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Optimum Conditions for the Formation of Ammonia as a Precursor of Tetramethylpyrazine by *Lactococcus lactis* ssp. *lactis* biovar. *diacetilactis* FC1

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To investigate the optimum conditions for the production of ammonia as a precursor of tetramethylpyrazine flavor compound from arginine by *Lactococcus lactis* ssp. *lactis* biovar. *diacetilactis* FC1, fermentation factors such as initial pH of culture media, fermentation temperature, concentration of arginine-HCl, and sugars were examined. The optimum conditions were initial pH 5.5 of the culture media, fermentation temperature of 34°C, 6% (w/v) of arginine-HCl, and 1% (w/v) of galactose as a carbon source. Under the optimum fermentation conditions, 40 mmole/l of ammonia was produced after 40 h.

In the microbial production of tetramethylpyrazine (TMP) flavor compound, it is desirable to produce ammonia because ammonia is one of the two precursors of TMP along with acetoin. The biosynthetic pathway of TMP in microorgaganism was proposed as that two moles of acetoin and two moles of ammonia are condensed into one mole of TMP (7). *Lactococcus lactis* ssp. *lactis* biovar. *diacetilactis* (*L. diacetilactis*) has been used as a lactic starter and a producer of diacetyl in the dairy industry, and can produce acetoin from citrate (6). In a previous study, the authors reported that optimum conditions for the formation of acetoin by *L. diacetilactis* FC1 were an initial pH 5.5 of the culture media, fermentation temperature of 34℃, concentration of Na-citrate 3%, addition of thiamin-HCl, and use of galactose as a carbon surce (1).

Some strains of *L. diacetilactis* can also produce ammonia by hydrolysis of arginine through the arginine deiminase pathway (2). Hence, this study was undertaken to investigate the optimum fermentation conditions of *L. diacetilactis* for the production of ammonia from arginine, so as to provide basic information for the microbial production of the TMP flavor compound by *L. diacetilactis* FC1.

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MATERIALS AND METHODS

Bacterial Strains and Media

Lactococcus lactis ssp. *lactis* biovar. *diacetilactis* FC1 (1) was preserved in litmus milk and investigation of the optimum conditions of ammonia production was carried out in lactose-citrate broth (4).

Fermentation Conditions

Medium containing $K_4Fe(CN)_6$ and ferric citrate (5) was used for the confirmation of the arginine hydrolytic ability of the strain. For the formulation of ammonia fermentation medium, the Na-citrate of the lactose-citrate broth was replaced with arginine-HCl and tryptone was added instead of peptone. Subcultures cultivated for 12 h were inoculated to the ammonia fermentation medium and cultivated at 34℃ for 24 h with agitation at 150 rpm.

Analytical Methods

Cell density was determined by measuring the absorbance at 600 nm of culture broth diluted 10 times and the ammonia concentratation was measured by the method of Wriston (10). One milliliter of the culture broth diluted 400 times, 5 ml of distilled water, and 1 ml of Nessler's reagent were mixed and left for 15 min. The absorbance of the solution at 425 nm was measured and this value was used to calculate the concentration of citrate.

RESULTS AND DISCUSSION

Arginine Hydrolytic Activity of the *L. diacetilactis* FC1

The arginine hydrolytic ability of *L. diacetilactis* is known to vary according to strains (2, 8), and to determine whether the *L. diacetilactis* FC1 strain could produce ammonia through the arginine metabolism, the strain was cultivated on the solid media containing arginine and bromocresol purple (BCP) as an indicator (9). When the strain was incubated on the control medium without arginine, the colonies and their halos were yellow, but the colonies on the medium containing both BCP and arginine were white and the size of colonies were larger than those on the control. The BCP color turns yellow when bacteria produce acid but turns white when the ammonia produced by bacteria neutralizes the acid (9). The larger size of the colonies was probably due to the fact that the ammonia minimized the growth inhibition by acid. From the above results, it was apparent that *L. diacetilactis* FC1 used in the present study could hydrolyze arginine and produce ammonia.

Factors Affecting Production of Ammonia from Arginine

Effect of initial pH of media: Effect of initial pH of media containing arginine-HCl on the growth and the production of ammonia are shown in Fig. 1. The cell growth, and ammonia production were low at pH 4 and 4.5, and this trend was similiar to the result of acetoin production (1). The ammonia production was stable in broad range of pH 5.0-8.5. Although the best pH for the ammonia production was 5.0, pH 5.5 was chosen as the optimum pH of ammonia production in order to match with the optimum pH of acetoin production which was pH 5.5 (1), and also to prevent the pH from falling below 4.5 where the ammonia production was very low.

Effect of the fermentation temperature: Fig. 2 shows the effect of fermentation temperature on the cell growth and ammonia production by *L. diacetilactis* FC1. The cell growth and the production of NH_3 followed the similar pattern, and the NH_3 production was very low at 22℃ and 42℃, but was high at temperatures ranging 30-38℃. Optimum temperature for the production of NH_3 and growth appeared to be 34℃ and it was the same as the optimum temperature for acetoin production (1).

Effect of arginine HCl concentration: The effect of arginine-HCl added to the media as a substrate of NH_3 on the NH_3 production is shown in Fig. 3. As shown in the figure, 48 mmole/*l* of NH_3 was produced even when no arginine was added, and the amount of NH_3 increased with the addition of arginine-HCl up to 6% but decreased at the arginine concentrations over 9%. In general, the pattern of the ammonia production was similar to that of cell growth. The maximum production of NH_3 and the cell growth was achieved at 6% arginine. There was a report that the optimum concentration of arginine for *L. lactis* cell growth was 1% (3), and this discrepancy may due to the difference in the strains and media compositions.

The addition of the arginine to the media resulted in the increase of cell growth and this can be explained by the report that the hydrolysis of arginine is an energy producing reaction accompanying ATP generation (2). The cell growth decreased rapidly at the concentrations

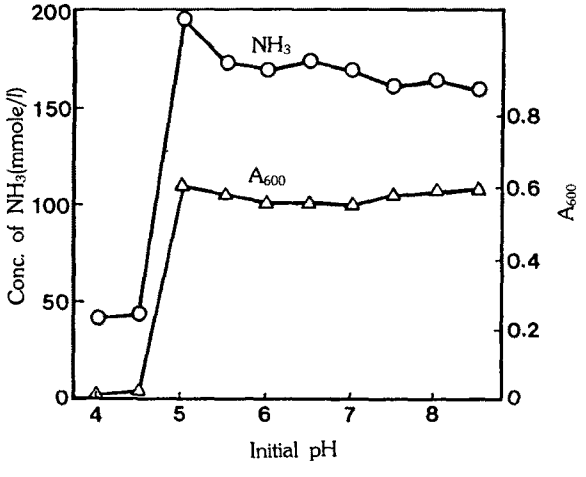

Fig. 1. Effect of initial pH of the medium on the production of NH_3 and cell growth (A_{600}) during the culture of *L. diacetilactis* FC1 in the Galactose-Citrate medium

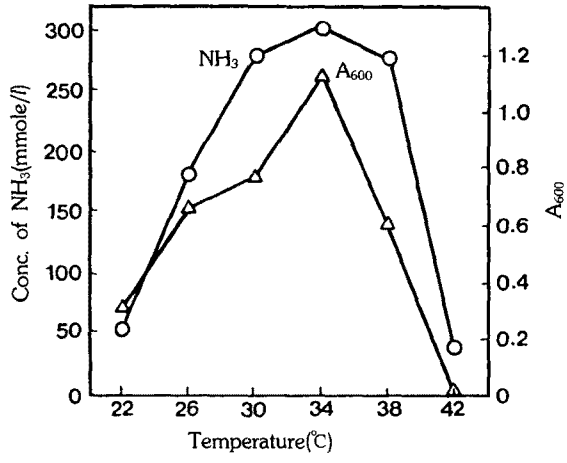

Fig. 2. Effect of temperature on the production of NH_3 and cell growth (A_{600}) during the culture of *L. diacetilactis* FC1 in the Galactose-Citrate medium

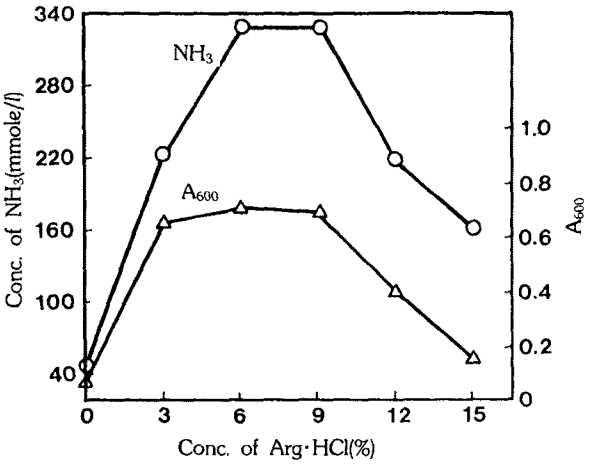

Fig. 3. Effect of initial concentration of arginine-HCl on the production of NH_3 and cell growth(A_{600}) during the culture of *L. diacetilactis* FC1 in the Galactose-Citrate medium

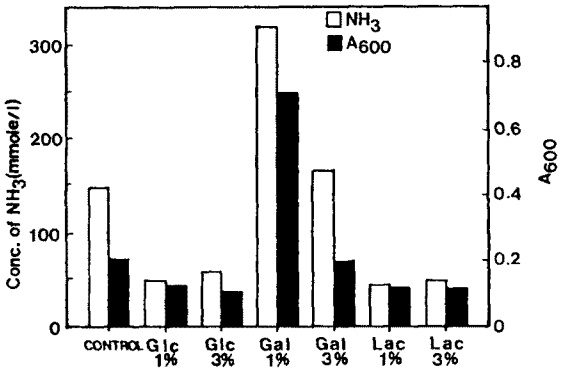

Fig. 4. Effect of various carbohydrates on the production of NH_3 and cell growth(A_{600}) during the culture of *L. diacetilactis* FC1

of arginine over 9%, and this was probably due to the fact that arginine-HCl increased the osmotic pressure or the ionic strength so that the growth was inhibited.

Effect of carbon sources: Fig. 4 shows the effect of the various carbon sources on the cell growth and the production of NH_3. A concentration of 3% galactose was the best for the cell growth and the NH_3 production. Regardless of concentration, glucose and lactose inhibited cell growth and the production of NH_3 when compared to the control that contained no sugar. Among three enzymes of *L. lactis* relevant to the hydrolysis of arginine, arginine deiminase and ornithine transcabamylase are inducible enzymes controlled by carbohydrates. Even when arginine is present, they are not induced before sugar is exhausted. However, when the sugar

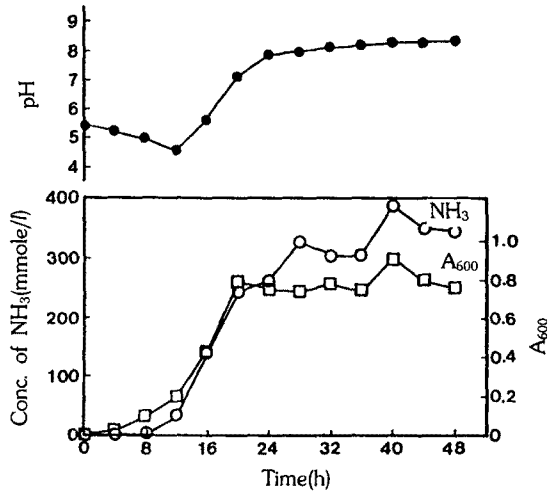

Fig. 5. Time course of NH_3 production and cell growth (A_{600}) during the culture of *L. diacetilactis* FC1 in the optimized NH_3 production medium

is galactose, the two enzymes are induced and they hydrolyze arginine irrespective of galactose concentration (2). The result showing that NH_3 production was low when glucose or lactose were added suggested that the enzymes related to arginine hydrolysis were not repressed by these sugars. However the production of NH_3 was good in the media containing galactose, and this may be due to the fact that the arginine hydrolysis was not influenced by the galactose.

Formation of NH_3 under the Optimum Conditions

According to the above results, the optimum conditions for the production of NH_3 from arginine HCl were an initial culture media pH of 5.5, fermentation temperature of 34℃, 6% concentration of arginine HCl and 1% galactose as a carbon source. Fig. 5 shows the production course of NH_3 by *L. diacetilactis* FC1 cultivated under the optimum conditions. The pattern of NH_3 production was similar to the growth curve of cells. It was after 20 h that the cell growth reached the stationary phase, and the increase in NH_3 stopped after 40 h. The pH of fermentation broth decreased until 12 h of fermentation, then the pH increased up to pH 8.0 together with the NH_3 production.

Among the optimum fermentation factors for the production of NH_3, fermentation temperature and galactose as a carbon source were the same optimum factors as those for the acetoin production (1). The amount of NH_3 produced after 40 h of fermentation was 40 mmole/l, and the conversion ratio of arginine into NH_3 was 70%. These results indicated that 24 h fermentation would be appropriate for the production of NH_3 because

the cell growth reached the stationary phase and the NH_3 production also reached the maximum level.

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