

THE EFFECT OF SORBIC ACID ON YEASTS ASSOCIATED
WITH CUCUMBER FERMENTATIONS

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ABSTRACT

The influence of sorbic acid on 24 cultures of yeasts representing 11 different species which are often isolated from cucumber fermentations was determined. The effectiveness of the acid as a yeast inhibitor depended greatly on the hydrogen ion concentration of the substrate. At pH 5.0 all yeasts tested were completely inhibited by 0.1 percent sorbic acid; but, as the pH was increased toward neutrality, a number of the species were found to grow. The addition of salt to the substrate in addition to sorbic acid aided the inhibition of some species. Survival studies of the yeasts in the presence of 0.05 and 0.1 percent sorbic acid at pH 4.5 showed that the death rate of most species was more rapid with the higher concentration. The results indicated that sorbic acid may be somewhat fungicidal as well as fungistatic at low pH levels. Sorbic acid was found to be quite stable in a laboratory medium. Spectrophotometric analysis of a medium inoculated with a yeast and of uninoculated medium after 2-weeks incubation showed no significant change in concentration of sorbic acid.

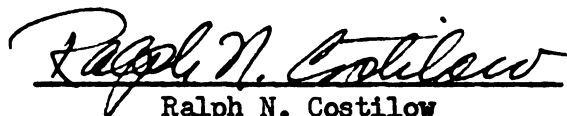

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INTRODUCTION

Yeasts are normally present in cucumber curing brines. The types present can be divided into 2 classes, sub-surface and film-forming yeasts. The former are gas producers and often cause a type of spoilage referred to as "bloaters" (hollow cucumbers). The film-forming yeasts, while not considered responsible for "bloater" spoilage, are undesirable in that they utilize the lactic acid produced during fermentation; thereby, partially destroying the preserving quality of the brine.

Unlike most other organisms associated with spoilage in brines, yeasts are both salt and acid tolerant. Therefore, some other means of control is required to prevent their development. A survey of the effect of sorbic acid, an alpha-beta unsaturated fatty acid, on pure cultures of the predominant species of these yeasts was made in this study.

REVIEW OF LITERATURE

Microbiology of cucumber fermentations. The identification of predominant types of microorganisms associated with the fermentation of cucumbers has been studied by different workers in recent years. Etchells et al. (5, 6) identified the film-forming and sub-surface yeasts normally found in such fermentation brines. Costilow and Fabian (1) in their studies of commercial cucumber fermentations found only 2 groups of active microorganisms — acid forming bacteria and yeasts. The acid forming bacteria were found to multiply rapidly and reach maximum numbers in 5 or 6 days. Yeasts decreased in numbers for 2 or 3 days after brining but then increased gradually with peak numbers found in 10 to 20 days. Rosen and Fabian (15) found a similar pattern of microorganism development in laboratory studies on cucumber fermentations. In addition to these 2 groups of microorganisms, Etchells et al. (7) in their studies of commercial cucumber fermentations in North Carolina noted a hydrogen fermentation due to Aerobacter. This occurred very frequently in high salometer brines.

Effect of sorbic acid on microorganisms. Gooding (8) in 1945 found that sorbic acid could be incorporated into

noted that this compound in concentrations effective against such organisms was tasteless, odorless and non-toxic. Smyth and Carpenter (18) confirmed the non-toxicity factor and more recently Deuel et al. (2, 3) proved its harmlessness as a dietary component. They found from rat feeding studies, that intermediary metabolism of sorbic acid is identical with such normally occurring fatty acids as caproic and butyric and that under normal conditions of alimentation sorbic acid is completely oxidized to CO_2 and H_2O . In these same studies they found sorbic acid considerably less toxic than sodium benzoate, a preservative used in many food products.

Phillips and Mundt (14) apparently were the first to suggest the use of sorbic acid to control the development of scum yeasts on pickle brines. They found that this compound in a concentration of 0.1 per cent would prevent mold and scum yeast development under conditions extremely favorable for their growth without harmful effect to the fermentation process. Jones and Harper (9) confirmed these findings. These papers dealt primarily with the film-forming yeasts in general. Apparently no work was done to evaluate this compound's effectiveness against pure cultures of yeasts including the sub-surface types considered more important as spoilage yeasts in the pickle industry.

Further indication that sorbic acid might be useful in cucumber fermentations was presented by Emard and Vaughn (4) who stated that this compound can be used advantageously in the isolation of lactic acid bacteria, particularly those belonging to the genus Lactobacillus. Their findings showed this compound to be selective for the catalase negative bacteria such as Lactobacillus, Leuconostoc, and Clostridium genera. All catalase positive organisms tested including yeasts, molds, actinomycetes and bacteria were inhibited by a concentration of 0.12 per cent sorbic acid when the pH of the medium was between 5.0 and 5.5 in the absence of phosphate salts. The degree of effectiveness of sorbic acid depended upon its concentration, the type of basal medium and the pH of the medium. York and Vaughn (19) found that sorbic acid could be used as an enrichment for media to isolate the more fastidious species of Clostridium if the hydrogen ion concentration of the medium was increased to pH 6.0.

Recently, extensive studies have been published on the efficiency of sorbic acid in controlling mold growth on cheese. Smith and Rollin (16, 17) found that 0.05 per cent sorbic acid mixed into processed cheese inhibited mold growth and that the application of this compound to cheese wrappers offered a practical method for packaging cheese. They estimated that its use would save 13 million pounds of

cheese annually in the United States. No mold growth and no organoleptic difficulties were encountered when $\frac{1}{4}$ to $\frac{1}{2}$ - pound packages of cheese were wrapped in a thermoplastic coated cellophane wrapper treated with 2.5 to 5.0 grams of sorbic acid per 1,000 square inches of paper. This concentration will furnish not more than 0.1 per cent sorbic acid to the cheese.

Although oxidizable by air, as are other polyunsaturated C₆ fatty acids, Melnick et al. (11, 12, 13) found that no oxidative deterioration of sorbic acid occurred in packages so wrapped during relatively long storage periods at 45°F. However, when cheese was wrapped with only one surface protected by the sorbic acid treated wrapper, molds grew on the other surfaces and the concentration of sorbic recoverable decreased. From this and other studies they concluded that in cheese with a high ratio of mold to sorbic acid concentration, the sorbic acid was utilized by the mold as a source of carbon. If the ratio was reversed, the sorbic acid effectively inhibited the dehydrogenase enzyme system of the molds; thereby, exhibiting fungistatic activity.

Melnick and Luckmann (10) presented a precise spectrophotometric method for the estimation of sorbic acid in cheese and on cheese wrappers. The sorbic acid was distilled at atmospheric pressure and the sorbic acid in the

absorption curve.

EXPERIMENTAL

Cultures used and method of handling. The pure cultures of yeasts used in this study were isolated by Etchells and Bell (5), Etchells et al. (6) and Costilow and Fabian (1) during their work on the identification of yeasts from cucumber brines. Cultures isolated by Etchells and Bell (5) and Etchells et al. (6) were obtained from Dr. L. J. Wickerham of the Northern Utilization Research Branch, United States Department of Agriculture, Peoria, Illinois.

All cultures were carried on V-8 agar slants and fresh transfers were made at about 2-week intervals. Two or three strains of each species were studied.

For each experiment, young actively-growing cultures used for inoculation purposes were obtained by repeated transfers in dextrose broth. The dextrose broth consisted of 0.5 per cent peptone, 1.0 per cent dextrose and 0.1 per cent yeast extract. All yeasts used in these studies grew well in this medium at pH levels between 4.5 and 7.0.

Source of sorbic acid and method of addition. The sorbic acid was obtained from the Carbide and Carbon Chemicals Company, New York 17, New York.

Sorbic acid was added to the media prior to pH adjustments and sterilization. The concentrations were calculated on a moisture free basis and added as per cent by weight per

on a moisture free basis and added as per cent by weight per unit volume of media.

Methods of estimating growth. In the preliminary work, yeast growth was estimated by visual observation of turbidity and scum formation as compared to uninoculated controls. Later the growth of the sub-surface yeasts was determined by measuring per cent light transmission in a photelometer.

In the study of yeast survival both plate counts on dextrose agar (Difco) plus 0.1 per cent yeast extract and acidified with 3 ml. of 5 per cent tartaric acid per 100 ml. and most probable numbers in dextrose broth were run.

RESULTS

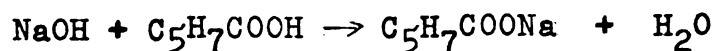
The Effect of Sorbic Acid and Sodium Sorbate on Growth of
Pure Cultures of Yeasts at pH 6.0.

Phillips and Mundt (14) stated that sorbic acid and its sodium salt appeared equally effective in laboratory tests in controlling pure cultures of film-forming yeasts on artificial media but showed no data to substantiate this statement. Due to the limited solubility of sorbic acid in water, the use of the soluble sodium salt, if equally effective, would obviously offer certain advantages. Deuel et al. (2) compared by rat feeding experiments the LD₅₀ for sorbic acid and sodium sorbate and found the latter considerably more toxic. However, sodium sorbate has not been available commercially and these workers have not yet given their method of preparation of the compound.

To compare the relative effectiveness of sorbic acid and sodium sorbate in controlling growth of yeasts from cucumber fermentations, a sample of sodium sorbate obtained from Mr. J. Sheneman, Department of Bacteriology, Michigan State University was used. This was prepared by Mr. Sheneman in the following manner:

Sodium hydroxide, sorbic acid and distilled water were mixed in the ratio of 5:14:50. This mixture was then thor-

oughly dried in a vacuum oven to remove all free water and leave sodium sorbate. Theoretically, this should yield 16.75 grams sodium sorbate when calculated according to the following reaction:



Since 14 grams of sorbic acid was used to make this 16.75 grams of sorbate, then 1.0 gram of sorbic acid is equivalent to approximately 1.2 grams of sodium sorbate.

To compare the relative effectiveness of the 2 compounds 0.1 per cent sorbic acid and 0.12 per cent sodium sorbate were added to separate lots of dextrose broth. A third lot of dextrose broth was used as a control medium. All 3 lots were dispensed in 10 ml. amounts into test tubes, sterilized, inoculated with 1 drop of each yeast culture studied and incubated at 30°C. Growth was assessed by visual examination only.

As shown in Table 1, neither sorbic acid nor sodium sorbate was very effective at pH 6.0 in inhibiting yeast growth. However, visible growth occurred consistently earlier in those tubes containing sodium sorbate for all cultures except strain Y-35 of Rhodotorula sp. This culture showed no growth in the presence of either compound. Sorbic acid under these conditions apparently caused complete inhibition of growth of both cultures of Rhodotorula and of Debaryomyces membranaefaciens var.

Table 1
Effect of Sorbic Acid and Sodium Sorbate on Yeasts at pH 6.0

Culture	T R E A T M E N T														
	Control			0.1% Sorbic Acid						0.12% Sodium Sorbate					
	Days			Days						Days					
	1	2	3	1	2	3	4	6	14	1	2	3	4	6	14
BRETTANOMYCES															
<i>B. versatilis</i> (Y-146)	+	5+		-	-	-	-	+	2+	-	+	3+	3+	4+	5+
<i>B. versatilis</i> (Y-176)	-	5+		-	-	-	+	+	2+	-	+	3+	3+	4+	4+
HANSENULA															
<i>H. subpelliculosa</i> (Y-72)	5+			-	-	+	+	+	+	-	2+	2+	3+	3+	5+
<i>H. subpelliculosa</i> (Y-58)	5+			-	-	-	-	-	+	-	+	2+	2+	3+	3+
<i>H. subpelliculosa</i> (Y-64)	5+			-	-	-	-	+	+	-	+	+	2+	3+	3+
RHODOTORULA															
<i>Rhodotorula</i> sp. (Y-35)	2+	4+	5+	-	-	-	-	-	-	-	-	-	-	-	-
<i>Rhodotorula</i> sp. (Y-103)	2+	3+	5+	-	-	-	-	-	-	-	+	+	2+	2+	3+
TORULASPORA															
<i>T. rosei</i> (Y-392)	5+			+	3+	3+	3+	4+	4+	3+	4+	4+	4+	4+	5+
<i>T. rosei</i> (Y-407)	5+			+	3+	3+	3+	4+	4+	3+	4+	5+	5+	5+	5+
<i>T. rosei</i> (Y-287)	5+			2+	3+	3+	3+	4+	4+	4+	4+	4+	4+	5+	5+
TORULOPSIS															
<i>T. holmii</i> (Y-109)	5+			2+	4+	4+	4+	4+	4+	4+	5+	5+	5+	5+	5+
<i>T. holmii</i> (Y-277)	5+			4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
<i>T. holmii</i> (Y-413)	5+			3+	4+	4+	4+	4+	4+	4+	4+	4+	5+	5+	5+
ZYGOSACCHAROMYCES s.g.															
<i>Z. halomembranis</i> (Y-1000)	3+	5+		-	-	+	+	+	2+	-	4+	4+	4+	4+	4+
<i>Z. halomembranis</i> (IS-864)	2+	5+		-	-	+	+	+	+	-	+	4+	4+	4+	5+
<i>Z. sp.</i> (IS-569)	-	5+		-	+	+	2+	3+	3+	-	2+	3+	4+	5+	5+
CANDIDA															
<i>C. krusei</i> (NRRLY-1487)	2+	5+		-	+	2+	4+	4+	4+	-	+	4+	5+	5+	5+
<i>C. krusei</i> (NRRLY-301)	4+	5+		+	2+	2+	3+	3+	3+	+	4+	4+	5+	5+	5+
DEBARYOMYCES															
<i>D. membranefaciens</i> var. <i>Hollandicus</i> (NFI-32)	+	3+	5+	-	-	-	-	-	-	-	+	2+	5+	5+	5+
<i>D. membranefaciens</i> var. <i>Hollandicus</i> (NFI-72)	+	3+	5+	-	-	-	+	+	+	-	-	2+	3+	5+	5+
ENDOMYCOPSIS															
<i>E. ohmeri</i> (FI-1)	5+			-	-	+	+	+	+	2+	4+	5+	5+	5+	5+
<i>E. ohmeri</i> (FI-15)	5+			-	-	-	+	+	+	2+	4+	5+	5+	5+	5+

* Number of plus signs indicates the extent of turbidity observed; 5+ = maximum turbidity; - = no growth observed.

Hollandicus culture NFY-32. Partial inhibition of Brettanomyces, Hansenula, Zygosaccharomyces and Endomycopsis species was evident but little or no inhibition occurred in those tubes inoculated with Torulaspora, Torulopsis and Candida species. However, later studies have proved that sorbic acid is much more effective against growth of all yeasts in a more acid medium. Also, it is quite conceivable that sodium sorbate might be more effective if the pH of the medium were lowered. This has not been confirmed at the present time.

Relationship of Size of Inoculum to Effectiveness
of Sorbic Acid at pH 6.0

Melnick et al. (13) showed that high mold populations would grow in the presence of sorbic acid while lower populations would not. Therefore, it was possible that lower yeast inocula might result in complete inhibition at pH 6.0. To test this possibility, serial dilutions were made of representative strains of the yeasts and the above experiment repeated.

Results of this study (Table 2) showed that the ability to survive and develop in the presence of 0.1 per cent sorbic acid varied considerably with different species of yeasts. However, it was apparent that with all species studied if growth occurred in the 10^{-2} dilution of the inoc-

Table 2
Relationship of Size of Inoculum to the Inhibitory Action of 0.1 Percent Sorbic Acid at pH 6.0

Culture	Cells/ml. original inoculum $\times 10^6$	Controls [#] Days	Dilution of Culture Used for Inoculation														
			10^{-2} Days					10^{-3} Days					10^{-4} Days				
			1	2	4	8	14	1	2	4	8	14	1	2	4	8	14
<u>Brettanomyces versatilis</u> (Y-115)	32	## 5+	-	+	3+	5+	5+	-	-	+	4+	4+	-	-	-	3+	3+
<u>Hansenula subpelliculosa</u> (Y-72)	48	3+	5+	-	-	+	3+	3+	-	-	-	2+	2+	-	-	-	+
<u>Rhodotorula sp.</u> (Y-35)	9	2+	5+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<u>Torulaspora rosei</u> (Y-392)	110	5+		4+	4+	4+	4+	4+	3+	4+	4+	4+	4+	+	4+	4+	4+
<u>Torulopsis holzii</u> (Y-109)	180	5+		4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	2+	3+	4+	4+
<u>Zygosaccharomyces balanosbranis</u> (Y-564)	25	2+	5+	-	+	+	3+	3+	-	-	+	+	+	-	-	-	+
<u>Candida krusei</u> (NRRL Y-301)	62	5+		+	3+	3+	4+	4+	+	3+	3+	4+	4+	-	+	2+	3+
<u>Debaryomyces membranefaciens</u> var. <u>Hollandicus</u> (NFI-72)	2	+	5+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<u>Endomycopsis obovata</u> (Y-1)	180	5+		+	+	+	3+	3+	-	-	-	2+	2+	-	-	-	+

[#] Controls were inoculated with the highest dilution of inoculum (10^{-5}) in each instance.

Number of plus signs indicates the extent of turbidity observed; 5+ equals maximum turbidity; - equals no growth observed.

ulum, then it also occurred in the 10^{-5} dilution. The time required for growth to appear in the 10^{-5} dilution varied according to the reproduction rate of the particular yeast. Species of Torulaspora and Torulopsis which reproduce very quickly showed comparable growth in all dilutions tested within 4 days. Brettanomyces, Hansenula, Zygosaccharomyces and Endomycopsis species reproduce more slowly and required a longer time to show visible growth in the higher dilutions of inoculum. The species of Rhodotorula and Debaryomyces studied were inhibited regardless of the size of inoculum used. These results indicate that some factor other than size of inoculum was responsible for the inability of 0.1 per cent sorbic acid to control growth of these yeasts.

The Relationship of pH to Inhibitory Action of Sorbic Acid

The inefficiency of sorbic acid in inhibiting growth of yeasts at pH 6.0 even when the inoculum was greatly diluted suggested that sorbic acid, like benzoic acid, might be useless as an inhibitory agent in low acid media. This was, also, indicated in the results of Emard and Vaughn (4). To test the pH effect, the yeasts were inoculated into the basal medium with and without sorbic acid at three pH levels — 6.0, 5.5, and 5.0.

In Table 3 the growth of one culture of each species at 3 pH levels is compared after incubation for 7 days. Growth in all control tubes was heavy. At pH 6.0, as previously found, growth was evident in all cultures except those of Rhodotorula in the presence of 0.1 per cent sorbic acid. As the pH of the medium decreased, greater inhibition occurred. At pH 5.5 only 3 of the 10 species (Torulaspora rosei, Torulopsis holmii and Candida krusei) grew to the extent that turbidity could be detected by visual examination. All cultures were apparently inhibited completely at pH 5.0.

Realizing the inaccuracy of evaluating growth of subsurface yeasts by visual examination, turbidity measurements were made of tubes inoculated with these yeasts using a Cenco-Sheard-Sandford photelometer with a filter having a maximum central transmission of 610 m μ . The pH 5.0 and 6.0 series of tubes were thus measured and compared for per cent light transmission. The photelometer was standardized for each series of tubes against a tube of similar medium which had not been inoculated. Since growth in the control medium at all pH levels was heavy, readings were taken on the more acid series only. As seen in Table 4, the transmission of light through this medium inoculated with the different yeasts varied from 5 to 13.5 per cent. Photelometer readings on media containing 0.1 per cent sorbic acid and inoculated with these yeasts confirmed the find-

Table 3

Effect of pH on the Inhibition of Yeasts by 0.1 Percent Sorbic Acid

<u>Culture</u>	pH 6.0		pH 5.5		pH 5.0	
	<u>Control</u>	<u>0.1% S.A.</u>	<u>Control</u>	<u>0.1% S.A.</u>	<u>Control</u>	<u>0.1% S.A.</u>
<u>Brettanomyces versatilis</u> (Y-1146)	5+ [#]	2+	5+	-	5+	-
<u>Hansenula subpelliculosa</u> (Y-72)	5+	4+	5+	-	5+	-
<u>Rhodotorula sp.</u> (Y-35)	5+	-	5+	-	5+	-
<u>Torulaspore rosei</u> (Y-392)	5+	4+	5+	4+	5+	-
<u>Torulopsis holmii</u> (Y-109)	5+	4+	5+	3+	5+	-
<u>Zygosaccharomyces</u> <u>halomembranis</u> (YS-864)	5+	3+	5+	-	5+	-
<u>Candida krusei</u> (NRRL Y-301)	5+	4+	5+	2+	5+	-
<u>Debaryomyces membranefaciens</u> var. <u>Hollandicus</u> (NFI-72)	5+	2+	5+	-	5+	-
<u>Endomycopsis ohmeri</u> (FI-1)	5+	2+	5+	-	5+	-

[#] Number of plus signs indicates the extent of turbidity observed; 5+ indicates maximum turbidity; - indicates no growth observed.

All readings taken after 7 days incubation.

Table 4

Effect of pH on Inhibition of Yeasts by 0.1 Percent Sorbic Acid

<u>Culture</u>	<u>% Light Transmission*</u>		
	<u>Control</u> pH 5.0	<u>0.1% Sorbic Acid</u>	
		pH 5.0	pH 6.0
<u>Brettanomyces versatilis</u> (Y-146)	12.5	98.5	74.0
<u>Hansenula subpelliculosa</u> (Y-72)	6.0	99.0	84.0
<u>Rhodoturula</u> sp. (Y-35)	13.5	98.5	96.0
<u>Torulasporea rosei</u> (Y-392)	5.0	98.0	48.0
<u>Torulopsis caroliniana</u> (RY-199)	7.5	99.0	92.5
<u>Torulopsis holmii</u> (Y-109)	5.5	98.5	62.0
<u>Zygosaccharomyces halomembranis</u> (YS-864)	5.0	98.0	73.5

* Readings made after 7 days incubation

ings. At pH 6.0 all cultures with the possible exception of Rhodotorula grew under these conditions but to a lesser degree than in the absence of the inhibitory compound. At pH 5.0 no significant change in light transmission was observed. The loss of 1.0 to 1.5 per cent transmission was attributed to the added inoculum and to variations in the photelometer cells. These results show very definitely that the hydrogen ion concentration of the substrate is critical in the inhibition of yeast growth by sorbic acid.

The Inhibition of Yeasts by Sorbic Acid in the Presence of Sodium Chloride

Since these yeasts are primarily a problem in cucumber fermentations, it was thought advisable to test the effectiveness of sorbic acid in controlling their growth in a substrate containing similar salt concentrations.

Salt was added to culture media in concentrations of 5 and 10 per cent and the media then adjusted to pH 5.0. Growth determinations were again made by photelometer measurements for sub-surface yeasts and by visual examination for the film-forming species. Readings were taken at the time of inoculation and at 2,6,9,12,16,24,30 and 60 hours.

The results of this study may be noted in Tables 5 and 6 and growth patterns of representative fast growing and slow growing yeasts are shown in Figure 1. The addition

Table 5
Effect of 0.1 Percent Sorbic Acid on Yeast Cultures in a Medium Containing 5 Percent Salt (pH 5.0).

Culture	Control								% Light Transmission								0.1% S.A.							
	0	2	6	9	12	16	24	60	0	2	6	9	12	16	24	60	0	2	6	9	12	16	24	60
EUZETIOMYCES																								
<i>E. versatilis</i> (Y-146)	100	99.5	98.0	98.0	98.0	98.0	98.5	36.0	100	99.5	-	99.5	-	99.5	99.5	99.5	100	99.5	-	99.5	-	99.5	99.5	99.5
<i>E. versatilis</i> (Y-176)	100	99.5	98.5	98.5	98.5	98.5	98.5	36.0	100	99.5	-	99.5	-	99.5	99.5	99.5	100	99.5	-	99.5	-	99.5	99.5	99.5
HAEMIDIA																								
<i>H. subpelliculosa</i> (Y-72)	98.5	99.0	98.5	98.0	93.0	84.0	35.5	6.0	99.5	99.5	-	99.5	-	99.0	99.5	99.0	98.5	99.0	-	99.0	-	99.0	99.5	99.0
<i>H. subpelliculosa</i> (Y-58)	99.5	99.0	99.0	97.5	95.0	91.0	56.5	8.0	99.5	99.0	-	99.0	-	99.0	99.5	99.0	98.5	99.0	-	99.0	-	99.0	99.5	99.0
<i>H. subpelliculosa</i> (Y-64)	98.5	98.5	98.5	98.5	97.0	94.5	84.5	13.0	99.5	99.5	-	99.0	-	99.0	99.5	99.5	98.5	99.0	-	99.0	-	99.0	99.5	99.5
RHODOTORULA																								
<i>Rhodotorula</i> sp. (Y-35)	100	99.0	98.0	97.5	97.0	97.5	88.5	36.0	100	99.0	-	99.0	-	99.0	99.5	99.5	100	99.0	-	99.0	-	99.0	99.5	99.5
<i>Rhodotorula</i> sp. (Y-103)	100	99.0	98.5	98.0	96.0	88.5	74.0	35.5	100	99.5	-	99.5	-	99.0	99.5	99.5	100	99.5	-	99.5	-	99.0	99.5	99.5
TOBULASPORE																								
<i>T. rosei</i> (Y-392)	99.5	98.5	96.0	93.5	84.0	58.0	12.0	5.5	99.5	99.0	-	99.0	-	99.0	98.0	98.0	99.5	99.0	-	99.0	-	98.0	98.0	98.0
<i>T. rosei</i> (Y-407)	98.5	98.0	97.0	92.5	82.0	56.0	11.0	5.5	99.0	98.0	-	98.0	-	98.0	98.0	98.0	99.0	98.0	-	98.0	-	98.0	98.0	98.0
<i>T. rosei</i> (Y-287)	99.5	98.0	98.0	96.5	89.5	72.5	18.5	5.5	99.5	99.0	-	99.0	-	98.5	98.0	98.5	99.5	99.0	-	99.0	-	98.5	98.0	98.5
TOBULOPOPSIS																								
<i>T. holmii</i> (Y-109)	99.0	98.5	98.0	96.0	92.5	87.0	54.5	10.0	99.0	98.0	-	99.0	-	98.5	98.0	98.0	99.0	98.0	-	99.0	-	98.5	98.0	98.0
<i>T. holmii</i> (Y-277)	99.0	98.0	98.0	97.0	97.0	97.5	94.0	9.0	99.0	98.5	-	98.5	-	98.0	98.5	98.5	99.0	98.5	-	98.5	-	98.0	98.5	98.5
<i>T. holmii</i> (Y-413)	99.0	99.0	97.5	97.5	95.0	92.0	83.0	11.0	99.0	99.0	-	98.5	-	98.5	99.0	98.5	99.0	99.0	-	98.5	-	98.5	99.0	98.5
<i>T. caroliniana</i> (RY-199)	99.5	98.0	97.5	94.0	85.0	57.5	16.5	8.0	99.5	99.5	-	99.5	-	99.0	99.0	99.0	99.5	99.5	-	99.5	-	99.0	99.0	99.0
<i>T. caroliniana</i> (RY-200)	99.0	98.5	98.0	93.5	83.0	50.0	16.0	7.5	99.5	99.5	-	99.0	-	99.0	99.0	99.0	99.5	99.5	-	99.0	-	99.0	99.0	99.0
ZYGOSACCHAROMYCES s.s.g.																								
<i>Z. halomembranis</i> (Y-1000)	100	99.0	98.0	99.0	98.0	98.5	98.0	7.5	100	99.0	-	99.5	-	99.5	99.5	99.5	100	99.0	-	99.5	-	99.5	99.5	99.5
<i>Z. halomembranis</i> (YS-864)	100	98.5	98.5	99.0	98.5	98.5	98.5	7.5	100	99.5	-	99.5	-	99.5	99.5	99.5	100	99.5	-	99.5	-	99.5	99.5	99.5
<i>Zygosaccharomyces</i> sp. (YS-569)	100	99.5	99.0	98.5	98.5	99.0	98.5	65.0	100	99.5	-	99.5	-	99.0	99.5	99.5	100	99.5	-	99.5	-	99.0	99.5	99.5

Table 6
Effect of 0.1 Percent Sorbic Acid on Yeast Cultures in a Medium Containing 10 Percent Salt (pH 5.0)

Culture	% Light Transmission													
	Control hours						0.1% S.A. hours							
	0	2	9	12	16	30	60	0	2	9	12	16	30	60
BRETTANOMYCES														
<u>B. versatilis</u> (Y-116)	99.5	98.5	98.5	99.0	99.0	98.5	39.5	100	-	98.5	-	99.0	98.5	99.0
<u>B. versatilis</u> (Y-176)	99.5	100	99.0	99.0	98.5	98.5	41.0	99.5	-	98.5	-	99.0	99.0	99.0
HANSENULA														
<u>H. subpelliculosa</u> (Y-72)	98.0	98.0	98.0	98.5	97.5	81.5	11.5	98.0	-	98.0	-	98.0	98.0	98.0
<u>H. subpelliculosa</u> (Y-58)	99.0	99.0	98.5	99.5	97.0	82.5	10.5	98.0	-	98.0	-	98.0	98.0	98.0
<u>H. subpelliculosa</u> (Y-64)	99.0	99.0	98.0	98.0	97.0	97.0	46.5	98.0	-	97.5	-	98.0	98.0	98.0
RHODOTORULA														
<u>Rhodotorula</u> sp. (Y-35)	99.5	99.0	98.0	98.0	98.0	86.0	72.0	99.5	-	97.0	-	97.5	97.5	97.5
<u>Rhodotorula</u> sp. (Y-103)	99.5	99.5	97.0	97.0	95.0	85.0	61.0	100	-	98.0	-	98.0	98.0	98.0
TORULASPORA														
<u>T. rosei</u> (Y-392)	98.0	96.0	94.0	83.0	69.0	39.5	7.0	98.0	-	98.0	-	98.0	98.0	98.0
<u>T. rosei</u> (Y-407)	98.0	98.0	97.5	89.0	78.0	28.5	7.0	98.5	-	98.0	-	98.0	98.0	98.0
<u>T. rosei</u> (Y-287)	98.5	98.0	97.5	95.0	91.0	57.5	7.0	98.5	-	98.0	-	98.0	98.5	98.0
TORULOPSIS														
<u>T. holmii</u> (Y-109)	97.0	94.0	94.0	96.0	93.5	94.0	95.0	97.5	-	98.0	-	98.0	98.0	98.0
<u>T. holmii</u> (Y-277)	97.5	97.0	97.0	97.0	96.0	97.0	95.5	98.0	-	96.5	-	97.0	97.0	97.0
<u>T. holmii</u> (Y-413)	97.5	97.0	97.0	96.0	95.0	96.0	95.5	98.0	-	97.5	-	98.0	97.5	98.0
<u>T. caroliniana</u> (RY-199)	98.0	97.0	91.0	84.5	60.0	18.0	9.0	98.0	-	97.5	-	97.5	97.5	97.5
<u>T. caroliniana</u> (RY-200)	98.0	97.0	89.0	75.0	45.0	16.5	9.0	98.0	-	96.5	-	97.0	97.0	97.0
ZYGOSACCHAROMYCES s.g.														
<u>Z. balneombrantis</u> (Y-1000)	99.0	99.0	98.5	99.0	97.5	96.0	25.0	99.5	-	99.0	-	99.0	99.0	99.0
<u>Z. balneombrantis</u> (YS-864)	99.0	99.5	98.5	99.0	97.0	96.0	21.0	99.0	-	98.5	-	98.5	98.5	99.0
<u>Z. sp.</u> (YS-569)	99.5	99.5	98.5	99.0	98.0	98.0	96.5	99.0	-	98.5	-	99.0	98.5	99.0

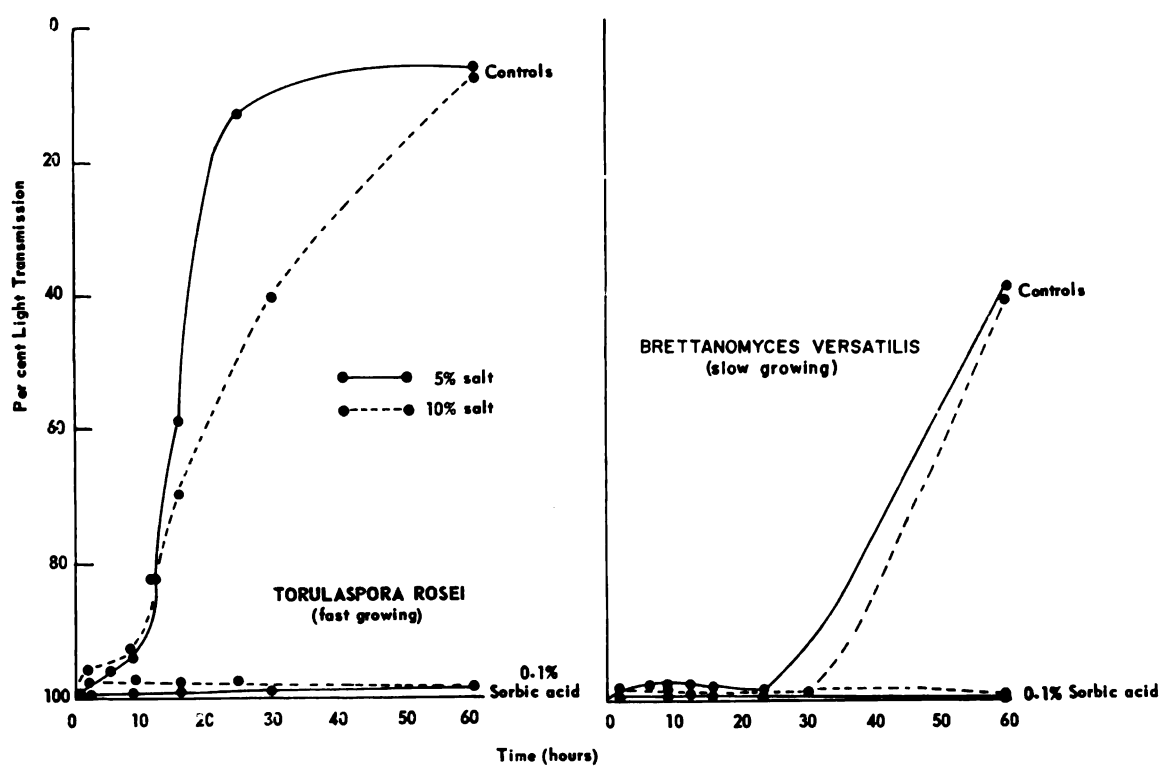


Fig. 1. Effect of 0.1 Per cent Sorbic Acid on Representative Yeast Cultures in Media Containing Salt

of salt did not reduce the inhibitory capacity of sorbic acid. Complete inhibition of all yeasts occurred in the presence of both salt concentrations in the sorbic acid medium. Also it was observed that the presence of 5 per cent salt in the dextrose broth without sorbic acid offered no retarding effect on the growth of these yeasts. With the exception of Brettanomyces and Zygosaccharomyces species growth of all yeasts was observed between 9 and 24 hours of incubation. Under similar conditions, but with the salt increased to 10 per cent, the growth pattern of most yeasts was similar but somewhat slower. Candida krusei and Torulopsis holmii, however, failed to grow in the higher salt concentration. Etchells and Bell (5) stated that the former yeast is susceptible to high salt concentrations with 10 per cent possibly the maximum concentration permitting growth. Of greater interest perhaps was the fact that all 3 cultures of Torulopsis holmii failed to grow in the higher salt broth. According to Etchells et al. (6) and Costilow and Fabian (1) this yeast predominates in the early fermentation of cucumbers. These results indicate that this may be due to its inability to tolerate the higher salt concentrations of the later stages. At any rate these findings indicated that salt did not interfere with the inhibitory action of the sorbic acid and, in some instances, may have aided the sorbic acid by exerting an inhibitory action of its own.

The Effect of Different Concentrations of Sorbic Acid
on Inhibition of Yeasts

Phillips and Mundt (14) stated that 0.1 per cent sorbic acid in pickle brines effectively controlled film-forming yeasts whereas 0.05 per cent failed to do so. Assuming the 0.1 per cent concentration equally effective against subsurface spoilage yeasts, its use would serve to eliminate some of the hazards of cucumber curing. However, the cost of adding this amount to large vats of cucumbers would discourage its use by some pickle manufacturers.

The fact that 0.1 per cent sorbic acid in the basal medium adjusted to pH 5.0 so effectively inhibited all these yeasts suggested that in the more acid pickle brines with a pH range of 3.9 to 4.3 within 3 days after brining and containing 7 to 8 per cent salt a lesser concentration might serve as efficiently. This possibility was investigated by comparing the inhibitory effect of 0.05 per cent sorbic acid in a medium containing 8 per cent salt and adjusted to pH 4.5. Again growth of subsurface yeasts was determined by photometer measurements and of film-forming yeasts by visual examination.

This experiment showed that both 0.05 and 0.1 per cent sorbic acid completely inhibited all yeasts tested under these conditions. The results were quite similar to those in Table 6. Representative results for two of the yeasts

these conditions. The results were quite similar to those in Table 6. Representative results for two of the yeasts are given in Table 7 which shows complete inhibition by both 0.05 and 0.1 per cent sorbic acid.

After 30 days incubation, the flasks containing sorbic acid medium were re-inoculated with the yeasts and incubated for an additional 30 days. No growth was evident in any of the flasks.

Survival of Yeasts in Presence of Sorbic Acid

This phase of study was carried out to determine the type of effect which sorbic acid has on yeast cells—fungistatic or fungicidal. A 1 ml. inoculum of a 24 hour culture of each yeast was inoculated into 3 flasks of dextrose broth plus 8 per cent NaCl (pH 4.5). One flask contained 0.05 and one 0.1 per cent sorbic acid and the other served as a control. Viable cell counts were made by the standard plate count and most probable numbers techniques.

Tables 8 and 9 show similar results obtained by plate counting and most probable numbers techniques. In the control tubes the numbers of yeasts increased in a manner typical of a normal growth curve. Flasks and tubes containing sorbic acid showed a reduction in viable yeast cells for both methods of counting. As seen in the plate counting method 0.1 per cent sorbic acid reduced the yeast count more

Table 7

Effect of Different Concentrations of Sorbic Acid on Growth of Yeasts

<u>Per cent Light Transmission</u>						
<u>Time</u>	<u>T. rosei</u>			<u>B. versatilis</u>		
	<u>Control</u>	<u>0.05%</u>	<u>0.1%</u>	<u>Control</u>	<u>0.05%</u>	<u>0.1%</u>
Initial	99.0	99.0	99.0	100	99.5	99.5
4 hours	98.5	99.0	99.0	100	100	99.0
8 hours	98.5	99.0	97.5	99.5	99.0	98.5
12 hours	93.5	98.0	98.0	99.0	98.0	98.0
16 hours	87.0	99.0	98.0	93.5	98.5	99.0
24 hours	51.5	97.5	97.5	86.5	97.5	99.0
35 hours	13.0	98.0	98.0	58.5	98.5	99.0
48 hours	8.5	98.0	97.5	16.0	98.5	99.0
60 hours	6.0	98.0	98.0	8.0	98.0	98.0
120 hours	5.5	97.5	98.0	4.0	98.5	98.5

Table 8

Survival of Yeasts in Media Containing Different Concentrations of Sorbic Acid as Determined by Standard Plate Counting Procedures

Culture	Treatment	T i m e						
		Initial (x 10 ³)	6 hrs (x 10 ³)	12 hrs (x 10 ³)	1 day (x 10 ³)	3 days (x 10 ⁶)	5 days (x 10 ⁶)	7 days (x 10 ⁶)
<u>Brettanomyces</u> <u>versatilis</u> Y-116	Control	.3	.3	.4	1.8	1.4	21	40
	0.05% S.A.	.3	0	0	0	0	0	0
	0.1% S.A.	.2	0	0	0	0	0	0
<u>Hansenula</u> <u>subpelliculosa</u> Y-72	Control	15	20	84	620	52	33	6
	0.05% S.A.	8	1.2	.3	.1	0	0	0
	0.1% S.A.	10	.1	0	0	0	0	0
<u>Rhodotorula</u> sp. Y-35	Control	11.4	46	83	880	5.4	15	22
	0.05% S.A.	8.4	1.4	1	.1	0	0	0
	0.1% S.A.	7.5	.7	0	0	0	0	0
<u>Torulaspora</u> <u>rosei</u> Y-392	Control	8.8	33	100	102	16	38	16
	0.05% S.A.	8.6	3.8	3.5	1.7	0	0	0
	0.1% S.A.	8.3	4.0	.5	.1	0	0	0
<u>Torulopsis</u> <u>caroliniana</u> RY-199	Control	23	78	680	9,300	25.8	.04	.0006
	0.05% S.A.	21	18	19	17.6	.01	.0006	0
	0.1% S.A.	19.6	21	16	8	0	0	0
<u>Torulopsis</u> <u>holmii</u> Y-109	Control	3.6	4.7	14.5	64	20	7.2	.19
	0.05% S.A.	2.2	0	0	0	0	0	0
	0.1% S.A.	1.2	0	0	0	0	0	0
<u>Zygosaccharomyces</u> <u>halomembranis</u> YS-864	Control	.3	1.3	11.5	78	8.3	3.7	1.8
	0.05% S.A.	.2	.2	.2	.1	0	0	0
	0.01% S.A.	.3	.3	.1	0	0	0	0
<u>Candida</u> <u>krusei</u> NRRL Y-301	Control	45	31	32	50	1.5	8.8	4
	0.05% S.A.	50	18	11.8	1	0	0	0
	0.1% S.A.	28	17	1	.1	0	0	0
<u>Debaryomyces</u> <u>membranaefaciens</u> var. <u>Hollandicus</u> NFF-72	Control	.9	.3	3	8	.34	1.6	7
	0.05% S.A.	1	.2	0	0	0	0	0
	0.1% S.A.	.5	0	0	0	0	0	0
<u>Endomycopsis</u> <u>ohmeri</u> FY-1	Control	120	143	570	5,300	77	53	54
	0.05% S.A.	80	17	3.3	.2	0	0	0
	0.1% S.A.	94	3.7	.1	0	0	0	0

Table 9

Survival of Yeast Cells in Medium Containing 0.1 Per cent Sorbic Acid
Using M.P.N. Technique

<u>Culture</u>	<u>Treatment</u>	T i m e				
		<u>Initial</u> ($\times 10^3$)	<u>1 day</u> ($\times 10^3$)	<u>3 days</u> ($\times 10^6$)	<u>5 days</u> ($\times 10^6$)	<u>8 days</u> ($\times 10^6$)
<u>Brettanomyces</u> <u>versatilis</u> <u>Y-116</u>	Control 0.1% S.A.	25 25	430 0	25 0	.92 0	.25 0
<u>Hansenula</u> <u>subpelliculosa</u> <u>Y-72</u>	Control 0.1% S.A.	43 92	14,700 0	14.7 0	92 0	43 0
<u>Rhodotorula</u> sp. <u>Y-35</u>	Control 0.1% S.A.	9.2 7.4	9,300 0	.92 0	.025 0	.0043 0
<u>Torulaspora</u> <u>rosei</u> <u>Y-392</u>	Control 0.1% S.A.	92 43	9,200 25	25 0	25 0	14.7 0
<u>Torulopsis</u> <u>caroliniana</u> <u>RY-199</u>	Control 0.1% S.A.	920 430	4,300 0	25 0	43 0	.147 0
<u>Torulopsis</u> <u>holmii</u> <u>Y-109</u>	Control 0.1% S.A.	92 92	92,000 25	430 0	250 0	25 0
<u>Zygosaccharomyces</u> <u>halomembrans</u> <u>Y5-864</u>	Control 0.1% S.A.	2.5 2.5	250 0	25 0	9.2 0	2.5 0
<u>Candida krusei</u> <u>NRRL Y-301</u>	Control 0.1% S.A.	4.3 9.2	25,000 2.5	92 0	43 0	92 0
<u>Debaryomyces</u> <u>membranefaciens</u> <u>var. Hollandicus</u> <u>RFY-72</u>	Control 0.1% S.A.	.9 .4	2.5 0	.025 0	.043 0	.043 0
<u>Endomycopsis</u> <u>ohmeri</u> <u>FY-1</u>	Control 0.1% S.A.	43 43	25,000 0	147 0	43 0	74 0

duction in viable yeast cells as compared with normal growth curves is illustrated in Figure 2 for cultures of Torulopsis caroliniana and Brettanomyces versatilis. The former yeast was apparently the most resistant and the latter the most susceptible to sorbic acid.

To determine whether the lack of growth was due to the absence of viable cells or to fungistatic action of sorbic acid carried over into the growth medium, further studies were made. Tubes used in the M.P.N. technique in the 8th day planting which contained 1, 0.1, 0.01 and 0.001 ml. inoculum respectively of the dextrose broth cultures of Torul- aspora rosei and Candida krusei were emptied into Petri plates and the plates poured heavily with dextrose agar. Plates were incubated at 30°C for 4 to 6 days after which time it was found that all plates were completely void of growth. This indicated that the yeast cells were destroyed rather than merely inhibited by residual sorbic acid.

Fate of Sorbic Acid in Media Inoculated with Yeasts

Melnick et al. (13) noted that sorbic acid was oxidized by air and its loss due to metabolic degradation by molds in cheese was complete when the ratio of molds to sorbic acid was initially high. A parallel case with yeasts in cucumber brines would not normally exist since the initial load of yeast cells would be small and the sorbic acid concentrat-

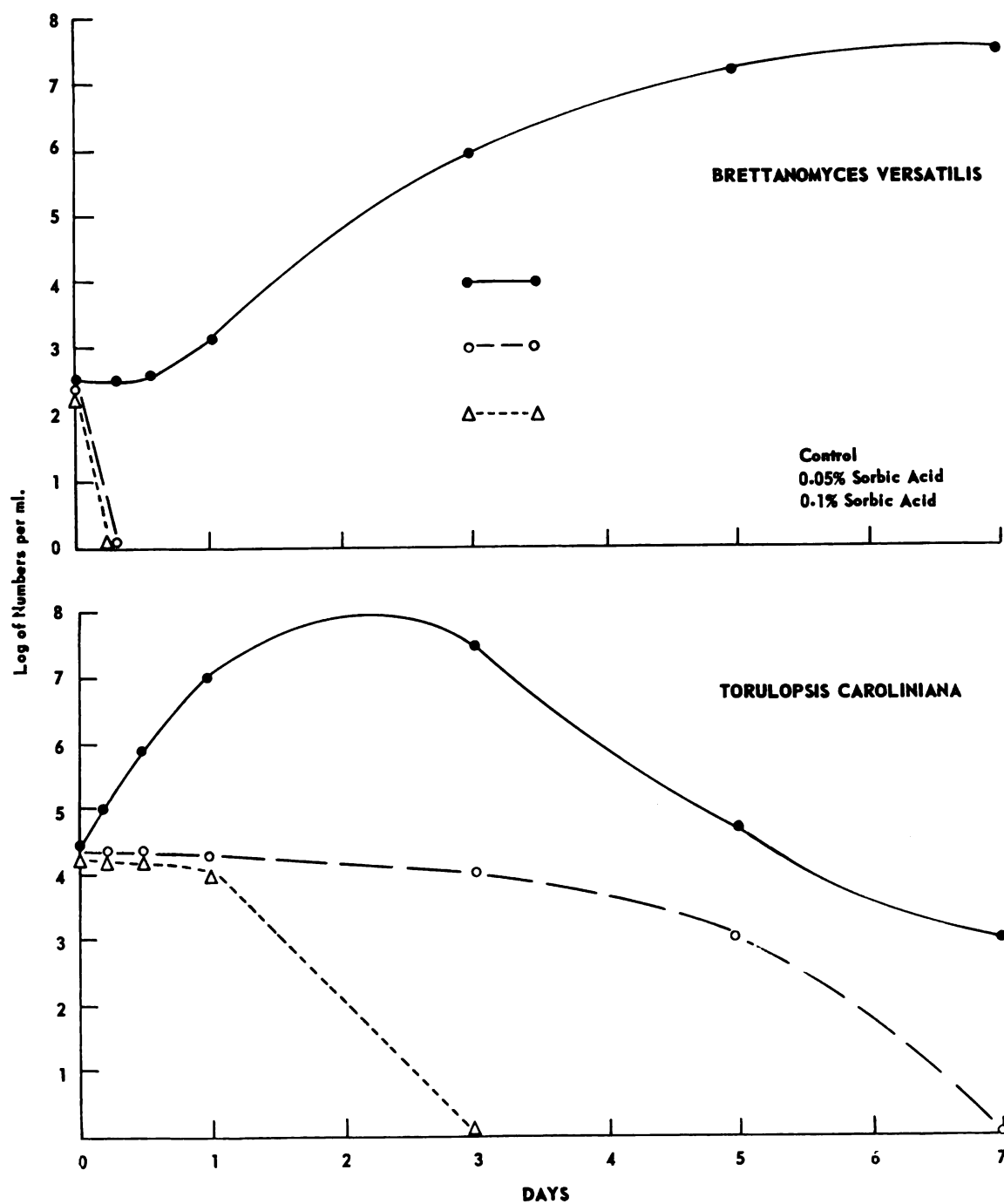


Fig. 2 Survival of Yeasts in the Presence of Sorbic Acid

ion would be uniform throughout the brine. However, to determine whether or not there was any loss of sorbic acid due to oxidation during incubation or by a normal inoculum of yeasts sorbic acid determinations were made of the following media;

1. Freshly prepared basal medium (dextrose broth) without sorbic acid; pH 4.60.
2. Freshly prepared medium containing 0.1 per cent sorbic acid; pH 4.51.
3. Sterile medium incubated 3 weeks at 30°C which contained initially 0.1 per cent sorbic acid; pH 4.63.
4. Medium similar to 3 except inoculated with 3 drops of an active culture of Torulopsis holmii per 100 ml. medium prior to incubation.

Measurements were made on a 1:250 dilution of each of the above media by the spectrophotometric method of Melnick and Luckmann (10). This method involves the plotting of ultraviolet absorbancy readings of the different media for a series of wavelengths with the maximum absorbancy readings converted to concentration of sorbic acid.

Results of these studies on the samples listed above are given in Table 10 and the absorbancy readings in Figure 3. Results from medium 1 give irrelevant sorbic acid readings since no sorbic acid as such had been added to this sample. Therefore, this reading was subtracted from absorb-

Table 10
Effect of Time and Organisms on Retention of Sorbic
Acid in Culture Media

<u>Sample #</u>	<u>Age of Medium</u>	<u>pH</u>	<u>Initial Sorbic Acid Content</u>	<u>Inoculum</u>	<u>Calculated % Sorbic Acid</u> *
1	Fresh	4.60	0.0%	none	.0025
2	Fresh	4.51	0.1%	none	.105
3	3 weeks	4.63	0.1%	none	.106
4	3 weeks	4.66	0.1%	<u>Torulopsis holmii</u>	.116

* Concentrations of sorbic acid were calculated from maximum absorbance at 258 - 260 mμ.

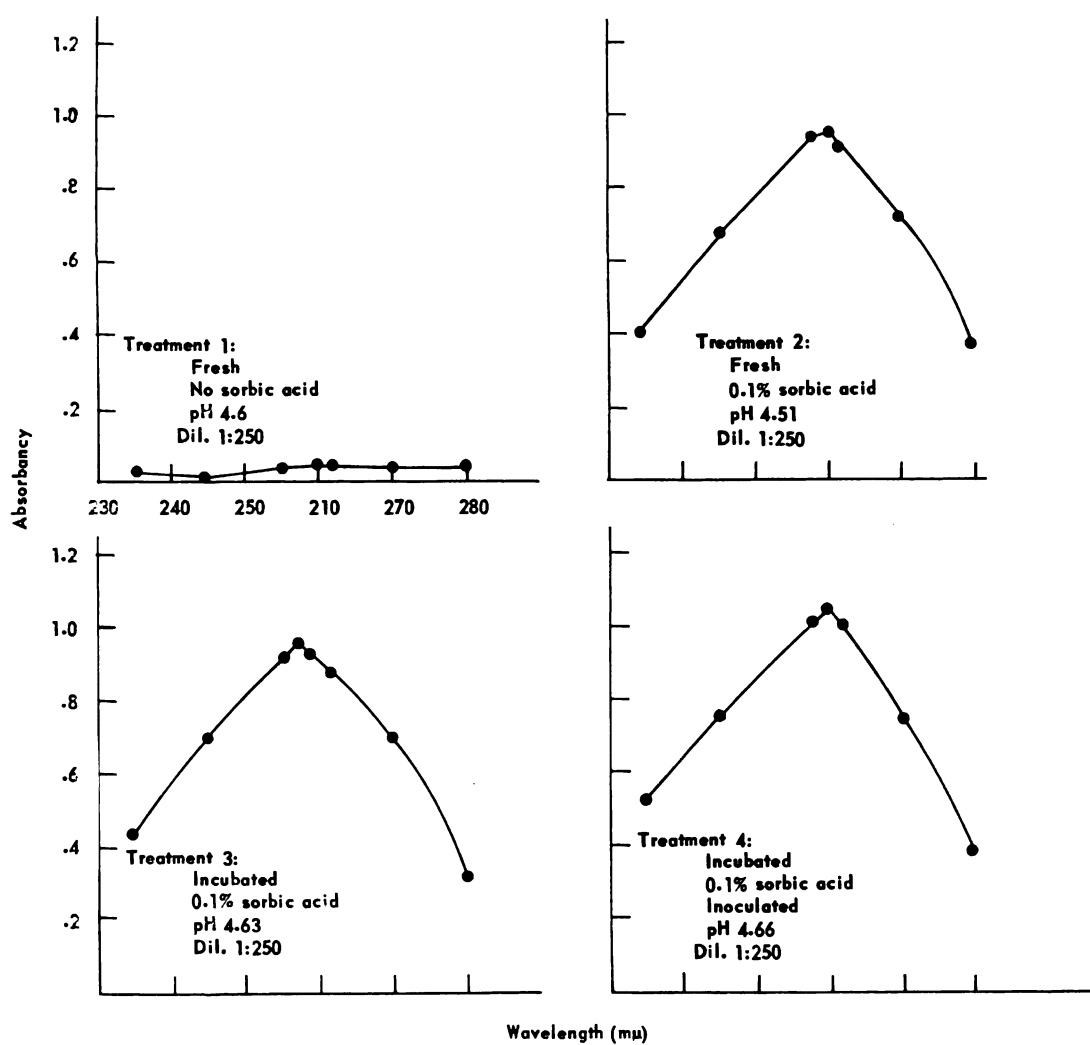


Fig. 3 Ultraviolet Absorption Curves of Media

readings since no sorbic acid as such had been added to this sample. Therefore, this reading was subtracted from absorbancy readings for the other media to correct for this error.

According to the method of calculating percentage sorbic acid from these ultraviolet light absorbancy readings as used by Melnick and Luckmann (10) it was apparent that no loss of sorbic acid occurred during incubation in any of the media. In fact concentrations of sorbic acid appeared slightly higher than those added. This apparent increase may have been due to inaccurate weighing of ingredients and to evaporation of water in the process of media sterilization. Further evaporation may have occurred in flasks (3) and (4) during the 3 week incubation period at 30 C.

DISCUSSION

Results of these studies are in complete agreement with reports of Emard and Vaughn (4) who state that the pH of the substrate is of major importance in the inhibition of yeasts by sorbic acid. However, in cucumber fermentations this factor requires no consideration since the hydrogen ion concentration of a cucumber brine reaches pH 4.0 or possibly lower within 2 or 3 days after brining. Yeast activity during these first few days is negligible in any case.

In contrast to reports by Phillips and Mundt (14) that 0.05 per cent sorbic acid was ineffective in the control of surface yeasts in fermentation brines, this concentration of sorbic acid in a medium containing 7.5 to 8 per cent salt was found to be as effective in inhibiting yeast growth as the 0.1 per cent concentration of sorbic acid.

That sorbic acid is fungistatic to yeasts, as Melnick et al. (13) found it was to molds, is unquestionable in media of pH 5.0 or lower. Survival studies of these yeasts in media containing 0.1 and 0.05 per cent sorbic acid showed a complete absence of viable cells in both media by the 7th day. The reduction, although faster in the 0.1 per cent concentration, was rather slow to claim much in the way of fungicidal qualities although Smith and Rollin (17) have

reported that 0.05 to 0.066 per cent sorbic acid in cheese actually kills molds.

Spectrophotometric studies showed that there was no loss of sorbic acid in media either from oxidation or yeast inoculation under the conditions of these experiments. Again this is in contrast to reports of Melnick et al. (12) who found sorbic acid oxidizable by air to form peroxides and aldehydes. These same workers (13) also found a degradation of sorbic acid when the mold population was high. Apparently yeast cultures do not use sorbic acid as a source of carbon which, according to York and Vaughn (19), some organisms are capable of doing.

Based on the excellent inhibition of yeasts obtained by the low concentrations of sorbic acid used in these studies and the apparent stability of sorbic acid under these conditions it would appear that its use in cucumber fermentations is practical and desirable.

SUMMARY AND CONCLUSIONS

The effectiveness of sorbic acid in the inhibition of yeasts normally associated with cucumber fermentations was primarily dependent on the hydrogen ion concentration of the substrate. At pH 6.0 sorbic acid and its sodium salt were ineffective in the control of yeasts although reproduction was slowed considerably. The size of inoculum was of minor significance since growth occurred in most instances whether this inoculum contained a few hundred or several million yeast cells. At pH 5.5 most yeasts were inhibited by a 0.1 per cent concentration of sorbic acid and at pH 5.0 or lower all yeasts were completely inhibited. At these lower pH levels a lower concentration (0.05 per cent) of sorbic acid proved equally effective.

The survival of these pure cultures of yeasts in media containing 0.05 per cent and 0.1 per cent sorbic acid respectively at 30°C showed that the death rate of most species tested was slower in the lower concentration but within 7 days viability was zero in both instances. Further platings from these media were void of growth indicating that the yeasts were non-viable rather than inhibited by residual sorbic acid.

Common salt in concentrations of 5, 8 and 10 per cent did not lessen the efficiency of the sorbic acid. In fact it was found that the 10 per cent concentration of salt exerted an inhibitory action of its own on pure cultures of Candida krusei

fact, it was found that the 10 per cent concentration of salt exerted an inhibitory action of its own on pure cultures of Candida krusei and Torulopsis holmii. Further studies are indicated on the effect of various concentrations of salt and sorbic acid in combination.

The effectiveness of sorbic acid in inhibiting yeast growth apparently is not transitory. Spectrophotometric studies showed that no loss of sorbic acid occurred in cotton-stoppered flasks of sterile or inoculated media over a 3-week period at room temperature. Furthermore, flasks of media of pH 5.0 containing sorbic acid and inoculated with pure cultures of the individual yeasts showed no growth of any yeast in the presence of either 0.05 per cent or 0.1 per cent sorbic acid after incubation for 30 days at 30°C. No growth was evident even after reinoculation and a further 30 days incubation period of these flasks.

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