Journal of Food Protection, Vol. 46, No. 7 Pages 601-604 (July 1983)

Acetoin and Diacetyl Formation by *Streptococcus lactis* subsp. *diacetylactis* DRC3

S. M. EL-GENDY*, H. ABDEL-GALIL, Y. SHAHIN and F. Z. HEGAZI

Department of Food Science, University of Assiut, Assiut, Egypt

(Received for publication August 23, 1982)

ABSTRACT

Cells of *Streptococcus lactis* subsp. *diacetylactis* DRC3 which had been grown on lactose in the presence of citrate were unable to form acetoin and diacetyl (AD) from citrate when suspended in succinate buffer, pH 4.4. Inclusion of both a source of energy and nitrogen in the buffer was necessary for AD formation. Concentrations of AD and D at pH 4.4 were about five and three times the concentrations at pH 6.4. The amounts of AD and D from citrate were about eight and two times those from pyruvate, calculated on a molar basis. It appears that D, at least, is formed not only from pyruvate arising during citrate cleavage, but also from acetyl-CoA resulting from a probable citrate breakdown in a reversible reaction of a citrate synthetase. The rate of AD formation, under optimum conditions, was 0.047 μmoles mg (dry wt^{-1}) cells ml^{-1} min^{-1}. Pyruvate-grown cells produced little AD from pyruvate. AD production was inhibited partly by acetate and completely by acetaldehyde. Cetylpyridinium chloride at a concentration higher than μg ml^{-1} suppressed AD production from citrate because of the absence of interfering compounds normally present in milk.

The quality of many fermented dairy products depends upon the ability of the citrate utilizing microorganisms in the starter culture to produce flavor compounds such as acetate and diacetyl. Diacetyl is responsible for the "buttery" aroma characteristic of butter, buttermilk and sour cream. Because of its ability to produce diacetyl from milk citrate, *Streptococcus lactis* subsp. *diacetylactis* represents an important component of many mixed-strain starter cultures either alone or together with *Leuconostoc* species (*11*).

Many workers (*10,24,25,27*) have investigated the mechanism of acetoin and diacetyl production by *S. lactis* subsp. *diacetylactis* and *Leuconostoc* species.

Magee et al. (*20*) used a cell-free extract of *S. lactis* subsp. *diacetylactis* encapsulated with pyruvate, thiamine pyrophosphate and $MgSO_4$ for generation of diacetyl and acetoin in cheese in an attempt to enhance and regulate flavor development.

Kempler and McKay (*16,17*) have begun to elucidate the enzyme systems and genetic make-up responsible for synthesis of diacetyl from citrate by *S. lactis* subsp. *diacetylactis*. It is the purpose of this work to study the ability of *S. lactis* subsp. *diacetylactis* DRC3 to produce acetoin and diacetyl as affected by some metabolites, pH and a cationic wetting agent.

MATERIALS AND METHODS

S. lactis subsp. *diacetylactis* was propagated routinely at 30°C in sterile litmus milk and subcultured in the tryptone-yeast extract-lactose (TYL) broth of Broome et al. (*3*).

Culture preparation

The organism was grown in TYL broth with 0.5% of either tripotassium citrate or sodium pyruvate for 17 h at 30°C.

Cell suspensions

Two hundred and fifty ml cultures (2.0% inoculum incubated for 17 h at 30°C) were harvested by centrifugation at 3000 × *g* for 20 min, washed once in 0.3 mmol /L KH_2PO_4 buffer, pH 7.0 and resuspended in the same buffer. The cells were added to the different assay reaction mixtures to give a dry weight of 0.4 to 0.5 mg ml^{-1} (*4*).

Effect of supplements on acetoin and diacetyl (AD) formation from citrate

The assay reaction mixture contained (in 3 ml): 150-200 μmoles succinic acid (adjusted to pH 4.4 with 0.2 mol/L NaOH); 1.2 to 1.5 mg (dry weight) of microorganism whole cells; 50 μmoles tripotassium citrate and one or more of the following components: 30 μmoles glucose, 30μmoles lactose, 10 mg tryptone, 5 mg yeast extract.

Effect of pH and substrate on AD formation

The reaction mixture contained (in 3 ml): 200 μmoles succinic acid, (adjusted to pH 4.4 with 0.2 mol/L NaOH) or 200 μmoles KH_2PO_4 (adjusted to pH 6.4 with 0.2 mol/L KOH); 1.2 to 1.5 mg (dry weight) of microorganism whole cells and 50 μmoles from either tripotassium citrate or sodium pyruvate.

Effect of each of acetate and acetaldehyde on AD formation from pyruvate

The reaction mixture contained (in 3 ml): 200 μmoles succinic acid, pH 4.4; 1.2 to 1.5 mg (dry weight) of microorganism whole cells; 50 μmoles sodium pyruvate and 50 μmoles sodium acetate or 11.4 μmoles acetaldehyde (100 μg ml^{-1} reaction mixture).

Effect of cetylpyridinium chloride on AD production from citrate

The reaction mixture contained (in 3 ml): 100 μmoles succinic acid, pH 4.4; 1.2 to 1.5 mg (dry weight) of microorganism whole cells; 50 μmoles tripotassiuim citrate; 1 mg tryptone; 5 mg yeast extract; 30 μmoles lactose and either 3, 15, 18, 21, 24, 27 or 30 μg cetylpyridinium chloride.

Reactions were initiated by addition of substrate. Incubation was at

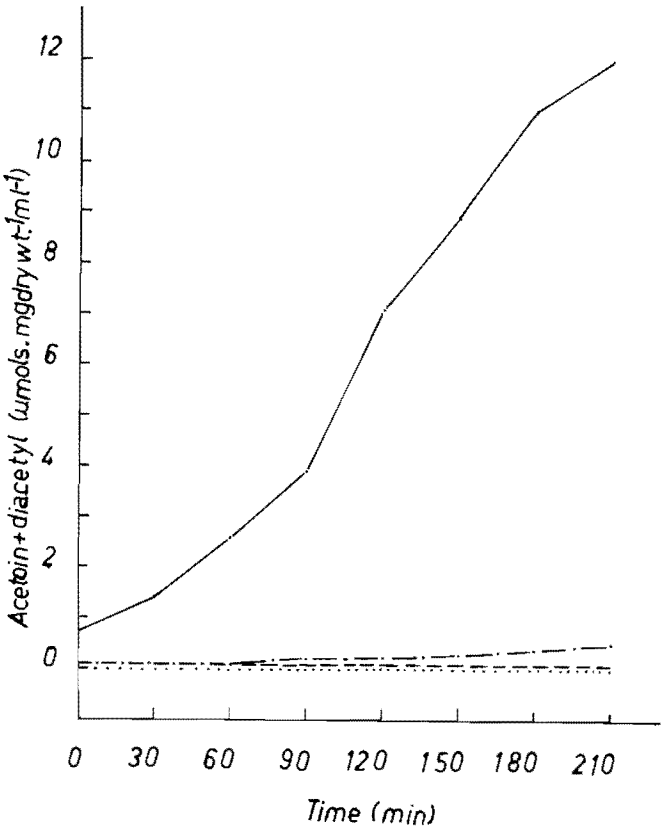

Figure 1. *Acetoin and diacetyl formation by whole-cell suspensions of citrate-grown* S. lactis *subsp.* diacetylactis *from: citrate + (tryptone + yeast extract + lactose), TYL———; citrate + yeast extract -.-.-.-.; citrate + lactose or glucose -------; citrate alone or + tryptone. . . . . .*

30°C for 30 to 210 min. The reaction was stopped by addition of 1.0 ml of 0.125 *N* NaOH and the content of diacetyl and acetoin was determined.

Determination of acetoin and diacetyl

As a rapid means of following the reaction, the diacetyl and acetoin content of the reaction mixture was determined by the colorimetric method of Westerfeld (*29*), as the sum of both, using a Bauch & Lomb Spectronic 21 Spectrophotometer at 520 nm. Results were calculated as acetoin.

Determination of diacetyl

The method of Krampitz (*18*) was adopted with adsorption at 470 nm.

RESULTS AND DISCUSSION

Although the optimum pH for the inducible citrate permease is around pH 5.0 (*13*), cells of *S. lactis* subsp. *diacetylactis* which had been grown on lactose in the presence of citrate were unable to form AD from citrate in succinate buffer, pH 4.4. If, however, tryptone-yeast extract-lactose (TYL) were included in the buffer, the cells were highly activated. The effect of TYL and each of its components on the activity of the cells is represented graphically in Fig. 1. One observes only a very slight enhancement of the rate of AD formation by addition of yeast extract, whereas the fermentable carbohydrate alone, or the nitrogenous source alone was completely ineffective. Magnesium and thiamine pyrophosphate used occasionally in the assay mixtures (*2,28*) were not examined. Failure of *S. lactis* subsp. *diacetylactis* to form AD until the addition of TYL may be due to the lack of inducible citrate permease since the enzymes citratase and ∝-acetolactate synthetase are constitutively present in the cells of this microorganism (*4,12*).

Examination of citrate-negative mutants of 2 strains of *S. lactis* subsp. *diacetylactis* 18 - 16 and DRCl by Kempler and McKay (*16*) revealed the loss of a 5.5 Mdal plasmid associated with citrate permease activity, whereas the mutants retained citratase activity. However, further examination by the same authors of a citrate-negative variant (DRCl-X) isolated by Collins and Harvey (*7*) revealed no loss of any plasmid species. Kempler and McKay (*17*) examined a spontaneous citrate-negative variant of *S. diacetilactis* DRC3, the organism under investigation here, and found it also was missing a 5.5 Mdal plasmid responsible for the ability of the cells to utilize citrate. It appears, however, that the inability of citrate-grown cells of strain DRC3 to use citrate, observed here, is due to loss of the ability to transport citrate into the cells (*7*) as a result of rapid disintegration of the inducible citrate permease rather than to loss of a plasmid or mutation in the permease gene.

The induced enzyme for the primary substrate (citrate permease) presumably reaches its peak of activity faster than those of secondary and tertiary substrates, which result from the breakdown of citrate into the cells, and as soon as the substrate is consumed or removed, the enzymatic activity starts to disappear. One can postulate, then, that resting cells of *S. lactis* subsp. *diacetylactis,* after growth in the presence of citrate in a TYL medium did not maintain their citrate permease, and could inducibly reform the enzyme in the presence of citrate only if both lactose and nitrogen sources were supplied. Pinsky and Stokes (*22*) found both types of substances to be essential for formation of the inducible formic hydrogenlyase of *E. coli.* Another probable reason for inability of *S. lactis* subsp. *diacetylactis* to use citrate is the species of buffer used. Cogan et al. (*4*) reported that more rapid use of citrate occurred in acetate than in phosphate buffer of the same pH. No detectable acetoin production occurred in phosphate buffer at pH 6.5 or 6.1, whereas acetoin production occurred in acetate at pH 6.1. However, the loss of citrate permease mentioned above seems to be more tenable.

Effect of pH and substrate

Effects of both pH and substrate on AD and D formation by citrate-grown cells are shown in Fig. 2 and 3. Raising the pH value to 6.4 away from about the optimum pH 4.4 for AD formation caused a marked reduction in the amounts produced. The total amount of AD and D at pH 4.4 was about five times and three times as high as those at pH 6.4, respectively. In *Leuconostoc lactis* NCWl, Cogan et al. (*4*) found the concentration of AD at pH 4.5 nine times the concentration at pH 6.3. Our results are also in agreement with those of Mizuno and Jezeski (*21*), who reported that in the presence of citric acid in dialysed milk

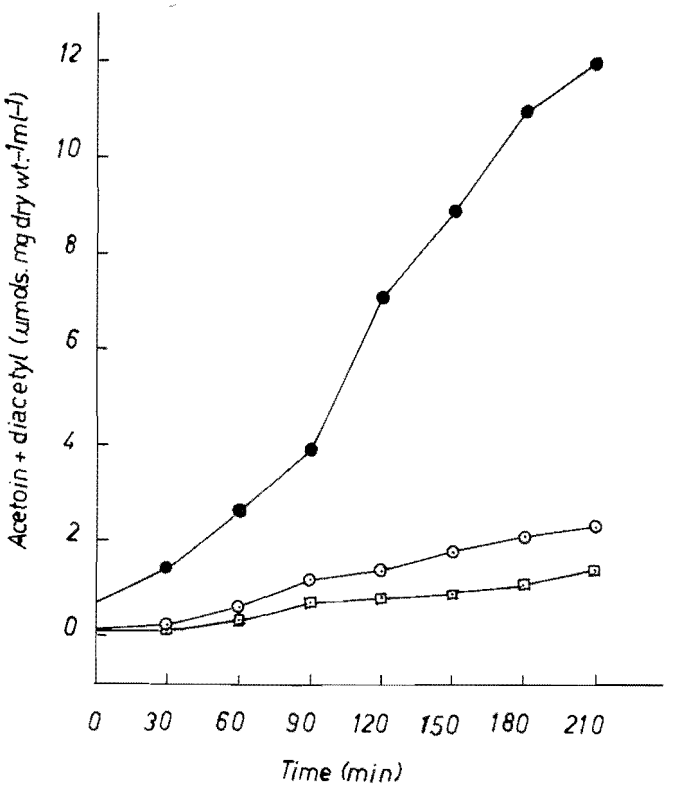

Figure 2. *Acetoin and diacetyl formation by whole-cell suspensions of citrate-grown* S. lactis *subsp.* diacetylactis *from: citrate + TYL at pH 4.4* ●——●; *citrate + TYL at pH 6.4* ○---○; *pyruvate + TYL at pH 4.4* □——□.

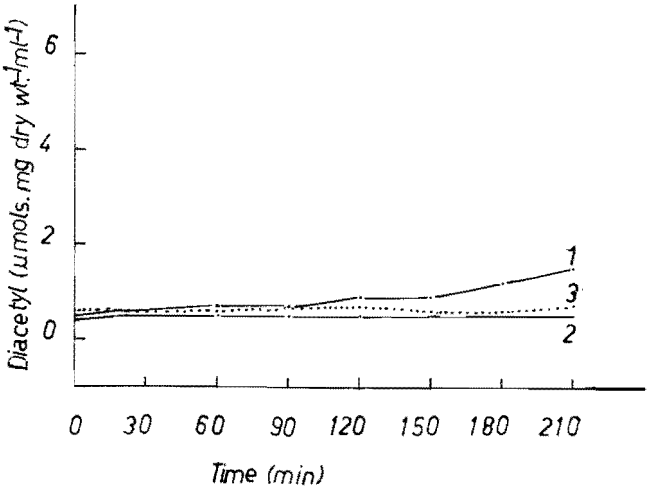

Figure 3. *Diacetyl formation by whole-cell suspensions of citrate-grown* S. lactis *subsp.* diacetylactis *from: 1, citrate + TYL at pH 4.4; 2, citrate + TYL at pH 6.4; 3, pyruvate + TYL at pH 4.4.*

a trace and a considerable amount of acetoin were formed at pH 7.0 and pH 5.0, respectively.

Production of AD and D from pyruvate by citrate-grown cells is also shown in Fig. 2 and 3. The amount of AD formed from citrate was about eight times as high as that formed from pyruvate, whereas the amount of diacetyl was about two times. According to the metabolism of citrate, if the organism is grown in the presence of citrate, then pyruvate also will be formed in the cell, and thus enzymes will be present not only for citrate, but also for pyruvate. If AD are formed only from pyruvate resulting from cleavage of citrate one would expect formation of equal amounts of these compounds from similar concentrations of citrate and pyruvate calculated on a molar basis. The results obtained in these experiments and in others revealed, however, that the amount from citrate are several times those from pyruvate. It appears, therefore, that acetyl-CoA arises during a probable citrate breakdown in a reversible reaction of a citrate synthetase. Because of its energy-rich bond, acetyl-CoA can readily condense with hydroxyethylthiamine pyrophosphate to form diacetyl (*25*). It can also be reduced to acetaldehyde, increasing the availability of hydroxyethylthiamine pyrophosphate for acetoin and diacetyl synthesis.

The calculated rate of AD formation by *S. lactis* subsp. *diacetylactis,* under nearly optimum conditions, was 0.047 μmoles mg dry wt^{-1} ml^{-1} min^{-1}. The rate of AD formation by citrate-grown *S. lactis* subsp. *diacetylactis* DRC3, in relation to pH, substrate and supplements, is shown in Table 1. The level of AD formation in the presence of tryptone alone and lactose alone represented only 0 and 14.9%, respectively, of that produced by the organism in the presence of both. The amount of AD formed from pyruvate at pH 4.4, and the amount from citrate at pH 6.4 were only 8.6 and 17.2%, respectively, of that given by the organism from citrate at pH 4.4 in the presence of TYL.

Effect of acetate and acetaldehyde on AD formation

The pyruvate-grown cells formed a small amount of AD from pyruvate (Table 2) although pyruvate is readily attacked by the enzymes of pyruvate metabolism. Pyruvate may have been converted to acetaldehyde and ethanol at pH 4.4, as was previously reported for this strain by Lees and Jago (*19*).

Inclusion of acetate or acetaldehyde in the assay mixture reduced AD formation. The production was inhibited partly in the presence of acetate and completely in the presence of acetaldehyde. Thomas et al. (*28*) reported that, while 30 μmoles sodium acetate did not inhibit pyruvate decarboxylase or lipoate acetyltransferase activity in *S. lactis* subsp. *diacetylactis* DRC2, it did inhibit acetate kinase activity by 50%. According to Thomas et al. (*28*) inhibition of acetate kinase could lead to accumulation of the acetaldehyde-TPP complex which is a substrate for the synthesis of AD. This could not be confirmed in the present investigation. Lactic streptococci are unable to synthesize lipoic acid and in its absence require acetate for growth (*6,8,23*). With lipoic acid in the medium, they do not require acetate and form acetyl-CoA from pyruvate. Incorporation of acetate into diacetyl by *S. diacetilactis* is only possible when lipoic acid is not present (*26*).

Mizuno and Jezeski (*21*) also found that acetaldehyde inhibited acetoin formation by a mixed culture of predominately *Streptococcus cremoris* and *Leuconostoc* sp. in a

TABLE 1. *The rate of acetoin and diacetyl formation (μmoles mg dry wt^{-1} ml^{-1} min^{-1}) by whole cell suspensions of citrate-grown* S. lactis *subsp.* diacetylactis *DRC3, in relation to pH, substrate and supplements.*

	Succinate buffer pH 4.4						KH_2PO_4 buffer pH 6.4
	Citrate + glucose	Citrate + lactose	Citrate + tryptone	Citrate + yeast extract	Citrate + TYL[a]	Pyruvate + TYL	Citrate + TYL
Amount	0.006	0.007	0	0.002	0.047	0.004	0.008
Activity level	12.8	14.9	0	4.3	100.0	8.6	17.2

[a]TYL = tryptone, yeast extract, lactose.

TABLE 2. *Acetoin and diacetyl formation (μmoles mg dry wt^{-1} ml^{-1}) from pyruvate, in the presence of acetate and acetaldehyde, by whole-cell suspensions of pyruvate-grown* S. lactis *subsp.* diacetylactis.

Time in minutes	Pyruvate alone	Pyruvate + acetate	Pyruvate + acetaldehyde
0	0.18	0.01	0.00
30	0.16	0.01	0.00
60	0.14	0.01	0.00
90	0.11	0.01	0.00
120	0.08	0.08	0.00
150	0.11	0.01	0.00
180	0.09	0.01	0.00
210	0.11	0.01	0.00

dialysed milk medium. In contrast, addition of acetaldehyde (100 μg ml^{-1}) to glucose-citrate broth stimulated growth of *Leuconostoc citrovorum (cremoris)* and approximately doubled the production of acetoin plus diacetyl (*9*), probably by increasing the availability of hydroxyethylthiamine pyrophosphate and acetyl-coenzyme A. *S. diacetilactis* produced acetaldehyde and diacetyl in milk, with the acetaldehyde peak height being higher than the diacetyl after 24 h (*1*). During continuous incubation the organism did not tend to remove acetaldehyde from cultures, whereas *S. lactis* and *S. cremoris* were able to (*15*).

Effect of cetylpyridinium chloride on AD formation

Cetylpyridinium chloride was included in the assay mixture in concentrations of 1, 5, 6, 7, 8, 9 or 10 μg ml^{-1}. The least inhibition of AD formation occurred at 1 μg ml^{-1}; being 21% after 30 min and increased to 54% after 60 min. Concentrations higher than 1 μg ml^{-1} were completely inhibitory. In milk, however, the inhibiting level of cationic wetting agents is higher than that in the buffer solution because of their probable reaction with the milk constituents and the protective action of milk proteins for the organism. Kaus and Kandler (*14*) observed, at a concentration of 5 μg ml^{-1}, quaternary ammonium compound only 28% inhibition of a growing culture of *S. diacetilactis* after 8 h and no inhibition after 24 h.

REFERENCES

1. Bassette, R., R. E. Bawdon, and T. J. Claydon. 1967. Production of volatile materials in milk by some species of bacteria. J. Dairy Sci. 50:167-171.
2. Branen, A. L., and T. W. Keenan. 1970. Diacetyl, acetoin and 2,3-butanediol production by *Lactobacillus casei*. J. Dairy Sci. 53:632.
3. Broome, M. C., M. P. Thomas, A. J. Hillier, and G. R. Jago. 1980. Pyruvate dehydrogenase activity in group N streptococci. Aust. J. Biol. Sci. 33:15-25.
4. Cogan, T. M. 1981. Constitutive nature of the enzymes of citrate metabolism in *Streptococcus lactis* subsp. *diacetylactis*. J. Dairy Res. 48:489-495.
5. Cogan, T. M., M. O'Dowd, and D. Mellerick. 1981. Effects of pH and sugar on acetoin production from citrate by *Leuconostoc lactis*. Appl. Environ. Microbiol. 41:1-8.
6. Collins, E. B., F. E. Nelson, and C. E. Parmellee. 1950. Acetate and oleate requirement of the lactic group of streptococci. J. Bacteriol. 59:69-74.
7. Collins, E. B., and R. J. Harvey. 1962. Failure in the production of citrate permease by *Streptococcus diacetilactis*. J. Dairy Sci. 45:32-35.
8. Collins, E. B., and J. C. Bruhn. 1970. Roles of acetate and pyruvate in the metabolism of *Streptococcus diacetilactis*. J. Bacteriol. 103:541-546.
9. Collins, E. B., and R. A. Speckman. 1974. Influence of acetaldehyde on growth and acetoin production by *Leuconostoc citrovorum*. J. Dairy Sci. 57:1428-1431.
10. De Man, J. C. 1959. The formation of diacetyl and acetoin from α-acetolactic acid. Rec. Trav. Chim. 78:480-486.
11. Galesloot, T. E., and F. Hassing. 1961. Some differences in behaviour between starters containing as aroma bacterium either *Streptococcus diacetilactis* or *Betacoccus cremoris*. Neth. Milk Dairy J. 15:225-247.
12. Harvey, R. J., and E. B. Collins. 1961. Role of citratase in acetoin formation by *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. J. Bacteriol. 82:954-959.
13. Harvey, R. J., and E. B. Collins. 1962. Citrate transport system of *Streptococcus diacetilactis*. J. Bacteriol. 83:1005-1009.
14. Kauss, U., and O. Kandler. 1962. Zerstören die QAV-Desinfektionsmittel wirklich das Aroma der Buttereisäurewecker? Deutsche

con't. p. 609