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Effects of pH and Sugar on Acetoin Production from Citrate by *Leuconostoc lactis*

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The relationship between acetoin production and citrate utilization in *Leuconostoc lactis* NCW1 was studied. In a complex medium the organism utilized citrate at neutral pH (initial pH, 6.3) and at acid pH (initial pH, 4.5) but produced nine times more acetoin at the latter pH. In resting cells the utilization of citrate was optimum at pH 5.3. Production of acetoin as a function of citrate utilization increased as the pH decreased, and at pH 4.3 all of the citrate utilized was recovered as acetoin. Glucose (10 mM) and lactose (10 mM) markedly stimulated citrate utilization but totally inhibited acetoin production in glucose- and lactose-grown cells. Addition of glucose to cells actively metabolizing citrate caused an immediate increase in citrate uptake and a reduction in the level of acetoin. The apparent K_m values of lactic dehydrogenase for pyruvate were 1.05, 0.25, and 0.15 mM at pH 7.5, 6.5, and 5.0, respectively. Several heterofermentation intermediates inhibited α-acetolactate synthetase and decarboxylase activities. The implications of these results in regulating acetoin formatin are discussed.

In most bacteria, acetoin (acetyl methyl carbinol; 3-hydroxy-2-butanone) is formed from pyruvate and is thus a product of carbohydrate metabolism. However, in the lactic acid bacteria little, if any, acetoin is produced from carbohydrates unless an additional source of pyruvate is also present. A common source of pyruvate is citrate, which is metabolized as shown in Fig. 1. This pathway is called the anaerobic or fermentation pathway of citrate metabolism to distinguish it from the aerobic citric acid cycle (14). The term citrate fermentation is an unfortunate choice as it implies that lactic acid bacteria use citrate as an energy source, which is not true. Citrate is transported into the cell by an inducible permease, where it is broken down to acetate and oxalacetate, which is decarboxylated to pyruvate. Two molecules of pyruvate condense to form α-acetolactate which is decarboxylated to form acetoin (7, 15, 17). Acetoin can also be formed from diacetyl (2,3-butanedione) by using reduced nicotinamide adenine dinucleotide (NADH) as a co-factor (15). Much larger amounts of acetoin than diacetyl are produced during growth (1, 6, 9). In *Streptococcus lactis* subsp. *diacetylactis* this has been ascribed to limited acetyl coenzyme A biosynthesis (3) which is involved in diacetyl, but not acetoin, formation. It has been suggested that the reason that acetoin is not produced from carbohydrate is because all of the pyruvate produced during fermentation is reduced to lactate to produce NAD^+ to continue the fermentation (8). However, NAD^+ can also be produced through reduction of diacetyl and acetoin (Fig. 1).

We have previously reported that several leuconostocs and heterofermentative lactobacilli do not produce acetoin (or diacetyl) at neutral pH, even though citrate is catabolized. However, considerable amounts of acetoin are produced from citrate under acidic conditions (2, 6). This communication reports our attempts to determine the reason(s) for these observations.

MATERIALS AND METHODS

Organism. The leuconostocs used were isolated in this laboratory from raw milk and cheese and were maintained by subculture every 2 weeks. They were identified by the methods of Sharpe et al. (16).

Medium. The medium used was modified from the MRS medium of de Man et al. (5) by reducing the glucose concentration to 1% (wt/vol) and omitting Tween 80 and sodium acetate. Glucose was sterilized with the medium. In the experiments in which lactose and galactose were used as energy sources, they were added aseptically to the medium (without glucose) after sterilization to give a final concentration of 1% (wt/vol).

Growth studies. The medium (glucose as energy source) was inoculated with 1% (vol/vol) inoculum grown overnight at 30°C from a 0.1% (vol/vol) inoculum, incubated at 30°C, and analyzed at intervals. For the acidified cultures, overnight cultures (0.1% [vol/vol] inoculum; incubated for 16 h at 30°C) were divided into two parts, to one of which was added 8.6 mmol of citric acid and 7.0 mmol of NaOH per liter of medium. The function of the NaOH was to maintain the pH within ±0.1 pH unit of the control. Incubation was

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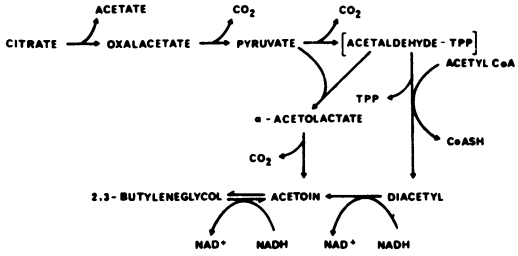

FIG. 1. *Citrate metabolism in lactic acid bacteria. ACETYL CoA, acetyl coenzyme A; TPP, thiamine pyrophosphate; CoASH, reduced coenzyme A.*

continued at 30°C, and samples were analyzed at intervals.

Cell-free extracts. To 250 ml of culture (0.1% [vol/vol] inoculum; incubated for 16 h at 30°C) was added 2.15 mmol of citric acid and 1.75 mmol of NaOH. Incubation was continued for 45 min before the cells were harvested at 5,000 × *g* for 15 min at 4°C. They were washed once with cold 0.05 M phosphate, pH 7.0, and resuspended in 10 ml of the same buffer. The cells were disrupted using an ultrasonic disintegrator (MSE Scientific Instruments, Sussex, England) operating at half-maximum amplitude for 18 min. Sonication was carried out in 30-s cycles, each followed by a 60-s cooling interval. The temperature was never allowed to rise above 16°C. After sonication, the crude extract was centrifuged at 15,000 × *g* for 30 min at 6°C to remove all debris. This supernatant was decanted and dialyzed overnight against 0.05 M phosphate, pH 7.0, at 4°C.

Enzyme assays. α-Acetolactate synthetase (EC 4.1.1.1) and α-acetolactate decarboxylase (EC 4.1.1.5) were assayed together by monitoring the conversion of pyruvate to acetoin. The assay system contained (in 1 ml) 20 μmol of sodium pyruvate, 0.2 μmol of $MnCl_2$, 80 μg of thiamine pyrophosphate, 0.06 mmol of different buffers, and 0.1 ml of cell-free extract. The reaction was initiated by addition of extract. After incubation for 15 min at 37°C, the reaction was stopped by placing the reaction mixture in an ice bath and immediately analyzing for acetoin. Diacetyl reductase (EC 1.1.1.5) was assayed by measuring the decrease in absorbance at 340 nm due to NADPH oxidation. The assay system contained (in 3.0 ml) 39 μmol of diacetyl, 0.39 μmol of NADPH, 0.27 mmol of different buffers, and 0.1 ml of cell-free extract. The reaction was started by addition of diacetyl. No NADPH oxidase activity was detected. NADPH was made up in 0.1 M carbonate buffer, pH 10.6, and stored at −20°C until required (10). Lactate dehydrogenase (LDH; EC 1.1.1.27) was assayed by measuring the decrease in absorbance at 340 nm due to NADH oxidation. The assay system contained (in 3.0 ml) 3.0 μmol of pyruvate, 0.18 μmol of NADH, 0.27 mmol of different buffers, and 0.1 ml of cell-free extract. The reaction was started by addition of pyruvate. NADH oxidase activity was especially high at low pH and was removed by treating the crude cell-free extract with $(NH_4)_2SO_4$ to 50% saturation, centrifuging at 5,000 × *g* for 15 min, and assaying the supernatant for LDH. All enzyme activities were calculated as micromoles of product formed (acetoin, $NADP^+$, or NAD^+) per milligram of protein per minute.

Cell suspensions. Cultures (0.1% [vol/vol] inoculum incubated for 16 h at 30°C) were harvested by centrifugation at 5,000 × *g* for 10 min, washed once in 0.3 mM phosphate, pH 7.0, and resuspended in the same buffer. The cells were added to the different buffers (final concentration, 0.05 M in buffer species) to give dry weights of 0.4 to 0.5 mg/ml. All experiments were carried out at 30°C.

Analyses. Protein was estimated by the Lowry method (11), using bovine serum albumin as a standard. Acetoin and diacetyl were separated by steam distillation (21) and determined by the methods of Westerfeld (23) and a modification of the Prill and Hammer method (22), respectively. Citrate was determined by the pyridine-acetic anhydride method (13); lactate was determined by titration of the acid developed in the culture (i.e., all values were corrected for the acidity of the uninoculated medium); and growth was determined by measuring the increase in absorbance at 600 nm and converting the values to dry weight. The specific growth rate (k, $hour^{-1}$) was determined by using the formula: $k = 2.303 (\log x_2 - \log x_1)/t_2 - t_1$, in which x_2 and x_1 are the dry weights at times t_2 and t_1, respectively, during the exponential phase.

RESULTS

Screening. Seven strains of *L. lactis* (7-1, N2, T1, 9-1, D12, NCW1, and P2) and two strains of *Leuconostoc mesenteroides* (S3 and X2) were screened for diacetyl and acetoin production from citrate at pH values between 4.1 and 4.7. The results showed that four cultures (viz. 7-1, N2, S3, and T1) produced no diacetyl and only traces of acetoin, even though citrate was metabolized (Table 1). The remaining five strains (9-1, D12, NCW1, P2, and X2) produced high levels of acetoin and diacetyl from citrate, except for strain P2 which produced no diacetyl (Table 1). Acetoin production was much greater than that of diacetyl. Maximum production of diacetyl and acetoin coincided with the almost complete disappearance of citrate, and accumulation then ceased, except in strain NCW1 in which reutilization was observed (data not shown). This strain was selected for further study. Recovery of acetoin from citrate ranged from 14 to 56% in the acetoin-producing strains (Table 1). Presumably, the remainder was reduced to 2,3-butylene glycol by acetoin reductase which may be proportionately less active in strains showing high recoveries.

Growth characteristics. Growth (glucose as energy source) and metabolism of citrate by strain NCW1 are shown in Fig. 2A. There was a gradual decrease in pH from an initial level of 6.3 to 4.7 as growth proceeded. Growth was exponential up to 0.2 mg/ml (dry weight), corresponding to pH 5.6, after which it slowed

down. The k value was 0.92 h^{-1}, equivalent to a doubling time of 45 min. The organism grew equally well with lactose as energy source (k, 0.87 h^{-1}) but poorly on galactose (k, 0.15 h^{-1}). Citrate utilization began as soon as growth was initiated, and the 8.2 mM citrate which was initially present in the medium was completely utilized at the point when growth slowed down. This decrease in growth rate is a reflection of the disappearance of citrate from the medium since this strain grew faster in the presence of citrate (k, 0.91 h^{-1}) than in its absence (k, 0.57 h^{-1}). The maximum level of acetoin produced was 0.04 mM which coincided with the almost complete disappearance of citrate from the medium. No acetoin was produced in the absence of citrate. Lactate reached a maximum of 20 mM. Both citrate utilization and acetoin production paralleled growth but lactate production did not.

TABLE 1. *Maximum amounts of diacetyl and acetoin produced by nine strains of leuconostocs under acid conditions in the presence and absence of 8.6 mM citrate*

Strain	pH[a]	Diacetyl (mM)		Acetoin (mM)		Recovery from citrate[b] (%)
		Without citrate	With citrate	Without citrate	With citrate	
7-1	4.2	0	0	0.02	0.03	0.7
N2	4.2	0	0	0	0.01	0.2
S3	4.2	0	0	0.02	0.04	0.9
T1	4.2	0	0	0	0.01	0.2
9-1	4.7	0	0.10	0.02	1.60	39.5
D12	4.2	0	0.18	0.01	2.22	55.8
NCW1	4.3	0	0.11	0	0.49	14.0
P2	4.2	0	0	0.01	1.50	34.8
X2	4.1	0	0.20	0.02	1.41	37.4

[a] All strains metabolized citrate at these pH values.
[b] Assuming 2 mol of citrate → 1 mol of acetoin + 1 mol of diacetyl.

The addition of a similar level of citrate (8.6 mM) to fully grown cultures at pH 4.5 resulted in production of nine times more acetoin (0.38 mM; Fig. 2B). No acetoin production occurred in the absence of added citrate, and no growth occurred in either the presence or absence of citrate. Maximum production of acetoin coincided with the almost complete disappearance of citrate. In both growing and nongrowing cultures a reduction in the level of acetoin was observed once citrate had been utilized.

Effect of pH. In cell suspensions, the rate of citrate utilization (in the absence of energy source) was dependent on pH (Fig. 3A). The optimum pH for utilization was 5.3 and little, if any, occurred at pH 6.5. Uptake was not linear,

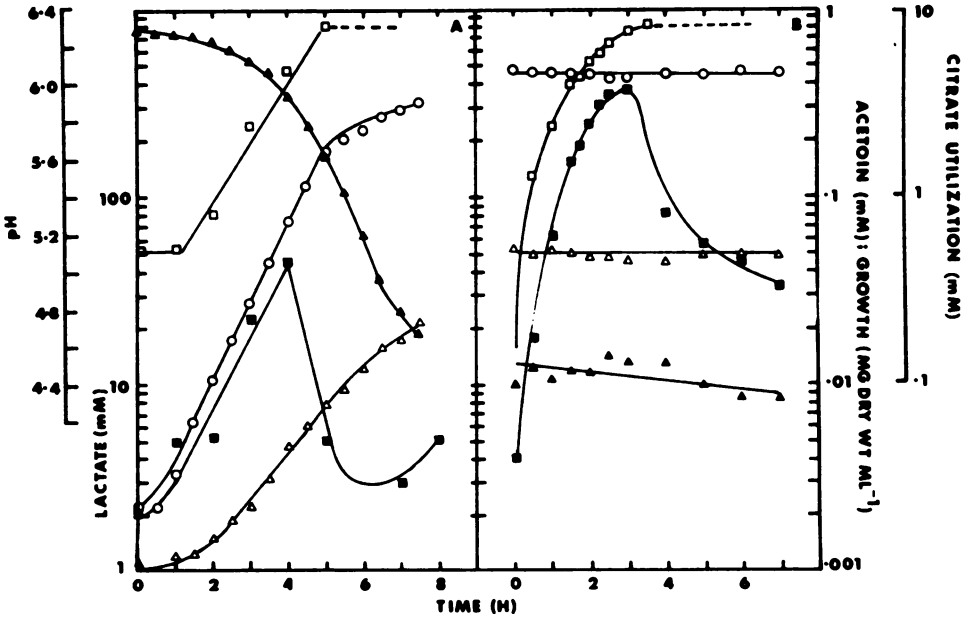

FIG. 2. *Growth and citrate metabolism of L. lactis NCW1 in modified MRS broth at 30°C at neutral pH (A) and acid pH (B). In (A) the medium was inoculated with 1% (vol/vol) culture and sampled periodically. In (B) 8.6 mM citrate was added to a culture which had been preincubated at 30°C for 16 h (0.1% [vol/vol] inoculum). The culture was reincubated at 30°C and sampled periodically. Symbols: ○, dry weight; △, lactate; □, citrate; ■, acetoin; ▲, pH. No acetoin was produced in the absence of citrate.*

and similar rates occurred at pH 6.1 and 5.7 (data not shown) and at pH 4.7 and 4.3 (data not shown). More rapid utilization occurred in acetate than in phosphate buffer of the same pH. The patterns of acetoin production as a function of citrate utilization were entirely different (Fig. 3B). No detectable acetoin production occurred in phosphate at pH 6.5 or 6.1. However, acetoin production occurred in acetate at pH 6.1, and the amount of acetoin produced increased as the pH decreased. At pH 4.3 the citrate utilized was quantitatively recovered as acetoin.

Effect of carbohydrate. The presence of sugar increased the rate of citrate utilization by cell suspensions at pH 5.3 (Fig. 4A). In glucose-grown cells, utilization was most rapid in the presence of 10 mM glucose, followed by 10 mM lactose and 10 mM galactose. Utilization of citrate by lactose-grown cells in the presence of 10 mM lactose was as rapid as that of glucose-grown cells in the presence of 10 mM glucose. The utilization of citrate by galactose-grown cells was not studied since the organism grew very poorly with galactose as an energy source.

Acetoin production as a function of citrate utilization was totally inhibited by 10 mM glucose in glucose-grown cells, by 10 mM lactose in lactose-grown cells, and almost completely by 10 mM lactose in glucose-grown cells (Fig. 4B). Galactose (10 mM) had little effect. The addition of 10 mM glucose to cell suspensions metaboliz-

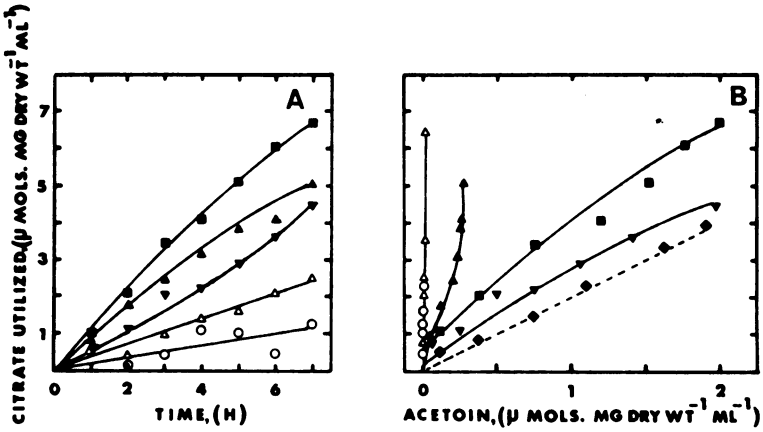

FIG. 3. *Effect of pH on citrate uptake (A) and acetoin production as a function of citrate uptake (B) by cell suspensions of L. lactis NCW1. Open symbols indicate phosphate buffer, and closed symbols indicate acetate buffer. Symbols: ---, theoretical amount of acetoin; ○, pH 6.5; △ and ▲, pH 6.1; ■, pH 5.3; ▼, pH 4.7; ◆, pH 4.3.*

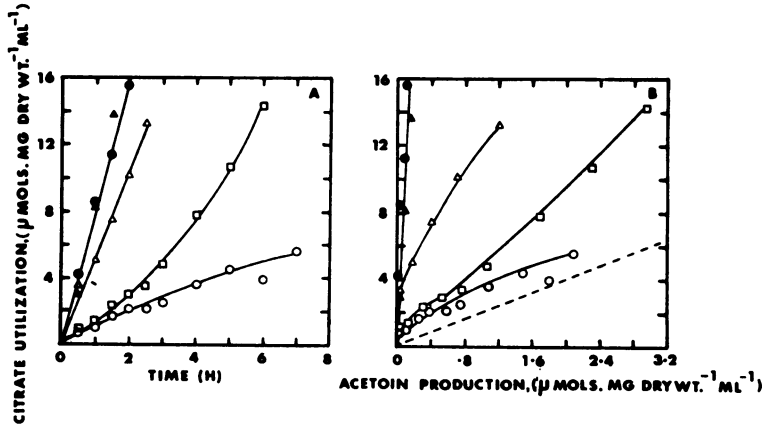

FIG. 4. *Effect of sugar on citrate uptake (A) and acetoin production as a function of citrate uptake (B) by resting cells of L. lactis NCW1 in 0.05 M acetate, pH 5.3. Symbols: ---, theoretical amount of acetoin; ○, no sugar added; ●, 10 mM glucose, glucose-grown cells; ▲, 10 mM lactose, lactose-grown cells; △, 10 mM lactose, glucose-grown cells; □, 10 mM galactose, glucose-grown cells.*

ing citrate caused an immediate increase in citrate utilization and an immediate decrease in acetoin production (Fig. 5).

The rapidity of citrate utilization by cell suspensions in the presence of sugar was dependent on concentration. The effect of glucose is shown in Fig. 6A. Glucose at a concentration of 1.0 mM was almost as effective as at 10 mM, whereas 0.1 mM glucose was only partially effective in increasing the rate of citrate uptake, but the lower concentrations of glucose decreased the inhibition of acetoin production as a function of citrate utilization (Fig. 6B), with 0.1 mM glucose showing little or no inhibition.

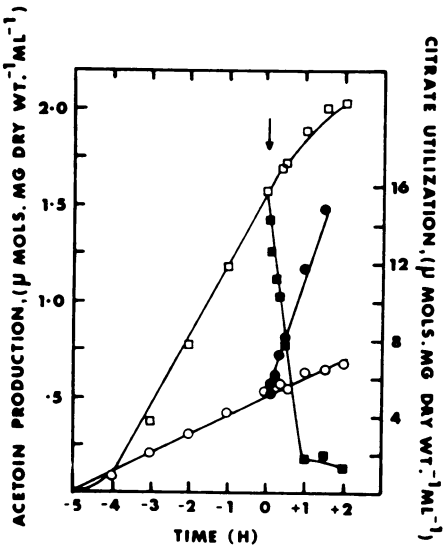

FIG. 5. *Effect of addition of 10 mM glucose on citrate uptake (○, ●) and acetoin production (□, ■) by resting cells of L. lactis NCW1 metabolizing citrate in 0.05 M acetate, pH 5.3. The cells were preincubated with citrate in the absence of glucose for 5 h before glucose was added (↓). Open symbols indicate absence of glucose, and closed symbols indicate presence of glucose.*

Growth parameters of the organism at pH 5.3 in the presence of low levels of glucose are shown in Table 2. Little growth was obtained at the lower levels of glucose (normal level, 55 mM) which was also reflected in the low levels of citrate utilized. However, acetoin production was inversely related to the glucose concentration in the medium.

Enzyme studies. The effect of pH on the activity of the acetoin-producing enzymes (α-acetolactate synthetase, α-acetolactate decarboxylase, and diacetyl reductase) is shown in Fig. 7. α-Acetolactate synthetase and decarboxylase activities were measured together because of the instability of α-acetolactate. These enzymes had a broad optimum at pH 5.4, whereas diacetyl reductase had a sharp optimum at pH 5.6. The inhibitory effect of several intermediates of glucose metabolism on α-acetolactate synthetase and decarboxylase activities is shown

TABLE 2. *Effect of glucose level on growth and acetoin production by L. lactis NCW1 at pH 5.3*

Glucose (mM)	Dry wt (mg/ml)	pH[a]	Citrate utilized (mM)[b]	Acetoin produced (mM)	Acetoin recovered (%)[c]
1	0.048	5.31	2.26	0.061	5.40
2	0.082	5.25	3.07	0.035	2.28
10	0.148	5.10	6.32	0.008	0.25

[a] pH value at the corresponding dry weight.
[b] Initial level, 8.2 mM.
[c] Assuming 2 mol of citrate → 1 mol of acetoin.

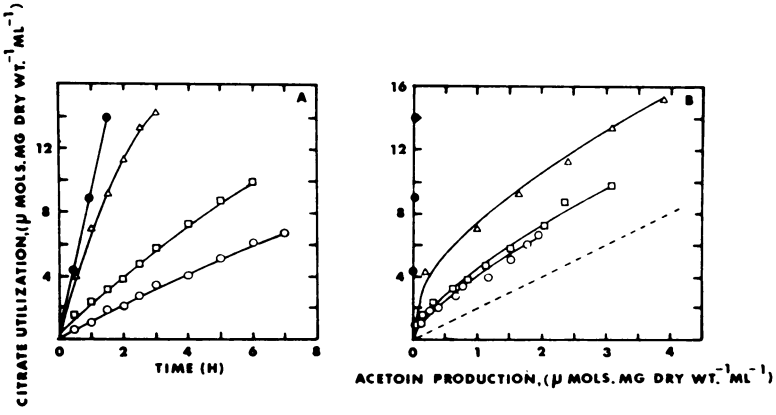

FIG. 6. *Effect of glucose concentration on citrate uptake (A) and acetoin production as a function of citrate uptake (B) by resting cells of L. lactis NCW1 in 0.05 M acetate, pH 5.3. Symbols: ---, theoretical amount of acetoin; ○, no glucose; □, 0.1 mM glucose; △, 1 mM glucose; ●, 10 mM glucose.*

in Table 3. These intermediates (10 mM) had no effect on LDH activity, except for adenosine triphosphate and adenosine diphosphate which were slightly inhibitory. Fructose-1,6-diphosphate is not an intermediate of glucose metabolism in this organism but it was included because it activates LDH in several streptococci (4, 19, 24, 25). NADH could not replace NADPH in the assay for diacetyl reductase.

Crude cell-free extracts had high NADH oxidase activity, especially at low pH, which interfered with the assay of LDH. Both activities could be separated by treatment with $(NH_4)_2SO_4$ at 50% saturation, when the oxidase activity was precipitated, and much of the LDH was retained in the supernatant. The apparent K_m values of LDH for pyruvate were 1.05, 0.25, and 0.13 mM at pH values of 7.5 (optimum pH), 6.5, and 5.0, respectively.

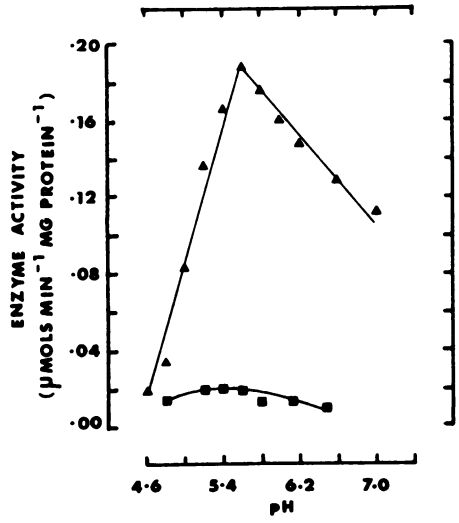

FIG. 7. *Effect of pH on the activity of diacetyl reductase (▲) and the combined activities of α-acetolactate synthetase and decarboxylase (■) in L. lactis NCW1.*

TABLE 3. *Concentration of several metabolites causing 50% inhibition of the combined activities of α-acetolactate synthetase and decarboxylase in L. lactis NCW1 at pH 5.4*

Metabolite	Concn (mM)
Adenosine triphosphate	2.5
Adenosine diphosphate	14
6-Phosphogluconic acid	5
2-Phosphoglyceric acid	8
3-Phosphoglyceric acid	10
Phosphoenolpyruvate	4.5
Dihydroxyacetone phosphate	20
Fructose-1,6-diphosphate	9

DISCUSSION

Citrate utilization occurred more rapidly in acetate than in phosphate buffer, suggesting that either phosphate inhibits or acetate stimulates citrate utilization. In addition, acetoin formation as a function of citrate utilization was greater in the presence of acetate than in phosphate buffer of the same pH (Fig. 3). These results are consistent with those of Stormer (18), who showed that acetate stimulated α-acetolactate synthetase and decarboxylase activities in *Enterobacter aerogenes.*

Diacetyl reductase was present in greater amounts than were the combined α-acetolactate synthetase and decarboxylase activities (Fig. 7), suggesting that diacetyl may be more important than α-acetolactate as a precursor of acetoin in *L. lactis.* This contrasts with the results of Collins and Bruhn (3), who felt that the low level of diacetyl produced by *S. lactis* subsp. *diacetylactis* was due to its limited ability to synthesize acetyl coenzyme A.

The results of this study show that low pH values and low sugar concentrations favor acetoin production. Thus, the lack of production of acetoin in growing cultures (Fig. 2A) was due to the high initial pH (pH 6.3) and the high glucose concentration (55 mM). Supporting evidence for the inhibitory role of glucose was obtained by growing the organism at pH 5.3 in media containing low concentrations of glucose. The results (Table 2) showed that acetoin formation was inversely related to glucose concentration. The low levels of acetoin recovered in these experiments were due to the relatively high pH values (pH 5.3) at which growth was monitored. In fully grown cultures, a lower pH and a lower glucose concentration (calculated to be 34 mM based on the amount of acid produced) were present, and acetoin production occurred (Fig. 2B). However, this concentration of glucose should have totally inhibited acetoin production. Certain constituents present in the medium (notably yeast extract) promote citrate utilization and acetoin production by cell suspensions (unpublished data). In the complete medium, net acetoin production is a reflection of the competition between the inhibiting effect of glucose and the promoting effects of the medium constituents and the decrease in pH. The pH and glucose effects on acetoin production are general effects, since several other leuconostocs and heterofermentative lactobacilli behaved in a similar manner (unpublished data).

The acetoin-producing enzymes (Fig. 7) were active at pH values (>pH 5.6) at which citrate was present in growing cultures. Thus, it was concluded that the effect of pH on inhibition of acetoin was not due to its effect on the activity of these enzymes. Between pH 7.5 and 5.0 there was an eightfold decrease in the apparent K_m of LDH for pyruvate. Similar results have also been reported in another strain of *L. lactis* (7). This decrease in the apparent K_m of LDH for pyruvate at low pH may be the reason for the limitation of acetoin production to low pH. At low pH, lower levels of pyruvate would be required to saturate LDH, and this would leave more pyruvate available for the expression of other products of its metabolism like acetoin. The intracellular concentration of pyruvate in leuconostocs is unknown but would probably be little different from that in other lactic acid bacteria. A value of 2.3 mM has been reported in glycolysing cells of *S. lactis* (20). This concentration is only twice the apparent K_m at pH 7.5 but 18 times the apparent K_m at pH 5.0. This argument presupposes that the internal and external pH values are similar. Such information has not been reported for leuconostocs, but it has been calculated that the internal pH is 0.2 pH units more acidic than the external pH in *S. lactis* (12). All strains of leuconostocs did not produce acetoin at low pH (Table 1). In these strains this may be due to the lack of an effect of pH on the K_m of their LDH for pyruvate or to lack of α-acetolactate decarboxylase and/or synthetase. An alternative explanation is that these strains contain a very active acetoin reductase.

The results of the effect of different sugars on the utilization of citrate (Fig. 4) imply that this is an energy-requiring process and that lactose metabolism is also inducible in this organism. Glucose caused inhibition of acetoin formation very rapidly in cell suspensions, in which little protein synthesis probably was occurring (Fig. 5), suggesting that catabolite repression is not involved. The inhibitory effect of intermediates of glucose metabolism on α-acetolactate synthetase and decarboxylase activities (Table 3) is a likely explanation for the effect of glucose on acetoin formation. The levels of these compounds in leuconostocs are again not known but would probably be like those in other lactic acid bacteria. Thompson (20) has reported similar concentrations of three of these inhibitors (viz., 2- and 3-phosphoglyceric acids and phosphoenol pyruvate) in glycolysing cells of *S. lactis*. The increase in acetoin production obtained at low levels of carbohydrate in both growing (Table 2) and resting (Fig. 4) cells is consistent with this notion, since lower levels of the inhibitory intermediates would probably be present in cells metabolizing low levels of glucose. Thus, the glucose effect on acetoin formation is a manifestation of the inhibitory effect of some of its metabolites on α-acetolactate synthetase and decarboxylase activities. More detailed studies on the kinetics of inhibition and the intracellular concentrations of glucose intermediates must be undertaken before the mechanisms for the pH and glucose effects on acetoin formation are considered proven. These aspects are currently being investigated.

ACKNOWLEDGMENTS

We thank F. Drinan and P. Thornhill for their technical assistance.

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ADDENDUM IN PROOF

Busse and Kandler (Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. **114**:675–682, 1961) also reported that glucose inhibited the production of acetoin from pyruvate at pH 5.6 in *Leuconostoc citrovorum* ATCC 8082. These workers suggested that the increased production of acetoin by leuconostocs under acid conditions was due to inhibition of LDH activity at low pH with consequent channeling of pyruvate to acetoin. This present data confirm the first statement but not the second. This strain of *L. citrovorum* has been reclassified as *L. mesenteroides* (Garvie, J. Dairy Res. **27**:283–292, 1960).

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