acetoin (Fig. 1, Fig. 2). Acetoin concentration was decreased in spite of VCR increase because equimolar amounts of acetoin and ammonia were converted into TMP (1).

Formation of TMP during Concentration

The effects of concentration of cell-free medium on the formation of TMP were investigated at 70°C and 80°C (Fig. 3). The culture supernatant could be concentrated only up to VCR 3.0 at 70°C when using an evaporator. When VCR increased, the productivity of TMP increased. The highest concentration (0.37 g/l) of TMP was obtained at VCR 3.0 and 70°C. TMP production was increased by 26% at VCR 3.0, at 70°C comparing with the unconcentrated cell-free medium (VCR 1.0). This result implies that the TMP formation was enhanced by concentration of the culture supernatant. TMP formation was better at 70°C than at 80°C. This may be due to the increase of the loss of acetoin and ammonia at the elevated temperature of 80°C. When VCR increased, the conversion ratio of acetoin to TMP increased (Fig. 4). When VCR was 4.0 at 80°C, the conversion ratio of acetoin to TMP was 8.6%, the highest percent conversion ratio throughout the experiment. This phenomenon indicates that the substrate (acetoin) utilization increased for the synthesis of TMP during concentration. At 70°C, cell-free medium could not be concentrated above VCR 3.0.

CONCLUSION

The results obtained in this study show that the increased VCR of the culture supernatant contributed to the increase of the TMP productivity and the conversion ratio of acetoin to TMP. Further studies to increase the TMP productivity using immobilized cell reactors, such as a packed bed reactor or a fluidized bed reactor, are necessary.

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