USE OF SORBIC ACID ENRICHMENT MEDIA FOR SPECIES OF CLOSTRIDIUM[1]

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The inhibitory properties of sorbic acid (2,4-hexadieonic acid) apparently were noted first by Gooding (1945). The first indication that sorbic acid caused a selective inhibition of microorganisms was reported by Phillips and Mundt (1950) who observed that this acid in 0.1 per cent concentrations effectively inhibited film yeasts in cucumber fermentations without undue disruption of the desirable lactic acid fermentation. This latter observation stimulated an attempt to develop a medium which would be useful for enrichment and presumptive identification of the lactic acid bacteria. Vaughn and Emard (1951) and Emard and Vaughn (1952) found that sorbic acid media could be used for selective enrichment and isolation of the catalase negative lactic acid bacteria. They also suggested that sorbic acid media might be useful for enrichment and isolation of the catalase negative clostridia. Further investigation has shown this to be true. Sorbic acid is tolerated well by representatives of both the proteolytic and saccharolytic clostridia as is described in the following pages.

EXPERIMENTAL METHODS AND RESULTS

Beef liver infusion prepared according to the method of the Committee on Bacteriological Technic (1950) *without added* K_2HPO_4 was the principal basal medium used to test the effect of sorbic acid on the growth of the clostridia. This same medium was used for the preparation of all inocula. Other media when used are described in the text.

Forty-seven cultures representing 20 species and types of *Clostridium* were used. The names of these species together with the number of cultures and the collection from which they were obtained are listed below.

Species of Clostridium tested

I. Species with both saccharolytic and proteolytic properties: A. Predominately proteolytic: *C. aerofoetidum*, 2 cultures[2]; *C. bifermentans*, 2 cultures[2]; *C. parabotulinum*, Type A, 3 cultures[3]; *C. parabotulinum*, Type B, 3 cultures[3]; *C. parabotulinum*, 1 nontoxic culture[3]; *C. sporogenes*, 3 cultures.[2, 3]

B. Predominately saccharolytic: *C. acetobutylicum*, 2 cultures[2]; *C. botulinum*, Type C, 1 culture[4]; *C. botulinum*, Type D, 1 culture[4]; *C. botulinum*, Type E, 1 culture[4]; *C. felsineum*, 2 cultures[2]; *C. novyi*, 2 cultures[2]; *C. perfringens*, 3 cultures[2, 5]; *C. septicum*, 2 cultures[2]; *C. tertium*, 1 culture.[2]

II. Species with proteolytic but no apparent saccharolytic properties: *C. histolyticum*, 2 cultures[2]; *C. lentoputrescens*, 1 culture[2]; *C. nigrificans*, 2 cultures[2]; *C. tetani*, 3 cultures.[2, 6]

III. Species with saccharolytic but no apparent proteolytic properties: *C. butyricum*, 2 cultures[2]; *C. carnis*, 2 cultures[2]; *C. tartarivorum*, 3 cultures from Food Technology, Davis; *C. tetanomorphum*, 2 cultures[2]; *C. thermosaccharolyticum*, 1 culture.[3]

Vegetative cells as well as spore suspensions of all cultures were tested for their resistance to sorbic acid. Suspensions in which vegetative cells predominated were prepared by growing the cultures of the different species in plain liver infusion up to about 72 hours, depending upon the activity of the individual strains.

Spore suspensions of all cultures except the species *C. perfringens* were prepared by first

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[2] From the collection of Dr. L. S. McClung.

[3] From the collection of the National Canners Association.

[4] From the collection of Dr. K. F. Meyer.

[5] From the collection of Dr. A. G. Marr.

[6] From the collection of Dr. H. S. Cameron.

growing the cultures in plain liver infusion until repeated microscopic examination showed that a maximum crop of spores had been produced. Then 1 ml of the sporulated culture was added to 9 ml of sterile distilled water, mixed and heated at 80 C (the more heat resistant species for 10 minutes, the others for 5 minutes) to destroy the remaining vegetative cells. Because sporulation in cultures of *C. perfringens* generally is difficult to demonstrate with the microscope, the three cultures of this species first were grown for 7 days in plain liver infusion. Then aliquots of these cultures were heated at 80 C for 5 minutes and inoculated into more plain liver infusion. If growth occurred, it was assumed spores were present. Then the original cultures were diluted as described above and heated at 80 C for 5 minutes.

An inoculum of vegetative cells always consisted of 0.2 ml of plain liver infusion culture. All spore inoculations were made with 1 ml of the diluted, heated culture. The mesophiles always were grown at 35 C and the thermophiles at 55 C throughout the course of these experiments.

All pH values were determined with the glass electrode. All pH adjustments were made by addition of N NaOH or N HCl as required.

The sorbic acid used in these experiments was purchased from Eastman Kodak Company. Its reference number is 5822.

The effect of concentration of sorbic acid. Although Emard and Vaughn (1952) reported that

TABLE 1

Effect of concentration of sorbic acid on growth of vegetative inocula of species of Clostridium

Species	Number of Cultures	Concentration of Sorbic Acid*										
		0.12	0.3	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
		Number growing in 7 days										
C. aerofoetidum	2	2	2	2	2	0	0	0	0	0		0
C. bifermentans	2	2	2	2	2	2	2	2	1	0	0	0
C. parabotulinum, Type A	3	3	3	3	3	3	3	3	3	1	0	0
C. parabotulinum, Type B	3	3	3	3	3	3	3	3	3	1	0	0
C. parabotulinum†	1	1	1	1	1	1	1	1	1	0	0	0
C. sporogenes	3	3	3	3	3	3	3	3	3	1	0	0
C. acetobutylicum	2	2	2	2	2	2	2	2	2	0	0	0
C. botulinum, Type C	1	1	1	0	0	0	0	0	0	0	0	0
C. botulinum, Type D	1	1	1	0	0	0	0	0	0	0	0	0
C. botulinum, Type E	1	1	1	0	0	0	0	0	0	0	0	0
C. felsineum	2	2	2	2	2	2	2	2	2	2	0	0
C. novyi	2	2	2	2	0	0	0	0	0	0	0	0
C. perfringens	3	3	3	3	0	0	0	0	0	0	0	0
C. septicum	2	2	2	2	1	0	0	0	0	0	0	0
C. tertium	1	1	1	1	1	1	1	0	0	0	0	0
C. histolyticum	2	2	1	1	0	0	0	0	0	0	0	0
C. lentoputrescens	1	1	1	1	1	1	1	0	0	0	0	0
C. nigrificans‡	2	2	2	0	0	0	0	0	0	0	0	0
C. tetani	3	3	3	3	3	3	3	3	3	1	0	0
C. butyricum	2	2	2	2	2	2	2	2	0	0	0	0
C. carnis	2	2	2	1	0	0	0	0	0	0	0	0
C. tartarivorum	3	3	3	3	3	3	3	3	2	0	0	0
C. tetanomorphum	2	2	2	2	2	2	0	0	0	0	0	0
C. thermosaccharolyticum	1	1	1	1	1	1	1	1	1	1	0	0
Total	47	47	46	40	35	29	27	25	21	7	0	0

* Per cent (grams per 100 ml in liver infusion). Reaction adjusted so pH after sterilization was 6.7 to 6.8.

† Nontoxic strain.

‡ Tested in sulfite agar as cultures did not grow in liver infusion.

0.12 per cent sorbic acid in liver infusion medium permitted the growth of clostridia, the 10 cultures they investigated represented but three saccharolytic types of the *C. butyricum* species complex. Therefore it was desirable to investigate the resistance of additional species of the genus to sorbic acid. The liver infusion broth without K_2HPO_4 was prepared with concentrations of 0.12, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 per cent sorbic acid, respectively. All portions were adjusted so that the pH value after sterilization was 6.7 to 6.8.

Both vegetative cell cultures and spore suspensions of the cultures representative of the different species were used to inoculate the media which were incubated for 7 days at 35 C or 55 C depending upon the requirements of each culture. Development of turbidity and gas were the criteria for growth.

Considerable variation in resistance to sorbic acid was observed with the different species. As expected, some of the species exhibited more tolerance to sorbic acid when vegetative cells were used as the inoculum (compare tables 1 and 2). However, it is obvious that, regardless of the type of inoculum, the cultures had the same resistance to 0.12 and 0.3 per cent sorbic acid in liver infusion. Furthermore, at the higher levels of sorbic acid, the tolerance of strains of *C. parabotulinum*, *C. sporogenes*, *C. acetobutylicum*, *C. felsineum*, *C. tetani*, *C. tartarivorum*, and *C. thermosaccharolyticum* was notable.

The effect of pH on germination of spores in the presence of 0.12 per cent sorbic acid. It was re-

TABLE 2

Effect of concentration of sorbic acid on germination of spores of species of Clostridium

Species	Number of Cultures	Concentration of Sorbic Acids*										
		0.12	0.3	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
		Number growing in 7 days										
C. aerofoetidum	2	2	2	2	2	0	0	0	0	0	0	0
C. bifermentans	2	2	2	2	2	2	2	1	0	0	0	0
C. parabotulinum, Type A	3	3	3	3	3	3	3	3	1	0	0	0
C. parabotulinum, Type B	3	3	3	3	3	3	3	2	1	0	0	0
C. parabotulinum†	1	1	1	1	1	1	1	1	0	0	0	0
C. sporogenes	3	3	3	3	3	3	3	3	3	0	0	0
C. acetobutylicum	2	2	2	2	2	2	2	2	2	0	0	0
C. botulinum, Type C	1	1	1	0	0	0	0	0	0	0	0	0
C. botulinum, Type D	1	1	1	0	0	0	0	0	0	0	0	0
C. botulinum, Type E	1	1	1	0	0	0	0	0	0	0	0	0
C. felsineum	2	2	2	2	2	2	2	2	2	0	0	0
C. novyi	2	2	2	1	0	0	0	0	0	0	0	0
C. perfringens	3	3	3	3	0	0	0	0	0	0	0	0
C. septicum	2	2	2	2	0	0	0	0	0	0	0	0
C. tertium	1	1	1	1	1	1	0	0	0	0	0	0
C. histolyticum	2	2	1	1	0	0	0	0	0	0	0	0
C. lentoputrescens	1	1	1	1	1	1	1	0	0	0	0	0
C. nigrificans‡	2	2	2	0	0	0	0	0	0	0	0	0
C. tetani	3	3	3	3	3	3	3	3	3	1	0	0
C. butyricum	2	2	2	2	2	2	2	2	0	0	0	0
C. carnis	2	2	2	1	0	0	0	0	0	0	0	0
C. tartarivorum	3	3	3	3	3	3	3	2	2	0	0	0
C. tetanomorphum	2	2	2	2	2	2	0	0	0	0	0	0
C. thermosaccharolyticum	1	1	1	1	1	1	1	1	1	1	0	0
Total	47	47	46	39	31	29	26	22	15	2	0	0

* Per cent (grams per 100 ml in liver infusion). Reaction adjusted so pH after sterilization was 6.7 to 6.8.

† Nontoxic strain.

‡ Tested in sulfite agar.

ported by Emard and Vaughn (1952) that pH values in the range of 5.0 to 5.5 enhanced the selectivity of the 0.12 per cent sorbic acid liver infusion medium for growth of the lactobacilli and suppression of catalase positive microorganisms. The species of the genus *Clostridium* are known to grow better in a more neutral environment. Therefore, it was desirable to investigate the effect of pH on germination and growth of spores of the different cultures of clostridia in the presence of sorbic acid.

The effect of pH on germination of spores of the various species in the presence of 0.12 per cent sorbic acid was determined by comparison with germination, and growth of an aliquot of the same suspension of spores in plain liver infusion adjusted to the same pH value. No attempt was made to train the cultures to grow at low pH values prior to preparation of the spore suspensions.

The limiting pH value for germination of the spores of the different species in the acid range was not appreciably influenced by the presence of 0.12 per cent sorbic acid. However, as shown in table 3, the ability of the spores of the different cultures to germinate in 7 days was markedly decreased as the pH value of the medium was reduced from 5.8 to 5.0. Therefore, the pH range must be increased to at least 6.0 or above if the sorbic acid medium is to be used for enrichment

TABLE 3

Effect of pH on germination of spores of species of Clostridium in 0.12 per cent sorbic acid liver infusion

Species	Number of Cultures	pH of Medium*										
		6.0†	5.8	5.6	5.4	5.2	5.0	4.9	4.8	4.7	4.6	4.5
		Number growing in 7 days‡										
C. aerofoetidum	2	2	2	2	1	1	0	0	0	0	0	0
C. bifermentans	2	2	2	2	2	1	0	0	0	0	0	0
C. parabotulinum, Type A	3	3	3	3	3	3	2	1	0	0	0	0
C. parabotulinum, Type B	3	3	3	3	3	3	2	1	0	0	0	0
C. parabotulinum§	1	1	1	1	1	1	0	0	0	0	0	0
C. sporogenes	3	3	3	3	3	3	2	2	1	0	0	0
C. acetobutylicum	2	2	2	2	2	2	2	2	2	2	2	2
C. botulinum, Type C	1	1	1	1	1	0	0	0	0	0	0	0
C. botulinum, Type D	1	1	1	0	0	0	0	0	0	0	0	0
C. botulinum, Type E	1	1	1	0	0	0	0	0	0	0	0	0
C. felsineum	2	2	2	2	2	2	2	2	2	2	2	0
C. novyi	2	2	2	2	2	0	0	0	0	0	0	0
C. perfringens	3	3	3	3	2	0	0	0	0	0	0	0
C. septicum	2	2	2	2	2	1	0	0	0	0	0	0
C. tertium	1	1	1	1	1	0	0	0	0	0	0	0
C. histolyticum	2	2	2	0	0	0	0	0	0	0	0	0
C. lentoputrescens	1	1	1	1	1	1	0	0	0	0	0	0
C. nigrificans‖	2	2	1	1	0	0	0	0	0	0	0	0
C. tetani	3	3	3	3	2	0	0	0	0	0	0	0
C. butyricum	2	2	2	2	2	1	0	0	0	0	0	0
C. carnis	2	2	2	1	0	0	0	0	0	0	0	0
C. tartarivorum	3	3	3	3	3	3	2	0	0	0	0	0
C. tetanomorphum	2	2	2	1	0	0	0	0	0	0	0	0
C. thermosaccharolyticum	1	1	1	1	1	1	1	1	1	1	1	1
Total	47	47	46	40	34	23	13	9	6	5	5	3

* pH after sterilization.

† All cultures also grew in the medium at pH 6.8.

‡ Growth in plain liver infusion at the various pH values paralleled growth in the sorbic acid medium.

§ Nontoxic strain.

‖ Tested in sulfite agar as cultures did not grow in liver infusion.

and isolation of the more fastidious species of *Clostridium*. In this higher range the 0.12 per cent sorbic acid liver infusion medium is not as effective in preventing growth of the catalase positive microorganisms as in the recommended range of pH 5.0 to 5.5.

Utilization of sorbic acid. It was thought advisable to investigate the possibility that sorbic acid was utilized by some cultures since a number of the species of the genus *Clostridium* tested were quite resistant to its effects. Individual cultures of the more resistant species including *C. parabotulinum*, type A, *C. sporogenes*, *C. acetobutylicum*, *C. tetani*, and *C. thermosaccharolyticum* were compared with a culture of *C. perfringens* which had little tolerance for sorbic acid. To test utilization the sorbic acid was added to a basal peptone broth in concentrations of 0.12 and 1.0 per cent. The basal peptone medium was composed of 0.25 per cent tryptone, 0.25 per cent neopeptone (Difco), and 0.01 per cent yeast extract. Controls included this basal medium without added carbohydrate and with 1.0 per cent glucose, respectively. The media, adjusted so that the pH after sterilization was between 6.7 and 6.8, were inoculated in duplicate with spore suspensions of the individual cultures and incubated for 7 days. Gas production and turbidity were used as criteria for utilization. Sorbic acid apparently was used as a carbon source by *C. parabotulinum*, type A, *C. sporogenes*, *C. acetobutylicum*, and *C. thermosaccharolyticum* but not by *C. tetani* and *C. perfringens*. However, it was obvious that sorbic acid was not as good a carbon source as glucose. Quantitative data obtained by the spectrophotometric method of Melnick and Luckmann (1954) that demonstrate utilization of sorbic acid in liver infusion broth by various species of Clostridium are shown in table 4.

Utility of the sorbic acid medium. Liver infusion broth or agar with 0.12 per cent sorbic acid has been used routinely since 1951 for isolation of species of the genus *Clostridium* as well as catalase negative lactic acid bacteria from various foods. Sorbic acid is quite effective in suppressing the growth of aerobic, sporeforming bacilli, as already reported (Vaughn and Emard, 1951; Emard and Vaughn, 1952; Lindberg *et al.*, 1954). Therefore, the medium is particularly satisfactory for enrichment of anaerobic spores or for rapid repurification of clostridial cultures contaminated with aerobic, sporeforming bacilli. However, since the medium also is suitable for selective isolation of the catalase negative lactic acid bacteria, no attempt has been made to evaluate the efficacy of the medium for isolation of vegetative cells of species of Clostridium from mixed microbial populations quite frequently found in foods.

TABLE 4

Utilization of sorbic acid by species of Clostridium

Culture	Sorbic Acid Content*	
	Apparent in medium, mg per 100 ml	Apparent utilization, per cent
C. parabotulinum, A, no. 69A	11.4	90.5
C. parabotulinum, A, no. 73A	9.8	91.9
C. parabotulinum, A, no. 246A	1.6	98.7
C. parabotulinum, B, no. 162B	9.8	91.9
C. parabotulinum, B, no. 112B	0.0	100.0
C. parabotulinum, B, no. 213B	14.6	87.8
C. parabotulinum, no. 3679	45.4	62.2
C. sporogenes, no. 225	1.6	98.7
C. novyi, no. 173	0.0	100.0
C. bifermentans, no. 135	77.9	35.9
C. thermosaccharolyticum, no. 3814	61.7	48.6
C. tartarivorum, no. T9-1	71.5	40.4
C. tartarivorum, no. T9-0	60.1	49.9
C. tartarivorum, no. S4-0	63.4	47.4
C. acetobutylicum, no. 636	66.6	44.5
C. acetobutylicum, no. 632	111.7	7.0
C. tertium, no. 258	110.9	9.1
C. tetani, no. 72	111.2	7.2
C. carnis, no. 1153	111.2	7.2
C. tetanomorphum, no. 256	120.0	0
C. butyricum, no. 624	116.9	2.6
C. perfringens, no. 814	125.0	0
C. histolyticum, no. 820	112.0	6.6
C. septicum, no. 1620	113.5	5.4
C. felsineum, no. 638	112.0	6.6
C. lentoputrescens, no. 1140	107.1	10.7
C. aerofoetidum, no. 1148	113.5	5.4
C. botulinum, C	113.5	5.4
Medium with sorbic acid	120.0†	0
Medium without sorbic acid	0	0

* Contained in liver infusion broth without phosphate or particles of liver at pH 6.7 and determined by the method of Melnick and Luckmann (1954) after 7 days' incubation at the required temperature.

† Average value. Cultures grown in the medium without sorbic acid exhibited no absorption.

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SUMMARY

It was found that the 0.12 per cent sorbic acid liver infusion medium was quite satisfactory for the growth of forty-seven cultures representing 20 species and types of the genus *Clostridium*. Considerable variation in resistance to sorbic acid was observed with the different species. Some cultures of the most tolerant species including *C. parabotulinum*, *C. acetobutylicum*, *C. felsineum*, *C. tetani*, and *C. thermosaccharolyticum* grew in the presence of 3.0 per cent sorbic acid. The limiting pH for growth of the different species in the acid range was not appreciably affected by the presence of 0.12 per cent sorbic acid in liver infusion without phosphate. The various species sporulated well, and their spores germinated without noticeable delay in the 0.12 per cent sorbic acid medium if the pH value was in the required range. Sorbic acid is utilized as a carbon source by some species.

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