Factors Affecting Diacetyl Production by Lactic Acid Bacteria[1]

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Recently it has been observed that the lactic acid bacteria are primarily responsible for the production of diacetyl during concentration of orange juice. The amount of diacetyl present is used as an indicator of the degree of contamination, even though it is not a major end product of fermentation. Its presence may be detected even though little change in total acidity or pH has been noted. A better understanding of factors influencing diacetyl production by lactic acid bacteria would be desirable.

The relationship of the lactic acid bacteria to diacetyl production evolved from an early date. Hammer (1920) concluded that the organisms in butter cultures other than *Streptococcus lactis* were responsible for the volatile acidity, aroma, and flavor of the fermented dairy product. Van Niel in 1929 as stated by Hammer and Babel (1943) recognized that diacetyl was either responsible for the aroma of butter or was the principal component of the aromatic material. Michaelian *et al.* (1933) noted that butter cultures with satisfactory character contained considerable quantities of acetylmethylcarbinol and diacetyl. This fact is generally accepted.

Hays (1951) and Hays and Riester (1952) identified spoilage organisms from orange juice as species of the genera *Leuconostoc* and *Lactobacillus*. Murdock *et al.* (1952) and Murdock and DuBois (1955) noted production of acetylmethylcarbinol and diacetyl by lactic acid bacteria grown in orange juice. Brokaw (1952) also employed plate counts and microscopic examination to follow the contamination of orange juice. Hill and Wenzel (1954b) developed a colorimetric method which has been used successfully as a quality control device.

Michaelian *et al.* (1938) and Glenn and Prouty (1955) studied the effects of citric acid on the growth of *Streptococcus lactis*, *Streptococcus citrovorum*, *Streptococcus cremoris*, and *Leuconostoc mesenteroides*.

While most reports recognize the role of the lactics in diacetyl production, conflicting reports do appear. Teunisson and Hall (1947) concluded that bacteria are probably not a major factor in influencing quality of orange juice since only one-third of their isolates were bacteria.

Wolford and Berry (1948) and Purko *et al.* (1956) noted the influence of coliform bacteria in orange juice concentrate. They isolated strains of the genera *Aerobacter*, *Escherichia*, and *Serratia*. Berry *et al.* (1954) felt that orange juice was high enough in acidity to prevent growth of most types of bacteria. They isolated strains of the genera *Leuconostoc* and *Lactobacillus*. Faville *et al.* (1951) found that yeasts do not appear to be the predominating organism and found that 40 per cent of their isolates were strains of the genus *Leuconostoc*. Murdock *et al.* (1952) and Rushing *et al.* (1956) isolated lactic acid bacteria.

Additional information on the ability of each of the lactic acid bacteria to produce acetylmethylcarbinol and diacetyl under a variety of conditions would augment the knowledge already available concerning specific characteristics of the lactic acid bacteria. This study involves species of the four genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus*.

Materials and Methods

The organisms used in this study were *Lactobacillus plantarum* strain B246, *Lactobacillus lactis* strain L33, *Lactobacillus bulgaricus* strain 7994, *Lactobacillus helveticus* strains L31, L85, and L43, *Lactobacillus brevis* strains B155 and 2S10, and the dextran producing variant L155, *Pediococcus cerevisiae* strain E66, *Leuconostoc mesenteroides* strain C29, and *Streptococcus faecalis* strain E166.

The basic medium A contained 1.5 per cent glucose, 0.5 per cent tryptone, and 0.25 per cent yeast extract. Medium B contained, in addition, 0.137 per cent citrate; medium C contained 0.1 per cent glucose and 0.137 per cent citrate. Medium AC contained only 0.1 per cent glucose, and medium CC contained 0.1 per cent glucose and 0.15 per cent citric acid. Media D through H were commercial orange juices adjusted to pH readings of 4.0, 5.5, 4.0, 5.5, and unadjusted, respectively. The orange juice media were sterilized in flowing steam for 30 min. Media A through E were incubated at 32 C whereas media F, G, and H were incubated at 21 C.

Diacetyl was determined by the method of Hill *et al.* (1954a). This determination measures total diacetyl, acetylmethylcarbinol, and 2,3-butylene glycol as diacetyl. Three hundred ml of media were inoculated with the representative culture from a 24 hr stab, incubated 96 hr, and then distilled. From each sample,

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25 ml of distillate were collected and from that a 10 ml aliquot was removed from below the surface to avoid small traces of peel oil which might interfere with colorimeter readings. This 10 ml aliquot was treated with 5 ml of 5 per cent α-naphthol in ethyl alcohol and 2 ml of 40 per cent potassium hydroxide (containing 0.3 per cent creatine), shaken vigorously for 15 sec, allowed to stand 10 min, and shaken again for 15 sec. The concentration of diacetyl was determined by measuring the color intensity in a Bausch and Lomb Spectronic 20 Model[2] colorimeter at 550 mμ using the reagent blank to zero the instrument. Standard calibration curves were prepared using solutions of 1 to 140 ppm diacetyl in distilled water, diluted until a suitable light transmission could be obtained with the instrument. The standard curve for samples requiring dilution of 1:20 is presented in figure 1. In the same way, after the color in the fractions was developed, those samples containing high concentrations of diacetyl were diluted in order to obtain suitable colorimetric readings.

Citric, lactic, and acetic acids were determined after separation by the column chromatographic technique of Bulen *et al.* (1952) and carbon dioxide was determined after titration of $Ba(OH)_2$ in a series of fermentations using Eldredge tubes (Christensen *et al.* 1958).

Results and Discussion

The production of diacetyl by the various lactic acid bacteria was found to vary, depending upon the medium, the pH, and the incubation temperature. The homofermentative species produced diacetyl more readily and in larger amounts than did the heterofermentative species, table 1. *Lactobacillus plantarum* strain 246, *Lactobacillus bulgaricus* strain 7994, *Lactobacillus helveticus* strain L31, *Pediococcus cerevisiae* strain E66, and *Streptococcus faecalis* strain E166 produced diacetyl even in the absence of citric acid. However, more diacetyl was produced when citric acid was added, medium B. The latter two species produced greater quantities when the sugar was limited to 0.1 per cent, medium C.

The heterofermenter, *Leuconostoc mesenteroides* strain C29, produced a small amount of diacetyl in the low

[2] Bausch and Lomb, Rochester, New York.

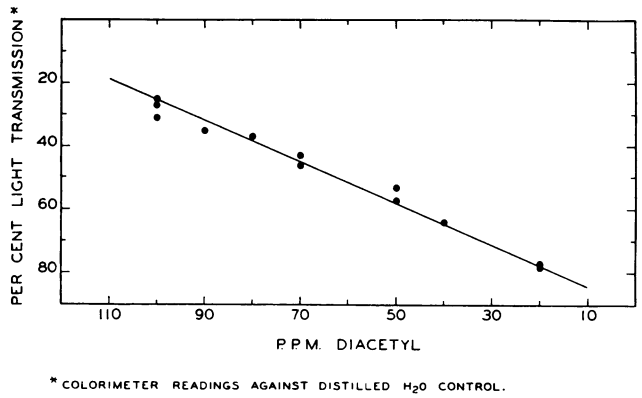

Figure 1. Diacetyl standard curve for sample diluted 1:20

TABLE 1

Production of diacetyl in various media by typical lactic acid bacteria

Organism	Diacetyl as ppm in Medium:*							
	A	B	C	D	E	F	G	H
Lactobacillus plantarum B246	2.18	9.27	6.72	4.55	0.73	5.45	6.18	11.27
Pediococcus cerevisiae E66	0.45	2.00	4.00	0.37	0.73	0.41	0.51	0.31
Streptococcus faecalis E166	0.27	0.27	0.47	3.09	0.73	6.63	4.45	0.29
Leuconostoc mesenteroides C29	0.00	0.00	0.64	6.45	3.18	6.09	0.51	0.29
Lactobacillus brevis B155	0.00	0.00	0.00	0.13	0.23	0.09	6.73	0.31
Lactobacillus brevis L155	0.00	0.00	0.00	0.38	0.73	0.15	9.09	0.35
Control	0.09	0.00	0.00	0.35	0.36	0.41	0.51	0.31
Lactobacillus helveticus L31	2.64	4.91	8.36					
Lactobacillus bulgaricus 7994	12.00	14.00	11.27					
Control	0.09	0.17	0.00					

* Medium A contained 1.5 per cent glucose, 0.5 per cent tryptone, 0.25 per cent yeast extract; medium B the same with 0.137 per cent citric acid; and medium C contained only 0.1 per cent glucose with 0.137 per cent citric acid. Media D to H were orange juices adjusted to: D and F, pH 4.0; E and G, pH 5.5; and H, unadjusted. Media F, G, and H were incubated at 21 C, all others at 32 C.

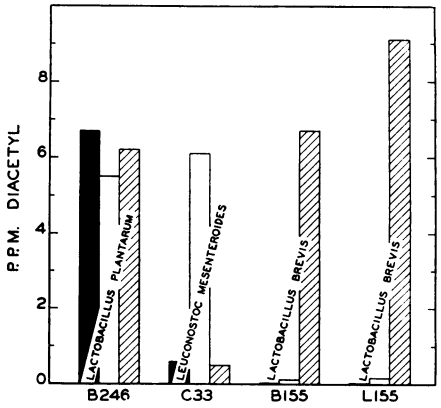

Figure 2. Production of diacetyl by strains of lactic acid bacteria grown in low sugar citrate medium and in orange juice. ■ = Medium C, contained 0.1 per cent glucose, 0.5 per cent tryptone, 0.25 per cent yeast extract, 0.137 per cent citrate, incubated at 32 C, pH unadjusted. □ = Medium F, contained commerically canned orange juice incubated at 21 C, pH adjusted to 4.0. ▨ = Medium G, contained commerically canned orange juice incubated at 21 C, pH adjusted to 5.5.

sugar, citric acid medium. However, the other heterofermenters *Lactobacillus brevis* strain B155 and the dextran producing variant L155 produced diacetyl only in orange juice media. The greatest amount was formed in orange juice adjusted to pH 5.5 and incubated at 21 C, conditions under which the least diacetyl was produced by *Leuconostoc mesenteroides* C33 (figure 2).

Since citric acid is necessary for production of diacetyl in some instances, it seemed desirable to determine how citric acid may affect other fermentation by-products. Instead of carrying on the fermentation in Erlenmeyer flasks, it was carried out in Eldredge tubes so that the carbon dioxide could be absorbed with barium hydroxide. From the same medium, larger quantities of diacetyl were produced in the Eldredge tubes and still larger amounts in the presence of citric acid (table 2, figure 3). However, neither of the strains of *Lactobacillus brevis* produced diacetyl regardless of whether or not citric acid was added or the sugar was reduced to 0.1 per cent. *Leuconostoc mesenteroides* produced diacetyl only in the low sugar citric acid medium CC.

It may be further noted that the citric acid was utilized, at least partially, by all cultures and that ordinarily carbon dioxide and acetic acid were produced in greater amounts when citric acid was added. In addition, the amount of lactic acid was usually somewhat greater showing partial conversion of citric acid to lactic acid. This occurred in both the high-sugar and low-sugar media.

The data indicate that diacetyl is produced in lesser quantities under optimum conditions for growth. *Leuconostoc mesenteroides* produced lesser amounts in media adjusted to pH 5.5 than pH 4.0 and little, if any, in laboratory media in contrast to the more acid orange juice. *Streptococcus faecalis* also produced less at pH 5.5 than at pH 4.0 and, when incubated at 21 C; *Lactobacillus brevis* produced diacetyl only in orange juice. Even *Lactobacillus plantarum* produced the least diacetyl in the orange juice adjusted to pH 5.5 and incubated at 32 C. In addition, it may be pointed out that citric acid is too poor a carbon source to allow initiation of growth of many of the lactics but will stimulate diacetyl production.

There is little doubt that differences would be found

TABLE 2

Diacetyl, acid and carbon dioxide production in Eldredge tubes by some typical lactic acid bacteria

Species and Strain No.	Media*	Diacetyl ppm	Carbon Dioxide	Acetic Acid	Lactic Acid	Citric Acid	Acetic/Lactic Acid
			Mg per cent carbon				
Lactobacillus plantarum, 246	A	31.5	9	14	612	—	0.02
	B	68.9	19	41	592	0	0.07
	AC	10.0	10	21	31	—	0.70
	CC	11.0	19	57	42	0	1.35
Lactobacillus bulgaricus, 7994	A	66.0	10	4	304	—	0.01
	B	75.5	15	9	505	—	0.02
Lactobacillus helveticus, L85	A	72.5	5	—	207	—	—
	B	61.0	11	13	620	30	0.02
Lactobacillus helveticus, L31	A	30.2	5	8	616	—	0.01
	B	37.0	7	15	572	33	0.03
Pediococcus cerevisiae, E66	A	12.5	7	20	362	—	0.05
	B	15.5	8	17	382	40	0.04
	AC	9.0	10	12	30	—	0.40
	CC	7.5	12	19	42	30	0.45
Streptococcus faecalis, E166	A	2.2	4	2	163	—	0.01
	B	2.2	3	3	212	39	0.01
	AC	0.9	5	9	44	—	0.20
	CC	0.9	5	9	50	39	0.18
Leuconostoc mesenteroides, C29	A	0	51	6	219	—	0.03
	B	0	57	52	266	0	0.20
	AC	0	2	13	28	—	0.47
	CC	19.0	35	47	39	0	1.20
Lactobacillus brevis, B155	A	0	71	39	294	—	0.13
	B	0	75	72	310	0	0.23
	AC	0	19	31	8	—	3.87
	CC	0	29	47	26	0	1.80
Lactobacillus brevis, L155	A	0	66	36	412	—	0.09
	B	0	66	63	428	0	0.14

* Medium A contained 1.5 per cent glucose, 0.5 per cent tryptone, and 0.25 per cent yeast extract and medium B contained the same plus 0.137 per cent citric acid. Medium AC contained only 0.1 per cent glucose, and medium CC contained 0.1 per cent glucose and 0.15 per cent citric acid.

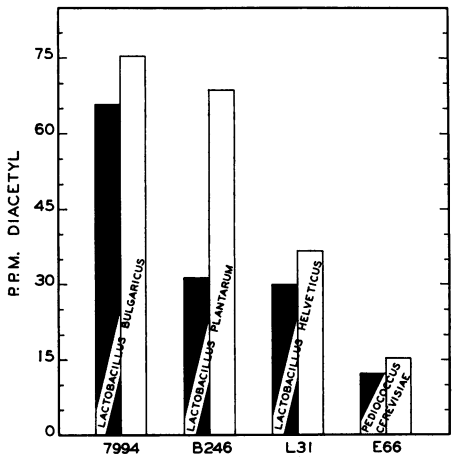

Figure 3. Production of diacetyl by strains of homofermentative lactic acid bacteria in Eldredge tubes using media with and without citric acid. ■ = Medium A, contained 1.5 per cent glucose, 0.5 per cent tryptone, 0.25 per cent yeast extract, incubated at 32 C, pH unadjusted. □ = Medium B, contained 1.5 per cent glucose, 0.5 per cent tryptone, 0.25 per cent yeast extract, 0.137 per cent citrate, incubated at 32 C, pH unadjusted .

between strains within each species as indicated by Berry *et al.* (1954). However, no marked difference was obtained between the nondextran producing strain of *Lactobacillus brevis* and the dextran producing strain.

SUMMARY

The production of diacetyl by various species of lactic acid bacteria is dependent upon several factors. The homofermentative species produce diacetyl more readily than do the heterofermentative species. Citric acid is essential for diacetyl production by some strains. In all species studied, diacetyl production in low-sugar medium is enhanced by the presence of citric acid. In addition, the greater quantities of diacetyl are produced under the less optimal conditions. From these results, one may conclude that, since certain strains of lactic acid bacteria, particularly the heterofermentative species, may develop without producing diacetyl, diacetyl production is not necessarily an indication of growth of such organisms.

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