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Production of Acetic Acid and Other Volatile Compounds by *Leuconostoc citrovorum* and *Leuconostoc dextranicum*[1]

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Single-strain milk cultures of *Leuconostoc dextranicum* are capable of reducing added acetaldehyde, propionaldehyde, and butanone to the corresponding alcohols at 30 C. *L. dextranicum* and *L. citrovorum* reduced propionaldehyde to *n*-propanol quantitatively in 30 hr, and the reduction of this compound paralleled culture growth. Under unagitated conditions, these organisms produced large amounts of acetic acid and ethyl alcohol. The yield of acetic acid increased when cultures were agitated during growth. This increase in acetic acid production was accompanied by a 20- to 70-fold decrease in ethyl alcohol. The addition of acetaldehyde to the fermentation caused a reduction in the final concentration of acetic acid.

Species of the genus *Leuconostoc* are important in the production of a number of fermented foods. Among the volatile flavor compounds produced by *Leuconostoc* species are diacetyl, acetaldehyde, acetic acid, and ethyl alcohol (T. W. Keenan and D. D. Bills, J. Dairy Sci., *in press*). Although diacetyl production and utilization by these organisms has been studied extensively, primarily because of the importance of this compound in the flavor of cultured dairy products (11, 13, 14), the metabolism of other flavor compounds has received limited attention.

Several workers have reported on the ability of *L. citrovorum*, *L. dextranicum*, and *L. mesenteroides* to utilize acetaldehyde (1, 4, 10, 11). Bills and Day (1) found that *L. citrovorum* and *L. dextranicum* reduced acetaldehyde to ethyl alcohol, but this conversion was not quantitative. Kandler (8) reported that heterofermentative lactic acid bacteria produced acetic acid and ethyl alcohol, in addition to lactic acid, as the terminal products of carbohydrate metabolism. Harvey and Collins (5) found *L. citrovorum* to contain an enzyme, citrate lyase, that splits citrate into acetic and oxalacetic acids.

The quantitative aspects of ethyl alcohol and acetic acid production by species of the genus *Leuconostoc* under different culturing conditions have not been studied. In view of the recent report demonstrating the importance of acetic acid to the flavor of cultured dairy products, a study of this matter seemed appropriate (7). Recently, Stamer and Stoyla (15) found that aeration of cultures of *Lactobacillus brevis* markedly increased acetic acid production at the expense of ethyl alcohol production. The present study was initiated to determine whether such an effect was operational in cultures of *L. citrovorum* and *L. dextranicum*. The quantitative aspects of carbonyl reduction by *L. dextranicum* were also investigated.

MATERIALS AND METHODS

Cultures and culturing conditions. All organisms used in this study were obtained from the departmental stock culture collection. Cultures were maintained in a reconstituted 11% solids nonfat milk medium prepared by dissolving the solids in distilled water and autoclaving at 121 C for 10 to 12 min. All incubations were at 30 C for 48 hr except as otherwise noted. Cultures were agitated on a reciprocating shaker operating at 240 strokes per min. Unagitated conditions refer to undisturbed incubation. For time course studies on propionaldehyde reduction and ethyl alcohol production, cultures were held static except at sampling times, when they were gently agitated. Plate counts were made by plating dilutions of cultures with Elliker agar (Difco).

Analytical methods. Cultures were analyzed for content of carbonyls and alcohols after a 48-hr incubation period by the on-column trapping, gas-liquid chromatographic (GLC) technique described by Morgan and Day (12). Conditions for quantitative GLC analyses were identical with those previously described (9). Acetic acid was determined by acidifying 50-ml samples of cultures to about *p*H 2 with phos-

[1] Journal paper no. 3459 from the Purdue Agricultural Experiment Station.

phoric acid, diluting to a total volume of 100 ml with distilled water, and steam distilling until 100 ml of distillate had been collected. Distillates were titrated to the phenolphthalein end point with 0.100 N sodium hydroxide. In order to obtain quantitative data and to determine the percentage of recovery, known amounts of acetic acid were added to uninoculated milk medium which was then acidified and subjected to distillation and titration. A linear relationship between titration values and acetic acid content was observed throughout the concentration range encountered in this study. Analysis of concentrated ether extracts of distillates by GLC showed acetic acid to be the only volatile acid present. This GLC analysis was performed on a column (⅛ inch by 7.5 ft) packed with 20% diethylene glycol succinate on Celite and operated isothermally at 90 C.

Sterile carbonyl solutions, prepared as described previously, were of sufficient concentration to permit the addition of 1.0 ml to the culture medium to give the desired concentration (2). Alcohol and acid production from these carbonyls was assayed as outlined above.

TABLE 1. *Alcohol production from added carbonyls by L. dextranicum in 48 hr at 30 C*

Culture	Alcohol concn (μg/g)			
	Ethyl alcohol[a]	Ethyl alcohol[b]	*n*-Propanol[b]	*n*-Butyl alcohol[b]
L. dextranicum 1-7....	708	8.1	102	31
L. dextranicum 7-6....	60	78	104	24.8
L. dextranicum 22-3...	640	24.3	99.2	38.8
L. dextranicum 26-3...	158	65	102	23.5
L. dextranicum 32-7...	743	102	102	41

[a] Amount of ethyl alcohol in control cultures. Cultures were devoid of other alcohols.

[b] Respective carbonyl added to cultures at 100 μg/g.

RESULTS AND DISCUSSION

The extent of carbonyl reduction by *L. dextranicum* strains in 48 hr at 30 C under unagitated conditions is shown in Table 1. Cultures without added carbonyl were incubated and analyzed concurrently with samples. All of these strains produced ethyl alcohol, but none produced detectable amounts of the other alcohols listed in Table 1. All organisms reduced acetaldehyde, propionaldehyde, and butanone to some extent, suggesting that their dehydrogenase system can reduce both aldehydes and methyl ketones. Chromatograms obtained were characterized by the absence of observable acetaldehyde and propionaldehyde peaks. Although propionaldehyde was reduced essentially quantitatively to *n*-propanol in all cases, only *L. dextranicum* 32-7 converted all of the added acetaldehyde to ethyl alcohol. Apparently the other *L. dextranicum* strains are capable of metabolizing acetaldehyde by means of other biosynthetic pathways, in addition to using it as a terminal hydrogen acceptor. All cultures were incubated in screw-capped tubes tightly sealed with Teflon-lined caps. Loss of added carbonyls under these conditions was negligible. With butanone, the total amount added could be accounted for as either 2-butanol or residual butanone. These strains of *L. dextranicum* produced relatively large amounts

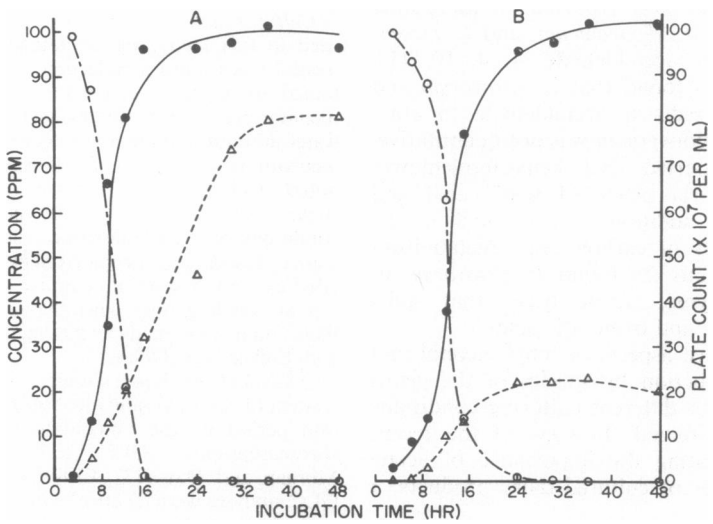

FIG. 1. *Reduction of propionaldehyde to n-propanol by L. dextranicum 1-7 (A) and L. citrovorum 8 (B). Propionaldehyde, ○: n-propanol, ●: plate count, △.*

of ethyl alcohol. With the exception of *L. dextranicum* 7-6, all cultures had accumulated over 150 μg/g of ethyl alcohol per g in 48 hr.

The quantitative nature of propionaldehyde reduction by both *L. citrovorum* 8 and *L. dextranicum* 1-7 is illustrated in Fig. 1. Propionaldehyde reduction was slow during the first 6 to 9 hr of incubation, after which concentrations of this compound decreased rapidly. Reduction paralleled culture growth in both cases, and the propionaldehyde concentration was reduced to levels below 1 μg/g in 24 hr. The conversion to *n*-propanol was essentially quantitative under these incubation conditions.

Production of ethyl alcohol by *L. citrovorum* 8 and *L. dextranicum* 1-7 lagged for the first 15 hr of incubation, after which it rapidly accelerated (Fig. 2). Concentrations of this compound increased throughout the incubation period, reaching 425 μg/g in the *L. citrovorum* 8 culture and 490 μg/g in the *L. dextranicum* 1-7 culture. This lag in ethyl alcohol production is similar to that observed by Galesloot (4) with diacetyl formation by *L. citrovorum*. A possible explanation for this lag is that, when pyruvate accumulates in levels above those needed for synthesis of cellular constituents, its rate of metabolism by other routes increases. Harvey and Collins (6) made this suggestion to explain diacetyl formation by *Streptococcus diacetilactis*. These investigators believe that diacetyl is formed as a means of preventing toxic levels of pyruvate from accumulating.

The effect of agitation upon cell growth and

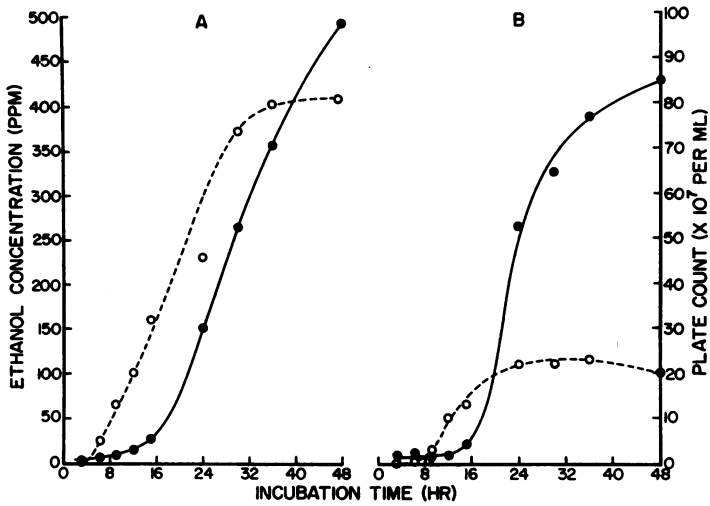

FIG. 2. *Ethyl alcohol production by L. dextranicum 1-7 (A) and L. citrovorum 8 (B). Ethyl alcohol,* ●*: plate count,* ○.

TABLE 2. *Effect of agitation on growth and production of acetic acid and ethyl alcohol by Leuconostoc species*[a]

Culture	Static			Shake		
	Acetic acid	Ethyl alcohol	Plate count	Acetic acid	Ethyl alcohol	Plate count
	μg/g	μg/g		μg/g	μg/g	
L. dextranicum 1-7	1,240	485	52×10^7	2,680	7.5	54×10^7
L. dextranicum 7-6	800	66	24×10^7	1,150	2.7	7×10^7
L. dextranicum 22-3	1,240	544	59×10^7	2,750	11.5	69×10^7
L. dextranicum 26-3	850	128	23×10^7	1,110	6.3	11×10^7
L. dextranicum 32-7	1,410	470	59×10^7	2,710	8.4	54×10^7
L. citrovorum Foster	1,260	705	58×10^7	2,530	9.8	10×10^7
L. citrovorum 91404	1,180	755	88×10^7	2,630	15.1	23×10^7
L. citrovorum 8	1,240	553	37×10^7	2,560	8.4	9×10^7

[a] Incubation for 48 hr at 30 C.

production of ethyl alcohol and acetic acid was studied (Table 2). In all cases, there was a marked increase in acetic acid production and a corresponding decrease in ethyl alcohol content of agitated cultures. In most cases, the acetic acid content of agitated cultures was twice as high as in the corresponding unagitated cultures. Acetic acid content increased at the expense of ethyl alcohol production. Still cultures contained 20 to 70 times as much ethyl alcohol as corresponding agitated cultures. This effect is similar to that observed by Stamer and Stoyla (15) with *L. brevis*.

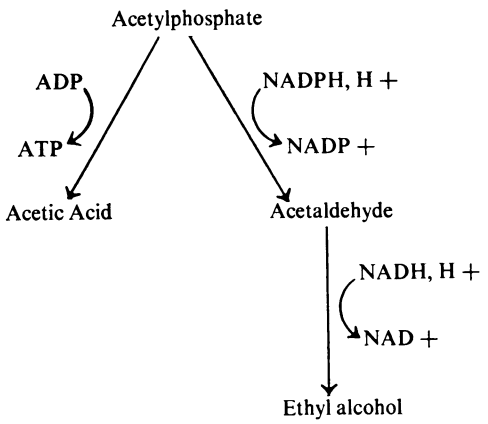

FIG. 3. *Terminal compounds of heterofermentative carbohydrate metabolism. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; NAD nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.*

The increased acetic acid content of aerobic cultures can be explained in the following manner. The terminal compounds of heterofermentative carbohydrate metabolism, in addition to D-(—)-lactic acid (8), are shown in Fig. 3. Galesloot (4) has stated that the reduction of acetylphosphate to ethyl alcohol is a waste of considerable energy, but *Leuconostoc* species are compelled to do this in order to oxidize the reduced pyridine nucleotides produced by the conversion of hexose phosphates to 6-phosphogluconic acid and the subsequent conversion of the latter to carbon dioxide and ribulose-5-phosphate. *Leuconostoc* organisms preferentially convert acetylphosphate to acetic acid with the concurrent production of adenosine triphosphate. This argument is supported by the observation of Lindsay et al. (11) that addition of acetaldehyde to cultures of *L. citrovorum* stimulates growth. Apparently aeration provides sufficient oxygen for *Leuconostoc* species to regenerate oxidized pyridine nucleotides without the formation of acetaldehyde. However, plate count data (Table 2) do not show increased cell numbers in agitated cultures. In fact, the trend was toward lower cell numbers in agitated cultures. In view of these results, a search for an electron transport system in these organisms may be illuminating.

The effect of added carbonyls on the production of acetic acid and ethyl alcohol in unagitated and

TABLE 3. *Effect of agitation on acetic acid and ethyl alcohol production by Leuconostoc species when grown in the presence of added carbonyls*[a]

Culture	Additive	Static			Shake		
		Acetic acid	Ethyl alcohol	*n*-Propanol	Acetic acid	Ethyl alcohol	*n*-Propanol
		μg/g	μg/g	μg/g	μg/g	μg/g	μg/g
L. dextranicum 7-6	None	1,200	69		940	6.8	
	Acetaldehyde (500)[b]	540	97.5		460	65.8	
	Propionaldehyde (500)	878	14.2	148	478	8.6	0
L. dextranicum 26-3	None	1,405	179		1,230	9.4	
	Acetaldehyde (500)	1,410	203		940	120	
	Acetaldehyde (250)				1,310	78	
	Propionaldehyde (500)	1,530	108	81.2	1,285	8.8	98
	Acetic acid (250)				1,500	7.5	
L. citrovorum 8	None	1,630	389		2,085	11.0	
	Acetaldehyde (500)	1,760	400		1,455	87.5	
	Propionaldehyde (500)	1,590	75.5	89.3	1,915	11.2	118

[a] Incubation for 48 hr at 30 C.
[b] Numbers in parentheses refer to the concentration of compound in micrograms per gram.

agitated cultures is shown in Table 3. Neither acetaldehyde nor propionaldehyde increased the final amount of volatile acidity of cultures to any appreciable degree. Instead, in several cases, the presence of these compounds decreased the production of acetic acid. Chromatograms were generally devoid of peaks for acetaldehyde and propionaldehyde. Although these compounds were lost slowly from agitated uninoculated controls, the amounts utilized could not be accounted for in alcohol or acetic acid. This observation is in contrast to the quantitative reduction of lower levels of propionaldehyde by *L. dextranicum* (Table 1). These organisms apparently metabolize acetaldehyde and propionaldehyde by a yet unknown route. These compounds may possibly be utilized in the synthesis of cellular material; this possibility is currently being investigated in our laboratory.

The observed inhibition of acetic acid production by acetaldehyde cannot readily be explained. This inhibition with 500 μg of acetaldehyde or propionaldehyde per g was noted in most trials, but these compounds at this level had no observable effect on culture growth as evidenced by *p*H and cell numbers. Acetaldehyde did not become toxic to *L. citrovorum* until the level exceeded 500 μg/g. One possible explanation for the inhibition of acetic acid production is that acetaldehyde in some manner controls the production of acetylphosphate. The addition of acetic acid did not concurrently inhibit ethyl alcohol production (Table 3, *L. dextranicum* 26-3).

Results presented herein show that culture conditions markedly affect the production of acetic acid and ethyl alcohol by *Leuconostoc* species. In view of a recent report on the desirable effects of high levels of acetic acid on the flavor of certain cultured dairy products (7), it may be profitable to investigate the use of aeration during culturing. This is especially true in light of the early report of Brewer et al. (3) that aeration of cultures results in a pronounced increase in diacetyl production.

ACKNOWLEDGMENT

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