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Tetramethylpyrazine Production by Immobilized Culture of *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis* FC1

LEE, JI-EUN, GUN-JO WOO[1], AND HYONG JOO LEE*

Department of Food Science and Technology, and Research Center for New Biomaterials in Agriculture, Seoul National University, Suwon 441-744, Korea
[1]Department of Foods and Nutrition, Ewha Woman's University, Seoul 120-750, Korea

Acetoin and ammonia, the precursors of tetramethylpyrazine (TMP) having "meaty" or "roasted" flavors, were produced by the culture of *Lactococcus lactis* ssp. *lactis* biovar. *diacetilactis* FC1 in free and immobilized cell systems. Cells were immobilized using *k*-carrageenan and then were incubated at 34°C. The TMP productivity (0.34 g/l) and the conversion ratio (9.3%) of acetoin to TMP of the immobilized cell system were higher than those (0.24 g/l, 7.0%) of the free cell system. When the beads were activated for 12 h, the productivity of acetoin and TMP increased slightly.

Pyrazines are heterocyclic nitrogen containing compounds contributing to the unique flavor related to roasting and toasting of numerous foods. Most pyrazines, except the methoxypyrazines in bell pepper, have been found in foods that are typically roasted or toasted (5). The food industry has used many kinds of pyrazine derivatives as important volatile components in food processing. In addition the demand for natural pyrazine is increasing in the food industry. The price of naturally derived pyrazines is as high as $3,000/kg (2). Biogeneration of natural pyrazine derivatives has been tried using microbial fermentation and enzyme technology, e.g., tetramethylpyrazine (TMP) from *Bacillus subtilis* (9), *Corynebacterium glutamicum* (7), and 2-methoxy-3-alkylpyrazine from *Pseudomonas perolens* (19). Pyrazine can be formed under mild conditions. Rizzi (22) showed that TMP was formed from acetoin and ammonia as precursors at 22°C with acidic pH, and proposed a mechanism for pyrazine formation from acyloin and ammonia. TMP is formed by condensation of acetoin and ammonia present in the cell free medium. *Lactococcus lactis* ssp. *lactis* biovar. *diacetilactis* FC1 (*L. diacetilactis*) produces both acetoin and ammonia (13, 14).

Previously, we reported the effects of the volume concentration ratio of the cell free medium on the concentrations of acetoin and ammonia, the formation of TMP, and the conversion ratio of acetoin to TMP, to enhance the utilization of acetoin as a precursor for TMP production (17).

*Corresponding author
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In the present study, immobilization of *L. diacetilactis* was also carried out to enhance the utilization of acetoin as a precursor for TMP formation. The immobilization of microbial cells has become a subject of increased interest, since immobilized cells have the following advantages over free cells: 1) reuse of cells, 2) higher conversion rate, 3) reduced susceptibility to contamination, 4) the ability to separate cells easily from products, 5) maintenance of stable and active cells, 6) ease of continuous cultivation, 7) improved process control, 8) protection of cells against mechanical damage (3, 4, 12, 26). Entrapment of cells in gel-forming materials, such as alginate or *k*-carrageenan, is the most commonly used immobilization method because of their non-toxicity, the simplicity of the cell immobilization method, the resulting high viability, and higher productivity of immobilized cells (4, 8, 12, 20). Rossi *et al.* produced diacetyl and acetoin by immobilized *L. diacetilactis* in Na-alginate (24). Here we report a comparison of TMP productivity by free and immobilized cells of *L. diacetilactis*.

MATERIALS AND METHODS

Microorganism and Fermentation Medium

L. diacetilactis was cultured on defined lactose-citrate medium as previously described (13-17). Galactose solution (1%, w/v) and thiamine-HCl (2 mg/l) were pre-filtered by passing through a Whatman membrane filter (pore size: 0.45 μm). The filtered solution was added to the fermentation medium.

Cell Immobilization

k-Carrageenan solution (4%, w/v) was autoclaved at 121°C for 15 min and then cooled to 45°C. The polymer solution was mixed with the cell slurry at volume ratio of 9 : 1. The mixture was poured into sterile soy oil (45°C) and stirred for 2 min at 450 rpm to create a strong vortex. The average diameter of the beads was 1.5 mm. The dispersion was cooled in cold water to promote gelation of the polymer droplets formed during stirring. The strength of the beads was then increased by soaking in 2% (w/v) KCl solution for 1 h.

Cultivation Method

Immobilized cells were cultivated in a 500 ml flask in a shaking incubator (Vision Scientific Co., Ltd., Korea) with an agitation rate of 50 rpm at 34°C. After 18 h of cultivation, arginine solution as the precursor of ammonia was added to be 50 mM. Beads were also activated by placing them in lactose-citrate medium for 12 h before cultivation to increase the productivity of acetoin, ammonia, and TMP.

TMP Formation by Condensation

The culture medium was clarified by centrifugation at 693 × g, at 4°C for 15 min. The supernatant was heated and condensed at 121°C, at pH 8.3 for 4 h following the previously reported procedure of optimum conditions for TMP formation (16).

Citrate, Acetoin, Arginine, and Ammonia Analysis

The identification and quantification of citrate, acetoin, arginine, and ammonia in the supernatant were followed according to the methods of Marier and Boult (18), Westerfeld (28), Rosenberg *et al.* (23), and Wriston (29).

Analysis of TMP

To isolate the flavor compound, TMP, a solvent extraction method was employed. The samples (pH 8.3) were extracted using diethylether as solvent in a continuous liquid-liquid extractor. The extraction was performed for 12 h. A gas chromatography PU4500 (Pye Unicam, Philips, Netherlands) equipped with a flame ionization detector was used for the analysis of TMP in the extract. A stainless steel column (phase: OV 101, support: diatomite CS 100-120 mesh size) was used to separate the volatile components in the samples. The oven temperature program was as follows: 80 to 190°C at 3°C per min. The temperatures of the injector and detector were 220 and 250°C, respectively. Quinoxaline as an internal standard was added to the samples for the quantification of TMP. TMP identification was based on the retention indices. The concentration of TMP was calculated by comparing the peak area of TMP with that of quinoxaline. The conversion ratio of acetoin to TMP was determined by the following equation,

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

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

RESULTS AND DISCUSSION

Productivity of Acetoin

Immobilized *L. diacetilactis* cells were cultivated at 34°C for 20 days as described in Materials and Methods. When using the alginate system, citrate disrupted the gel structure by chelating calcium (12, 25). *k*-Carrageenan was more efficient than alginate because its matrix was hardened by K^+ ions instead of Ca^{2+} ions (for alginate) which was chelated by lactate and citrate leading to the disruption of the gel (4).

The utilization of citrate as the precursor of acetoin and the productivity of acetoin in free and immobilized cell systems are shown in Fig. 1. The concentration of citrate decreased during cultivation because citrate is the precursor of acetoin. *L. diacetilactis* have the citrate permease activity linked to a 5.5 megadalton plasmid (11), so they utilize citrate to produce diacetyl and acetoin (10, 27). After 5 days of cultivation, 68% and 78% of citrate was consumed in free and immobilized cell systems, respectively. The acetoin concentration reached maximum at 5 days of cultivation. The productivity of acetoin in immobilized cell system was higher than that in the free cell system after 5 days of fermentation. This result was similar to that reported by Schmitt *et al.* (25). They reported that the citrate bioconversion ratio was 42.8 and 80% in free and immobilized *L. diacetilactis*

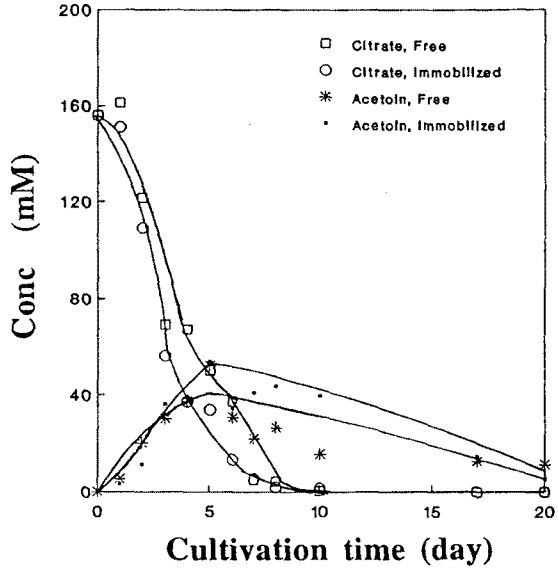

Fig. 1. The utilization of citrate and the concentration of acetoin in free and immobilized *L. diacetilactis* cell systems. The cells were incubated at 34°C for 20 days in a shaking flask.

cells, respectively. However, it has been observed that diffusion limitation occurring in immobilized cell systems might reduce citrate utilization (4, 20, 26). The immobilization of *L. diacetilactis* cells enhanced the productivity of acetoin in spite of diffusion limitation. One possible explanation for this phenomenon was that the gel matrix system employed in this study contributed to prevent microorganisms from contacting the accumulated product (acetoin) causing a product inhibition, which might lower the citrate uptake efficiency.

After 6 days of cultivation, the concentration of acetoin had decreased. Kim and Lee (15) observed that TMP concentration increased as acetoin concentration decreased implying that the substrate (acetoin) utilization and the formation of product (TMP) occurred simultaneously.

Production of Ammonia

The utilization of arginine and the formation of ammonia in free and immobilized *L. diacetilactis* cell systems are presented in Fig. 2. The concentration of arginine in the medium decreased rapidly to 17-20% for 3 days of cultivation, and was maintained at 7-8 mM after 5 days of fermentation. *L. diacetilactis* metabolizes arginine via the arginine deiminase pathway producing ammonia (6).

The production of ammonia increased rapidly for 3 days of cultivation. The production of ammonia in the immobilized cell system was higher than that of the free cell system throughout the period of cultivation after 3 days. It has been reported that immobilization constitutes a change in microenvironment and influences optimum pH and optimum temperature (26). This might be the reason why the production of ammonia of the immobilized cell system was greater than that of the free cell system.

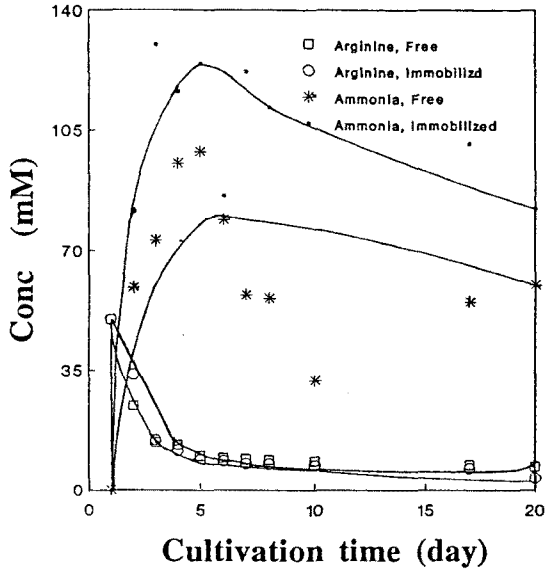

Fig. 2. The utilization of arginine and the concentration of ammonia in free and immobilized *L. diacetilactis* cell systems. The cells were incubated at 34°C for 20 days in a shaking flask. After 18 h of cultivation, arginine was added to be 50 mM.

The concentration of ammonia slowly decreased after 3 days of cultivation. This could be attributed to the formation of TMP during cultivation (15). The concentration of ammonia declined more rapidly in the free cell system than in the immobilized cell system. The decreased concentration of ammonia will have no significant effect on the productivity of TMP because ammonia is not a limiting substrate for TMP formation. Two moles of ammonia are converted into one mole of TMP (1). After cultivation, the concentration of ammonia was much higher than that of acetoin.

Productivity of TMP

As depicted in Table 1, no significant difference in acetoin productivity resulted from changing the cell cultivation system. The immobilized cell system (2.21 g/l, 130 mM ammonia), however, was shown to be better than the free cell system (1.62 g/l, 95 mM) for ammonia production. It is possible that the carrageenan matrix prevented microorganisms from contacting ammonia and increased cell viability. The yield of TMP in the immobilized cell system was 95 mg/l higher than that in the free cell system. The increased yield of TMP in the immobilized system was attributed to the increased concentration of substrates, i.e., acetoin and ammonia, in the culture supernatant.

Effect of Activation on TMP Productivity in the Immobilized Cell System

Immobilized *L. diacetilactis* cells were cultivated for only 5 days after activation because the concentration of acetoin and ammonia decreased after 5 days of cultivation as shown in the preceeding experiments (Fig. 1, 2). As shown in Fig. 3, the concentration of citrate decreased to 21-23% after 5 days of cultivation because citrate was consumed by cells to produce acetoin. The

Table 1. Productivity of TMP from acetoin and ammonia, and the conversion ratio of acetoin to TMP in free and immobilized *L. diacetilactis* cell systems.

	Concentration of acetoin		Concentration of ammonia		TMP		Conversion ratio of acetoin to TMP
	(g/l)	(mM)	(g/l)	(mM)	(g/l)	(mM)	(%)
Free cell system	4.49	51	1.62	95	0.241	1.8	7.0
Immobilized cell system	4.76	54	2.21	130	0.336	2.5	9.3

The cells were incubated at 34°C for 20 days, and then the culture supernatant was heated at 121°C for 1 h.

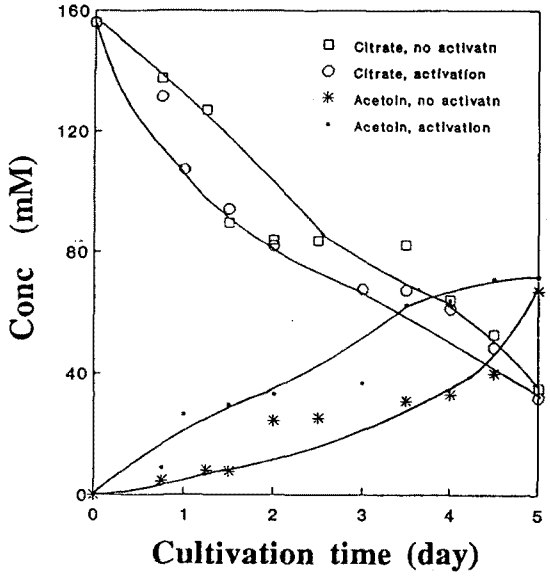

Fig. 3. The utilization of citrate and the concentration of acetoin in immobilized *L. diacetilactis* cell system with and without activation.

The immobilized cells were activated by placing the beads in a nutrient medium for 12 h. The cells were incubated at 34°C for 5 days in a shaking flask.

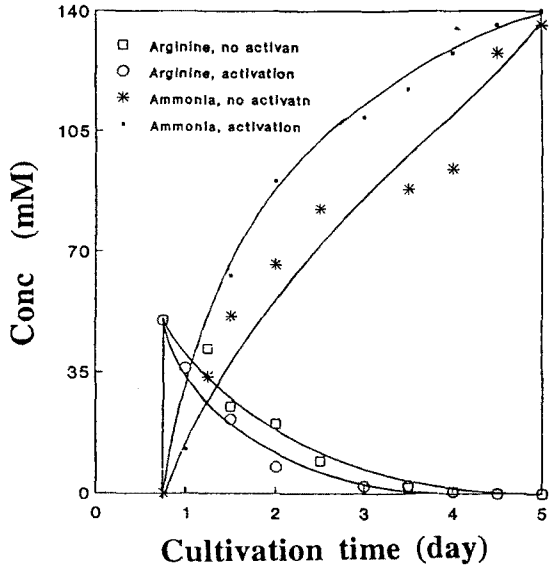

Fig. 4. The utilization of arginine and the concentration of ammonia in immobilized *L. diacetilactis* cell system with and without activation.

The immobilized cells were activated by placing the beads in a nutrient medium for 12 h. The cells were incubated at 34°C for 5 days in a shaking flask. After 18 h of cultivation, arginine was added to be 50 mM.

acetoin production was also slightly increased, up to 107%, by activation. Nilsson *et al.* (21) observed that the formation of ethanol was increased by activation and also observed that small colonies have been formed without the polymer matrix during activation of immobilized yeast cells. This result indicates increased cell numbers within the beads.

Fig. 4 shows the utilization of arginine and the productivity of ammonia in immobilized cell systems with or without activation. The concentration of arginine decreased during cultivation because arginine was consumed by *L. diacetilactis* to produce ammonia (6). After 5 days of cultivation, arginine was consumed completely. The productivity of ammonia increased proportionally with the consumption of arginine. The results showed that ammonia concentration with activation was higher than that without activation up to 4 days of cultivation implying the effect of activation on the increase in production of ammonia. When the arginine was almost utilized by the cells after four and a half days of cultivation, there was no major difference in ammonia production with activation (139.5 mM, 5 days) and without activation (136 mM, 5 days).

As presented in Table 2, the concentration of TMP was 0.31 g/l with activation and 0.29 g/l without activation, respectively. The yield of TMP increased slightly up to 107% by activation. This was due to the increase in acetoin concentration (from 66 to 72 mM) in the fermentation medium by activation. No significant difference in the conversion ratio of acetoin to TMP resulted with activation (Table 2) because acetoin is a rate limiting substrate in TMP formation.

Table 2. Productivity of TMP from acetoin and ammonia and the conversion ratio of acetoin to TMP in immobilized *L. diacetilactis* cell system with and without activation.

	Concentration of acetoin		Concentration of ammonia		TMP		Conversion ratio of acetoin to TMP
	(g/l)	(mM)	(g/l)	(mM)	(g/l)	(mM)	(%)
With activation	6.34	72	2.36	139	0.309	2.3	6.4
Without activation	5.82	66	2.31	136	0.288	2.1	6.3

The immobilized cells were activated by placing the beads in a nutrient medium for 12 h. The cells were incubated at 34°C for 5 days in a shaking flask, and then the culture supernatant was heated at 121°C for 1 h.

In summaries acetoin concentration, ammonia concentration, and TMP productivity of the immobilized cell system was higher than that of the free cell system. The productivity of acetoin and TMP was increased by activation. The concept of TMP production was successfully demonstrated in a *k*-carrageenan/*L. diacetilactis* immobilized system. Further studies to increase TMP pro-

ductivity using immobilized cell reactors, such as a packed bed reactor or a fluidized bed reactor are necessary.

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