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Optimum Conditions for the Formation of Acetoin as a Precursor of Tetramethylpyrazine during the Citrate Fermentation by *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis* FC1

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To produce acetoin as a precursor of the tetramethylpyrazine flavor compound from citrate by *Lactococcus lactis* ssp. *lactis* biovar. *diacetilactis* FC1, fermentation factors such as inital pH of culture media, temperature, concentration of Na-citrate, thiamin-HCl and sugars were examined. The best acetoin production was achieved with initial pH in the culture media of 5.5, fermentation temperature of 34°C, Na-citrate concentration of 3%, addition of thiamin-HCl at 2 mg/l and galactose as a carbon source. When fermentation was carried out under the optimum conditions, the exhaustion of Na-citrate and the production of acetoin took simultaneously and acetoin reached the maximum content, 80 mmole/l after 20 hours.

Pyrazines are heterocyclic compounds containing two nitrogen atoms that are distributed in more than 100 different foods and their flavor characteristics are described as nutty, meaty, and roasted (13). Pyrazine derivatives are known to be formed with substrates such as reducing sugars and amino acids at temperature over 100℃ (12). Microorganisms such as *Bacillus subtilis* (8), a mutant of *Corynebacterium glutamicum* (4), *Pseudomonas perolens* (11), *Psedomonas taetrolens* (17) could also produce pyrazines, but the synthetic pathways have not been elucidated and their productivities were very low except for a mutant of *C. glutamicum*.

Tetramethylpyrazine (TMP), as one of the pyrazine compounds, was first isolated from the culture of *B. subtilis* (8). TMP that has the flavor characteristics of roasted nut (23) and fermented soybean (10) is listed as a GRAS (generally recognized as safe) food additive (13). TMP discovered naturally in meat, nut, and marine products has been reported to be formed in model systems containing reducing sugars or carbonyls and amino acids or ammonia salts (12).

Studies on the microbiological production of TMP have seldom been reproted like other pyrazines, but the biosynthetic pathway of TMP proposed by Adachi (1) suggested that two moles of acetoin derived from microbial metabolism and two moles of ammonia are condensed into one mole of TMP. A recent report (19) that TMP was also formed from acetoin and ammonium salts under mild conditions supported Adachi's postulation, and TMP originating from microbial culture was thought to be produced by condensation of acetoin and ammonia (10). It was known that *B. subtilis* (8) and a mutant of *C. glutamicum* (4) also produced TMP through this mechanism.

Lactococcus lactis subsp. *lactis* biovar. *diaectilactis* (*L. diacetilactis*) used in the dairy industry as a lactic starter and a producer of diacetyl is known to be able to produce acetoin from citrate due to a 5.5 Mdal plasmid pertaining to citrate permease (9) and some of its strains can produce two moles of ammonia by hydrolysis of arginine through the arginine deiminase pathway (3). Therefore the citrate and arginine metabolisms of *L. diacetilactis* can be used in the production of acetoin and ammonia as TMP's precursors and the conversion of them into TMP could be possible. In the present study, optimum conditions for producing acetoin as a precursor of TMP by *L. diacetilactis* were investigated.

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MATERIALS AND METHODS

Strain and Media

The microoganism used in this experiment was *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis* FC1 preserved at the Department of Food Science and Technology, Seoul National University. Litmus milk was used for preserving the strain and Lactose-citrate medium (5) was used in the subculture. To investigate the optimum conditions of acetoin production, the Lactose-citrate broth was used as a basal medium.

Fermentation Conditions

For the experiment of acetoin fermentation, the Na-citrate concentration of the Lactose-citrate broth was increased to 3% (w/v) and peptone was also replaced by tryptone. Subcultures cultivated for 12 h were inoculated to 100 ml of the acetoin fermentation media at the level of 1% (v/v) and cultured in the shaking flasks for 24 hours at 34℃ and at 150 rpm.

Analysis

Culture broth taken from the flasks was diluted 10 times and the absorbances of the diluted broth at 600 nm were measured as the cell density.

Westerfeld's method (24) for the measurement of acetoin concentration was modified in this study. Culture broth was centrifuged at 10,000 ×g for 10 min to remove cells and the supernatant was diluted 100 times. Five milliliters of distilled water, 1 ml of 0.5% creatine solution, and 1 ml of 5% α-naphthol solution were added to 1 ml of the diluted supernatant and it was left for 1 h at room temperature for color development. The absorbance of the solution was measured at 530 nm by a spectrophotometer and converted into the concentration of acetoin by the standard curve. For the determination of Na-citrate, the cells of culture broth were removed and diluted 10 times. To 1 ml of the diluted supernatant, 1 ml of pyridine and 5 ml of acetic anhydride were added and it was left in a 32℃ water bath for 30 min. After the color was developed, the absorbance at 420 nm was measured and converted into the concentration of Na-citrate (14).

RESULTS AND DISCUSSION

Factors Affecting Production of Acetoin

Effect of initial pH of media: Effect of initial pH on the production of acetion is shown in Fig. 1. The production was parallel with cell growth and the best production of acetoin was obtained at pH 5.5. At pH 6.5, the production was also good but it was very poor at pH 6.0 and pH 7.0.

It was known that the production of acetoin was very low at pH 6.0 and 7.0 but high at pH 5.0 in milk media

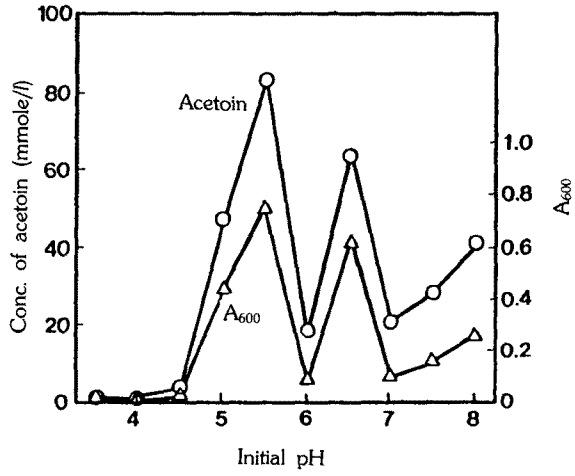

Fig. 1. Effect of initial pH on the production of acetoin and cell growth (A_{600}) during the culture of *L. diacetilactis* FC1 in lactose-citrate medium.

containing citrate (16). It was also reported that the conversion activity of the citrate into actoin was enhanced due to the increase of citrate transport system when the pH was lowered from 7.0 to 5.5 (6). The optimum pHs of enzymes relevant to the citrate fermentation, i.e., citrate permease, acetolactate synthase, and diacetyl reductase have been known to be 5.0 (6), 5.5 (6), and 5.5 (2), respectively. Schmitt reported (20) that acetoin was produced maximally at pH 4.8, and the result of this research showed the same tendency in that the uptake of citrate and the production of acetoin was enhanced at an acidic pH of 5.5. Although another peaks of cell growth and acetoin production were appeared at pH 6.5 (Fig. 1), this would not be applicable in the citrate fermentation because most of fermentations by lactic acid bacteria are carried out at much acidic conditions.

Effect of fermentation temperature: Fig. 2 shows the effect of fermentation temperature on the cell gorwth and the production of acetoin. The acetoin production was relatively good at 30~38℃ but very low at other temperatures. In general, the optimum temperature for the gorwth of lactococci is near 30℃ (18), however the optimum temperature for the growth and acetoin production in this experiment was somewtat higher at 34℃.

Effect of Na-citrate concentration: To investigate the optimum concentration of Na-citrate, the substrate of acetoin, fermentation was carried out at different concentrations of Na-citrate. As shown in Fig. 3, when citrate was not added to the culture media, acetoin was not produced, and acetoin production increased in proportion to the concentration of citrate up to 3%. At higher

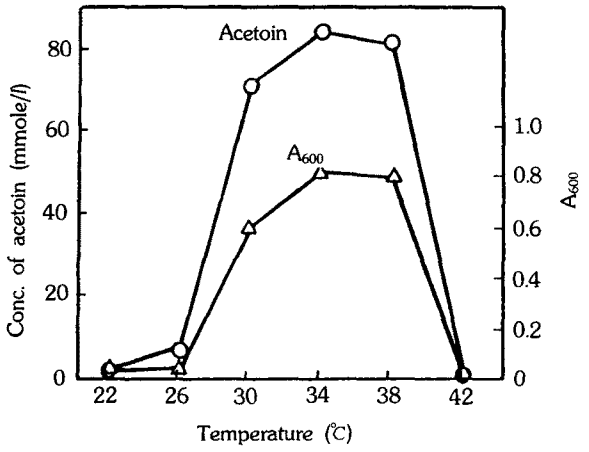

Fig. 2. Effect of temperature on the production of acetoin and cell growth (A_{600}) during the culture of *L. diacetilactis* FC1 in lactose-citrate medium.

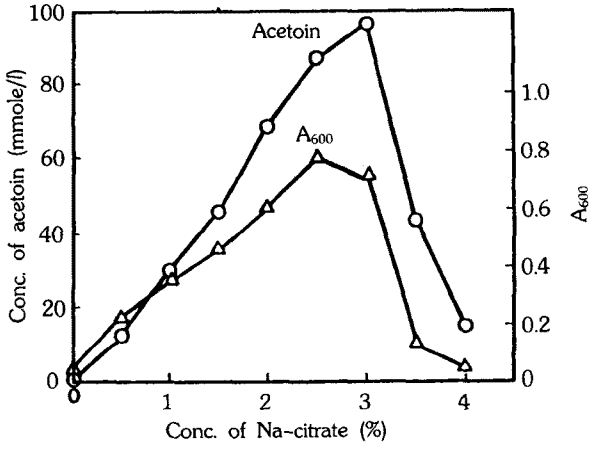

Fig. 3. Effect of initial concentration of Na-citrate on the production of acetoin and cell growth (A_{600}) during the culture of *L. diacetilacis* FC1 in lactose-citrate medium.

concentrations however, the acetoin production and the cell growth decreased. The production of acetoin was maximum at 3% citrate and the conversion ratio into acetoin was 70~100% when citrate was added at 0~3 % levels. The conversion ratio reached near 100% at 2% and 2.5% citrate. The acetoin was not produced when citrate was not added to the medium, and this was in accord with the fact that pyruvate derived from glycolysis is not converted into acetoin but only the pyruvate from citrate is converted into acetoin (7). The result showing that the ratio of conversion into acetoin was maximum at 2.5% (85 mmole/l) Na-citrate was similiar to Schmitt's report (20) that the availavility of citrate was the highest when the concentration of citrate was

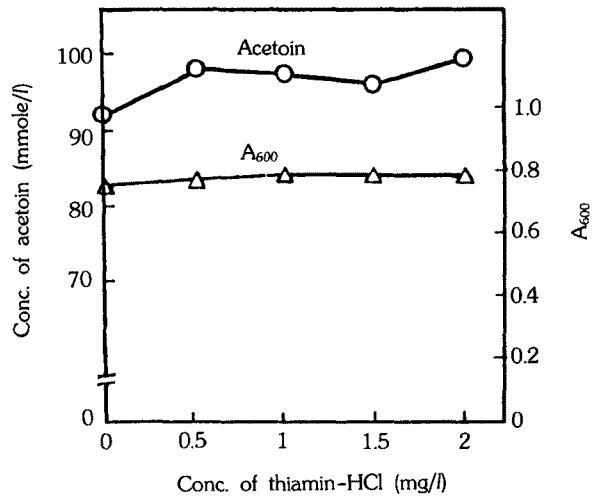

Fig. 4. Effect of thiamin-HCl on the production of acetoin and cell growth (A_{600}) during the culture of *L. diacetilactis* FC1 in lactose-citrate medium.

75 mmole/l.

Effect of thiamin-HCl: Fig. 4. illustrates the effect of thiamin-HCl on the acetoin production when the different concentrations of thiamin were added to the media. The effect was very slight on the cell growth and the production of acetoin. In citrate metabolism of *L. diacetilactis*, pyruvate is decarboxylated into TPP-acetaldehyde and this active acetaldehyde is condensed with pyruvate into α-acetolactate which is transformed into acetoin (15). Therefore it is obvious that TPP (thiamin pyrophosphate), a coenzyme in the decarboxylation of pyruvate is necessary, but thiamin scarcely showed any effect on the acetoin production. This suggested that since the media used in this experiment were complex media including many undefined components such as yeast extract, thiamin could be supplied from such components in the media. However, thiamin-HCl at 2 mg/l was added to the media for the stable production of acetoin.

Effect of carbon sources: As in Fig. 5, galactose rather than lactose and glucose, appeared to be a good carbon source for the cell growth and the production of acetoin. According to Thompson *et al.* (22), when *L. lactis* was cultivated in the medium containing glucose, lactose and galactose, glucose and lactose were consumed first and the galactose was utilized after the exhaustion of glucose and lactose. This sequential consumption of sugars was due to the catabolite repression. It was also reported that the cellular activities of lactate dehydrogenase (LDH) and pyruvate formate lyase (PFL) are dependent upon the kinds of sugars in lactococci (21). In the presence of galactose, the activity of LDH decreased but that of PFL increased due to the switch from

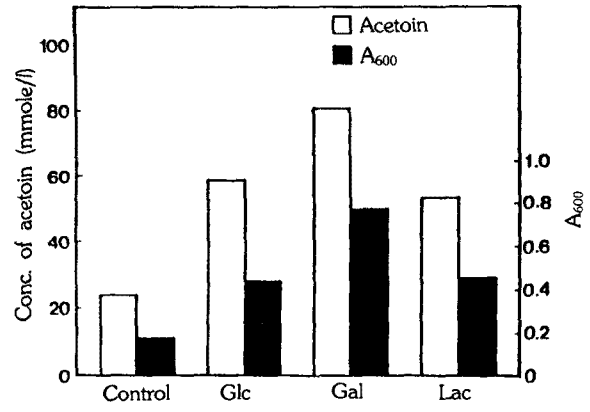

Fig. 5. Effect of various carbohyrates, added 3% in citrate medium, on the production of acetoin and cell growth (A_{600}) during the culture of *L. diacetilactis* FC1.

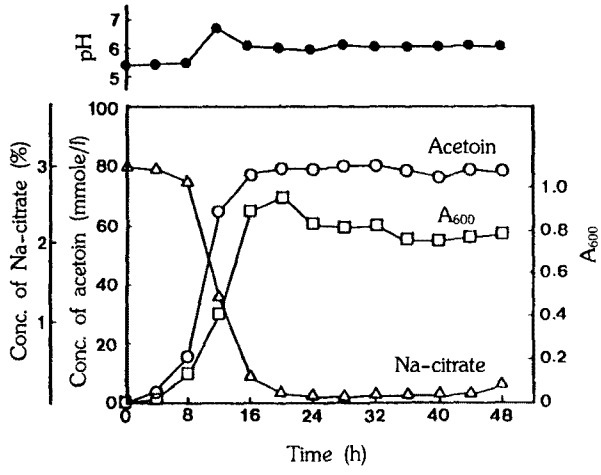

Fig. 6. Time course of acetoin production during the culture of *L. diacetilactis* FC1 in the optimized acetoin production medium.

the homolactic fermentation to the heterolactic pathway in which pyruvate is the main product. However the homolactic fermentation in which the lactate is the main product takes place when glucose or lactose is the carbon source. Of two types of lactic fermentations, the cell growth is higher in heterolactic fermentation because ATP is produced while forming acetate (21).

Therefore when glucose, lactose and galactose are supplied together as carbon surces, glucose or lactose will be consumed first, considering with the result of Thompson (22). However the heterolactic frementation of galactose will be more advantageous to the cell growth and the uptake rate of galactose alone will not be different from that of glucose or lactose. This could also explain the result of Fig. 5 that galactose was better for the cell growth.

Fermentation of Acetoin under the Optimum Conditions

According to the above results, the optimum fermentation conditions of *L. diacetilactis* FC1 for the production of acetoin from citrate were an initial pH of 5.5, 34℃, concentration of 3% Na-citrate, addition of thiamin-HCl at 2 mg/l and galactose as a carbon source. Fig. 6 is the acetoin production course of *L. diacetilactis* FC1 cultivated under the optimum conditions. The cell growth reached the stationary phase and most of citrate was exhausted after 8~16 hours and acetoin also increased cocurrently to the maximum after 20 hours. This result suggested that 20-hour fermentation would be proper for the production of acetoin.

Acknowledgement

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