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Use of Gas-Liquid Chromatography to Determine the End Products of Growth of Lactic Acid Bacteria

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A simple gas-liquid chromatographic procedure for analyzing ethanol, acetic acid, acetoin, and racemic and *meso*-2,3-butylene glycol in broth media is described. Overnight broth cultures were filtered or centrifuged, and the filtrate or supernatant was treated with formic acid to aid separation of volatile fatty acids. Samples were then directly analyzed by gas-liquid chromatography on a 20% Tween 80–Chromosorb W-AW column and propionic acid as an internal standard. A complete analysis took ca. 8 min. The method can be used to distinguish homofermentative from heterofermentative lactic acid bacteria based on the level of ethanol produced and citrate-utilizing from non-citrate-utilizing lactic acid bacteria based on the levels of acetic acid produced. The method also has potential in distinguishing other bacterial fermentations. Of the 13 species of lactic acid bacteria tested, *Streptococcus lactis* subsp. *diacetylactis* was the major producer of 2,3-butylene glycol (total range, 0.3 to 3.5 mM), and, except for strain DRC1, both the racemic and *meso* isomers were produced in approximately equal amounts.

The pathway of glucose catabolism is a fundamental distinguishing characteristic of lactic acid bacteria (LAB). Homofermentative organisms catabolize glucose by the glycolytic pathway almost completely to lactic acid, whereas heterofermentative organisms catabolize glucose by the phosphoketolase pathway to equimolar quantities of lactic acid, ethanol, and CO_2. Traces of acetate may also be formed. These two groups of bacteria are usually distinguished by measuring CO_2 production in a complex, well-buffered, semisolid medium containing a high concentration of glucose (11, 27). Citrate catabolism is also an important distinguishing characteristic of many LAB, especially those found in dairy starter cultures (*Streptococcus lactis* subsp. *diacetylactis* and *Leuconostoc* spp.). The end products of citrate metabolism are CO_2, acetic acid, diacetyl, acetoin (acetylmethylcarbinol), and 2,3-butylene glycol (2,3-BG). Colorimetric measurement of mixtures of diacetyl, acetoin, and 2,3-BG is difficult because 2,3-BG must be oxidized to acetoin and diacetyl before measurement and because diacetyl interferes with the common method for determining acetoin. These difficulties are obviated to some extent if the compounds are first separated by either salting-out chromatography or steam distillation (16, 25, 29).

Gas-liquid chromatography has been used by numerous workers to measure the fermentation products produced by bacteria (for reviews, see references 10 and 22). Most methods suffer from the disadvantage that pretreatment of samples with an acidification-extraction procedure followed in many cases by a derivatization procedure is necessary. Obviously, recovery rates from such procedures would vary with the nature of the compound being extracted. In addition, there are conflicting reports on the extractability of some compounds (e.g., acetoin) with diethyl ether (15, 19). Gas-liquid chromatography of volatile fatty acids is especially useful in identifying anaerobic bacteria (3, 14) and in measuring microbial activity in silage (28) and in the rumen (7). Since neutral products such as diacetyl, acetoin, 2,3-BG, and ethanol often accompany volatile fatty acids in fermentations, it would be useful if both neutral and acidic end products could be analyzed directly, without extraction, on the same column. Such a method is described and evaluated in the present paper.

MATERIALS AND METHODS

Organisms. Twenty-two strains of 13 different species of LAB from our stock collection and *Escherichia coli* NCDO 1689 and *Enterobacter aerogenes* NCDO 711 were used. The LAB were grown in MRS broth (8), modified by reduction of the glucose concentration to 1% (wt/vol) and omission of Tween 80 and sodium acetate, and the coliform bacteria were grown in MR VP broth (Oxoid Ltd., Basingstoke, England). All incubations were at 30°C, except for *Streptococcus thermophilus* Y1, *Lactobacillus bulgaricus* NYL2, and *Lactobacillus lactis* BYL1, which were incubated at 37°C.

Sample preparation. Static cultures were grown overnight with a 0.1% (vol/vol) inoculum. After incubation they were clarified either by centrifugation at 5,000 × *g* for 10 min or by membrane filtration with a 0.45-μm-pore-size filter. To 0.5 ml of supernatant or filtrate was added 0.2 ml of 0.036 M propionic acid as an internal standard and 20 μl of AristaR-grade formic acid (see below). A 0.5-μl portion of this mixture was injected into the gas chromatograph. Uninoculated broths were run as controls to correct for any metabolites which might be present. Standards were treated in the same way except that they were dissolved in water.

Analyses. Growth was measured by the decrease in pH from the initial value of 6.4 (modified MRS broth) or 7.5 (MR VP broth). Citrate was determined in the supernatant or filtrate by the pyridine-acetic anhydride method (21), and heterofermentation was determined by the methods of Gibson and Abd-el-Malek (11, 27).

Chemicals. All chemicals used were obtained from BDH Ltd., Poole, England. The formic acid was AristaR grade (see below), and the other chemicals used were of AnalaR grade if available; otherwise, they were of laboratory reagent grade. In the development work, all were made to the required concentration, and then 20 μl of formic acid (AristaR grade) was added to 0.5 ml of solution to give a final concentration of 1 M formic acid.

Gas chromatograph. A dual-column gas chromatograph (model Sigma 3; The Perkin-Elmer Corp., Norwalk, Conn.)

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TABLE 1. Chromatographic conditions and RTs of ethanol, acetic acid, diacetyl, acetoin, and 2,3-BG on eight different columns[a]

Phase	Support	Mesh size	% H_3PO_4	Temp (°C)		RTs (s)				
				Column	Injector and detector	Ethanol	Acetic acid	Diacetyl	Acetoin	2,3-BG
20% Tween 80	Chromosorb W-AW	80-100		125	160	20	93	20	70	170; 196[b]
10% SP 1000	Chromosorb W-AW	100-120	1	110	180	33	228	31	108	360; 434[b]
10% SP 1200	Chromosorb W-AW	80-100	1	125	180	34	225	34	114	402; 491[b]
3% Carbowax 20M	Chromosorb W-HP	100-120		120	190	55	196[c]	57	116	305
0.3% Carbowax 20M	Carbopack C	60-80	0.1	110	180	18	58	39	94	470[d]
Chromosorb 101		80-100		170	200	ND[e]	132	106	303	560[c]
Porapak Q		100-120		205	235	ND	100	144	299	449[c]
Porapak QS		100-120		205	235	22	75	115	233	341[c]

[a] For all columns, the air pressure was 30 lb/in^2; H_2 was at 19 lb/in^2, and N_2 was at 69 lb/in^2. The flow rate for N_2 was 40 ml/min.
[b] Two peaks were obtained (see text).
[c] Tailing.
[d] Nonsymmetrical peak.
[e] ND, Not determined.

fitted with a flame ionization detector was used. A computing integrator (model 304-50; Laboratory Data Control, Ltd., Shannon Airport, Ireland) was coupled to the chromatograph, and a chart recorder (model 023; The Perkin-Elmer Corp.) was connected to the integrator. The glass columns used were 2 m long and 6.25 mm in diameter. The packings used and the chromatographic conditions are summarized in Table 1. All columns were run isothermally. During selection, the N_2 flow rate was 40 ml/min at 69 lb/in^2; H_2 was used at 19 lb/in^2; and air was used at 30 lb/in^2. For routine analysis on the 20% Tween 80–Chromosorb W-AW column, the N_2 flow rate was reduced to 20 ml/min (at 69 lb/in^2), and the column temperature was reduced to 120°C (isothermal) unless otherwise stated. Other parameters remained the same.

RESULTS AND DISCUSSION

Column selection. One of the major problems in the resolution of nonderivatized volatile fatty acids is adsorbtion of the acids on the column, which is manifested as tailing of peaks, irregularly shaped peaks, and ghosting (for a review, see reference 5). This is effectively solved by bubbling the carrier gas through concentrated formic acid (5). An alternative, simpler, and equally effective method is to add formic acid (final concentration, 5%) to the samples being analyzed (7). Consequently, all solutions in the present study were made 1 M in formic acid. The AnalaR-grade formic acid used (90% purity) was contaminated with about 1 mM acetic acid, which was one of the compounds we wanted to measure. Replacing it with AristaR-grade formic acid (98% purity) obviated this difficulty. The chromatographic columns, their conditions of use, and the retention times (RTs) for ethanol, acetic acid, diacetyl, acetoin, and 2,3-BG are summarized in Table 1. Ethanol coeluted with diacetyl on the first four columns and was well separated from it on the other two columns tested (0.3% Carbowax 20M and Porapak QS). The position of acetic acid varied from before diacetyl (Porapak Q and QS) to between acetoin and 2,3-BG (the first four columns). In all columns, the sequence of resolution of the C_4 compounds was diacetyl, acetoin, and 2,3-BG.

TABLE 2. RTs and reproducibilities of the gas-liquid chromatographic method for measuring ethanol, acetoin, acetic acid, and 2,3-BG in broth cultures

Compound	RT (s)	n	Range (mM)	SD of differences
Ethanol	33	11	0–2.7	0.109
Acetoin	115	9	0–4.4	0.043
Acetic acid	157	11	0–6.7	0.057
2,3-BG				
Racemic	290	9	0–2.2	0.067
meso	340	9	0–4.5	0.067
Propionic acid[a]	226			

[a] Internal standard.

2,3-BG gave an irregularly shaped peak on the 0.3% Carbowax column and tailed on the three porous polymers (Chromosorb 101 and Porapak Q and QS) regardless of whether they were silanized. Jansen et al. (15) reported the separation of ethanol, diacetyl, acetoin, and 2,3-BG on Chromosorb 101 but made no mention of tailing, perhaps because temperature programming was used. In the present study, tailing of acetic acid also occurred on the 3% Carbowax column. Thus, these five columns were of little value for separating 2,3-BG and acetic acid. The remaining three columns (namely; 20% Tween 80, 10% SP 1000, and 10% SP 1200) did not separate ethanol from diacetyl but did separate acetoin, 2,3-BG, and acetic acid into well-resolved peaks; in addition, 2,3-BG was resolved into two peaks.

Three isomers of 2,3-BG are possible, namely, the L and D enantiomers and the *meso* form. Sufficiently pure solutions of each isomer were not available, but the identity of both 2,3-BG peaks was determined indirectly. According to Hohn-Bentz and Radler (13), 2,3-BG-producing microorganisms are of two types: one type producing mainly *meso*-2,3-BG with small amounts of L-2,3-BG (*Enterobacter aerogenes*, *Erwinia carotovera*, *Serratia marcescens*, and *Staphylococcus aureus*) and the other type producing mainly D-2,3-BG with small amounts of *meso*-2,3-BG (*Bacillus polymyxa*, *Acetobacter suboxydans*, and *Aeromonas hydrophila*). In the present study, *Enterobacter aerogenes* NCDO 711 produced mainly peak 2 of 2,3-BG, so that this must be the *meso* isomer. Thus, the first 2,3-BG peak must represent the D and L or racemic isomer. This assumes that there is no difference in the gas-liquid chromatographic behavior of the L and D isomers. The racemic and *meso* isomers have also been separated on other columns (12, 20).

The solvent (1 M formic acid) gave two peaks on the 10% SP 1200 column at RTs of 65 and 305 s, respectively, suggesting that this column may be less useful than the other two. The RT of the solvent on the 10% SP 1000 column was 64 s, whereas it gave several small peaks at RTs of 20 to 25 s on the 20% Tween 80 column. Flame ionization detectors are

TABLE 3. Growth (pH), citrate utilization, and concentrations of ethanol, acetic acid, acetoin, and 2,3-BG production by several species of LAB in modified MRS broth

Species	Strain	pH	Hetero-fermen-tation	Ethanol concn (mM)	Citrate utili-zation	Acetic acid concn (mM)	Acetoin concn (mM)	2,3-BG concn (mM)	
								Racemic	*meso*
Streptococcus thermophilus	Y1	5.00	−	0.24	−	0.90	ND[a]	ND	ND
Lactobacillus bulgaricus	NYL2	4.90	−	0.14	−	0.02	ND	ND	ND
Lactobacillus lactis	BYL1	4.40	−	0.33	−	1.36	ND	ND	0.12
Streptococcus cremoris	P2	4.89	−	0.40	−	0.27	ND	ND	ND
Streptococcus lactis	ML8	4.34	−	0.39	−	0.20	ND	ND	ND
Lactobacillus casei	A148C	3.95	−	0.10	−	0.48	0.49	ND	0.11
Streptococcus lactis subsp. *diacetylactis*	16B	4.74	−	4.36	+	13.94	ND	1.68	1.68
	18B	4.37	−	0.59	+	8.29	1.67	0.42	0.41
	19B	4.57	−	2.49	+	12.90	0.06	1.69	1.84
	DRC1	4.79	−	1.42	+	10.98	0.07	1.72	1.25
	DRC3	4.41	−	0.66	+	7.37	2.27	ND	0.33
	DNCW4	4.39	−	0.43	+	8.38	1.76	0.45	0.44
Lactobacillus casei	C2	3.85	−	0.20	+	9.00	ND	ND	ND
Lactobacillus plantarum	P5	3.85	−	0.15	+	9.50	2.57	ND	0.04
	G1	3.80	−	0.15	+	8.20	2.40	ND	0.18
Leuconostoc lactis	7-1	4.27	+	30.51	−	1.30	ND	ND	0.21
	D12	4.30	+	30.50	−	1.89	ND	ND	0.20
Leuconostoc mesenteroides	X2	4.85	+	15.70	−	1.35	ND	ND	0.14
Leuconostoc lactis	NCW1	4.28	+	29.03	+	10.51	ND	ND	0.26
Leuconostoc paramesenteroides	9-1	4.42	+	23.69	+	9.80	ND	ND	0.38
Lactobacillus fermenti	D1	4.72	+	11.14	+	12.62	ND	ND	ND
Lactobacillus viridescens	7-7	5.22	+	7.49	+	9.88	ND	ND	0.26

[a] ND, Not detected.

usually considered to be insensitive to formic acid; the peaks obtained here are probably due to the high concentration of formic acid present in the samples. The RTs of all compounds were much shorter on the 20% Tween 80 column; therefore, this one was chosen for further study. This column has been recommended by other workers for volatile fatty acid determinations in rumen fluid (7, 23).

Quantitation. On the 20% Tween 80 column, ethanol and diacetyl had similar RTs (20 s) at 120°C. Reduction of the gas flow to 20 ml/min partially resolved both peaks, and complete resolution was possible by additionally lowering the column temperature to 100°C. At this temperature, racemic and *meso*-2,3-BG had RTs of 796 and 945 s, respectively. Because these RTs were excessively long and because diacetyl is only produced in trace (<0.2 mM) amounts by LAB (6, 9), it was decided to forego the separation of diacetyl and ethanol and operate the column isothermally at 120°C and an N_2 flow rate of 20 ml/min. Presumably, temperature programming would resolve all five components. For our purpose, propionic acid was used as an internal standard as it is not a known end product of LAB and its RT did not overlap with the other compounds of interest (Table 2).

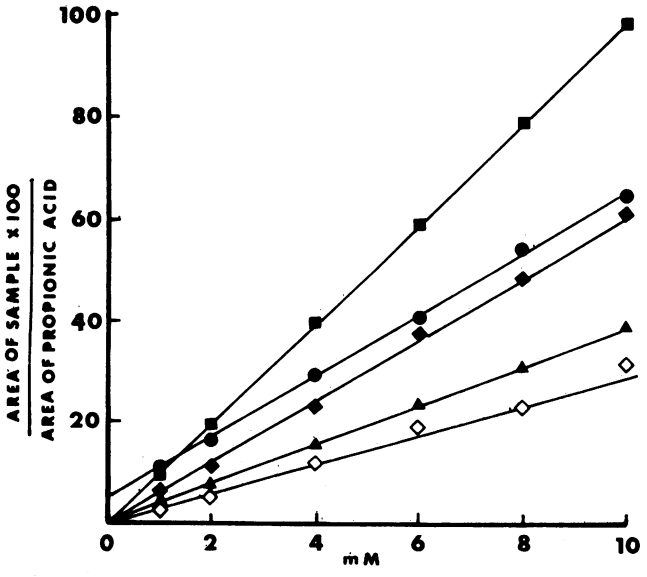

FIG. 1. Standard curves for ethanol (●), acetoin (■), acetic acid (▲), and racemic (◇) and *meso*- (◆) 2,3-BG.

The response of ethanol, acetoin, acetic acid, and racemic and *meso*-2,3-BG at 120°C was linear up to at least 10 mM, the highest concentration tested; acetic acid was the least responsive and acetoin was the most responsive (Fig. 1). It is presumed that the responses of racemic and *meso*-2,3-BG to the flame ionization detector were similar; the racemic and *meso* isomers represented 33 and 67% of the total, and the standard curves are not corrected for this but the data in Tables 3 and 5 are. The standard curve for ethanol was linear but did not go through the origin since its RT was close to that of the solvent. In practice, the solvent value was subtracted from the ethanol value to give the true ethanol concentration. For the standard curves and unknown samples, the concentration of the AristaR-grade formic acid was reduced to 0.63 M in the injected sample as a result of dilution with the internal standard, but this did not result in any apparent tailing or ghosting (5).

Reproducibilities were good as measured by the standard deviation of differences between duplicates (Table 2). Re-

peatabilities of standard curves were erratic when the standards (and the internal standard and formic acid) were stored in plastic vials, but storage of the standards in glass vials overcame this difficulty. Column stability was excellent. No apparent loss in sensitivity was observed after hundreds of injections of the medium, which contained several complex C and N sources and various inorganic salts. Except for two peaks at the RTs of ethanol and acetic acid, the uninoculated broth gave a linear base line, presumably because the chromatograph was not operating at its maximum sensitivity. Direct injection of acidified media has been reported previously (3, 24), although in one case (3) the broth was pretreated by passing it through a cation-exchange resin.

Distinguishing LAB. Homofermentative LAB produce 2 mol of lactate per mol of glucose metabolized, whereas heterofermentative LAB produce 1 mol each of lactate, ethanol, and CO_2. Thus, it should be possible to distinguish between homofermentative and heterofermentative LAB on the basis of the amount of ethanol produced. This is shown in Table 3. The uninoculated broth contained the equivalent of 0.6 mM ethanol and 1.1 mM acetate, and the data are corrected for this. The homofermentative strains produced low levels (<3 mM) of ethanol, except *Streptococcus lactis* subsp. *diacetylactis* 16B, which produced 4.4 mM, whereas the heterofermentative strains produced high levels (>11 mM) of ethanol with one exception, *Lactobacillus viridescens* 7-7, which produced 7.5 mM. Strain 16B is an unusual strain of *Streptococcus lactis* subsp. *diacetylactis* since it produces low levels of lactate (6) and may therefore be compensating for this by producing high levels of ethanol. Strain 7-7 grew poorly, as shown by the high pH, and so would produce low levels of ethanol in any case.

Metabolism of citrate by LAB theoretically produces 1 mol of acetate and a total of 0.5 mol of diacetyl, acetoin, and 2,3-BG per mol of citrate consumed. Under the present chromatographic conditions, diacetyl coeluted with ethanol, but since diacetyl is only produced in trace amounts by LAB (6, 9), it can be ignored. Thus, it should be possible to distinguish citrate-utilizing from non-citrate-utilizing LAB on the basis of the amounts of acetate, acetoin, and 2,3-BG produced. Only acetate was of any value, since the levels of acetoin and 2,3-BG varied too much (Table 3). The noncitrate utilizers produced low levels (<1.4 mM) of acetate, whereas the citrate utilizers produced high levels (>8 mM). The concentration of citrate in modified MRS broth is ca. 8 mM; therefore, the theoretical concentration of acetate which can be produced is 8 mM. Some citrate-utilizing strains produced greater than the theoretical level (Table 3) owing to the additional production of small amounts from glucose. In general, the levels of ethanol and acetate produced by the different species of LAB are similar to those reported by other workers (1, 2, 4, 6, 18), although in some studies (2, 18) it is not clear whether citrate is being metabolized.

Considerable variation in acetoin and 2,3-BG production occurred within the different groups of LAB. In the homofermentative group N streptococci, the citrate-utilizing *Streptococcus lactis* subsp. *diacetylactis* could be differentiated from the non-citrate-utilizing *Streptococcus lactis* and *Streptococcus cremoris* by the production of acetoin and 2,3-BG by *Streptococcus lactis* subsp. *diacetylactis*. In contrast, non-citrate-utilizing *Lactobacillus casei* A148C produced acetoin and a trace of 2,3-BG, whereas citrate-utilizing *Lactobacillus casei* C2 produced neither compound. In the heterofermentative group, there was no acetoin and little or no 2,3-BG produced regardless of whether citrate was utilized. The end products of citrate metabolism in the citrate-utilizing strains which produce no acetoin and little or no 2,3-BG (namely, *Lactobacillus casei* C2, *Leuconostoc lactis* NCW1, *Leuconostoc paramesenteroides* 9-1, *Lactobacillus fermenti* D1, and *Lactobacillus viridescens* 7-7) have not been identified at this time.

TABLE 4. RTs at 100°C of several compounds representative of bacterial fermentations

Compound	RT (s)
Acetone	42
Ethanol	47
Isopropanol	48
Diacetyl	57
n-Butanol	104
Acetoin	252
Acetic acid	375
Propionic acid	586
Racemic 2,3-BG	796
Butyric acid	938
meso-2,3-BG	945

There is little information on 2,3-BG production by LAB. Values have been reported in the early literature, but the levels are suspect because of the methodology used. In the present study, *Streptococcus lactis* subsp. *diacetylactis* produced the greatest levels of 2,3-BG, ranging from 0.3 mM in strain DRC3 to 3.5 mM in strain 19B (Table 3). An inverse relationship existed between 2,3-BG production and acetoin production, with high levels of 2,3-BG coinciding with low levels of acetoin and vice versa. Thus, the strains could be divided into low (strains 18B, DRC3, and DNCW4) and high (strains 16B, 19B, and DRC1) 2,3-BG producers or, alternatively, into high and low acetoin producers. Keen and Walker (17) have shown that *Streptococcus lactis* subsp. *diacetylactis* DRC1 produced 1.8 mM 2,3-BG in cheddar cheese. This value is much lower than that found for the same strain in the present study, suggesting that there may be other factors in cheese regulating its production. Speckman and Collins (26) reported 2,3-BG production by *Streptococcus lactis* subsp. *diacetylactis* 1816 in sweet (pH 6.0) whey and based calculations for production on the amount of lactose utilized. By using their data and assuming that 0.25% lactic acid was produced from lactose, it can be calculated that 0.8 mM 2,3-BG was produced in 3 days at 30°C. Neither of these reports distinguishes the isomer of 2,3-BG produced. The present data show that, except in strain DRC3, approximately equimolar amounts of racemic and *meso*-2,3-BG are produced by *Streptococcus lactis* subsp. *diacetylac-*

TABLE 5. Concentrations of ethanol, acetic acid, acetoin, and 2,3-BG produced by *Escherichia coli* NCDO 1989 and *Enterobacter aerogenes* NCDO 711 in MR VP medium after 24 h at 30°C

Organism	Concn (mM) of:				
	Ethanol	Acetic acid	Acetoin	2,3-BG	
				Racemic	*meso*
Escherichia coli NCDO 1989	6.1	5.8	ND[a]	ND	ND
Enterobacter aerogenes NCDO 711	5.5	5.1	0.8	1.5	17.4

[a] ND, Not detected.

tis. This contrasts with the results of Hohn-Bentz and Radler (13), who indicate that 2,3-BG-producing bacteria produce either mainly *meso*-2,3-BG with small amounts of L-2,3-BG or mainly D-2,3-BG with small amounts of *meso*-2,3-BG . On this basis, it is probable that the racemic 2,3-BG produced by *Streptococcus lactis* subsp. *diacetylactis* is the D isomer. Obviously, this conclusion needs independent confirmation.

It should be emphasized that the levels of the different metabolites found in the present study (Table 3) refer only to the growth conditions and medium used. Presumably, the use of different media and incubation conditions would influence the levels of some, if not all, of these products.

Other metabolic products. Several metabolic products representative of other bacterial fermentations were analyzed. All were made up in 1 M formic acid. Some of the RTs were very short, and therefore the column temperature was reduced to 100°C; other conditions remained the same. The RTs are summarized in Table 4. The response of the column to these compounds was not evaluated further. Butyric acid and *meso*-2,3-BG had similar RTs (Table 4), but they are unlikely to be produced by the same organism. However, in silage, in which both lactic and butyric fermentation may occur, 2,3-BG may interfere with the detection of butyric acid. We conclude that with the correct choice of internal standard and with temperature programming the column would appear to have considerable potential for the detection of the end products of several bacterial fermentations, including the mixed acid, BG, propionic acid, butyric acid, and solvent fermentations.

This was confirmed for the mixed acid and BG fermentations by growing *Escherichia coli* NCDO 1989 and *Enterobacter aerogenes* NCDO 711 in MR VP medium for 24 h at 30°C and measuring metabolite production. As expected, only *Enterobacter aerogenes* produced acetoin and 2,3-BG (Table 5). A time course study showed that these concentrations did not change significantly during incubation from 12 to 54 h. That the ethanol peak was in fact ethanol and was not contaminated with any diacetyl was confirmed by repeating the analysis at 100°C. The Voges-Proskauer test is normally used to detect the presence of the 2,3-BG fermentation even though it does so only indirectly as it actually measures diacetyl and acetoin production. The present technique is much simpler to use and more specific since 2,3-BG is actually measured.

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