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INFLUENCE OF SORBIC ACID ON THE GROWTH OF CERTAIN SPECIES OF BACTERIA, YEASTS, AND FILAMENTOUS FUNGI[1]

THOMAS A. BELL, JOHN L. ETCHELLS, AND ALFRED F. BORG[2]

United States Food Fermentation Laboratory,[3] North Carolina State College, and Departments of Botany and Animal Industry, North Carolina Agricultural Experiment Station, Raleigh, North Carolina

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Interest in the use of sorbic acid (2,4-hexadienoic acid) as a food preservative and fungistatic agent was stimulated by Gooding in 1945 and by the report of its harmlessness as a dietary component by Deuel *et al.* (1954). The numerous reports which followed, especially those which dealt with sorbic acid as a selective inhibitor of certain microorganisms (Emard and Vaughn, 1952; York and Vaughn, 1954, 1955; Hansen and Appleman, 1955; Borg *et al.*, 1955, 1956; Costilow *et al.*, 1955) prompted this study.

The present report is concerned with the inhibitory action of sorbic acid in cultural media at different pH levels for certain species of bacteria, yeasts, and filamentous fungi which, for the most part, are associated with the growing cucumber or the commercial cucumber fermentation.

Sorbic acid falls into a group of short-chain organic acids, which, together with their salts, have been shown to exhibit antimicrobial properties. Such properties, for many of these acids, have been demonstrated for over fifty years; for reviews see: Wyss *et al.* (1945), Wyss (1948), Schelhorn (1953), Ingram *et al.* (1956), Grubb (1957).

Hoffman *et al.* (1939) investigated the fungistatic properties of normal saturated and unsaturated fatty acids containing from 1 to 14 carbon atoms and used as test organisms a mixed culture of common bread molds such as *Aspergillus niger*, *A. glaucus*, *Rhizopus nigricans*, and *Penicillium frequentans*. Sorbic acid was not included in this study. These workers reported that the effectiveness of the acids as fungistatic agents increased with chain length and concentration, and varied with the pH. Hoffman *et al.* (1941) continued this study with emphasis on the effect of pH and added to the list of chemicals tested such common food preservatives as benzoic and salicylic acids. Their work showed that these antimicrobial compounds are effective only when added in sufficient concentration to foods or culture media with a pH below 7.

Rahn and Conn (1944) compared the pH and the concentrations of undissociated benzoic and salicylic acids with the inhibitory action on *Saccharomyces ellipsoideus* and concluded that the toxic principle of such acids was the undissociated fraction and not the acid anion. As early as 1928, Rogers and Whittier reported that *Streptococcus lactis* ceased to make lactic acid when the concentration of the undissociated lactic acid reaches 0.017 M, regardless of pH. Cowles (1941) studied the action of acetic, propionic, butyric, valeric, caproic, and caprylic acids on *Staphylococcus aureus* and *Escherichia coli* and reported the bactericidal action was due to the undissociated acid fraction. Spoehr *et al.* (1949) also investigated the short-chain fatty acids as to antibacterial activity and showed a relation of activity to chain length, pH, and concentration.

Emard and Vaughn in 1952 studied the effect of sorbic acid in cultural media on microbial activity and reported that all the catalase positive cultures tested were inhibited and most of the catalase negative cultures grew without noticeable inhibition. On the other hand, these authors indicated an influence of pH on the growth of both catalase positive and negative organisms. Sorbic acid was used by Beneke and Fabian (1955) to inhibit a number of fungi isolated from tomato and strawberry fruits at pH 4.0. They also reported on the influence of sorbic acid con-

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[2] Present address: Department of Bacteriology, Kansas State College, Manhattan, Kansas.

[3] One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, United Stated Department of Agriculture.

TABLE 1

Yeasts and filamentous fungi tested for growth in basal broth A with and without 0.1 per cent sorbic acid at pH 7.0 and at pH 4.5. All cultures grew in the control basal media at both pH levels, and in the sorbic acid media at pH 7.0. All cultures were inhibited from growing in the basal media containing sorbic acid at pH 4.5

	A. Yeasts	
Candida	*Endomycopsis*	*Saccharomyces*
C. albicans (2, UNC)*	*E. ohmeri*	*S. cerevisiae* (2)
C. krusei	*Hansenula*	*S. fragilis* (UC)
C. tropicalis (UNC)	*H. subpelliculosa* (2)	*S. delbrueckii*
Candida sp. A	*Pichia*	*S. lactis* (NRRL-1140)
Candida sp. B	*P. alcoholophila*	*Saccharomyces* sp.
Brettanomyces	*Pichia* sp.	*Torulaspora*
B. versatilis (2)	*Rhodotorula*	*T. rosei* (2)
Cryptococcus	*R. flava*	*Torulopsis*
C. neoformans (MH)	*R. glutinis*	*T. candida* (NRRL-211)
Cryptococcus sp. (MH)	*R. rubra* (NRRL-1592)	*T. caroliniana* (2)
Debaryomyces	*Rhodotorula* sp. A	*T. lipofera* (NRRL-1351)
D. membranaefaciens	*Rhodotorula* sp. B	*T. minor* (NRRL-341)
var. *Hollandicus*	*Rhodotorula* sp. C	*Zygosaccharomyces*
Debaryomyces sp.		*Z. globiformis* (2)
		Z. halomembranis
	B. Filamentous Fungi	
Alternaria	*Geotrichum*	*Pestalotiopsis*
A. tenuis (5)	*Geotrichum* sp. (2)	*P. macrotricha* (2)
Ascochyta	*Gliocladium*	*Phoma*
A. cucumis (4)	*G. roseum*	*Phoma* sp.
Ascochyta sp.	*Helminthosporium*	*Pullularia*
Aspergillus	*Helminthosporium* sp. (2)	*P. pullulans*
A. elegans	*Heterosporium*	*Rhizopus*
A. fumigatus (3)	*H. terrestre*	*R. arrhizus*
A. niger	*Humicola*	*R. nigricans*
A. sydowi	*H. fusco-atra*	*Rosellinia*
A. terreus	*Mucor*	*Rosellinia* sp.
A. unguis	*M. silvaticus*	*Sporotrichum*
A. versicolor (2)	*Mucor* sp. (3)	*S. pruinosum* (2)
Cephalosporium	*Myrothecium*	*Stagonospora*
Cephalosporum sp. 1 (4)	*M. roridum*	*Stagonospora* sp.
Cephalosporum sp. 2	*M. verrucaria*	*Stysanus*
Cercospora	*Myrothecium* sp.	*Stysanus* sp.
Cercospora sp.	*Papularia*	*Thielavia*
Chaetomium	*P. arundinis*	*T. basicola* (2)
C. globosum (2)	*Penicillium*	*Trichoderma*
Cladosporium	*P. atromentosum*	*T. viride* (5)
C. cladosporioides (5)	*P. chermesinum*	*Truncatella*
Colletotrichum	*P. duclauxi* (2)	*Truncatella* sp.
C. lagenarium (2)	*P. expansum* (2)	
Cunninghamella	*P. frequentans*	
C. echinulata	*P. funiculosum* (2)	
Curvularia	*P. gladioli*	
C. trifolii (2)	*P. herquei*	

TABLE 1—*Continued*

B. Filamentous Fungi

Fusarium	*P. implicatum*	
F. roseum (3)	*P. janthinellum* (5)	
F. oxysporum (3)	*P. oxalicum* (7)	
F. solani	*P. piscarium*	
F. moniliforme	*P. restrictum*	
F. episphaeria	*P. rugulosum*	
	P. steckii (2)	
	P. sublateritium (2)	
	P. thomii	
	P. urticae	
	P. variabile	
	Penicillium sp.	

* Where more than one strain in the species was tested, this is indicated by the number in parentheses following the species. Also, the sources of yeast cultures are indicated as follows: UNC = University of North Carolina, Chapel Hill; MH = Mercy Hospital, Charlotte, North Carolina; NRRL = Northern Utilization Research and Development Division, Peoria, Illinois; UC = University of California, Davis. All other yeast cultures and all the filamentous fungi were from the authors' laboratory.

centration and pH of the media in relation to inhibition of growth.

Additional information is presented herein on the factors controlling the effectiveness of sorbic acid as a growth inhibitor for certain species of microorganisms; particular attention is given to the calculated undissociated acid concentration. A summary of part of the work has appeared earlier (Etchells *et al.*, 1955).

TABLE 2

Growth of two yeast species in agar plates at pH 5.0 and pH 8.0 with different levels of sorbic acid*

pH	Total Sorbic Acid		Undissociated Sorbic Acid	*Torulopsis caroliniana*		*Saccharomyces delbrueckii*	
				Growth density†	Size of colonies	Growth density†	Size of colonies
	%	*mg/100 ml*	*mg/100 ml*				
5.0	0	0	0	5+	Large	5+	Large
5.0	0.05	50	18	3+	Medium	5+	Large
5.0	0.10	100	37	–	None	2+	Small
8.0	0	0	0	5+	Large	5+	Large
8.0	0.05	50	0	4+	Large	5+	Large
8.0	0.10	100	0	3+	Large	5+	Large
8.0	1.00	1000	1	2+	Small	2+	Small
8.0	2.00	2000	2	2+	Small	1+	Small

* Broth A with 2.0 per cent agar.

† Growth density of colonies on agar plates; 5+ = extremely heavy; 4+ = moderately heavy; 3+ = heavy; 2+ = moderately light; 1+ = light; – = no growth.

MATERIALS AND METHODS

Cultures tested. The filamentous fungi (table 1) represented 66 species in 32 genera. These were obtained from studies by Raymond *et al.* (1959) and Etchells *et al.* (1958) which dealt with the populations, identification, and softening enzyme activity of fungi isolated from blossoms, ovaries, and fruit of pickling cucumbers. The yeasts (table 1) represented 32 species in 12 genera and, with few exceptions, were isolated during studies on the fermentation of cucumbers (Etchells and Bell, 1950*a*, *b*; Etchells *et al.*, 1953; Borg, Etchells, and Bell, 1958, *unpublished experiments*). The lactic acid bacteria (table 3) represented 6 species in 3 genera and were obtained from the three following sources: the authors' collection, isolated from cucumber fermentations by Borg *et al.* (1956); the collection of the Dairy Manufacturing Section, North Carolina State College, Raleigh; and the collection of the Northern Utilization Research and Development Division, Peoria, Illinois.

Media. The stock cultures were maintained, with frequent transfers, on the following solid media: potato-dextrose agar slants for the filamentous fungi; vegetable-juice agar slants (Etchells and Bell, 1950*a*) for the yeasts; and deep liver agar stabs (Haynes *et al.*, 1955) for the lactic acid bacteria. Growth tests were made

TABLE 3

Influence of 0.1 per cent sorbic acid at different pH levels on the growth of certain lactic acid bacteria and yeasts

Organism Tested	Growth with 0.1% Sorbic Acid* at pH						
	6.8	6.0	5.5	5.0	4.5	4.0	3.5
Bacteria							
Lactobacillus							
L. brevis (7)†	+	+	+	+	+	+	−
L. brevis (NCS)‡	+	+	+	+	+	−	−
L. plantarum (7)†	+	+	+	+	+	+	−
L. plantarum (NRRL-531)	+	+	+	+	+	+	−
L. arabinosus (NCS)	+	+	+	+	−	−	−
L. fermenti (NCS)	+	+	+	+	−	−	−
Pediococcus							
P. cerevisiae (19)†	+	+	+	+	+	−	−
P. cerevisiae (NRRL-1325)	+	+	+	+	+	−	−
Streptococcus							
S. lactis (NCS)	+	+	+	+	−	−	−
Yeasts							
Candida							
C. albicans	+	+	+	−	−	−	−
C. krusei	+	+	+	+	−	−	−
Brettanomyces							
B. versatilis	+	+	+	−	−	−	−
Cryptococcus							
C. neoformans	+	−	−	−	−	−	−
Debaryomyces							
D. membranaefaciens var. *Hollandicus*	+	+	−	−	−	−	−
Endomycopsis							
E. ohmeri	+	+	−	−	−	−	−
Hansenula							
H. subpelliculosa	+	+	−	−	−	−	−
Rhodotorula							
R. glutinis	+	−	−	−	−	−	−
Saccharomyces							
S. fragilis	+	+	+	−	−	−	−
S. delbrueckii	+	+	+	−	−	−	−
Torulaspora							
T. rosei	+	+	+	−	−	−	−
Torulopsis							
T. caroliniana	+	+	+	−	−	−	−
Zygosaccharomyces							
Z. globiformis (No. 29)	+	+	+	+	−	−	−
Z. globiformis (No. 466)	+	+	+	−	−	−	−
Z. halomembranis	+	+	−	−	−	−	−

* All organisms listed grew in the control broths.

† Indicates number of cultures tested from cucumber brines; in all other cases, single isolates were tested.

‡ NCS refers to cultures obtained from Dairy Manufacturing Section, North Carolina State College.

in basal broths with and without sorbic acid (Carbide and Carbon Chemicals Corp., New York). In one test, an agar medium was used and is so indicated. The three broths used were as follows: broth A, for testing fungi and yeasts consisted of 0.5 per cent peptone (Difco), 0.25 per cent yeast extract (Difco), 0.5 per cent sodium chloride, and 2.0 per cent glucose; broth B, for yeasts (Wickerham, 1951) contained 0.67 per cent yeast nitrogen-base (Difco), 0.1 per cent yeast extract, and 2.0 per cent glucose; broth C, for lactic acid bacteria was that recommended by Speck (1955, *personal communication*) and was composed of 1.5 per cent trypticase-soy (BBL), 0.5 per cent phytone (BBL), 0.1 per cent sodium citrate, 0.4 per cent sodium chloride, and 0.02 per cent L-cystine (dissolved first in a few drops of 2 N HCl). The basal broths with and without sorbic acid were adjusted to the desired pH values with 6 N HCl or 2.5 N NaOH using a model H-2, Beckman pH meter. Ten-ml amounts of the broths in 18 mm by 150 mm culture tubes were autoclaved at 15 psi for 15 min.

Inoculation and measurement of growth. For the yeasts and bacteria, one drop per tube from a 24- to 72-hr broth culture, grown in the corresponding medium under test, was used as inoculum. The cultures were incubated at 30 C and their growth measured as optical density at 1, 3, 7, and 14 days using a Lumetron colorimeter with 650 mμ filter. For the fungi, bits of mycelia from 7 to 14-day old potato-dextrose agar slants were transferred directly to the surface of the broths. The fungi were incubated at 30 C and the growths (mycelial mats) were observed visually at 5, 15, and 30 days.

RESULTS

Effect of sorbic acid concentration at pH 5.0 and pH 8.0. Two yeasts, *Torulopsis caroliniana* and *Saccharomyces delbrueckii*, were selected for testing the influence of sorbic acid concentration on growth at two widely separated pH levels. These yeasts are associated with the early, rapid fermentation of brined cucumbers (Etchells and Bell, 1950*a*; Etchells *et al.*, 1952) and have the property of growing over a wide pH range. The two yeast species were grown in basal broth A for about 72 hr, then 1 ml of a 1:1000 dilution was seeded into agar containing two levels of sorbic acid and at pH 5.0, and four levels of the acid at pH 8. The results of these agar plate tests are given in table 2. At pH 5.0, *T. caroliniana* was completely inhibited with 0.1 per cent sorbic acid; with 0.05 per cent there was definite growth but the colonies were restricted in size. *S. delbrueckii* was not inhibited at pH 5.0, but some restriction in colony size was observed with 0.1 per cent acid. At pH 8.0, the two yeast species were only slightly inhibited in sorbic acid concentrations from 0.05 to 2.00 per cent; however, there was definite restriction in colonial growth. It is probable that this chemical in high concentrations at pH 8.0 has little or no inhibiting effect on these two organisms. The restriction of colony growth observed was probably caused by a shift in pH of the medium toward the acid side caused by growth of the test yeasts. Such a pH change would increase the effectiveness of the sorbic acid as an inhibitor.

Screening test on yeasts and higher fungi. The 172 cultures of yeasts and fungi (see table 1) were inoculated into the following 4 test broths: (a) broth A with 0.1 per cent sorbic acid, at pH 4.5 (b) broth A, control, at pH 4.5; (c) broth A with 0.1 per cent sorbic acid, at pH 7.0; (d) broth A, control, at pH 7.0. The yeasts and filamentous fungi grew well in the control broths at both pH values. In the sorbic acid broths, all of the organisms tested grew equally well at pH 7.0 and conversely, none grew at pH 4.5.

Results of experiments on the influence of various pH levels on the growth-inhibitory properties of sorbic acid for certain species of lactic acid bacteria and yeasts in liquid media are presented in table 3. Additional information pertinent to this portion of the study follows.

Lactic acid bacteria. Thirty-nine cultures, representing 6 species in 3 genera were selected for the sorbic acid tests. Seven cultures each of *Lactobacillus brevis* and *L. plantarum*, and 19 cultures of *Pediococcus cerevisiae*, all isolated from commercial cucumber fermentations, were included in the tests because of conflicting reports (Phillips and Mundt, 1950; Emard and Vaughn, 1952; Borg *et al.*, 1955; Costilow *et al.*, 1955, 1956, 1957) on the effect of sorbic acid on the growth of these species, both in cucumber fermentations and in selective media studies.

The lactic acid bacteria grew in broth C with 0.1 per cent sorbic acid at the higher pH levels, i. e., pH 5.0 and above. None of the species tested grew at pH 3.5 with 0.1 per cent sorbic acid; only two species, *L. brevis* and *L. plantarum* grew at pH 4.0; *P. cerevisiae* (20 cultures) grew at pH 4.5. *L. arabinosus*, *L. fermenti*, and *S. lactis*

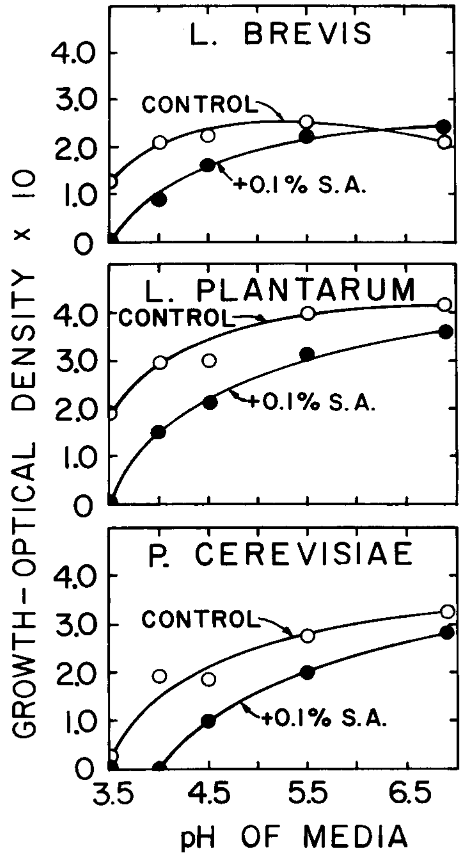

Figure 1. Effect of sorbic acid (S.A.) and pH of the broth media on growth of lactic acid bacteria isolated from commercial cucumber fermentations. *Top: Lactobacillus brevis*, average of 7 cultures. *Middle: Lactobacillus plantarum*, average of 7 cultures. *Bottom: Pediococcus cerevisiae*, average of 19 cultures. Growth measured at 14 days.

were the most susceptible of the acid-forming bacteria tested and did not grow below pH 5.0 in the broth containing 0.1 per cent sorbic acid.

The cell growths as measured by optical density of the broth cultures were recorded for those organisms listed in table 3. Growth curves for the three predominant species of lactic acid bacteria from commercial cucumber fermentations, namely, *L. brevis*, *L. plantarum*, and *P. cerevisiae*, are shown in figure 1. In the presence of 0.1 per cent sorbic acid, a definite and increasing inhibition was observed with the above species over the pH range from 6.8 to 3.5. There was one exception however, *L. brevis* at pH 6.8; this species grew better in the sorbic acid broth at this pH level than in the control.

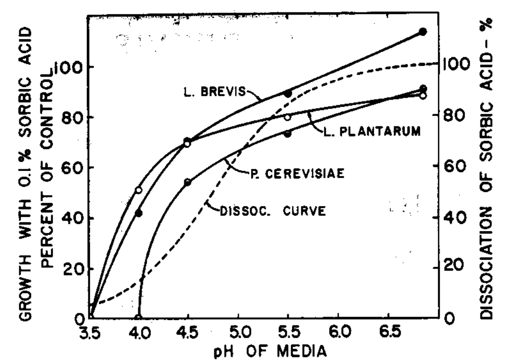

Figure 2. Growth of 3 species of lactic acid bacteria in sorbic acid broth, expressed as per cent of control, and the calculated dissociation curve of sorbic acid plotted from pH 3.5 to 6.8.

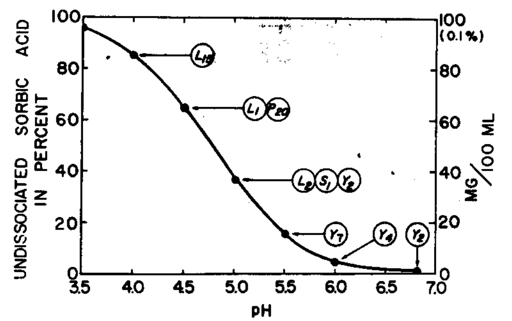

Figure 3. The calculated undissociated sorbic acid, shown in per cent and mg per 100 ml of 0.1 per cent solution, is drawn from pH 3.5 to 6.8. The lowest pH values where growth was observed for the organisms tested are indicated on the curve in the circles by *L* for *Lactobacillus* spp., *P* for *Pediococcus* sp., *S* for *Streptococcus* sp., and *Y* for yeast spp. The number of cultures tested is also indicated in the circles. See table 3 for names of organisms tested.

Yeasts. Sixteen cultures representing 14 species of yeasts were tested in broth B with 0.1 per cent sorbic acid at seven pH levels from 3.5 to 6.8 (table 3). All of the cultures grew at pH 6.8, 14 of them at pH 6.0, 10 at pH 5.5, 2 at pH 5.0, and none at pH 4.5 and below. The 2 species most tolerant to sorbic acid were *Candida krusei* and *Zygosaccharomyces globiformis*; the 2 most susceptible species were *Rhodotorula glutinis* and the pathogen, *Cryptococcus neoformans*. All yeast cultures grew in the control broths without sorbic acid at all pH levels employed.

Relationship of undissociated sorbic acid concentration to antimicrobial activity. When the growth data for three species of lactic acid bacteria (*L. brevis*, *L. plantarum*, and *P. cerevisiae*) in sorbic acid broth are plotted with the dissociation curve for the acid from pH 3.5 to 7.0, the reduction in growth of these species parallels the dissociation of the acid (figure 2). Earlier evidence of this was presented in table 2 for the yeast *T. caroliniana*. The toxic action of sorbic acid correlates with the concentration of undissociated acid. As indicated earlier in this paper, this relationship between undissociated acid and antimicrobial activity has been reported by investigators a number of times with other organic acids used as fungistatic agents.

The concentration of undissociated sorbic acid required to inhibit growth for certain of the organisms tested, may be derived from the results given in table 3. Growth of some of the organisms at the lowest pH value is indicated on the undissociated curve presented in figure 3. As an example, there were 15 Lactobacilli cultures which grew in broth media containing 0.1 per cent sorbic acid (100 mg per 100 ml) at pH 4.0 but did not grow at pH 3.5. At pH 4.0, 85 per cent of the sorbic acid (85 mg per 100 ml) is undissociated; this amount permitted the growth of the 15 cultures. At pH 3.5, 95 per cent of the sorbic acid (95 mg per 100 ml) is undissociated; this amount inhibited growth.

Further observation of the undissociated sorbic acid curve (figure 3) with respect to yeast growth reveals that only two cultures grew at pH 5.0 with 37 per cent undissociated acid; 14 yeast cultures were inhibited with this level of the acid. A good separation zone on the curve to inhibit the yeasts, yet permit good growth of the lactic acid bacteria, is about 30 to 40 mg per 100 ml of undissociated sorbic acid.

DISCUSSION

The efficiency of sorbic acid as a selective inhibitor of certain microorganisms or as a food preservative would have to be based upon the undissociated acid concentration and not the total concentration. A suggested efficiency pattern of sorbic acid for microbial inhibition, based upon increased hydrogen ion concentration, is as shown in table 4. As shown in the table, a drop in pH from 5.3 to 4.3 causes a threefold increase in the percentage of undissociated sorbic acid.

It has been shown herein that 0.1 per cent sorbic acid did not inhibit the growth of certain species of fungi, yeasts, and lactic acid bacteria

TABLE 4

Efficiency pattern of sorbic acid for microbial inhibition based upon increased hydrogen ion concentration

pH	Undissociated Sorbic Acid	Antimicrobial Efficiency
	%	
7.0	1	None
6.0	5	Minimal range
5.3	25	
5.1	33	
4.8	50	Maximal range
4.3	75	
3.8	90	
3.1	99	

at pH 7. From a search of the literature it is apparent that sorbic acid and other short chain fatty acids are not effective as antimicrobial agents at neutral pH values or above. York and Vaughn (1954, 1955) and Hansen and Appleman (1955) studied the action of sorbic acid in culture media at such pH levels using certain species of *Clostridium* and reported no inhibition of growth but rather, a utilization of the acid as a carbon source.

Jacobs (1940) and Anderson (1945), in studies on cell permeability to weak electrolytes, have pointed out that it is the undissociated molecules that pass through the cell membranes. Samson *et al.* (1955) have demonstrated that, based upon phosphate uptake, acetate (0.2 M) inhibits glucose fermentation by *Saccharomyces cerevisiae* much more at pH 4.0 than at pH 7.0. In view of these findings, it seems probable that the inhibitory property of sorbic acid increases with decreasing pH because the acid is permeable to the cell only in the undissociated form of the latter. Once inside the cell, the mechanism of sorbic acid inhibition is not understood, although Melnick *et al.* (1954) have suggested that this chemical inhibits dehydrogenation of fatty acids.

SUMMARY

The influence of sorbic acid on the growth of cultures, representing 66 species of filamentous fungi, 32 species of yeasts, and 6 species of lactic acid bacteria cultures, was studied. These microorganisms, for the most part, were associated with the growing cucumber or the commercial cucumber fermentation.

The pH of the culture medium was found to be the principal factor controlling the effectiveness of sorbic acid as an inhibitor for microbial growth. All of the organisms studied grew in media containing 0.1 per cent sorbic acid at pH 7.0. The yeasts and filamentous fungi were inhibited in media containing 0.1 per cent sorbic acid at pH 4.5; the lactic acid bacteria were inhibited at this concentration of the chemical at pH 3.5.

Certain species of yeasts and lactic acid bacteria were studied in media with 0.1 per cent sorbic acid over a pH range from 3.5 to 6.8. The reduction in growth of these organisms paralleled the dissociation of sorbic acid over the pH range studied. Thus, the toxic action of sorbic acid appears to be directly related to the concentration of undissociated acid.

The efficient use of sorbic acid as an antimicrobial agent would be at a pH value which resulted in the highest concentration of undissociated acid. At pH 4.8, 50 per cent of the acid is undissociated; thus, at this pH or below, one half of the total sorbic acid or more would be effective.

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