Note

Diacetyl Formation and Degradation by *Streptococcus lactis* subsp. *diacetylactis* 3022

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Over the past years many workers have investigated the mechanism of diacetyl and acetoin formation by *Streptococcus lactis* subsp. *diacetylactis*.¹⁻⁵⁾ In addition, Kempler *et al.*⁶⁾ demonstrated that citrate metabolism is plasmid-linked in *S. lactis* subsp. *diacetylactis*, which

relates to the formation of these compounds. However, more extensive work is needed in the regulation of these compounds' formation to obtain uniform fermented products of desired quality. In the present study, we examined the influences of citrate and redox potential (*Eh*) on diacetyl formation by *S. lactis* subsp. *diacetylactis*.

S. lactis subsp. *diacetylactis* 3022 cells were incubated in 500 ml of MRS⁷⁾ with and without citrate at 30°C, respectively. A sample was withdrawn periodically during incubation and harvested from the culture broth by centrifugation at 5,000 rpm at 4°C for 15 min and washed twice with 0.1 M phosphate buffer (pH 6.0). These cells were re-suspended to 30 ml of phosphate buffer and then disrupted using a SONIFIER Model 200 (Branson Sonic Power Co.). These crude extracts were centrifuged at 25,000 rpm at 4°C for 30 min and the supernatant was used as a cell-free extract. Determination of the diacetyl was performed by the headspace gas chromatographic technique on a Perkin-Elmer Dual FID Chromatograph SIGMA 300 with 1.8 m × 3 mm O.D. steel column packed with Unicarbon A200, 60~80 mesh. One gram of the sample was transferred into a headspace vial (Perkin-Elmer) and the vial was closed by a rubber sep-

FIG. 1. Effects of Citrate on Diacetyl Formation and Degradation by *Streptococcus lactis* subsp. *diacetylactis* 3022.

A: Diacetyl accumulation in MRS (—●—) and MRS without citrate (—○—) and utilization of citrate (—●—) by *S. lactis* subsp. *diacetylactis* 3022.

B: Diacetyl formation activity of the cells grown in MRS (—●—) and MRS without citrate (—○—). The reaction mixture contained 20 mM sodium pyruvate, 0.2 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 0.1 M acetate buffer (pH 5.5) and 0.1 ml of cell-free extract in a total volume of 1.1 ml. After incubation at 30°C for 30 min, the amount of diacetyl was determined.

C: Diacetyl reductase activity of the cells grown in MRS (—●—) and MRS without citrate (—○—). The reaction mixture contained 0.25 mM NADH, 13 mM diacetyl, 0.2 M phosphate buffer (pH 6.0) and 0.1 ml of cell-free extract in a total volume of 3.3 ml. The reaction was started by addition of cell-free extract and the rate of NAD^+ formation was measured by recording the initial decrease in absorbance at 340 nm at 30°C.

tum and an aluminium cap. After equilibration at 80°C for 30 min, the diacetyl was analyzed under the following conditions. After holding the column temperature at 70°C for 4 min, the temperature rise was programmed at a rate of 30°C per min from 70 to 130°C. The nitrogen-flow rate was 30 ml per min and the temperatures of the injection port and detector were 150°C. Activities of diacetyl formation from pyruvate and diacetyl reductase (EC 1.1.1.5) were assayed as described under Fig. 1. One unit of diacetyl formation activity was defined as the amount of cell-free extract which catalyzes the production of one μmol diacetyl from pyruvate per min at 30°C and one unit of diacetyl reductase activity was defined as the amount of enzyme oxidizing one μmol of NADH. The molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm at 30°C for NADH was used for the calculation of diacetyl reductase activity. Protein concentration was determined by the method of Lowry *et al.*⁸⁾ using bovine albumin as a standard. The redox potential was measured at 25°C with a combination electrode PS-115C (TOA Electronics Ltd.) connected to a pH Meter HM-5B (TOA Electronics Ltd.). E_h was calculated by the formula $E_h = E_0 + 199 \text{ mV}$, E_0 being the potential difference between the platinum and a saturated silver-chloride electrode.

At 4 hr incubation, 49% of the citrate added in the medium was utilized by the cells and almost all of the citrate was utilized at 8 hr incubation. Diacetyl formation activity of the cells grown in MRS was higher than that of the cells grown in MRS without citrate, and the former cells produced a larger amount of diacetyl than the latter. On the other hand, *S. lactis* subsp. *diacetylactis* 3022 cells grown in MRS had significantly high activity of diacetyl reductase even in the exponential period when a considerable amount of citrate was present in the medium, though the enzyme activity of the cells grown in MRS was lower than that of the cells grown in MRS without citrate. These results are shown in Fig. 1. Furthermore, we found that diacetyl reductase activity was lowered by the addition of citrate to the reaction mixture (Fig. 2). Citrate had disappeared from the medium, through utilization by the cells, at 8 hr incubation, and the amount of diacetyl decreased with succeeding incubation. These results suggest that diacetyl accumulation in the culture grown in the presence of citrate is caused both by the inhibition of diacetyl reductase and by repression of enzyme synthesis by the citrate, and that the degradation of diacetyl increases rapidly after the disappearance of the citrate. This speculation partly differs from that of Cogan.³⁾

In order to investigate the effect of E_h on diacetyl formation activity, 5 mM sodium ascorbate and 0.08 mM cysteine were added to the reaction mixture. Addition of sodium ascorbate and cysteine reduced the E_h of the reaction mixture from 342 mV to 276 mV and 307 mV, and 3.2-fold and 2.4-fold increases in diacetyl formation activity were found respectively (Table I). On the other hand, when *S. lactis* subsp. *diacetylactis* 3022 cells were in-

FIG. 2. Effect of Citrate on Diacetyl Reductase Activity. Diacetyl reductase activity was assayed as described under Fig. 1 except that the reaction mixture contained sodium citrate (0 ~ 10.2 mM).

TABLE I. EFFECT OF REDOX POTENTIAL ON DIACETYL FORMATION ACTIVITY FROM PYRUVATE

Diacetyl formation activity was assayed as described under Fig. 1 except that the reaction mixture contained 5 mM sodium ascorbate or 0.08 mM cysteine.

Addition	E_h (mV)	Diacetyl formation activity (nmol/min/mg protein)
None	342	12.6
5 mM Sodium ascorbate	276	40.3
0.08 mM Cysteine	307	29.8

cubated at 30°C for 20 hr in 10% reconstituted skim milk medium with 5 mM sodium ascorbate, they produced 2-times the amount of diacetyl as did the cells grown in a medium without sodium ascorbate (unpublished data). Reducing the E_h of the reaction mixture would seem to stimulate diacetyl formation activity.

These results indicate that *S. lactis* subsp. *diacetylactis* 3022 should be cultivated in a medium of low E_h until the citrate has disappeared from the medium for the purpose of increasing the diacetyl content.

REFERENCES

- 1) R. J. Harvey and E. B. Collins, *J. Bact.*, **82**, 954 (1961).
- 2) R. J. Harvey and E. B. Collins, *J. Bact.*, **83**, 1005 (1962).

- 3) T. M. Cogan, *J. Dairy Res.*, **48**, 489 (1981).
 - 4) R. A. Speckman and E. B. Collins, *J. Bact.*, **95**, 174 (1968).
 - 5) E. W. Seitz, W. E. Sandine, P. R. Elliker and E. A. Daty, *Can. J. Microbiol.*, **9**, 431 (1963).
 - 6) G. M. Kempler and L. L. McKay, *Appl. Environ. Microbiol.*, **37**, 316 (1979).
 - 7) J. C. De Man, M. Rogosa and M. E. Sharpe, *J. Appl. Bact.*, **23**, 130 (1960).
 - 8) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
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