Journal of Microbiology and Biotechnology
Vol. 4, No. 4, 327-332, 1994

Optimum Conditions for the Production of Tetramethylpyrazine Flavor Compound by Aerobic Fed-batch Culture of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* FC1

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Optimum conditions for the production of acetoin and ammonia as the precursors of tetramethylpyrazine (TMP) were determined using *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* FC1 in a modified Lactose-citrate broth containing galactose, citrate, and arginine. The cell growth and the productivity of acetoin and ammonia were remarkably increased in an aerobic culture with 10 μM of hematin. For the optimum conditions of acetoin and ammonia production, the concentration of citrate and arginine were adjusted to 156 mM and 50 mM after 18 hr cultivation, and citrate and galactose to 156 mM and 50 mM after 36 hr cultivation, respectively. In these conditions, acetoin and ammonia were produced to the final concentration of 127 mM and 195 mM, which were the highest concentations, respectively. The optimum conditions of the TMP production were also determined as follows; 4 hours at 121, pH 8.3, and the maximal yield of TMP under these conditions was 0.81 g/l.

The tetramethylpyrazine (TMP) provides the fermented soybean flavor in soy sauce, natto, and miso, and has been used as a sweetness enhancer for beverage, candy, and other food products (10). After the first isolation of TMP from a strain of *Bacillus subtilis* (6), a mutant of *Corynebacterium* has been known to produce the TMP (4). It has also been reported that *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (*L. diacetylactis*) is a TMP producer (9).

The biosynthetic pathway of the TMP in microorganisms is explained to be two moles of acetoin and two moles of ammonia condensed into one mole of TMP (1, 12). Aerobic condition may change sugar metabolism from homo- to hetero- lactic fermentation and this leads to additional ATP generation and consequently yields more growths (2). Therefore, aerobic culture of *L. diacetylactis* with citrate may maximize the formation of acetoin which is one of the two precursors of TMP flavor compound (5, 7).

In this paper, we report the optimum conditions for the production of the two precursors of TMP (acetoin and ammonia) and the TMP flavor compound in aerobic fed-batch culture.

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Key words: Microbial flavor, tetramethylpyrazine, aerobicculture, fed-batch culture, *L. lactis* subsp. *lactis* biovar. *diacetylactis*

MATERIALS AND METHODS

Strains and Media

The microorganism used in this experiment was *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* FC1 (*L. diacetylactys*) which has been used in the previous studies (9). Litmus milk was used for the propagation of the strain and Lactose-Citrate medium was used as a basal medium for the citrate fermentation.

Basic Culture Conditions

For the batch culture of *L. diacetylactis* FC1, Lactose-Citrate medium was modified by increasing the concentration of Na-citrate from 26 mM to 150 mM and replacing peptone and lactose with tryptone and galactose (1% w/w), respectively (9). Galactose and thiamine were sterilized separately and added to the culture media. In the case of aerobic culure, 10 μM of hematin was added to the medium to prevent the toxicity of H_2O_2 (14, 18). The liquid cultures were grown in 5 L fermentors with working volumes of 2 L at 34°C with pH 5.5± 0.3.

An aerobic fed-batch culture was done with 0.6 VVM aeration rate and 200 rpm of impeller speed, while an

anaerobic culture was done under static conditions.

Analysis of the Culture

The concentrations of acetoin and citrate were determined quantitatively by the method of Westerfeld (17) and Marier (11), respectively. The ammonia concentration was measured by the method of Wriston (19). For the determination of arginine concentration, the method of Rosenberg (13) was used.

The TMP content in the oleoresin was analyzed with gas chromatography (GC, Pye Unicam, PU 4500) with a stainless steel column (OV 101) and a flame ionizaion detector.

After the TMP concentration was determined, the conversion efficiency of acetoin into TMP was calculated by the following equation.

$$\% \text{ conversion efficiency} = \frac{\text{TMP concentration (mM)}}{\text{1/2 Acetoin concentration (mM)}}$$

[The "1/2" term in the denominator was introduced because two moles of acetoin are converted into one mole of TMP.]

RESULTS AND DISCUSSION

Effect of Aeration on the Fed-batch Culture of *L. diacetylactis* FC1

Effect of aeration on the cell growth: The effect of aeration on the cell growth is shown in Fig. 1. The cell growth of *L. diacetylactis* FC1 under anaerobic or aerobic conditions showed similar patterns, but the final cell concentration in the aerobic culture was higher with about 0.1 difference in $A_{600\ nm}$.

The better cell growth in the aerobic culture may have resulted from the following two factors: galactose as a main carbon source and the aerobic culture condition. It was reported that a sugar fermentation of lactococci in the presence of galactose tends to switch the pathway from homo- to hetero- lactic because the cellular activity of lactate dehydrogenase (LDH) decreases whereas that of pyruvate formate lyase (PFL) increases (2).

It is also well known that the lactococci produces more acetate and less ethanol from sugar in aerobic condition than in anaerobic condition (2). The acetyl CoA formed in aerobic condition is more likely to be converted to acetate via acetyl phosphate with ATP synthesis than to ethanol via acetaldehyde (2, 14).

Acetoin production during the aerobic fermentation: The effects of aeration on the production of acetoin and the utilization of citrate are shown in Fig. 2. The production of acetoin was higher under aerobic condition and the maximal concentration of acetoin reached 69 mM, while it was only 40 mM under anaerobic condition. The increase of the acetoin production in aerobic culture may have resulted from the increase in biomass.

It was reported that hematin which has been added to induce catalase-activity for H_2O_2 breakdown stimulated the activity of diacetyl synthase and NAD-dependent diacetyl reductase in the cells of *L. diacetylactis* (8). Therefore, much larger amounts of acetoin was produced instead of diacetyl during growth due to the addition of hematin.

Effect of Citrate on the Fed-batch Culture of *L. diacetylactis* FC1

As shown in Fig. 3, the cell growth decreased when a large amount of citrate was added at the early stage of fermentation. The minimum cell growth was obtained when 312 mM of citrate was added after 18 hr cultivation (Fig.3, D) and the maximum cell growth was obtained with the addition of 156 mM citrate at 36 hr cultivation (Fig.3, B).

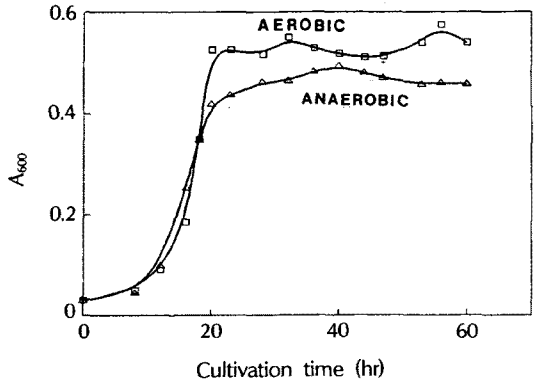

Fig. 1. Effect of aeration on the cell growth ($A_{600\ nm}$) in the fed-batch culture of *L. diacetylactis* FC1.
Anaerobic culture was done under static condition and aerobic culture was done with 0.6 VVM aeration rate and 200 rpm of impeller speed.

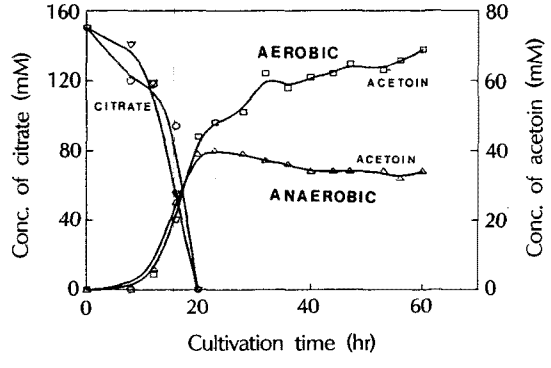

Fig. 2. Effect of aeration on the utilization of citrate and the production of acetoin in the fed-batch culture of *L. diacetylactis* FC1.
Anaerobic culture was done under static condition and aerobic culture was done with 0.6 VVM aeration rate and 200 rpm of impeller speed.

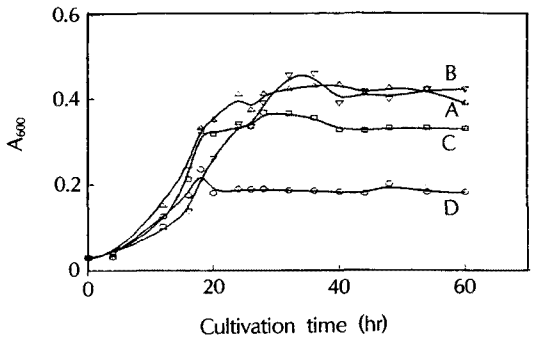

Fig. 3. Effect of citrate on the cell growth ($A_{600\ nm}$) in the fed-batch culture of *L. diacetylactis* FC1.
Concentrated citrate was added to the concentration of 156 mM after 18 hr cultivation (A), to 156 mM after 36 hr (B), to 156 mM after 18 hr and after 36 hr (C) and to 312 mM after 18 hr (D).

The effect of citrate on the acetoin production is also shown in Fig. 4. The citrate was more rapidly utilized in the exponential and stationary phase than in the lag phase, and the high concentration of citrate inhibited both citrate utilization and acetoin production (Fig. 4, D). The production of acetoin was high in the cultures with citrate of 156 mM after 18 hr or with citrate of 156 mM after 18 and 36 hr cultivation (Fig. 4, A and C), and the maximum concentration of acetoin was 108 mM. It is also known that the culture can produce more acetoin if the cells are grown for much longer time (Fig. 4, C), and this result indicates the presence of intermediates of acetoin which remained in the medium.

The results in Fig. 4 showed that the maximum production of acetoin was achieved in the later stage of cultivation at which the citrate was exhausted, and this phenomena can be explained by the previous reports (15, 16). It has been reported that the addition of citrate induced citratase and acetolactate synthase and repressed the synthesis of diacetyl and acetoin reductase. When citrate was exhausted, diacetyl reduction by diacetyl reductase occured, which leads to acetoin formation (15, 16).

In conclusion, the highest amount of acetoin was produced, when the cells were grown aerobically in the medium with hematin, and citrate was added to the media to bring its concentration to 156 mM after 18 hr and 36 hr cultivation.

Effect of Arginine on the Fed-batch Culture of *L. diacetylactis* FC1

Arginine was added to the culture as a source of ammonia which was used as a precursor of TMP formation through deiminase pathway (3). The effect of arginine on the cell growth is shown in Fig. 5. When arginine was added during the initial stage of growth, the lag

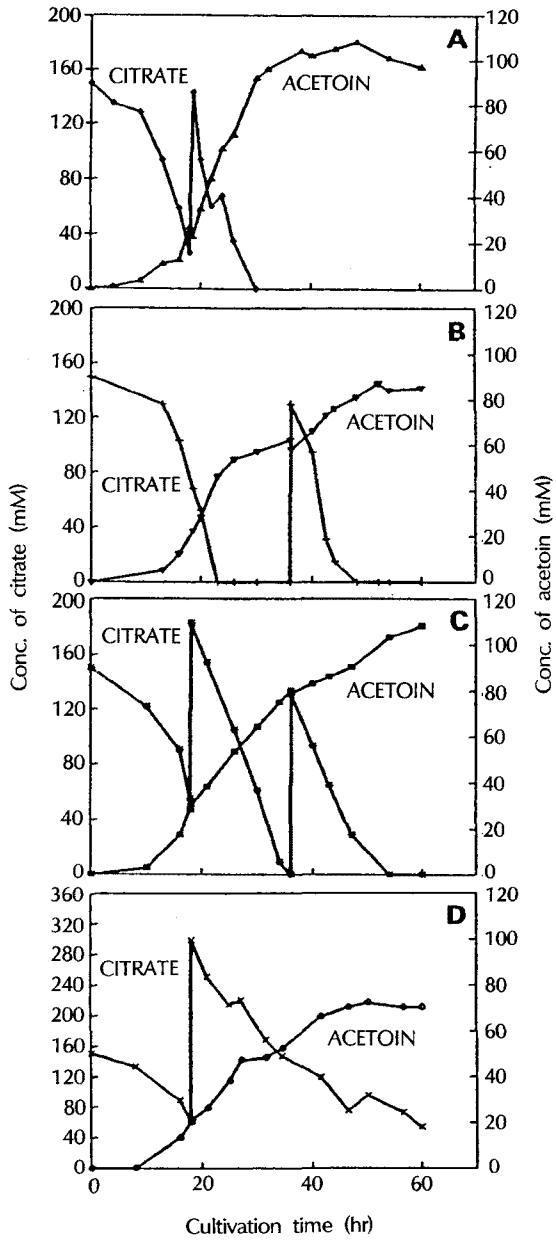

Fig. 4. Effect of citrate on the utilization of citrate and the production of acetoin in the fed-batch culture of *L. diacetylactis* FC1.
Concentrated citrate was added to the concentration of 156 mM after 18 hr cultivation (A), to 156 mM after 36 hr (B), to 156 mM after 18 hr and after 36 hr (C) and to 312 mM after 18 hr (D).

phase was extended and cells did not grow until after 50 hr cultivation (Fig. 5, A). Therefore, it can be said that the high concentration of arginine in the medium inhibits cell growth. However, when the arginine was added after 18 or 36 hr cultivation, the lag phase was not extended (Fig. 5, B and C).

The effect of arginine on ammonia production is

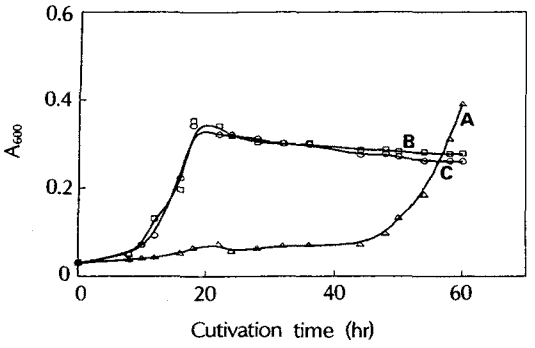

Fig. 5. Effect of arginine on the cell growth ($A_{600\ nm}$) in the fed-batch culture of *L. diacetylactis* FC1.
Concentrated arginine was added to the concentration of 50 mM at 0 hr (A), after 18 hr (B) and after 36 hr cultivation (C).

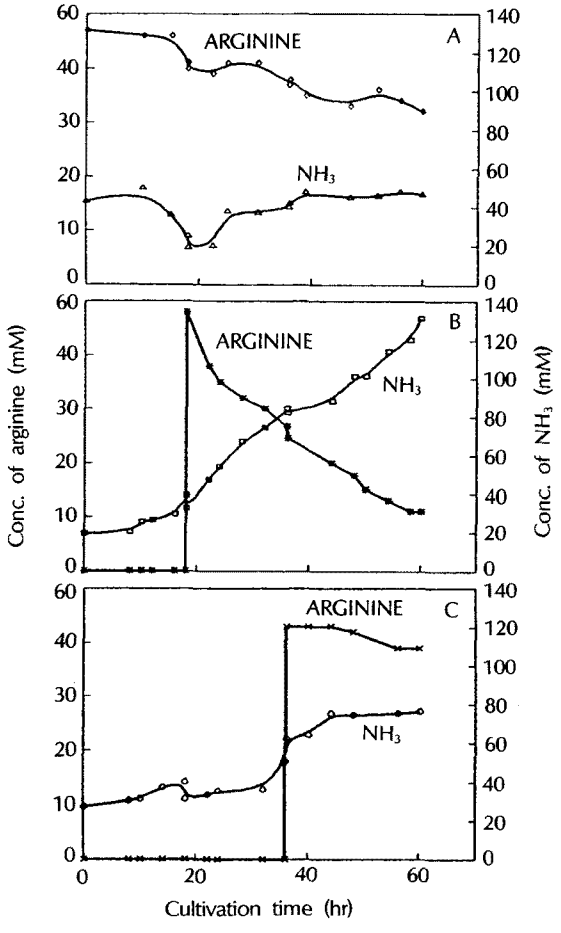

Fig. 6. Effect of arginine addition on the utilization of arginine and the production of ammonia in the fed-batch culture of *L. diacetylactis* FC1.
Concentrated arginine was added to the concentration of 50 mM at 0 hr (A), after 18 hr (B) and after 36 hr cultivation (C).

shown in Fig. 6. When the arginine was added after 18 hr cultivation, at which the cells were growing rapidly, arginine was rapidly utilized and the production of ammonia reached 131 mM after 60 hr of cultivation (Fig. 6, B). However, the arginine addition after 36 hr did not affect the production of ammonia significantly (Fig. 6, C). Therefore, it can be concluded that 50 mM arginine should be added to the culture after 18 hr cultivation for the optimum conditions of ammonia production.

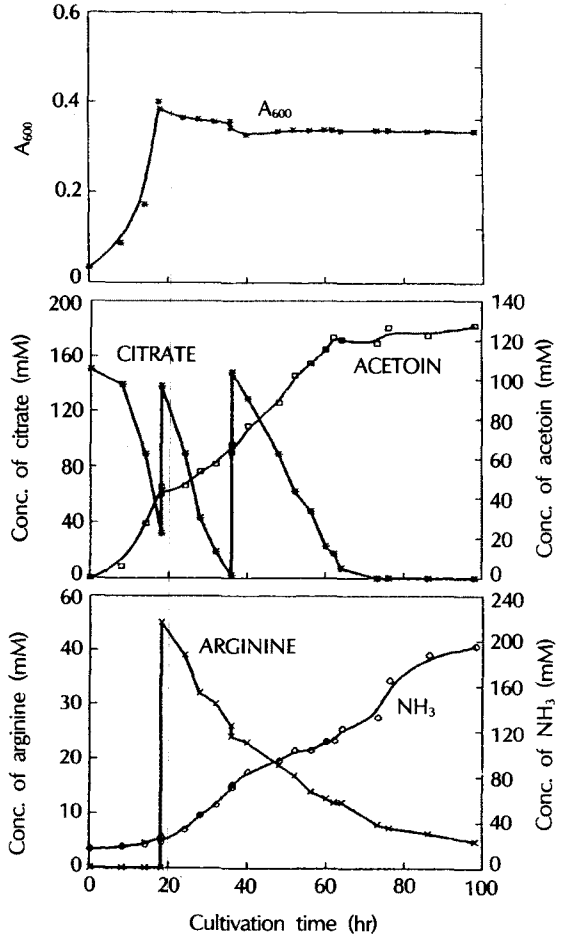

Fig. 7. Effect of galactose addition and extension of cultivation time on the cell growth in the fed-batch culture of *L. diacetylactis* FC1 (A).
The effect of these conditions on the utilization of citrate and arginine and on the production of acetoin and ammonia are also shown (B, C).

Effect of Galactose on the Fed-batch Culture of *L. diacetylactis* FC1

Upon addition of galactose after 36 hr cultivation, the cell numbers reached the stationary phase as shown in Fig. 7. This was different from the pattern of the culture without galactose addition, in which the cell numbers decreased gradually after 36 hr (Fig. 5).

It seems that galactose provides additional carbon-source to increase cell growth, leading to the increase of acetoin production. The maximum concentration of

Table 1. Determination of the optimum conditions for TMP production

Exp. No.	Rx temp. (°C)	pH	Rx time	TMP production (g/l)	TMP production (mM)	Acetoin (mM) Total.	Acetoin (mM) Util.	Conversion efficiency (%) A	Conversion efficiency (%) B
1	80	6.5	30 min	0.115	0.84	108	8	1.5	22
2	100			0.181	1.3		13	2.4	20
3	121			0.272	2		18	5	22
4		5.5	1 hr	0.287	2.1		15	5.3	28
5		8.3		0.465	3.4		17	8.6	38
6			2 hr	0.457	3.3		26	8.4	27
7			3 hr	0.603	4.4		33	11	27
8			4 hr	0.81			43	15	27

Utilized acetoin=the concentration of acetoin before thermal reaction−the concentration of acetoin after thermal reaction.
Conversion efficiency from acetoin in the culture (A) and utilized acetoin (B).

acetoin reached 115 mM, which was the highest concentration of acetoin observed in this study. When *L. diacetylactis* FC1 was grown for 98 hr, the acetoin and ammonia levels reached 127 mM and 195 mM respectively (Fig. 7). Therefore, it can be concluded that the addition of galactose is necessary for the maximum production of TMP precursors.

Effectors of the TMP Production

The effect of reaction temperature on the TMP production from acetoin and ammonia in the culture liquid is shown in Table 1. The concentration of TMP were 0.115, 0.181 g/l and 0.272 g/l in experiments No. 1, 2 and 3, respectively. The formation of TMP from acetoin and ammonia was affected by heat, and a higher amount of the TMP was produced at higher temperatures. From these data, it was known that reaction temperature of 121°C gave the highest yield of TMP.

The effect of pH on the TMP production, when the samples were treated for 1 hr at 121°C (Exp. No. 4 and 5) is also shown in Table 1. Although the utilization of acetoin was similar in each experiment, the yield of TMP was higher (0.465 g/l) at pH 8.3 than at pH 5.5 (0.287 g/l).

The results from experiments 5~8 in Table 1 showed that the production of TMP at 121°C and initial pH of 8.3 increased in proportion to the length of the heating period. The yield of TMP were 0.465, 0.457, 0.603 g/l and 0.81 g/l with the length of heating period of 1, 2, 3 hr and 4 hr, respectively. The maximum yield was obtained after four hours of heating and the conversion efficiency rate of acetoin into TMP was 15%. The yield did not increase although the heating was done over 4 hr. Therefore, from the above results, the optimum conditions for TMP production was determined to be as follows: reaction temperature, 121°C; pH 8.3; and reaction time, 4 hr.

REFERENCES

1. Adachi, T., H. Kamiya and T. Kasuge. 1962. Studies on the metabolic products of *Bacillus subtilis*. IV Determination and mechanism of formation of tertramethylpyrazine. *Yakugaku Zasshi.* **84**: 545-548.
2. Condon, S. 1987. Responses of lactic acid bacteria to oxygen. *FEMS Microbial. Rev.* **46**: 269-280.
3. Croco, V.L. and T.D. Thomas. 1982. Arginine metabolism in lactic Streptococci. *J. Bacteriol.* **150**: 1024-1032.
4. Demain, A.L., M. Jackson and N.R. Trenner. 1967. Thiamine-dependent accumulation of tetramethylpyrazine accompanying a mutation in the isoleucine-valine pathway. *J. Bacteriol.* **94**: 323-326.
5. Hickey, M.W., A.J. Hiller and G.R. Japo. 1983. Metabolism of pyruvate and citrate in lactobacilli. *Aust. J. Biol. Sci.* **36**: 487-496.
6. Kamiya, H., T. Adachi and T. Kosuge. 1964. Studies on the metabolic products of *Bacillus subtilis* I. Isolation and identification of tetramethylpyrazine. *Yacugaku Zasshi.* **84**: 448-451.
7. Kaneko, T., H. Suzuki and T. Takahashi. 1986. Diacetyl formation and degradatin by *Streptococcus lactis* subsp. *diacetylactis* 3022. *Agric. Biol. Chem.* **50**: 2639-2641.
8. Kaneko, T., H. Suzuki and T. Takahashi. 1990. Acetoin fermentation by citrate-positive *Lactococcus lactis* subsp. *lactis* 3022 grown aerobically in the presence of hemin or Cu^{2+}. *Appl. Environ. Microbiol.* **56**: 2644-2649.
9. Kim, K.H. and H.J. Lee. 1991. Opimum condition for the formation of acetoin as a precursor of tetramethylpyrazine during the citrate fermentation by *L. diacetylactis* FC1. *J. Microbiol. Biotechnol.* **1**: 202-206.
10. Kosuge, T., H. Zenda, K. Tsuji, T. Yamato and H. Narita. 1971. Studies on flavor components of foodstuff. Part I. Distribution of tetramethylpyrazine in fermented foodstuffs. *Agric. Biol. Chem.* **35**: 693-696.
11. Marier, J.R and M. Boult. 1958. Direct determination of citric acid milk with an improved pyridine-acetic anhydride method. *J. Dairy Sci.* **41**: 1683-1692.
12. Rizzi, G.P. 1988. Formation of pyrazines form acyloin precursors under mild conditions. *J. Agric. Food Chem.* **36**: 349-352.
13. Rosenberg, H., A.H. Ennor and J.F. Morrison. 1956. The estimation of arginine. *J. Biochem.* **63**: 153-159.
14. Smart, J.B. and T.D. Thomas. 1987. Effect of oxygen on Lactose metabolism in lactic *Streptococci. Appl. Environ. Microbial.* **53**: 533-541.
15. Vilches, C., C. Pitt and R. Marczak. 1990. Citrate metabo-

lism in starved cells of *Streptococcus lactis* subsp. *diacetylactis*. *J. Basic Microbiol.* **30**: 609-615.
16. Walsh, B. and T.M. Cogan. 1973. Diacetyl, acetoin and acetaldehyde production by mixed species of lactic starter cultures. *Appl. Environ. Microbiol.* **26**: 820-825.
17. Westerfeld, W.W. 1945. A colorimetric determination of blood acetoin. *J. Biol. Chem.* **161**: 495-502.
18. Whttenburry, R. 1964. Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. *J. Gen. Microbial.* **35**: 13-26.
19. Wriston, J.C., Jr. 1970. Asparaginase. p. 732-736 in Tabor, H., C.W. Tabor (eds). *Methods in Enzymology*. Vol. 17 part A Academic press.

(Received August 2, 1994)