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Microbial Degradation of Sorbic Acid

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MICROBIAL DEGRADATION OF SORBIC ACID

By

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ABSTRACT

MICROBIAL DEGRADATION OF SORBIC ACID

By

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The main objective of the study was to investigate the microbial decomposition of sorbic acid in cultural media. Initial screening tests on selected microorganisms were conducted to ascertain their resistance to sorbate, and to select some for further studies.

Of the fungi tested, the molds Aspergillus flavus, Aspergillus niger, Penicillium sp., Fusarium sp., Trichoderma viride, and Geotrichum candidum and the yeasts Candida lipolytica, Hansenula saturnus, Brettanomyces claussenii and Schwanniomyces occidentalis exhibited relatively high resistance to sorbate. These fungi were not inhibited by 3000 mg/kg of potassium sorbate at pH 5.5 on YPG medium.

Of the bacteria tested, Lactobacillus bulgaricus, Lactobacillus plantarum, Pediococcus cerevisiae and a strain of Pseudomonas fluorescens were the most resistant. Lactic acid bacteria were inhibited by 3000 mg/kg of potassium sorbate on YTG medium at a pH of 5.5.

All of the sorbate-resistant molds apparently were able to utilize sorbate as a carbon source. The yeasts Candida lipolytica and Hansenula californica and the bacteria Pseudomonas vulgaris, Staphylococcus aureus and Salmonella senftenberg also used sorbate as a carbon source.

Growth of some microbes which utilized sorbate as a carbon source was retarded by the presence of glucose beside sorbate. This phenomenon was designated as the "glucose inhibitory effect."

The fate of sorbate in media inoculated with some selected microbes was followed both qualitatively and quantitatively by chromatographic

procedures. Data indicate that the extent of depletion of sorbate varied, ranging from no significant loss with the lactic acid bacteria to total loss with molds such as Penicillium sp., Trichoderma viride, Rhizopus stolonifer and yeasts such as Candida lipolytica. All Penicillium and Aspergillus species and Trichoderma viride depleted sorbate when present in sub-lethal concentrations. 1,3-Pentadiene has been shown to be associated with growth of the above molds on sorbate-containing media.

Sorbaldehyde, sorbyl alcohol, and 4-hexenol were produced by species of Rhizopus, Mucor and some species of Fusarium. Sorbaldehyde was detected only while sorbate was still in the system. Sorbyl alcohol was produced in the early stages of incubation. 4-Hexenol was detected in high amounts at late stages of growth. Therefore, it appears that sorbic acid is reduced to sorbaldehyde which is in turn reduced to sorbyl alcohol. This alcohol is further reduced to 4-hexenol.

4-Hexenoic acid and trace amounts of ethyl sorbate were produced by Geotrichum candidum, a yeast-like mold, and yeasts such as Candida lipolytica.

Clostridium perfringens, Clostridium sporogenes and Clostridium tertium were grown on a prereduced medium containing 1000 mg/kg of sorbate with a pH of 6.0. All of these Clostridia grew well on this medium. Clostridium sporogenes and Clostridium tertium metabolized sorbate principally to 4-hexenoic acid, 4-hexenol and sorbyl alcohol. Clostridium perfringens apparently did not metabolize sorbate.

In order to confirm that the above metabolites originated from sorbic acid, [U-^{14}C]-labelled sorbic acid was introduced into the media. Data obtained indicate that sorbic acid was the precursor of these metabolic products.

To my late father, Saad Al-Mohizea

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TABLE OF CONTENTS

	Page
LIST OF TABLES	vi
LIST OF FIGURES	vii
INTRODUCTION	1
REVIEW OF LITERATURE	3
Physical and Chemical Properties of Sorbic Acid	3
Sorbic Acid as an Antimicrobial Agent	4
Nitrites and Sorbates in Food Systems	7
Effect of pH on Antimicrobial Properties of Sorbic Acid	11
Mechanism of Inhibition	12
Physiological Properties of Sorbic Acid	14
Decomposition of Sorbic Acid	16
1. Chemical degradation of sorbic acid	16
2. Microbial degradation of sorbic acid	18
MATERIALS AND METHODS	26
Microorganisms	26
Screening Tests on Molds, Yeasts and Bacteria	26
Media preparation	26
Incubation conditions	27
Growth evaluation	28
Utilization of Potassium Sorbate as a Carbon Source	28
Growing Cells Studies	28
Non-Growing (Resting) Cells Studies	29
Sample Preparation for GC Analysis	31
Extraction of metabolites	31
Preliminary concentration	34
Esterification and final concentration	34
Headspace Analysis	34
Entrapment of Pentadiene	35
Gas Liquid Chromatographic Analysis of Sorbic Acid Metabolites	35
Apparatus and operation conditions	35
Gas Liquid Chromatography-Mass Spectrometry (GC-MS)	36
Quantitative Analysis of Residual Sorbate by High Performance Liquid Chromatography	36
Preparation of sample	36
High performance liquid chromatography conditions	37
Fate of Labelled Sorbic Acid	38
Thin layer chromatography of neutral metabolites of sorbic acid	38
Further confirmation by GC Analysis	39

Page

RESULTS AND DISCUSSION 40

Microbial Resistance to Sorbic Acid 40
Molds 40
Yeasts 43
Bacteria 47
Microbial Degradation of Sorbic Acid 50
Sorbic Acid as a Carbon Source 55
Metabolites of Sorbic Acid 61
Sorbic acid metabolism by molds 61
Sorbic acid metabolusm by yeasts 81
Sorbic acid metabolism by bacteria 85
Fate of Labelled Sorbic Acid 88

SUMMARY AND CONCLUSIONS 89

RECOMMENDATIONS FOR FURTHER STUDIES 92

APPENDICES 94

BIBLIOGRAPHY 98

LIST OF TABLES

Table		Page
1.	Inhibitory concentrations of potassium sorbate for some mold cultures on YPG plates at pH 5.5, 25°C	41
2.	Inhibitory concentrations of potassium sorbate for some yeasts on YPG plates at pH 5.5, 25°C	45
3.	Inhibitory concentrations of potassium sorbate for some bacterial cultures on YTG broth at pH 5.5, 25°C	48
4.	The effect of the presence or absence of glucose (two percent) on the growth of different mold cultures on yeast extract-peptone-agar media (pH 6.0) containing 3000 mg/kg of potassium sorbate and incubated at 25°C for six days	56
5.	The effect of the presence or absence of glucose (two percent) on the growth of yeast cultures on yeast extract-peptone-agar media (pH 6.0) containing 3000 mg/kg of potassium sorbate and incubated at 25°C for six days	58
6.	The effect of the presence or absence of glucose (two percent) on the growth of bacterial cultures on yeast extract-peptone-agar media (pH 6.0) containing 3000 mg/kg of potassium sorbate and incubated at 25°C for six days	60
A.1.	Mold cultures used for screening tests	94
A.2.	Yeast cultures used for screening tests	95
A.3.	Bacterial cultures used for screening tests	97

LIST OF FIGURES

Figure		Page
1.	Wieland's scheme for fatty acid oxidation (Melnick et al., 1954)	22
2.	Flow chart diagram for the isolation and purification of acidic and neutral metabolites of sorbic acid	33
3.	Utilization of sorbate by growing cells of different fungi cultures cultivated on YPG containing 1000 mg/kg of potassium sorbate, at pH 5.5, and an incubation temperature of 25°C	52
4.	Utilization of sorbate by resting cells of different fungi cultures suspended in a phosphate buffer containing 2.0 percent glucose and 1000 mg/kg of potassium sorbate, at pH 5.5 and an incubation temperature of 25°C	53
5.	A partial gas chromatogram of the neutral metabolites of sorbate-grown Rhizopus oligosporus	62
6.	Mass spectrum of 4-hexenol (retention time, 13.0 minutes) isolated from a medium containing sorbate and inoculated with Rhizopus oligosporus	64
7.	Mass spectrum of 4-hexenol (retention time, 13.2 minutes) isolated from a medium containing sorbate and inoculated with Rhizopus oligosporus	65
8.	Mass spectrum of 2,4-hexadienol (retention time, 17.5 minutes) isolated from a medium containing sorbate and inoculated with Rhizopus oligosporus	66
9.	Mass spectrum of 2,4-hexadienol (retention time, 18.1 minutes) isolated from a medium containing sorbate and inoculated with Rhizopus oligosporus	67
10.	Mass spectrum of 2,4-hexadienol isolated from a medium containing sorbate and inoculated with Rhizopus oligosporus	68
11.	Mass spectrum of methyl ester of 4-hexenoic acid isolated from a medium containing sorbyl alcohol and inoculated with Rhizopus oligosporus	71

Figure		Page
12.	A partial gas chromatogram of both neutral and acidic metabolites of sorbaldehyde-grown Mucor sp.	72
13.	Mass spectrum of methyl ester of sorbic acid isolated from a medium containing sorbaldehyde and inoculated with Mucor sp.	73
14.	Mass spectrum of 1,3-pentadiene isolated from a medium containing sorbate and inoculated with Penicillium sp.	77
15.	GC chromatogram of both neutral and acidic metabolites of sorbate-grown Geotrichum candidum	79
16.	Mass spectrum of ethyl sorbate isolated from a medium containing sorbate and inoculated with Geotrichum candidum	80
17.	A partial gas chromatogram of both neutral and acidic metabolites of sorbyl alcohol-grown Candida lipolytica	82
18.	A partial gas chromatogram of both neutral and acidic metabolites of sorbate-grown Clostridium sporogenes	86

INTRODUCTION

Sorbic acid and its potassium salt are very popular food additives and are used routinely by certain elements of the food industry. Sorbates have been cleared for use and were approved as food preservatives by the United States Food and Drug Administration almost a quarter of a century ago (Anonymous, 1978). Sorbates are utilized in more than 70 food products designated by standards of identity (Anonymous, 1978). Sorbates are officially listed as GRAS food additives by the Food and Drug Administration, which means that they are "generally recognized as safe." Consequently, sorbates may be used freely, provided that the quantities do not exceed the amount reasonably required to achieve the desired function (inhibition of microorganisms), and that the application is also in conformance with state or local regulations.

With the growing concern over the use of nitrites in cured meat systems, and with the possibility that nitrite may be partially replaced by sorbate in meat systems, sorbates are attracting considerable attention. As a result, sorbates are undergoing intensive studies to justify the usefulness of their new applications. However, there are unexplained problems associated with the application of sorbic acid and its potassium salt in the food industry. One major problem is the loss or degradation of sorbic acid over a relatively short period of time. It is well established that there are some sorbic acid-resistant microorganisms which can grow in the presence of this acid, and, in some cases, metabolize it. As a

consequence of this partial or total degradation of sorbic acid, the protection of foods against food spoilage microorganisms is decreased. Hence, microbial degradation of sorbic acid has been cited in many cases for the loss of this preservation activity.

It is the scope of the present study to investigate some aspects of the microbial degradation of sorbic acid, using some representative microbes. In order to accomplish the main goal, the following objectives were considered:

1) To investigate microbial resistance to sorbic acid.
2) To ascertain which microorganisms utilize sorbic acid as a sole source of carbon.
3) To study the rate of sorbic acid decomposition, in cultural media or buffer systems by growing and/or resting cells and to identify the metabolites thus formed.
4) To follow the fate of labelled sorbic acid to confirm that the metabolites originate from sorbic acid.
5) Finally, to attempt to categorize microorganisms according to their behavior towards sorbic acid.

REVIEW OF LITERATURE

Physical and Chemical Properties of Sorbic Acid

Sorbic acid is a naturally occurring food acid and was discovered in 1859 by Hoffman, a German chemist (Luck, 1976). He treated rowan berry oil, an acid distillation product from the juice of unripe rowan berries with strong alkali, and named the new substance "sorbic acid" after the scientific name of the mountain ash, Sorbus aucuparia Linne, the parent plant. It is now prepared synthetically by many methods (Luck, 1976).

Sorbic acid (trans, trans-2, 4-hexadienoic acid, CH_3-CH=CH-CH=CH-COOH) is an α, β-unsaturated, aliphatic, straight-chain monocarboxylic acid (Anonymous, 1978). It is a white, crystalline compound and has a very low solubility in water, especially at room temperature where the solubility is only about 0.16 percent. However, it dissolves more easily in hot water, ethanol, or diethyl ether. In boiling water, a solution of 3.8 percent sorbic acid can be obtained (Gershenfeld, 1968). Fortunately, solutions of more than 50 percent can be prepared from its potassium salt (potassium sorbate) in cold water. It has been shown that the pH of the medium greatly affects the solubility (Pfizer, as cited by Bradley, 1960), especially potassium sorbate which tends to hydrolyze to sorbic acid at low pH. Other factors such as high concentrations of glucose, sucrose or sodium chloride, have also been shown to affect the solubility of sorbic acid in water (Gershenfeld, 1968).

Sorbic acid has a melting point of 134.5°C and starts to decompose at 228°C. However, above 60°C , the acid begins to sublime (Luck, 1976).

Muller (1939) and Gooding (1940) independently discovered that sorbic acid has an antimicrobial effect (Luck, 1976). Later, Gooding (1945) was granted the original patent on the use of sorbic acid as a food preservative. This finding stimulated further studies on the inhibitory effect of sorbic acid against microorganisms, physiological and chemical properties of this acid, and the possible mechanism of this inhibition.

Sorbic Acid as an Antimicrobial Agent

Phillips and Mundt (1950) observed that sorbic acid at a concentration of 1000 mg/kg effectively inhibited film yeasts in cucumber fermentations without noticeable inhibition of the desirable lactic acid fermentation. Vaughn and Emard (1951) reported that sorbic acid selectively favored the growth of Lactobacillus and Leuconostoc strains and inhibited all of the other test cultures of bacteria, molds, and yeasts. Emard and Vaughn (1952) tested 229 different cultures of bacteria, molds, yeasts, and actinomycetes on two cultural media containing sorbic acid. They found that all of the catalase positive cultures were inhibited to such a degree that growth was not detected in the test media after seven days, while the catalase negative cultures grew without noticeable inhibition. The selective inhibitory effect of sorbic acid was thus suggested. The pH's of the media were not specified. However, when potassium dihydrogen phosphate was added to the media, the selectivity of the media was reduced, i.e. both catalase negative as well as catalase positive cultures were inhibited. In an attempt to determine the effect of pH on the

selectivity of sorbic acid, the same authors concluded that the pH of the media containing sorbic acid should be adjusted so that, after sterilization, the pH is between 5 and 5.5.

In an attempt to develop a selective medium for the isolation of the catalase negative bacteria, York and Vaughn (1954a) reported that, at a concentration of 1200 mg/kg of sorbic acid, the ability of the spores of the different cultures of Clostridia to germinate in seven days was markedly decreased as the pH value of the medium was reduced from 5.8 to 5.0. Consequently, they recommended that, if the sorbic acid medium is to be used for enrichment and isolation of Clostridia, the pH range must be increased to at least six or above. However, at this higher range, sorbic acid is not as effective as in the recommended range of pH 5 to 5.5. In another study, the same investigators (York and Vaughn, 1954b) noticed a marked resistance to sorbic acid by Clostridium parabotulinum type A in the acid range (pH 4.8-5.0). They observed that Clostridium parabotulinum type A was not appreciably affected by the presence of 1200 mg/kg of sorbic acid in liver infusion broth at this pH.

Hansen and Appleman (1955) initiated a study to determine whether sorbic acid, caproic acid and propionic acid are growth stimulants for Clostridia. They found that, at pH 6.7 and on two media, liver-infusion broth and glucose-yeast extract broth, these acids do not inhibit or stimulate the growth of Clostridium parabotulinum type A and B, and Clostridium sporogenes. They recommended sorbic acid for its anti-mycotic activity, but concluded it was not suitable to prevent growth of catalase negative organisms.

Costilow (1957) reported that sorbic acid-treatments of cucumber fermentations reduced the occurrence of bloater-type spoilage; it was significantly reduced by the addition of sorbic acid to the brine in concentrations as low as 200 mg/kg. It has been found that lactic acid fermentation was inhibited by 1000 mg/kg of sorbic acid when the initial brine strengths ranged from 20° to 40° salometer. Concentrations of sorbic acid as low as 500 mg/kg were sufficient to inhibit the fermentation process when the initial brine strength was 40° salometer (Costilow et al., 1957).

Klis et al. (158) examined the inhibitory effect of sorbic acid on cultural media using 16 common food-spoilage fungi. They found that all of the fungi tested except Aspergillus niger and Zygosaccharomyces barkeri, were completely inhibited by 500 mg/kg of sorbic acid. These two fungi were not inhibited by 1000 mg/kg of sorbic acid.

A study was conducted by Weaver et al. (1957) to determine whether sorbate would be as effective in preserving commercial apple cider as benzoate which imparts an undesirable flavor to cider. They found that sodium sorbate (500 mg/kg) controlled molds and yeasts, but was not effective against bacteria at high storage temperatures. Similar results were obtained by Ferguson and Powrie (1957) who pointed out that the spoilage was due to Acetobacter. Bradley et al. (1962) demonstrated that the presence of potassium sorbate usually caused a "definite lag" in the growth curve of organisms commonly associated with cottage cheese spoilage, resulting in the extension of the shelf life of the cheese by one to eight days dependent upon the concentration of the sorbate.

From the previous studies on the antimicrobial activity of sorbic acid, the following conclusions can be made:

1. Sorbic acid is effective only against catalase positive bacteria and fungi.
2. The inhibitory effect of sorbic acid is very pH dependent.
3. Sorbic acid is considered ideal for the pickling industry since it inhibits only undesirable microorganisms.

Nitrites and Sorbates in Food Systems

Over the past two decades, there has been much concern over the use of nitrite in cured meats. This concern has originated from reports implicating nitrite as a precursor of carcinogenic N-nitrosamines in cured meats, and in particular, fried bacon. Excellent papers and comprehensive reviews on this subject have been published (Fiddler, 1975; Mirvish, 1975; Shank, 1975; Wogan and Tannenbaum, 1975; Crosby and Sawyer, 1976; Gray, 1976; Mirvish, 1977; Gray and Randall, 1979; Sofos et al., 1979a). It is worthy to mention that nitrite itself has been incriminated as a possible direct carcinogen in recent reports (USDA, 1978; Newberne, 1979). It is for this reason that considerable effort has been directed towards finding a potential substitute for nitrite in meat systems. Sorbic acid and its potassium salt have attracted the most attention as a possible alternative to nitrite, or adjunct to nitrite, i.e. reducing the ingoing nitrite to the lowest level which permits the development of a satisfactory color and flavor, while adding sorbate at a concentration of 2600 mg/kg to provide adequate protection against Clostridium botulinum.

As a result of studies prior to 1974 (Phillips and Mundt, 1950; Vaughn and Emard, 1951; Emard and Vaughn, 1952; York and Vaughn, 1954a; 1955; Costilow et al., 1955; Hansen and Appleman, 1955; Costilow, 1957; Costilow et al., 1957), there was no attempt to utilize potassium sorbate in meat systems, except when the presence of fungi were expected. For this reason, potassium sorbate was approved for use in dry stuffed sausages to retard mold growth on the surface of the sausages by dripping the casings in a 2.5 percent solution of potassium sorbate (Sofos and Busta, 1979).

More recently, Tompkin et al. (1974) conducted a study where they added 1000 mg/kg of potassium sorbate to skinless precooked, uncured sausage links which were then inoculated with Staphylococcus aureus, Salmonellae and Clostridium botulinum. They found that potassium sorbate not only delayed the growth of the normal spoilage flora, but also the growth of Staphylococcus aureus and Salmonellae was remarkedly retarded. The growth of Clostridium botulinum was considerably reduced and the production of toxin was delayed for six days. The pH of the system was high enough to exclude the effect of hydrogen ion concentration. These results were contrary to those of previous investigations with regard to the inhibitory effect of sorbic acid on Clostridia. This new finding stimulated further studies. Consequently, several reports have been published recently which reveal that the combination of reduced levels of nitrite (e.g. 40 mg/kg) and 2600 mg/kg of potassium sorbate gave the same level of protection against Clostridium botulinum and the same desired organoleptic characteristics as that achieved by nitrite in the regularly permitted amounts. Tanaka et al. (1977) demonstrated that in frankfurters, potassium sorbate (2700 mg/kg) produced an antibotulinal effect

similar to that of 100 mg/kg of nitrite. Robach (1980) reported that the effectiveness of potassium sorbate in preventing or delaying the outgrowth of Clostridium sporogenes PA3679 was increased by adding one to five percent sodium chloride. Robach et al. (1980c) also studied the effect of sorbates on microbiological growth in cooked turkey products. They reported that 1000 to 2000 mg/kg of potassium sorbate depressed microbiological growth and delayed the formation of the pink color and off odors in uncured, cooked, vacuum packed poultry products, without affecting the organoleptic quality. Robach et al. (1980b) demonstrated that bacon processed with 40 mg/kg of sodium nitrite and 2600 mg/kg of potassium sorbate contained an average of 8.7 µg/kg of N-nitrosopyrrolidine (NPYR) compared to 4.4 µg/kg and 28.1 µg/kg of NPYR for bacon made without and with 120 mg/kg of nitrite, respectively. Similar results were obtained by Ivey et al. (1978), Pierson et al. (1979) and Price and Stevenson (1979).

It has been shown by many workers that nitrite-sorbate combinations are more effective in retarding Clostridium botulinum growth and toxin production than either nitrite or sorbate when used individually (Tanaka et al., 1977; Ivey and Robach, 1978; Ivey et al., 1978; Robach et al., 1978). However, the mechanism by which nitrite-sorbate combination interacts to inhibit the growth and toxin production by Clostridium botulinum is not known. Sofos et al. (1979a) suggested the possibility of an additive nitrite-sorbic acid effect and the production of a more inhibitory substance(s) through reaction of nitrite and sorbic acid.

It has been reported that the addition of potassium sorbate to cured meat delayed the disappearance of nitrite (Sofos et al., 1979a,b).

Tompkin et al. (1978) pointed out that the lower the residual nitrite at the time of incubation, the faster the rate of swelling indicating the involvement of nitrite in the inhibition of growth of Clostridium botulinum. Sofos et al. (1979a) suggested that sorbic acid depresses spore germination to an insignificant level, while nitrite retards the growth of germinated spores. Namiki and Kada (1975) reported the isolation of ethylnitrolic acid from the reaction of sorbic acid with sodium nitrite. This compound was considered by the authors to be a strong antibacterial agent.

Some controversies have been created when Kada (1974) reported that a mixture of sorbic acid and nitrite (130 g/kg) in aqueous medium, when heated, gave a positive result in the Bacillus subtilis recombination assay. This indicates the possible formation of DNA-damaging substance(s) in this reaction. Hayatsu et al. (1975) and Namiki and Kada (1975) confirmed the findings of Kada (1974). Ethylnitrolic acid was identified as the mutagenic principle arising from the reaction of nitrite and sorbic acid (Namiki and Kada, 1975).

More recent studies have failed to demonstrate the mutagenicity of ethylnitrolic acid in both model system and food system (Difate, 1978; Robach et al., 1980a). Difate (1978) attributed the apparent mutagenicity of ethylnitrolic acid to possible free nitrite contamination.

Tanaka et al. (1978) reported that, when lower levels of nitrite (13,000 mg/kg) reacted with sorbic acid (2000 mg/kg) at different pH values (1-5), reaction product(s) were produced which were negative in the Ames Salmonella assay and Bacillus subtilis recombination assay. Furthermore, they found that the presence of sorbic acid inhibited the formation of N-nitrosamines in in vitro tests under a wide variety of conditions.

Effect of pH on Antimicrobial Properties of Sorbic Acid

Sorbic acid is one of a group of short chain organic acids which, together with their salts, have been shown to exhibit antimicrobial properties (Bell et al., 1959). Hoffman et al. (139) studied the antimicrobial properties of a number of normal saturated and unsaturated fatty acids containing from one to 14 carbon atoms, and used as test organisms, a mixed culture of common food spoilage molds. Their study revealed that the effectiveness of the acids as fungistatic agents increased with chain length and concentration, and varied with the pH. Rahn and Conn (1944) postulated that preservative acids such as benzoic acid are effective only when present in the undissociated state, i.e. when the pH is low enough to prevent dissociation. Samson et al. (1955) observed that the effectiveness of fatty acids as fungistats was dependent on the pH of the system. The inhibitory effect increased with decreasing pH because the cell was permeable to the acid in the undissociated form only. Beneke and Fabian (1955) observed the influence of both sorbic acid concentration and pH of the media on the inhibition of a number of fungi isolated from tomato and strawberry fruits. Bell et al. (1959) reported that the toxic action of sorbic acid was found to be directly related to the concentration of undissociated acid. They concluded that sorbic acid would be more effective as an antimicrobial agent when used at a low pH which would permit more of the undissociated form to be present in solution. Raevori (1976) stated that the free acid enters the bacterial cell and inhibits several enzyme systems.

As previously discussed, it is apparent that the undissociated form of the acid plays the most important role as the antimicrobial agent. The

reason is that the microbial cell carries a net negative charge, so the uncharged form (undissociated) will enter the cell, while the charged form (dissociated) which carries a negative charge will be repelled.

$$A^- + H^+ \longleftrightarrow AH \longleftrightarrow AH \longleftrightarrow A^- + H^+$$

Outside Cell | Cell Membrane | Inside Cell

Once the acid is inside the cell, the mechanism of inhibition is not quite clear, although many studies have been conducted to elucidate the nature of this inhibition.

Mechanism of Inhibition

The inhibition of various enzymatic reactions by sorbic acid has been suggested as the possible mechanism by which the growth of microorganism is inhibited (Woodford and Adams, 1970). Melnick et al. (1954) suggested that the accumulation of α, β-unsaturated fatty acids can inhibit the dehydrogenase system in molds. Sorbic acid already exists in this form, so the dehydrogenase enzymes which are vital for cell metabolism are inhibited by sorbic acid. Furthermore, Melnick et al. (1954) reported that when molds are present in high numbers, sorbic acid is metabolized without inhibition of the dehydrogenase enzymes. However, when molds are present in low numbers, sorbic acid remains in the original effective form.

Azukas et al. (1961) demonstrated that the fermentation of glucose by resting cells of baker's yeast was greatly inhibited by sorbic acid and that the inhibition was very much pH dependent. However, when the same workers used a cell-free extract of the same yeast, they found no significant difference in the percent inhibition between pH five and six.

York and Vaughn (1955b) reported that fumarase activity is suppressed by sorbic acid. Whitaker (1959) demonstrated that the sulfhydryl-containing enzymes, ficin and alcohol dehydrogenase, were inhibited by sorbic acid, and suggested that sorbic acid and the other α, β-unsaturated acids were non-specifically effective against sulfhydryl containing enzymes, with which the acids form complexes. York and Vaughn (1964) found that the sulfhydryl enzymes, fumarase, aspartase, and succinic dehydrogenase were inhibited by sorbic acid. They also observed the loss of activity of sorbic acid after reacting with cysteine which led them to conclude that a thiol addition occurred, the mechanism which was believed to be involved in the action against sulfhydryl enzymes. Rehm (1967) reported that sorbic acid strongly inhibited a number of dehydrogenases including malate dehydrogenase, α-ketoglutarate dehydrogenase and succinate dehydrogenase.

Pellaroni and Pritz (1960) suggested that the reduction in respiration rate of Saccharomyces cerevisiae var ellipsoideus was the result of the formation of sorbyl coenzyme A. In an attempt to elucidate the mechanism by which sorbate inhibits baker's yeast, Harada et al. (1968) suggested that sorbate competitively combines with coenzyme A and acetate and consequently inhibits the enzymatic reaction involving coenzyme A. It has been reported that lipophilic acids, including sorbic acid, uncouple both substrate transport and oxidative phosphorylation from the electron transport system (Freese et al., 1973).

More recently, Przybylski and Bullerman (1980) observed the depletion of the ATP level of the conidia of Aspergillus parasiticus upon exposure to sorbic acid. They suggested that the depletion of ATP levels of conidia may be a possible mechanism for the action of sorbic acid in fungal inhibition.

Physiological Properties of Sorbic Acid

Luck (1976) stated that "as an aliphatic carboxylic acid, the consideration of the structure of sorbic acid leads to the expectation that sorbic acid is utilized in the body similarly to other fatty acids." Physiological studies on sorbic acid using laboratory animals proved that the above hypothesis is correct.

Witter et al. (1950) reported that sorbic acid, as well as caproic acid, is quantitatively transformed to acetone bodies, since both, on a molar basis, yield 1.5 moles of acetoacetate when treated with rat liver homogenate or washed liver particulate. A comprehensive study on the metabolism of sorbic acid has been carried out by Deuel et al. (1954a) using rats and dogs in their study. Their findings can be summarized as follows:

1. The gain-in-weight of rats receiving diets containing up to eight percent of sorbic acid was similar to that of rats on a basal diet free of sorbic acid.
2. There was no evidence of abnormality in liver or kidney weights or pathological conditions in other tissues of rats receiving four percent of sorbate. Rats on the eight percent sorbic acid diet exhibited a slight but "statistically significant" increase in liver weight.
3. Sorbic acid did not act as an antimetabolite for essential fatty acids in rats.
4. Sorbic acid is utilized by the animals as a source of calories.
5. Sorbic acid is much less toxic than sodium benzoate as indicated by the ratios of LD_{50} (sorbate) :LD_{50} (benzoate), 1.7-1.9.

Physiological Properties of Sorbic Acid

Luck (1976) stated that "as an aliphatic carboxylic acid, the consideration of the structure of sorbic acid leads to the expectation that sorbic acid is utilized in the body similarly to other fatty acids." Physiological studies on sorbic acid using laboratory animals proved that the above hypothesis is correct.

Witter et al. (1950) reported that sorbic acid, as well as caproic acid, is quantitatively transformed to acetone bodies, since both, on a molar basis, yield 1.5 moles of acetoacetate when treated with rat liver homogenate or washed liver particulate. A comprehensive study on the metabolism of sorbic acid has been carried out by Deuel et al. (1954a) using rats and dogs in their study. Their findings can be summarized as follows:

1. The gain-in-weight of rats receiving diets containing up to eight percent of sorbic acid was similar to that of rats on a basal diet free of sorbic acid.
2. There was no evidence of abnormality in liver or kidney weights or pathological conditions in other tissues of rats receiving four percent of sorbate. Rats on the eight percent sorbic acid diet exhibited a slight but "statistically significant" increase in liver weight.
3. Sorbic acid did not act as an antimetabolite for essential fatty acids in rats.
4. Sorbic acid is utilized by the animals as a source of calories.
5. Sorbic acid is much less toxic than sodium benzoate as indicated by the ratios of LD_{50} (sorbate) : LD_{50} (benzoate), 1.7-1.9.

Deuel et al. (1955b) conducted another study with rats to elucidate how sorbic acid is metabolized in the presence and absence of glucose (a glucogenic substance). They found that the intermediate metabolism of sorbic acid is identical to that of the normally occurring fatty acids, caproic and butyric. They concluded that, under normal conditions of alimentation, sorbic acid is completely oxidized to CO_2 and H_2O and thus yields its potential energy as calories. Stavkuv and Petrova (1964) reported that when sorbic acid was administered daily at levels of 0.5 to 1.0 percent over a prolonged period, there was no sign of toxicity to the test animals. However, doses of five to ten percent of sorbic acid when taken daily for four months produced some signs of toxicity. Westos (1964) followed the fate of labelled sorbic acid during metabolism by mice. He found that, of the sorbic acid given to mice during a four day period, 81 percent of the radioactivity was released as $^{14}CO_2$ and about four percent in the urine, mainly as muconic acid (HOOC-CH=CH-CH=CH-COOH).

Gaunt et al. (1975) conducted a long term toxicity study of sorbic acid and found no signs of toxicity or carcinogenicity when sorbic acid was fed to rats at levels up to ten percent of the diet. This represented an intake of approximately five g/kg/day. They concluded that, on the basis of the 100X safety factor, the daily intake would be 50 mg/kg/day, a value in excess of the unconditional level established by the joint FAO/WHO Expert Committee on Food Additives (FAO, 1967). In a similar study on the toxicity of sorbic acid on mice, Hindy et al. (1976) reported that when sorbic acid was fed at levels of up to ten percent of the diet for 80 weeks, there was no evidence of carcinogenicity. They observed no adverse effects although a slight enlargement of the kidney and a

reduction in body weight gain were detected in mice fed with 5.0 to 10.0 percent sorbic acid. These changes were not observed when sorbic acid was given at a concentration of one percent. Lishund (1969), cited by Gaunt et al. (1975) calculated that if sorbic acid were used at the "technically acceptable levels" in cheese, preserves, dried fruits, nuts, bread, cakes and pastries, pickles and sauces, wine, soft drinks, and fruit, the average daily intake would be 293 mg. This is well below the unconditional acceptable daily intake of 12.5 mg/kg (750 mg/day for a 60-kg adult male) established by the joint FAO/WHO Expert Committee on Food Additives (FAO, 1967).

Decomposition of Sorbic Acid

The decomposition of sorbic acid has been reported earlier by several workers (Melnick and Luckmann, 1954a and b; Melnick et al., 1954; Costilow et al., 1957; Alderton and Lewis, 1958). In spite of the fact that the loss may become severe in some cases, considerably little is known about the mechanism of the decomposition of sorbic acid in biological systems.

Losses may be classified into two types: 1) loss due to chemical degradation, and 2) loss due to biological degradation (mainly microbial).

1. Chemical degradation of sorbic acid

Although sorbic acid and its salts are stable in the dry pure form, their solutions are known to be relatively unstable (McCarthy and Eagles, 1976). It has been reported that 35 to 65 percent of the original sorbic acid in the pickle brine solution was lost during storage (Costilow et al., 1957). Similar losses have also been reported by Alderton and Lewis

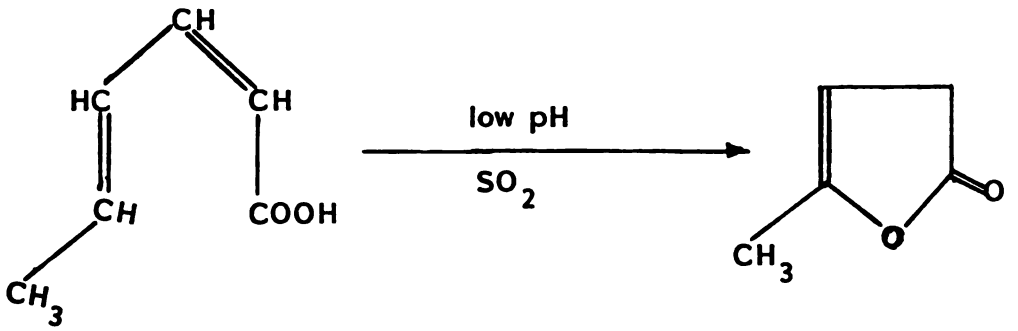
(1958). However, Costilow (personal communication with Alderton and Lewis, 1958) strongly suggested the possibility of chemical degradation of sorbic acid during cucumber fermentation.

McCarthy et al. (1973) investigated the stability of a sorbic acid solution (pH 4.2) when stored in a variety of plastic and brown glass bottles which had previously been found to transmit no light between the wavelengths of 220 to 450 nm and only 5.5 percent transmission at 500 nm. They reported that up to 50 percent of the original sorbic acid (1000 mg/kg) was lost in a period of twelve weeks. They pointed out that the temperature at which the bottles were stored greatly affected the rate of sorbic acid degradation. Furthermore, they found that in most cases, the decay was first-order, whereas in a few cases, the decay was first-order for a period of time followed by a rapid drop suggesting catalytic decomposition. The latter was associated with high temperature (50°C).

Recently, Arya et al. (1978) demonstrated the effect of some food ingredients on the rate of sorbic acid degradation in aqueous solutions. They reported that sugar, salt, glycerol, and some metallic ions (Cu^{++}, Fe^{++} and Ni^{++}) retarded the rate of degradation of sorbic acid. Furthermore, they stated that all of the amino acids tested, other than histidine, accelerated the degradation. Thus, they concluded that the autoxidative degradation of sorbic acid differs from the autoxidation of unsaturated fatty acids.

More recently, Saxby et al. (1979) conducted a comprehensive study on the chemical degradation of sorbic acid in a model system where some food ingredients were examined for their effects on the rate of degradation.

They demonstrated that citric acid and ascorbic acid catalyzed to some extent the degradation of sorbic acid. Their study also demonstrated that light strongly influences the rate of degradation at pH 2.6, but not at pH 1.2 or higher values of pH (4.3 or 5.4). Furthermore, Saxby et al. (1979) detected the presence of ∝-angelicalactone, albeit in relatively low concentrations, i.e., 0.1 percent of the total decomposed sorbic acid. The fate of the remaining sorbic acid was not determined.



Sorbic acid ∝-angelicalactone

McCarthy and Eagles (1976) reported that sorbic acid in solutions is oxygen labile. However, no further explanation as to how oxygen affects the stability of sorbic acid was given.

In an attempt to explain the loss of sorbic acid from sorbic acid-preserved high moisture prunes, Bolin et al. (1980) demonstrated that the extent of sorbic acid loss was a function of moisture and temperature. They also concluded that microorganisms did not play any role in the loss. However, no attempts were made to isolate and identify the degradation products.

2. Microbial degradation of sorbic acid

In spite of the fact that sorbic acid is commonly used as an antimicrobial agent in food systems, there are certain microorganisms which can grow in

the presence of relatively high concentration of sorbic acid or its salts. Degradation of sorbic acid by microorganisms has been reported by many investigators (Melnick et al., 1954; York and Vaughn, 1954b; Luckas, 1964; Rehm et al., 1964). Melnick and Luckmann (1954a) noticed that sorbic acid disappeared partially or totally from sorbic acid-preserved cheese when stored at 45°F for six weeks. Neither autoxidation nor sublimation were deemed responsible for this loss (Melnick and Luckmann, 1954b). Melnick et al. (1954) further demonstrated that the loss from the sorbic acid-treated cheese wrapper was due to metabolic oxidation of sorbic acid by molds. Perry and Lawrance (1959) stated that sorbic acid would not suppress heavy contaminations, but would effectively retard small contaminations of microorganisms.

Sorbic acid may be used by microorganisms as a source for carbon and energy. Clostridium parabotulinum type A, Clostridium sporogenes, Clostridium acetobutylicum and Clostridium thermosaccharolyticum have been shown to utilize sorbic acid as a carbon source (York and Vaughn, 1954a). Furthermore, York and Vaughn (1954b) reported that all the cultures of Clostridium parabotulinum type A and B tested utilized 1200 mg/kg to 10000 mg/kg of sorbic acid in a basal medium at pH 5.6. However, not all of the microorganisms which can tolerate sorbic acid can use it as a carbon source.

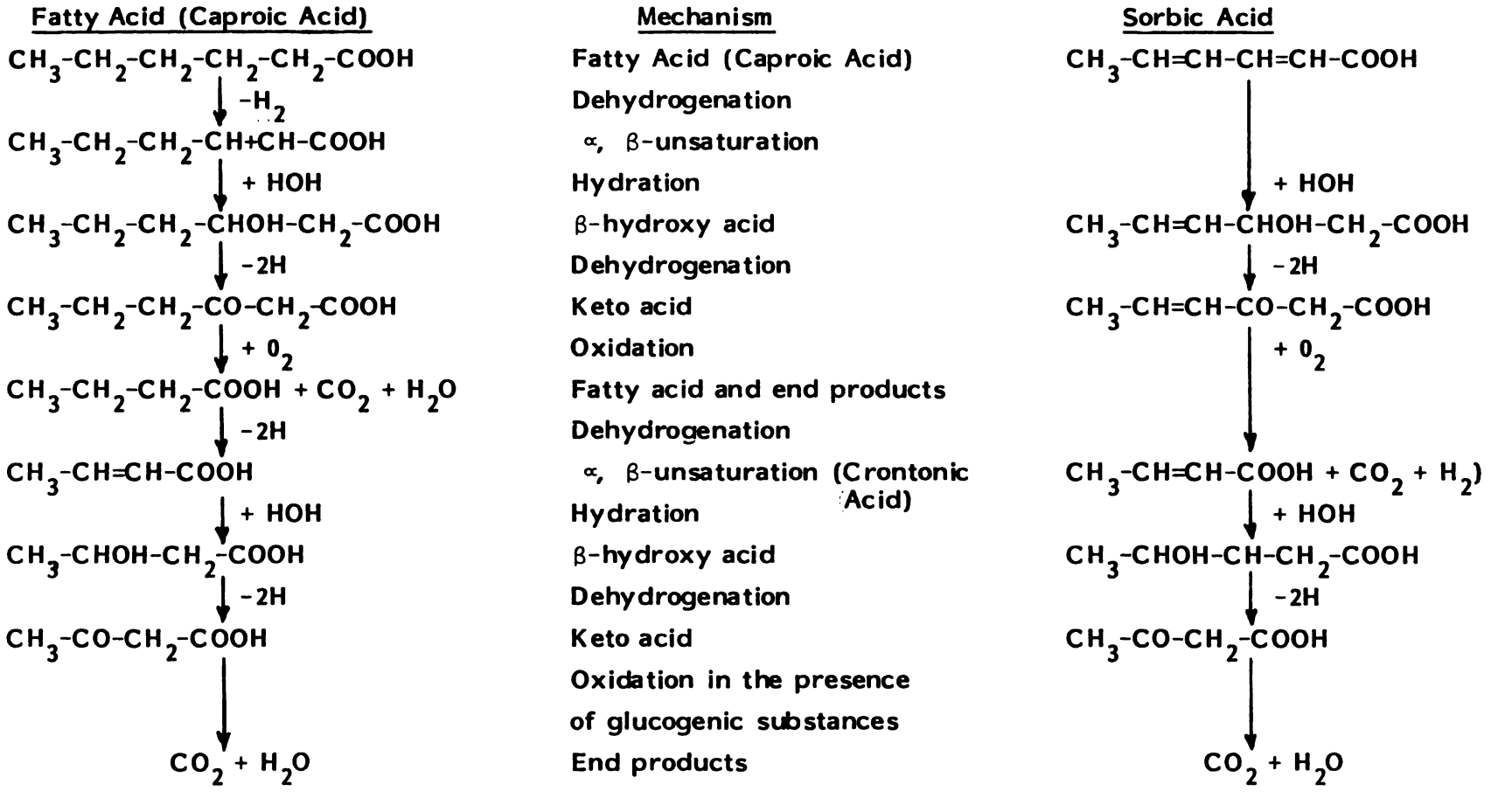
Many of the data reported regarding the loss of sorbic acid have been associated with products with high initial microbial counts or with cases where microbial action is involved in the processing, e.g. pickles (Deak and Novak, 1970; Deak et al., 1972). However, to rule out the possibility of the utilization of sorbic acid in sorbic acid-treated pickle brine by microorganisms commonly present during the fermentation process, Costilow

et al. (1955) tested a number of lactic acid bacteria and a sorbic acid-tolerant yeast, Torulopsis holmii. They found that neither the lactic acid bacteria nor the heavy inoculum of the sorbic acid-tolerant yeast, could grow on a basal medium containing sorbic acid as the sole source of carbon. Rehm et al. (1964) found that Aspergillus niger and Pseudomonas fluorescens destroy and metabolize sorbic acid in sublethal concentrations. Luckas (1963) reported that Aspergillus niger can decompose sorbic acid and that mycelia growth was inhibited by 1500 mg/kg of sorbic acid.

Little is known about the mechanisms by which microorganisms degrade sorbic acid. However, because sorbic acid shares a lot of physical and chemical characteristics with fatty acids, it has been thought that it is metabolized in a similar way to that of fatty acids. The degradation of fatty acids by microorganisms have prompted many investigations over the past four decades. Many of these studies have been devoted to the elucidation of the mechanism of microbial degradation of fatty acids.

Thaler and Geist (1939a, b), Mukherjee (1951, 1952a, b), and Melnick et al. (1954) have contributed to our knowledge in this field. Mukherjee, in a series of papers, established a "definite reaction mechanism" for the degradation of fatty acids by molds, using representative species of Penicillium and Aspergillus. Melnick et al. (1954) investigated Wieland's scheme, i.e., fatty acid oxidation of saturated fatty acids proceeds through beta-oxidation involving dehydrogenation at the α, β-position, hydration and dehydrogenation. The authors suggested the scheme, outlined in Figure 1, as a possible mechanism for the degradation of sorbic acid by molds.

Figure 1. Wieland's scheme for fatty acid oxidation (Melnick et al., 1954)


Fatty Acid (Caproic Acid)
CH3-CH2-CH2-CH2-CH2-COOH
-H2
CH3-CH2-CH2-CH+CH-COOH
+ HOH
CH3-CH2-CH2-CHOH-CH2-COOH
-2H
CH3-CH2-CH2-CO-CH2-COOH
+ 02
CH3-CH2-CH2-COOH + CO2 + H2O
-2H
CH3-CH=CH-COOH
+ HOH
CH3-CHOH-CH2-COOH
-2H
CH3-CO-CH2-COOH
CO2 + H2O
Mechanism
Fatty Acid (Caproic Acid)
Dehydrogenation
∝, β-unsaturation
Hydration
β-hydroxy acid
Dehydrogenation
Keto acid
Oxidation
Fatty acid and end products
Dehydrogenation
∝, β-unsaturation (Crontonic Acid)
Hydration
β-hydroxy acid
Dehydrogenation
Keto acid
Oxidation in the presence
of glucogenic substances
End products
Sorbic Acid
CH3-CH=CH-CH=CH-COOH
+ HOH
CH3-CH=CH-CHOH-CH2-COOH
-2H
CH3-CH=CH-CO-CH2-COOH
+ 02
CH3-CH=CH-COOH + CO2 + H2)
+ HOH
CH3-CHOH-CH-CH2-COOH
-2H
CH3-CO-CH2-COOH
CO2 + H2O

This scheme results in a fatty acid containing two carbon atoms less than the original fatty acid, ketones, carbon dioxide and water as end products of one cycle. Thaler and Geist (1939a) noticed that the presence of glucose or any glucogenic material in the test medium prevents ketone formation. Melnick et al. (1954) observed that ketone formation was associated with sorbic acid degradation. The same authors reported that in the presence of added carbohydrates such as glucose, metabolic degradation of fatty acids, as illustrated by sorbic acid, proceeds at a more rapid rate, but without an accumulation of ketones. Consequently, the fatty acids are rapidly and completely metabolized to carbon dioxide and water as end products. This mechanism appears to be similar to that of the mammalian system (Deuel et al., 1954a, b). Luckas (1963) demonstrated that sorbic acid was taken up by the cells and metabolized mainly to carbon dioxide and water and partly to methyl ketones. There was no mention as to whether sorbic acid was used as the sole source of carbon or with a glucogenic substance such as glucose. Rehm et al. (1964) stated that the destruction of sorbic acid is similar to that of fatty acids.

More recently, it has been shown that a Mucor sp. can promote a biological hydrogenation of sorbic acid at the second carbon, before or after the reduction of the carboxyl group, resulting in the formation of 4-hexenol (Kurogochi et al., 1974). Furthermore, the same authors reported that a Geotrichum sp. metabolized sorbic acid partly into 4-hexenoic acid and ethyl sorbate. No explanation was given for the mechanism of formation or the possible intermediate metabolites. However, in another study, Kurogochi et al. (1975) extensively studied the fungal metabolites of sorbic acid and analogous compounds in an attempt to elucidate the mechanism of sorbic acid degradation. They identified a new

intermediate metabolite, sorbyl alcohol (2,4-hexadienol) but only in the early stages (one to two days) of growth of the fungus. However, when the mold, Mucor sp., was grown in the presence of sorbyl alcohol or 4-hexenoic acid, the two substrates were rapidly reduced to 4-hexenol. Hence, it was concluded that the fungus, Mucor sp., converted sorbic acid into 4-hexenol in two independent steps:

1) Reduction of the carboxylic group

CH_3-CH=CH-CH=CH-COOH ⟶ CH_3-CH=CH-CH=CH-CH_2OH

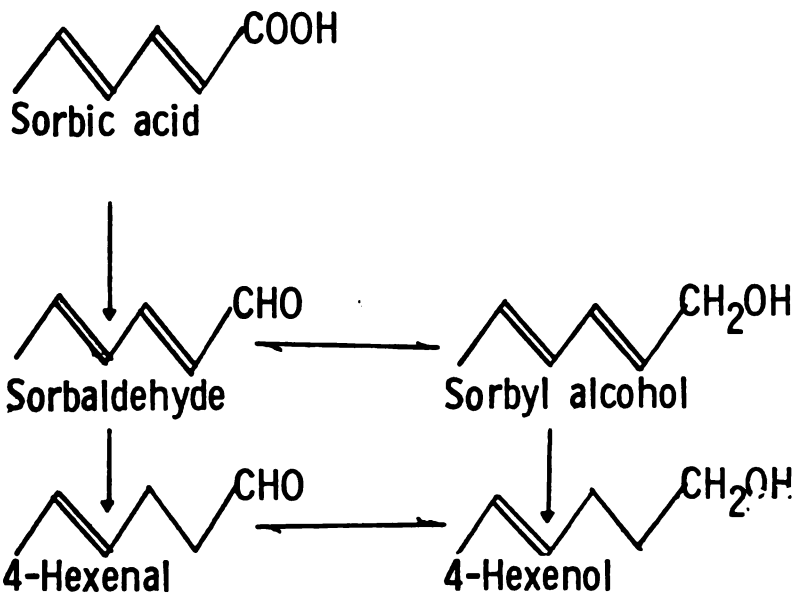
Sorbic acid — Sorbyl alcohol

2) Hydrogenation of one olefinic bond in 2-alkenol

CH_3-CH=CH-CH=CH-CH_2OH ⟶ CH_3-CH=CH-CH_2-CH_2-CH_2OH

Sorbyl alcohol — 4-Hexenol

Tahara et al. (1977) identified an intermediate metabolite, sorbaldehyde (2,4-hexadienal) in the early stages of growth of Mucor sp. Tahara et al. (1979) used the cell free system of Mucor griseo-cyanus to clarify the reduction of sorbic acid to 4-hexenol. They suggested the following scheme:



Tahara et al. (1977) examined 26 species of molds including twelve species of Mucor for their ability to reduce sorbic acid to 4-hexenol. They found that most of the Mucor and Rhizopus species tested were capable of reducing sorbic acid to hexenol.

Marth et al. (1966) indicated that a hydrocarbon-like odor appeared in sorbate-fortified mold cultures isolated from cheeses fortified with potassium sorbate. They identified that volatile substance as 1,3-pentadiene. They postulated the following mechanism:

Potassium sorbate+tartaric acid $\longrightarrow$ Potassium tartrate+sorbic acid

Sorbic acid $\xrightarrow{\text{decarboxylation}}$ 1,3-pentadiene + CO_2

Furthermore, the same authors pointed out that potassium sorbate was degraded by some species of Penicillium, especially if it was in a highly nutritious substrate.

More recently, Crowell and Guymon (1975) reported the isolation of ethyl sorbate, 2,4-hexadien-1-ol , 1-ethoxyhexadiene, 3,5-hexadien-2-ol and 2-ethoxyhexa-3,5-diene from a sorbic acid-containing wine which was spoiled by lactic acid bacteria. They pointed out that the latter compound has an intense odor and is responsible for the geranium-like off-odor associated with spoilage in sorbate-containing wines.

MATERIALS AND METHODS

Microorganisms

All of the microorganisms tested, with the exception of a few molds, were obtained from the culture collection of the Food Microbiology Laboratory, Department of Food Science and Human Nutrition, Michigan State University. Some of the molds were obtained from the Department of Botany and Plant Pathology, Michigan State University.

Some food and air-borne molds were isolated from different sources and were included in the preliminary studies. Only those molds which showed a marked resistance to sorbic acid were identified as to genus using the scheme described by Beneke and Stevenson (1978).

Screening Tests on Molds, Yeasts and Bacteria

Media preparation

Screening tests were carried out by growing selected molds (Table A.1) and yeasts (Table A.2) on yeast extract-peptone-glucose (YPG) (yeast extract, Difco Laboratories, Detroit, MI, 0.05 percent; peptone, Difco, 0.5 percent; and glucose, 2.0 percent) as the basal medium for molds and yeasts. Sorbic acid was added as its potassium salt (Sigma Chemical Co., St. Louis, MO) in the appropriate concentration (500-5000 mg/kg). For the solid medium, two percent of Bacto agar (Difco) was added. Yeasts were tested on both solid and liquid media while only the solid medium was used for molds.

Two media, yeast extract-tryptone-glucose (yeast extract, Difco, 0.2 percent; tryptone, Difco, 0.5 percent; and glucose, 2.0 percent) and trypticase soy broth (TSB), which was prepared according to the Difco manual (Difco, 1963) with the exception that the pH was adjusted to 5.5 were used for growing bacteria. The latter medium was mainly used for the fastidious bacteria. Sorbic acid, as its potassium salt, was added in the appropriate concentration (500-5000 mg/kg). Both solid and liquid media were used for cultivating selected bacteria (Table A.3).

The initial pH was adjusted by adding 1N HCl or 1N NaOH to give the required pH (5.5) after sterilization and addition of sorbic acid. The pH was recorded by a Beckman Research pH meter (Beckman Instruments, Inc., Fullerton, California).

The medium was autoclaved at 15 psi for 15 minutes, cooled to about 60°C, and potassium sorbate was added at the required concentration under aseptic conditions. The medium was poured into petri dishes, left to solidify at room temperature, and then inoculated with the appropriate microorganisms.

The liquid media were prepared as previously described, except that agar was not added and that potassium sorbate was added before sterilization. The complete media were dispensed (five ml) into screw cap culture tubes (15 x 150 mm) and the tubes sterilized as previously described.

Incubation conditions

Unless otherwise stated, molds and yeasts (both plates and tubes) were incubated at room temperature (25°C).

For bacteria, the tubes and plates were incubated at 37°C for the pathogenic bacteria and coliform group members, and at 32°C for the remainder of the bacteria.

Growth evaluation

Both plates and tubes were visually examined for growth at predetermined time intervals and compared with the growth of the control cultures (grown on basal media only).

At the end of the incubation period, growth on sorbate-treated media were reported as no growth (-), weak growth (±), moderate growth (+), good growth (++), and vigorous growth (+++).

Utilization of Potassium Sorbate as a Carbon Source

In all of the utilization studies for molds and yeasts, the media used were the same as for the screening tests, except that glucose was not present in the medium. Instead, potassium sorbate was added as the sole source of carbon. For bacteria, the medium used was yeast extract and tryptone, to which potassium sorbate was added in order to test for their ability to utilize sorbate as the sole source of carbon. Potassium sorbate was used at concentrations ranging from 3000 to 5000 mg/kg. The initial pH was adjusted to 6.0 in all utilization experiments.

The remainder of the media preparations, inoculation, incubation, and growth evaluation were carried out in the same manner as in the screening tests.

Growing Cells Studies

Molds and yeasts were precultured for 24 to 72 hours at 25°C on YPG agar plates fortified with 500 mg/kg of potassium sorbate. The cultures were suspended in sterilized, distilled, deionized water and the suspensions were used as inocula.

In all growth studies, unless otherwise stated, the medium was the same as that used in the screening tests except that the potassium sorbate concentration and pH were varied in some cases.

All growth experiments were carried out in 500 ml Erlenmeyer flasks containing 200 ml of medium. The flasks were inoculated with the cell suspension and incubated in a gyratory shaker (Model G-25, New Brunswick Scientific Co., New Brunswick, NJ) at the same temperature as used in the screening tests. The lactic acid bacteria were not shaken, but instead were incubated by standing at the appropriate temperature. In some cases, very slow shaking was provided shortly before the termination of the incubation period.

Representative samples were aseptically drawn at predetermined time intervals and analyzed for residual potassium sorbate according to the method described by Robach et al. (1978). These samples were also analyzed for metabolites using gas liquid chromatography (GC) and mass spectrometry (MS).

The Clostridia were cultivated on thioglycollate broth without dextrose (Difco). Potassium sorbate (1000 mg/kg) and glucose (2.0 percent) were added. The pH was adjusted to 5.5. The medium was inoculated with vegetative cell suspensions (suspension in which vegetative cells were predominant). This suspension was prepared by growing the culture under study in thioglycollate broth for 72 hours. Aliquots of one to two ml of these cultures were used as inocula.

Non-Growing (Resting) Cells Studies

The resting cell suspensions of molds and yeasts were prepared according to the method described by Tahara et al. (1977) with some

modifications. Aliquots (200 ml) of the basal medium plus 500 mg/kg of potassium sorbate in 500 ml Erlenmeyer flasks were inoculated from fresh stock cultures and incubated for 24 to 72 hours, depending on the strain and the inoculum.

For molds, mycelia and spores were separated from the broth by filtration on a Buchner filter with Whatman filter paper No. 42 and washed several times with distilled water to yield a high moisture paste ready for inoculation.

For yeast and bacteria, cells were separated from the broth by centrifugation at 14000 x g using a Sorvall Super speed RC-2 automatic refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Conn.) for 20 minutes, and washed at least one time with distilled water. The high moisture paste which was obtained was used for inoculating the buffer system.

The reaction medium was prepared according to the method described by Tahara et al. (1977), except that glucose was used at a lower concentration. This medium contained a phosphate buffer, glucose (two percent) and 1000 mg/kg of potassium sorbate. Phosphate buffer was prepared from stock solutions of 0.2 M monobasic sodium phosphate and 0.2 M dibasic sodium phosphate in the proper proportions according to the method described by Sorensen (1909a and b).

One to two gram portions of the cell pastes were added to 100 ml of medium in 500 ml Erlenmeyer flasks. Incubation was carried out at room temperature for all molds and yeasts in a slow shaker developing about 50 rpm (Eberbach Corp., Ann Arbor, Michigan).

Representative samples were drawn every hour, and analyzed for residual sorbate and metabolites of sorbic acid according to the procedure described later herein.

Sample Preparation for GC Analysis

Extraction of metabolites

At the end of incubation period, the flask contents (200 ml) were either filtered through Whatman No. 42 filter paper using a Buchner filter or centrifuged. Molds usually were filtered, whereas yeast and bacteria were centrifuged at 14000 x g for ten to 20 minutes.

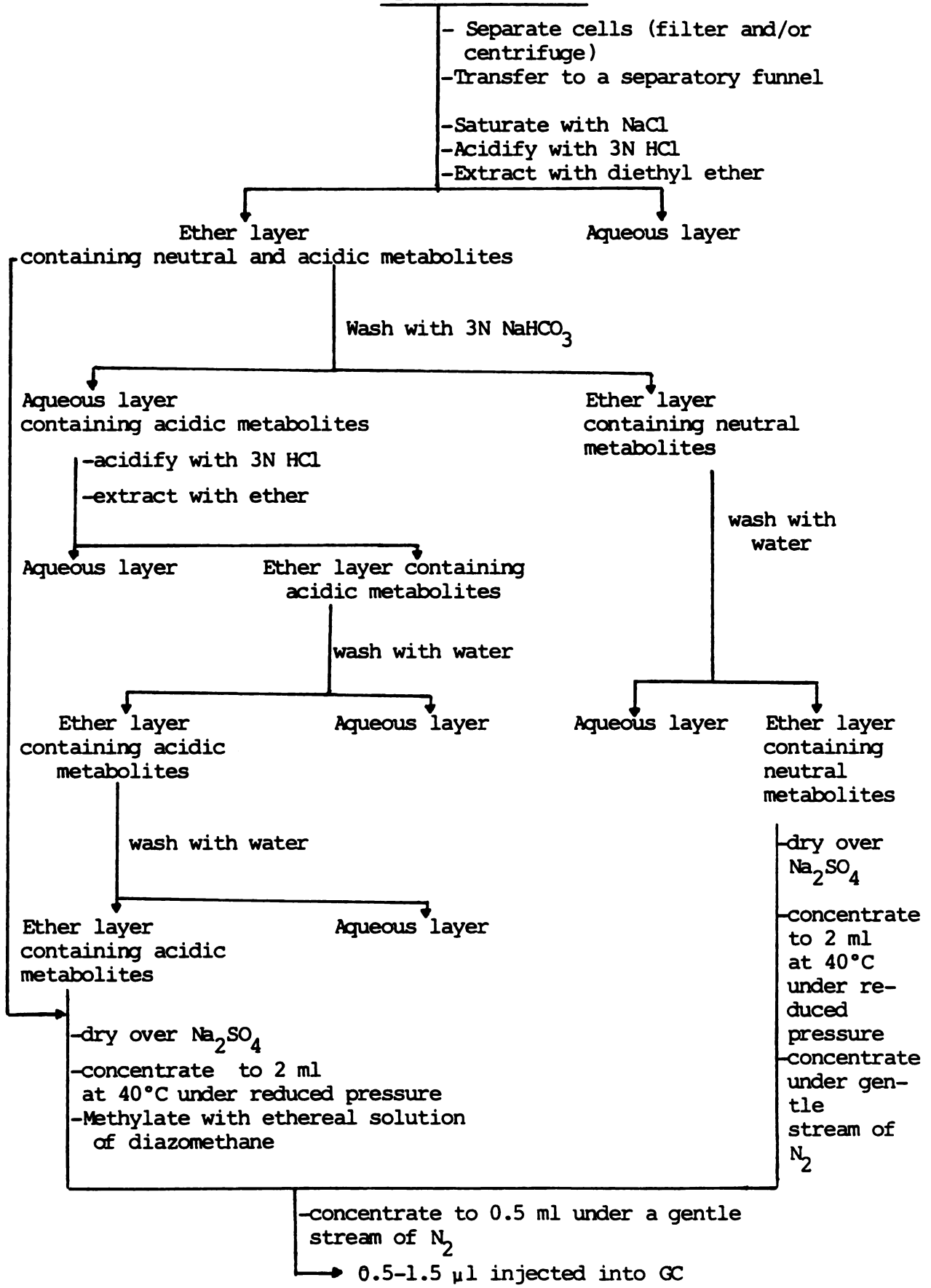
Generally speaking, the extraction was carried out according to Tahara et al. (1977). The filtrate or the supernatant was saturated with analytical reagent (AR) grade sodium chloride, acidified with 3N HCl to a pH of 1-2, transferred into a separatory funnel, extracted with two 50 ml portions of redistilled diethyl ether (original analytical reagent grade diethyl ether was obtained from Mallinckrodt Inc., St. Louis, MO).

The combined ether extracts were washed with distilled, deionized water and dried over anhydrous sodium sulfate. This extract was used for the detection of neutral and acidic metabolites at the same time after methylation.

To divide the above extract into acidic and neutral metabolites, the ether extract was washed with a 3N solution of sodium carbonate. This extract was designated as "wash #1." The aqueous phase was saved, reacidified, and extracted with redistilled ether. The ether layer was washed, dried over anhydrous sodium sulfate and saved for the analysis of acidic metabolites after methylation.

The ether layer from wash #1 was rewashed with water, dried over anhydrous sodium sulfate and saved for analysis for neutral metabolites. The extraction procedure is outlined in Figure 2.

Figure 2. Flow chart diagram for the isolation and purification of acidic and neutral metabolites of sorbic acid


Microbial Culture
- Separate cells (filter and/or centrifuge)
-Transfer to a separatory funnel
-Saturate with NaCl
-Acidify with 3N HCl
-Extract with diethyl ether
Ether layer containing neutral and acidic metabolites
Aqueous layer
Wash with 3N NaHCO3
Aqueous layer containing acidic metabolites
Ether layer containing neutral metabolites
-acidify with 3N HCl
-extract with ether
wash with water
Aqueous layer
Ether layer containing acidic metabolites
wash with water
Ether layer containing acidic metabolites
Aqueous layer
Aqueous layer
Ether layer containing neutral metabolites
wash with water
Ether layer containing acidic metabolites
Aqueous layer
-dry over Na2SO4
-concentrate to 2 ml at 40°C under reduced pressure
-Methylate with ethereal solution of diazomethane
-dry over Na2SO4
-concentrate to 2 ml at 40°C under reduced pressure
-concentrate under gentle stream of N2
-concentrate to 0.5 ml under a gentle stream of N2
0.5-1.5 µl injected into GC

Preliminary concentration

The extracts were transferred into 500 ml round bottom flasks and concentrated under reduced pressure at 40°C, using a Rotavapor-R (Buchi) to a volume of two ml. For further concentration, a nitrogen stream was used to concentrate the extract to 0.1 to 0.5 ml, depending on the concentrations of the metabolites.

Esterification and final concentration

Esterification of the acidic metabolites was carried out with an ethereal solution of diazomethane which was prepared from Diazald (Aldrich Chemical Co.) according to the manufacturer's instructions.

The esterification was carried out using the method described by Wick et al. (1969). An ethereal solution of diazomethane was added drop by drop to the concentrate until a yellow color persisted. The solution was permitted to stand at room temperature for ten minutes. To remove the excess diazomethane and to further concentrate the extract, a stream of nitrogen was directed toward the surface at a rate of 300 to 500 ml/minute.

Headspace Analysis

Headspace analysis was carried out specifically for the detection of 1,3-pentadiene since it is very volatile and is very difficult to extract from aqueous solutions, and to concentrate without entailing significant losses.

After incubation of the microorganism had been terminated, the flasks were tightly stoppered, heated to about 60°C, and a gas-tight syringe was used to draw a sample (one ml) from the headspace. This sample was injected directly into the gas chromatograph or gas chromatograph/mass spectrometer.

Entrapment of Pentadiene

To entrap pentadiene, the method described by Marth et al. (1966) was essentially followed. The flask contents were heated to about 70°C in a water bath and the volatiles were distilled into a small tube containing approximately 0.5 ml of AR grade methanol. The tube was immersed in a dry ice-acetone mixture. A slow stream of air was used to flush the volatiles out of the flask. The tube contents were transferred into a vial kept in a dry ice-acetone mixture. One to two µl portions of the vial contents were injected into the gas chromatograph on the same day.

Gas Liquid Chromatographic Analysis of Sorbic Acid Metabolites

Apparatus and operation conditions

A Hewlett Packard gas chromatograph (Model 5830A) equipped with a flame ionization detector (FID) and a Hewlett Packard 18850A GC terminal (Hewlett Packard Corp., Avondale, PA) was used for the analysis of sorbic acid metabolites. The column used was glass (2 m x 2 mm i.d.) and was packed with ten percent Carbowax 20 M coated on 80/100 mesh acid-washed (AW) Chromosorb W (Supelco, Inc., Bellfonte, PA). The instrument was operated under the following conditions:

Initial temperature (T_1)	:	60°C (30°C for 1,3-pentadiene analysis)
Time at T_1 (t_1)	:	one minute
Rate	:	5°C per minute
Final temperature (T_2)	:	160
Time at T_2	:	5-10 minutes
Injection port temperature	:	200°C

Flame ionization detector temperature	:	275°C
Chart speed	:	one cm per minute
Attenuation	:	variable
Slope sensitivity	:	.02
Carrier gas	:	Nitrogen
Carrier gas flow rate	:	30 ml per minute
Hydrogen flow rate	:	30 ml per minute
Air flow rate	:	200 ml per minute

For injection, a Hamilton syringe of ten µl capacity (701N) (Hamilton Co., Reno, Nevada) was used to inject volumes of between 0.5 to 1.5 µl.

Gas Liquid Chromatography-Mass Spectrometry (GC-MS)

The GC/MS system used was a Hewlett Packard 5985 A Gas Chromatograph/Hewlett Packard Mass Spectrometer (Hewlett Packard Corp.). The column was the same as that used for GC analysis. Helium was the carrier gas with a flow rate of 25 ml/minute. The analyses were carried out using a temperature program from 60°C to 160°C at 5°C/minute, with a one minute hold at 60°C.

The ion source and analyzer temperature of the mass spectrometer were maintained at 200°C. The electron multiplier voltage was 2000 V and the ionizing potential was 70 eV.

Quantitative Analysis of Residual Sorbate by High Performance Liquid Chromatography

Preparation of sample

For the analysis of residual sorbate, five to ten ml samples were drawn aseptically from the growing culture or from the resting cells

reaction medium at certain time intervals, filtered through a Whatman No. 42 filter paper and/or centrifuged as previously described, refiltered through a Millipore filter (0.45 µl) and stored in tightly closed amber vials at 4°C for analysis.

High performance liquid chromatography conditions

A Water Associates high performance liquid chromatograph, Model ALC/GPC 202/R401 equipped with a septumless Model U6K universal injector, a Model M-6000 pump, and a Model 440 absorbance detector (Water Associates, Inc., Milford, MA) was used for the analysis of residual sorbate. The response was recorded on a Model 232 Linear instruments recorder (Linear Instruments Corp.).

The method described by Robach et al. (1978) was followed, except that acetate buffer (pH 4.4) was used in place of phosphate buffer. According to this method, a reverse phase column, µBondapak C_{18} (3.9 mm x 20 cm) (Water Associates), was used.

A degassed mixture of acetonitrile and acetate buffer (20:80, (v/v)) was used as the eluent, with a flow rate of 1.5 ml per minute.

Ten to 40 µl portions of the samples were injected into the chromatograph using a 50 µl syringe.

For quantitation, 20 µl portions of different concentrations (five to ten mg/kg) of a fresh aqueous solution of potassium sorbate were injected, and a standard curve established by plotting peak heights against sorbate concentrations. From this standard curve, the concentration of potassium sorbate was determined. However, in some cases, the sample had to be diluted to fall within the sensitivity range of the instrument.

Fate of Labelled Sorbic Acid

Labelled sorbic acid was utilized in this study to confirm that the metabolites isolated from media containing sorbic acid inoculated with selected microorganisms were in fact derived from sorbic acid. Since only a limited amount of uniformly ^{14}C-labelled sorbic acid (supplied by Monsanto Chemical Company, St. Louis and originally obtained from New England Nuclear, Boston, Mass.) was available, the study was limited to the thin layer chromatographic analysis of the neutral metabolites of Rhizopus stolonifer grown on a medium containing labelled and nonlabelled sorbic acid. For the same reason, only one metabolite in the reaction system was analyzed for the presence of radioactivity. Sorbyl alcohol was chosen for this purpose since a standard sample was commercially available and it had been detected in appreciable amounts over a relatively long period of time among the metabolites of Rhizopus stolonifer grown on sorbate containing medium.

Thin layer chromatography of neutral metabolites of sorbic acid

Precoated 20 x 20 cm silica gel G plates (Fisher Scientific Co., Pittsburgh, PA) were used to separate the neutral metabolites of sorbic acid. The plates were activated at 105°C for one hour. Approximately five µl portions of the neutral metabolite concentrate were spotted on the plates about 1.5 cm from the lower edge. On the same plant, five µl portions of an ethereal solution of standard sorbyl alcohol were also spotted.

The mobile phase was a mixture of petroleum ether, diethyl ether and acetic acid (90:50:1, v/v). The plates were developed in one

dimension for a distance of about 15 cm (approximately 75 minutes), after which the plates were removed, dried at 60°C for about 20 minutes and sprayed with a 50 percent aqueous solution of sulfuric acid. The average R_f values for sorbyl alcohol were recorded.

The neutral extract which was to be examined for the presence of radioactivity was spotted on a preparative TLC plate and developed as previously described. The spot having the same R_f value as the standard sorbyl alcohol was located with the aid of the predetermined R_f value and was removed from the TLC plate and placed in scintillation vials. Ten ml of a liquid scintillation cocktail (Aquasol) (New England Nuclear) were added to the sample. The vial was then placed in a Packard Tri Carb Liquid Scintillation Spectrometer Model 3310 (Packard Instruments Co., Inc., Downers Grove, Ill.) and counts per minute were recorded.

Further confirmation by GC Analysis

Another preparative TLC plate was spotted with the same neutral metabolite extract as above. The compound with an R_f value equal to that of sorbyl alcohol was scraped off the plate, extracted from the silica gel with diethyl either, concentrated and injected into the gas chromatograph. The retention time was compared with that of a standard sorbyl alcohol sample.

RESULTS AND DISCUSSION

Microbial Resistance to Sorbic Acid

One of the main objectives of this study was to test microorganisms for their resistance to sorbic acid. For this reason, various species of molds, yeasts and bacteria were examined.

Molds

Molds in general, exhibited a wide variation in their resistance to sorbic acid. Results of the screening tests for molds using concentrations of potassium sorbate ranging from 500 to 3000 mg/kg are presented in Table 1. Most of the cultures grew in the presence of 500 mg/kg of potassium sorbate at pH 5.5. Only a few molds were inhibited under these conditions and included a strain of Rhizopus oligosporus, two strains of Cladosporium cladosporiodes, and a strain of Alternaria tenuis. As the concentration of potassium sorbate was increased to 1000 mg/kg, a number of other molds were also inhibited (Table 1). However, most of the inhibition occurred when the concentration of potassium sorbate was increased above 2000 mg/kg (Table 1).

Some of the mold cultures, however, were found to be relatively resistant to sorbic acid and included Aspergillus flavus NRRL 2999, Aspergillus flavus NRRL 6550, Fusarium sp., Geotrichum candidum, Penicillium sp. and Trichoderma viride. These mold cultures grew well in the presence of 3000 mg/kg of potassium sorbate. Unfortunately, some of the resistant molds are potential food spoilage agents, and

Table 1. Inhibitory concentrations of potassium sorbate for some mold cultures on YPG plates at pH 5.5, 25°C

500 mg/kg potassium sorbate	2000 mg/kg potassium sorbate
Rhizopus oligosporus ATCC 2295	Alternaria sp.
Cladosporium cladosporiodes QM 489	Mucor pusillus
Cladosporium cladosporiodes QM 9485	Mucor humiculus
Alternaria tenuis NRRL 2169	Mucor sp.
	Rhizopus oligosporus NRRL 2710
	Rhizopus stolonifer
	Penicillium janthinellum NRRL 2016

1000 mg/kg potassium sorbate	3000 mg/kg potassium sorbate
Fusarium roseum	Aspergillus oryzae
Fusarium oxysporum NRRL 1943	Aspergillus niger
Fusarium sp.	Penicillium roqueforti
Calvatia gigantica	
Penicillium oxalicum NRRL 790	

in addition, Aspergillus flavus can produce aflatoxins in foods under certain conditions. In this regard, it has been reported that sorbic acid will inhibit aflatoxin B_1 production by Aspergillus flavus when used at a concentration of 100 mg/kg. However, much higher levels (1700 mg/kg) of sorbic acid are necessary for complete inhibition of the growth of the mold.

Species of both genera, Aspergillus and Penicillium, were found to be, in general, more resistant than the other species of other genera. Most of these cultures required 2000 mg/kg or more for complete inhibition. This observation agrees with the findings of York (1960) who reported that 25 mg of undissociated acid per 100 ml of medium (250 mg/kg) was necessary to inhibit all but the most resistant Penicillium. This concentration is equivalent to approximately 2000 mg/kg of potassium sorbate at pH 5.5. It was also reported that some species of Penicillium were resistant to the highest sorbic acid concentration (300 mg/kg) used (York, 1960).

On the other hand, Klis et al. (1959) who studied the effect of several antifungal agents including sorbic acid on the growth of common food spoilage fungi, reported that a number of fungi including Alternaria sp., Botrytis cinerea, Fusarium sp., Mucor mucedo, Oidium lactis (Geotrichum candidum), Penicillium digitatum, Penicillium expansum, Penicillium sp., and Rhizopus sp. were completely inhibited for 96 hours by 500 mg/kg of sorbic acid at pH about 5.6. However, Aspergillus niger was not inhibited by 1000 mg/kg of sorbic acid. In the present study, Aspergillus niger was found to require 3000 mg/kg for complete inhibition. Similarly, two strains of Geotrichum candidum were found to resist a concentration of potassium sorbate as high as 3000 mg/kg. A preliminary study on a strain of Penicillium digitatum indicated that this mold can tolerate at least 1500 mg/kg of potassium sorbate.

When the concentration of potassium sorbate was raised to 5000 mg/kg (600 mg/kg of undissociated acid), none of the mold cultures tested grew over a period of five days. Likewise, Bell and Borg (1959) tested 66 species of mold in 32 genera for growth in basal media with or without 0.1 percent sorbic acid (1000 mg/kg) at pH 4.5 and 7.0. All of the cultures grew in the control basal media at both pH values and in the sorbic acid media at pH 7.0, but none grew in the basal medium containing sorbic acid at pH 4.5. At this latter pH, the concentration of the undissociated acid is about 650 mg/kg.

Contrary to previous reports, Marth et al. (1966), found that a number of Penicillium species including Penicillium cyano-fulcum, Penicillium notatum, Penicillium frequentans, Penicillium roqueforti and a mutant of Penicillium roqueforti were able to grow in the presence of up to 1800, 2300, 2800, 5400 and 7100 mg/kg of potassium sorbate at pH 5.5, respectively. These mold cultures were isolated by the authors from potassium sorbate-preserved cheese.

According to the results of the present study and the previous literature reports, variations in the susceptibility to sorbic acid exists between different mold species of a single genus, and to a lesser extent between the cultures of the same species. The latter variation is possibly due to the effect of their previous habitats.

Yeasts

Although both solid and liquid media were used for the screening tests, only those of the solid media will be shown here. Slight differences, however, were observed between the two media, i.e., slightly higher concentrations of potassium sorbate were required to inhibit the growth

of yeast cells in liquid media compared to those required to achieve the same effects in solid media. These findings are in agreement with those of York (1960). He ascribed this to the possible syneresis which occurs at the surface of media upon autoclaving and in subsequent drying of the surface, coupled with the slower rate of diffusion of solutes through agar than through a liquid medium.

Results of the screening tests on selected yeasts using concentrations of potassium sorbate ranging from 500 to 3000 mg/kg at pH 5.5 are presented in Table 2. Most of the yeasts tested possessed some resistance to sorbic acid. The majority of these yeasts are resistant to concentrations of potassium sorbate ranging from 500 to 1000 mg/kg (60-120 mg/kg of undissociated acid). Rhodotorula rubra, Endomycopsis selenospora, Schizosaccharomyces pombe and Sporobolomyces coralliformis were very susceptible to as low as 500 mg/kg of potassium sorbate (Table 2). These cultures either did not grow at all or grew very weakly. Thus, this concentration was considered inhibitory to this group. The growth of another group of yeasts was found to be severely retarded or completely inhibited by 1000 mg/kg of potassium sorbate (Table 2). The members of the last two groups required concentrations of potassium sorbate ranging from 2000 to 3000 mg/kg for complete inhibition of growth. This range corresponds to 237 to 355 mg/kg of the undissociated acid.

It is apparent that the members of the four groups of yeasts do not have many things in common, except the way they behave toward sorbic acid, i.e., the four groups include cultures of different criteria. York (1960) pointed out that yeasts with a predominantly oxidative metabolism, e.g. Pichia, Debaryomyces, Rhodotorula, and Cryptococcus are more

Table 2. Inhibitory concentrations of potassium sorbate for some yeasts on YPG plates at pH 5.5, 25°C

500 mg/kg potassium sorbate	2000 mg/kg potassium sorbate
Rhodotorula rubra	Saccharomyces carlsbergensis ATCC 9080
Endomycopsis selenospora NRRL Y-1357	Hansenula anumala
Schizosaccharomyces pombe	Hansenula anumala
Sporobolomyces coralliformis ATCC 16039	Hansenula californica NRRL Y-1427
	Saccharomyces cerevisiae
	Saccharomyces cerevisiae
	Saccharomyces rouxii
	Saccharomyces cerevisiae var elliposoideus
	Torulopsis sphaerica
	Hansenula wingei ATCC 14355
	Hansenula wingei ATCC 14355
	Endomycopsis bispora
	Saccharomyces kluyveri
	Saccharomyces kluyveri (C-26)

1000 mg/kg potassium sorbate	3000 mg/kg potassium sorbate
Endomycopsis bispora ATCC 14628	Candida krusei
Torulopsis sake NRRL Y-1622	Schizosaccharomyces octosporus
Nadsonia fulvescens NRRL Y-991	Debaryomyces membranaefaciens NRRL Y-489
Rhodotorula sp.	Saccharomyces oleaginosus
Rhodotorula glutinis var glutinis	Pichia fermentans
Candida utilis NRRL Y-900	
Saccharomyces sp.	
Schizosaccharomyces aponicus var virsatilis	
Trigonopsis variabilis ATCC 10679	
Candida steatolytica	
Trichosporon fermentans NRRL Y-1492	
Trichosporon cutaneus ATCC 13445	

sensitive to sorbic acid than many of the yeasts which have fermentative properties. In the present study, to the contrary, several yeasts with strong fermentative activities were shown to be very susceptible to sorbic acid (Table 2). It appears that there are factors other than the fermentative properties that influence yeast behavior towards sorbic acid.

In contrast to the results of this study, Emard and Vaughn (1954) tested ten cultures representing ten species of yeasts and concluded that the cultures tested did not exhibit marked resistance to sorbic acid. The same authors tested Debaryomyces membranaefaciens on a glucose broth containing a 0.07 percent sorbic acid at different pH values. They found that the growth of this yeast was severely retarded at pH 6.4, while growth was completely inhibited when the pH was lowered to 5.4 (170 mg/kg of undissociated acid). Results of the present study indicate that the same yeast species required 355 mg/kg of undissociated acid.

Some of the yeast cultures investigated in the screening tests were found to possess relatively high resistance to sorbic acid and included Candida lipolytica, Hansenula saturnus, Schwanniomyces occidentalis, and Brettanomyces claussenii. These yeast cultures could grow well in the presence of 3000 mg/kg of potassium sorbate at pH 5.5 (355 mg/kg of undissociated acid). This concentration is well above the concentration (236 mg/kg undissociated acid) which Costilow, et al. (1955) reported was necessary to inhibit the most tolerant strains of yeasts, Torulopsis holmii and Candida krusei. In the present study, 355 mg/kg of undissociated acid was necessary to inhibit the latter yeast, Candida krusei.

When the concentration of potassium sorbate was raised to 5000 mg/kg, none of the tested yeast cultures grew. However, Splittstoesser et al.

(1978) reported that a strain of Saccharomyces bisporus var bisporus exhibited exceptional resistance to sorbic acid. This yeast, which was isolated from grape juice, grew well in a grape juice broth containing over 800 mg/kg of the undissociated acid. This concentration is equivalent to about 6700 mg/kg of potassium sorbate at pH 5.5.

From the results obtained in present study and from previous literature reports, it can be concluded that there is a variation within members of a single species in their behavior toward sorbic acid. One explanation for this variation could be the type of media used in the experiments. For example, Marth et al. (1966) and Emard and Vaughn (1952) have shown that the media greatly affect the microbial response to sorbic acid. Another explanation is the previous habitat of the yeast strain.

Bacteria

Both liquid and solid media were used for the preliminary screening tests on bacteria. Essentially, no significant difference was found between liquid and solid media as far as the inhibitory action of sorbic acid is concerned. Thus, the liquid medium was chosen for this study and only those observations will be presented here.

Results of the screening tests on bacterial cultures when grown in different concentrations of potassium sorbate (500-3000 mg/kg) at pH 5.5 are presented in Table 3. As was the case with molds and yeasts, the resistance of bacteria to sorbic acid varies widely. Most of the bacterial cultures tested in the study were not inhibited in culture media fortified with 500 mg/kg of potassium sorbate at pH 5.5.

Bacillus cereus can cause food borne illnesses and is readily isolated from nearly all plant foods and dehydrated foods (Ayres et al., 1980).

Table 3. Inhibitory concentrations of potassium sorbate for some bacterial cultures on YTG broth at pH 5.5, 25°C

500 mg/kg potassium sorbate	2000 mg/kg potassium sorbate
Bacillus cereus	Salmonella typhimurium
Bacillus coagulans	Serratia marcescens
Bacillus polymyxa	Pseudomonas fluorescens
Sarcina lutea	Pseudomonas schuylkilliensis ATCC 15916
	Pseudomonas schuylkilliensis ATCC 15917
	Pseudomonas vulgaris
	Staphylococcus aureus
	Streptococcus zymogenes
	Salmonella senftenberg
	Micrococcus flavus
1000 mg/kg potassium sorbate	**3000 mg/kg potassium sorbate**
Aerobacter aerogenes	Lactobacillus plantarum
Bacillus subtilis	Lactobacillus bulgaricus
E. coli	Leuconostoc mesenteroides
Pseudomonas fragi	Pedicoccus cerevisiae
Staphylococcus epidermidis	Pseudomonas fluorescens
Salmonella pollorum	Lactobacillus brevis

This bacterium was shown to be very sensitive to sorbic acid in this study. Potassium sorbate at a concentration of 500 mg/kg was very effective in inhibiting a strain of this bacterium in culture medium at pH 5.5.

When the concentration of potassium sorbate was increased to 1000 mg/kg, the growth of a number of bacterial species were markedly retarded or completely inhibited (Table 3). This group included some enteric bacteria such as Aerobacter aerogenes, Escherichia coli and Salmonella pollorum.

As the concentration of potassium sorbate was increased to 2000 mg/kg, more bacteria were inhibited (Table 3). Two cultures of Salmonellae (Salmonella senftenberg and Salmonella typhimurium), which are well-known food illness agents, were effectively inhibited as was Staphylococcus aureus which is also known to cause food poisoning. Emard and Vaughn (1952) reported that strains of Staphylococcus are considerably more resistant to sorbic acid than other catalase positive microorganisms, including some species of Salmonella. According to their data, Staphylococci grew in 1000 mg/kg of sorbic acid in liver infusion media but were inhibited by 2000 mg/kg of sorbic acid in liver infusion media and by 700 mg/kg of sorbic acid in glucose yeast extract media. Recently, Tompkin et al. (1974) reported that growth of the Salmonellae (Salmonella anatum, Salmonella infantis, Salmonella senftenberg, Salmonella choleraesuis, and Salmonella newport) in cooked, uncured sausage with a pH of 6.2 were remarkedly retarded by 1000 mg/kg (<50 mg/kg of undissociated acid) of potassium sorbate. This concentration is much lower than the concentration (120-140 mg/kg) which was found to be necessary to inhibit some of these bacterial cultures in the present study. Tompkin et al.

(1974) reported that Staphylococcus aureus was not inhibited by 1000 mg/kg of potassium sorbate, which agreed with the results obtained in this study for the same microorganism.

Some of the food spoilage bacteria such as strains of Pseudomonas fluorescens and Pseudomonas vulgaris were also effectively inhibited by this concentration (2000 mg/kg) (Table 3).

The members of the third group (Table 3) were found to possess considerable resistance to sorbic acid. They required 3000 mg/kg of potassium sorbate for marked retardation or complete inhibition of growth. Of the most resistant bacteria among the test bacteria were lactic acid bacteria. It is interesting to note that when these bacteria were cultivated in a rich medium such as trypticase soy broth, the degree of inhibition was less, i.e., more potassium sorbate (5000 mg/kg) was needed to accomplish the same degree of inhibition. These results confirmed those of Emard and Vaughn (1952), Costilow et al. (1955), and York (1960) with respect to the tolerance of the catalase negative lactic acid bacteria to sorbic acid, especially in highly nutritious media.

Microbial Degradation of Sorbic Acid[1]

Melnick et al. (1955) were the first to report that mold enzymes accomplish metabolic degradation of sorbic acid by reducing it to carbon dioxide and water. In spite of the importance of sorbic acid as a food preservative, few studies have been carried out on the metabolism of sorbic acid, and especially the identification of the metabolites of this additive. One of the objectives of the present study was to qualititatively follow the fate

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[1]The terms sorbic acid and potassium sorbate will be used interchangeably throughout the text.

of sorbic acid in the presence of microorganisms in terms of the metabolites produced, and quantitatively in terms of how much sorbic acid was consumed. In order to follow the fate of sorbic acid in the presence of different microbes, both resting and growing cells studies were performed using some selected microbes. Observations on only some of these microbes will be presented herein.

Data regarding the utilization of sorbate by growing cells of different fungi cultivated on YPG media containing 1000 mg/kg of potassium sorbate are presented graphically in Figure 3. As indicated, some microbes such as Trichorderma viride and Penicillium sp. degrade sorbic acid completely in a relatively short time. Other molds such as Rhizopus stolonifer were relatively slower in degrading sorbic acid, and thus took a longer time to bring about a total destruction of sorbic acid. The reason for this delay was that mold growth was initially retarded for at least one day, after which growth proceeded normally.

Geotrichum candidum and Candida lipolytica were though to be very effective in degrading sorbic acid, especially Geotrichum candidum, since they started to grow vigorously in a relatively short time. Results indicated that the growing cells of Geotrichum candidum required two to three days to bring about total degradation of sorbic acid, while Candida lipolytica required three days to achieve the same effect (Figure 3). The resting cells of both organisms were also much slower than the other fungi in bringing about the total destruction of sorbic acid (Figure 4). This behavior may be ascribed to the catabolic repression or the "glucose effect." This phenomenon is usually encountered when a certain microbial culture is grown in a medium containing pairs of compounds as carbon sources,

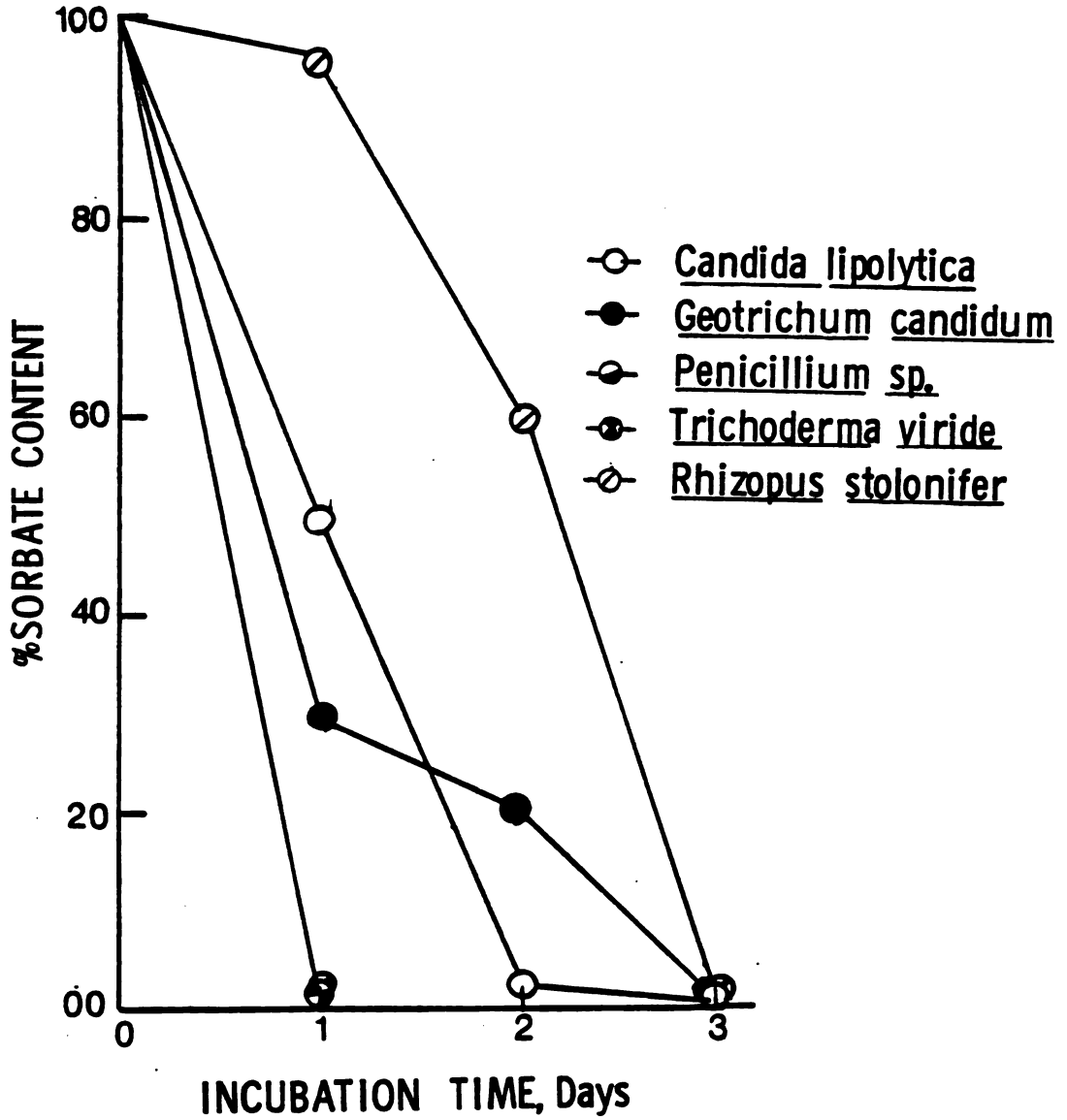


Figure 3. Utilization of sorbate by growing cells of different fungi cultures cultivated on YPG containing 1000 mg/kg of potassium sorbate, at pH 5.5, and an incubation temperature of 25°C

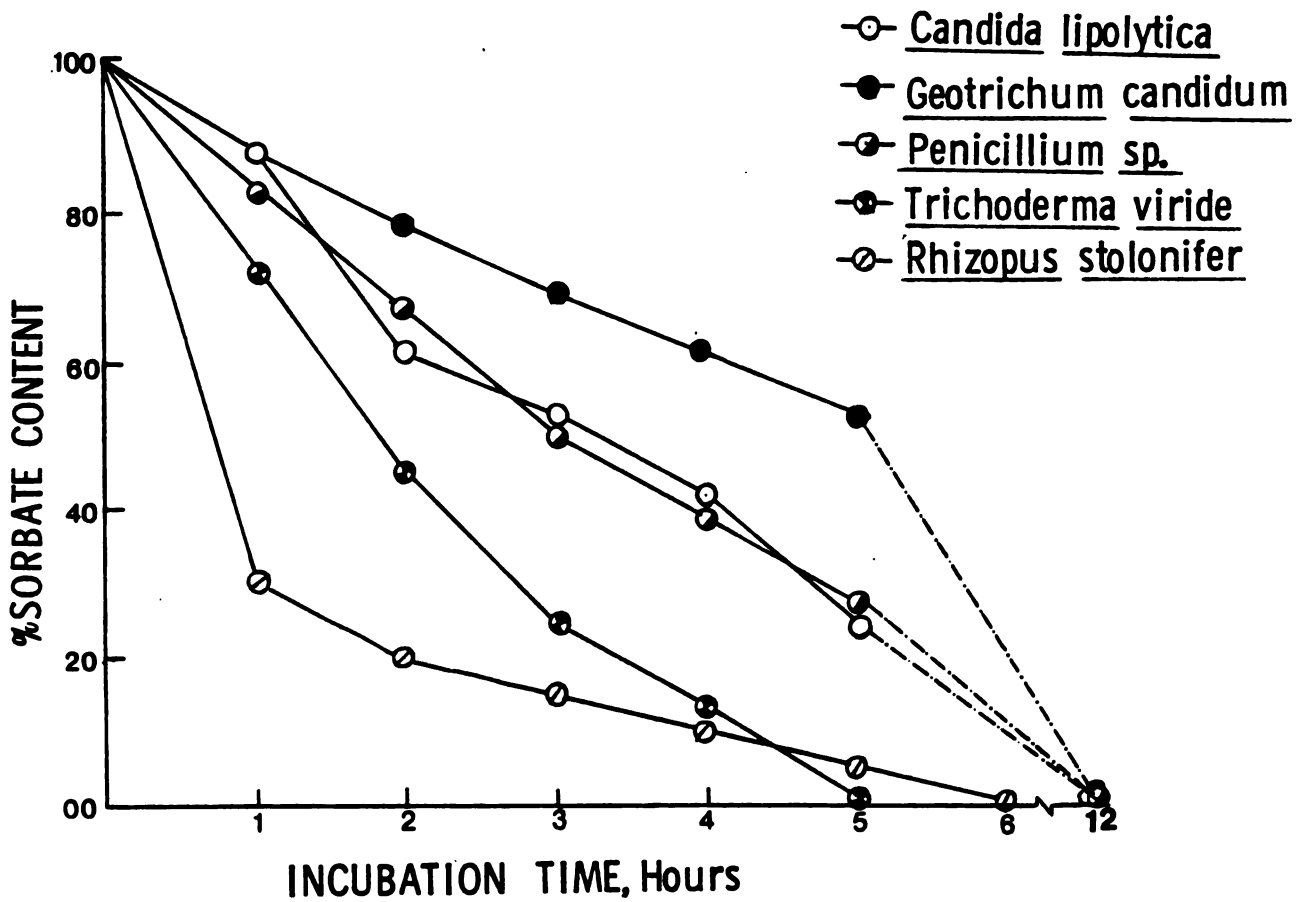


Figure 4. Utilization of sorbate by resting cells of different fungi cultures suspended in a phosphate buffer containing 2.0 percent glucose and 1000 mg/kg of potassium sorbate, at pH 5.5 and an incubation temperature of 25°C

one of which is more easily metabolized than the other (Stanier et al., 1976). It appears that these two microorganisms depleted the glucose after which growth ceased. The microorganisms enter a short lag phase where they start to synthesize the inducible enzymes necessary for sorbic acid metabolism.

In order to exclude the complications of growing cells, and to follow the fate of sorbic acid in a relatively short period, experiments were also carried out with resting cells (Figure 4). Since the experiments were initiated with heavy inocula, total destruction of sorbic acid was accomplished in a relatively short time. Resting cells of Rhizopus stolonifer depleted sorbic acid in six hours. This was in contrast to the growing cells of the same mold which took a relatively long time (three days). These observations appear to be consistent with those of Kurogochi et al. (1975) and Tahara et al. (1977) on Mucor sp.

Species of Aspergillus and Mucor behaved toward sorbic acid in a similar way to that of Penicillium and Rhizopus, respectively. Thus, results with only the latter genera were included in this study.

Results, in general, indicate that the species of the genera Penicillium, Aspergillus, Mucor, and Rhizopus were very effective in degrading sorbic acid when present in sublethal concentrations. Trichoderma viride also very effective in degrading sorbic acid, but since only one species was tested, one cannot make any generalizations about the entire genus. These findings are in general agreement with those of Lukas (1963) who reported that sorbic acid was decomposed by Aspergillus niger, Aspergillus oryzae, Penicillium sp., Penicillium expansum, Alternaria sp., Trichoderma legnorum, Mucor racemorus and Rhizopus nigricans (Rhizopus stolonifer).

To rule out the possibility of loss due to chemical and/or physical means, uninoculated samples treated the same way, were analyzed for residual sorbate. These samples, however, did not exhibit any loss of sorbate during the incubation period of the inoculated samples. Hence, the microbial degradation of sorbic acid was confirmed.

The bacteria, Lactobacillus bulgaricus, Lactobacillus plantarum, Pediococcus cerevisiae and Pseudomonas fluorescens were grown on trypticase soy broth fortified with 1000 mg/kg of potassium sorbate. Sorbate concentration were monitored over a period of six days. All of the above bacteria, except Pseudomonas fluorescens, did not exhibit any significant loss. Pseudomonas fluorescens showed about 28 percent loss after three days.

Sorbic Acid as a Carbon Source

Having established that sorbic acid could be depleted by some of the microorganisms tested, further studies were conducted to establish which microorganisms could use sorbic acid as a sole source of energy and carbon. Results of this study help in understanding the pathway of degradation and the mechanism by which microorganisms detoxify sorbic acid. For this reason, most of the microbial cultures were grown on basal media containing 3000 mg/kg of potassium sorbate as the only carbon source. Growth on this medium was the criterion for utilization of sorbic acid as a carbon source.

The state of growth of selected mold cultures on the medium containing both sorbic acid and glucose and the basal medium containing only sorbic acid is shown in Table 4. Trichoderma viride grew vigorously on both media as did Penicillium sp., Geotrichum candidum, Aspergillus niger and

Table 4. The effect of the presence or absence of glucose (two percent) on the growth of different mold cultures on yeast extract-peptone-agar media (pH 6.0) containing 3000 mg/kg of potassium sorbate and incubated at 25°C for six days

Mold	Growth on yeast extract-peptone-agar+3000 mg/kg potassium sorbate	Growth on yeast extract-peptone-agar+ 3000 mg/kg potassium sorbate + glucose
Aspergillus flavus NRRL 2999	++	+
Aspergillus flavus NRRL 6550	++	+
Penicillium roqueforti	+	++
Aspergillus niger	+	+
Trichoderma viride	+++	+++
Penicillium sp.	++	++
Geotrichum candidum	++	++
Aspergillus oryzae	+	+
Fusarium sp. (2)	++	+

\+ Moderate growth

++ Good growth

+++ Vigorous growth

Aspergillus oryzae. Only Penicillium roqueforti exhibited more vigorous growth on the basal medium containing both sorbic acid and glucose than it did on the medium containing only sorbic acid. Marth et al. (1966) also reported that the depletion of sorbic acid was enhanced by a highly nutritious substrate and retarded by a minimal growth medium.

Unexpectedly, two strains of Aspergillus flavus and one strain of Fusarium sp. exhibited better growth on the basal medium containing only sorbic acid. This behavior was designated as the "glucose inhibitory effect." This may be explained by the assumption that these organisms, once grown on media containing both sorbic acid and glucose, can produce glucose metabolites in the early stages of growth. These metabolites may enhance the inhibitory action of sorbic acid. In this regard, Phaff et al. (1966) stated that metabolites of glucose may reduce the pH, thus accentuating the inhibitory effect of sorbic acid.

When the concentration of potassium sorbate was raised to 5000 mg/kg in both media, none of the mold cultures could grow.

The yeasts which could grow when sorbic acid was used as the sole source of carbon are listed in Table 5. As indicated, Candida lipolytica grew vigorously on media containing sorbic acid as the sole source of carbon. Unexpectedly, sorbate (3000 mg/kg) in the presence of glucose (2.0 percent) markedly inhibited growth of Candida lipolytica. An almost similar result was observed with Hansenula californica, although the inhibition here was more apparent (Table 5). Contrary to the observations in this study with regard to the inhibitory effect of glucose, Pitt (1974) reported that increasing the glucose concentration from 0.5 to 1.0 percent in a yeast nitrogen base broth increased the maximal levels of sorbate permitting growth of Sacchanomyces bailii from 380 mg/kg to 600 mg/kg. This

Table 5. The effect of the presence or absence of glucose (two percent) on the growth of yeast cultures on yeast extract-peptone-agar media (pH 6.0) containing 3000 mg/kg of potassium sorbate and incubated at 25°C for six days

Yeast	Growth on yeast extract-peptone-agar + 3000 mg/kg potassium sorbate	Growth on yeast extract-peptone-agar + 3000 mg/kg potassium sorbate + glucose
Candida lipolytica	+++	±
Hansenula californica NRRL Y-1425	++	-
Candida steatolytica	+	-
Trichosporon cutaneus	+	-
Saccharomyces occidentalis ATCC 2320	+	+
Brettanomyces claussenii NRRL Y-149	+	+
Candida krusei	±	±
Debaryomyces membranaefaciens NRRL Y-149	±	±
Hansenula wingei ATCC 14355	±	±
Hansenula wingei ATCC 14356	±	±
Schizosaccharomyces octosporus	±	-
Saccharomyces cerevisiae	±	±

- No growth

± Weak growth

+ Moderate growth

++ Good growth

+++ Vigorous growth

case, in particular, could be explained by the fact that this yeast is an osmophilic one (Ayres et al., 1980).

Interestingly, the yeasts which grew vigorously in the presence of 3000 mg/kg of potassium sorbate at pH 5.5 grew weakly when the pH was raised to 6.0 (Table 5). One possible reason for this behavior is that these yeasts were maintained on a medium with a pH of approximately 5.0. Thus, it appears that they adapted to this low pH. The other possibility is that these yeast cultures possess pH optima close to 5.5.

It is apparent from the present study that only yeasts with high tolerances to sorbic acid can metabolize sorbic acid as the sole source of carbon. However, one cannot make any positive conclusions about these observations since other yeasts which are sensitive to sorbic acid may utilize it, if present in sublethal concentrations. Hansenula wengei, when grown in a liquid medium fortified with 1500 mg/kg of potassium sorbate as the only sole source of carbon, was effective in metabolizing sorbic acid in a very short time. It appears, however, that at high concentrations of sorbate (3000 mg/kg), sorbic acid no longer serves as a substrate, but instead becomes an inhibitory substrate. In this regard, Deak and Novak (1972) reported that sorbic acid, at high concentration, exerted an inhibitory effect on yeast species capable of metabolizing it, while at low concentration it acted as a substrate.

Bacteria, which grow in the presence of 3000 mg/kg of potassium sorbate, both in the presence or absence of glucose are listed in Table 6. Staphylococcus aureus, Salmonella senftenberg and Pseudomonas vulgaris grew well in the presence of sorbic acid when used as the sole source of carbon. However, growth of these bacteria was retarded by the presence of glucose and sorbic acid together. Aerobacter aerogenes grew when

Table 6. The effect of the presence or absence of glucose (two percent) on the growth of bacterial cultures on yeast extract-peptone-agar media (pH 6.0) containing 3000 mg/kg of potassium sorbate and incubated at 25°C for six days

Bacteria	Growth on yeast extract-tryptone-agar + 3000 mg/kg potassium sorbate	Growth on yeast extract-tryptone-agar + 3000 mg/kg potassium sorbate + glucose
Staphylococcus aureus	++	±
Staphylococcus epidermidis	±	-
Lactobaccilus plantarum	-	+
Lactobacillus bulgaricus	-	+
Leuconostoc mesenteroides	±	±
Enterobacter aerogenes	+	-
Stretococcus lactis	±	±
Salmonella senftenberg	++	-
Pseudomonas vulgaris	++	-
Pediococcus cerevisiae	-	±
Serratia marcescens	+	-

- No growth

± Weak growth

+ Moderate growth

++ Good growth

+++ Vigorous growth

sorbic acid was the only source of carbon, but in the presence of both glucose and sorbic acid, this bacterium was completely inhibited. The previous explanation to this phenomenon, i.e., the glucose inhibitory effect, in connection with molds may also apply here.

The lactic acid bacteria did not grow when sorbic acid was used as the sole source of carbon (Table 6). Leuconostoc mesenteroides, however, grew weakly under similar conditions.

Costilow et al. (1955) tested a number of lactic acid bacteria for their abilities to deplete sorbic acid and found that none could metabolize sorbic acid. Rehm et al. (1964) reported that Lactobacillus buchneri and Lactobacillus arabinosus (Lactobacillus plantarum) can grow in high concentrations of sorbic acid but no metabolites of sorbic acid were observed. Results of this study are in agreement with those of previous reports. In contrast to these observations, Crowell and Guymon (1975) reported the isolation of several sorbic acid metabolites from sorbic acid-preserved red wine spoiled by lactic acid bacteria. These authors did not mention which lactic acid bacterium or bacteria was/were responsible for the metabolites.

Metabolites of Sorbic Acid

Sorbic acid metabolism by molds

Selected molds were grown in liquid media containing sublethal concentrations of sorbate, and the media analyzed for the presence of metabolites of sorbic acid. Figure 5 shows a partial GC chromatogram of the neutral metabolites of sorbic acid produced by the mold, Rhizopus oligosporus. Four major peaks were observed on this chromatogram. Two of these metabolites (retention time, 13 and 13.2 minutes) gave mass spectral fragmentation patterns (Figures 6 and 7) which were found to be characteristic

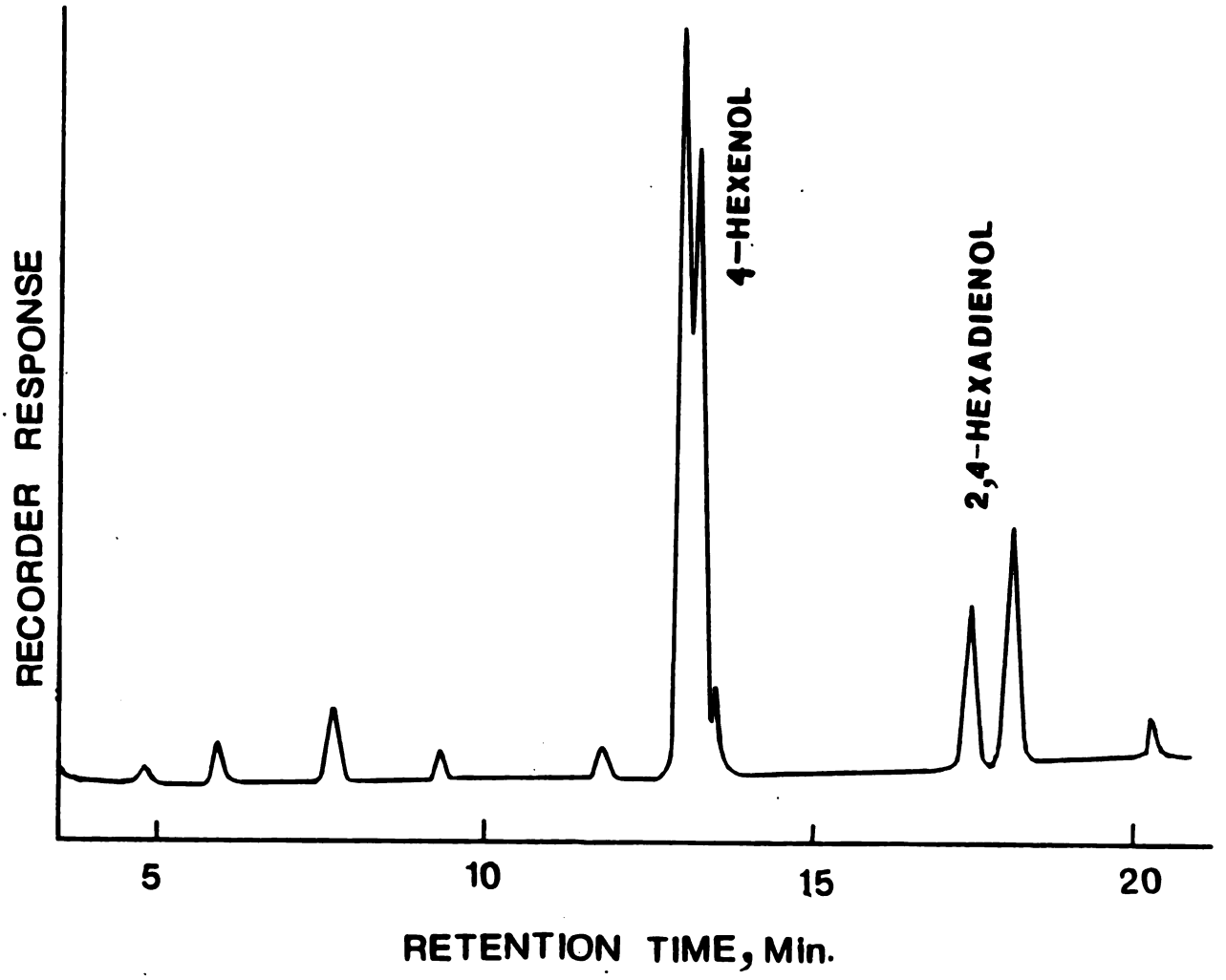


Figure 5. A partial gas chromatogram of the neutral metabolites of sorbate-grown Rhizopus oligosporus

of trans-4-hexenol (Wick et al., 1969; Kurogochi et al., 1975). This product was detected only in the late stages of growth of this mold. The other two major metabolites (retention time, 17.5 and 18.1 minutes) exhibited similar fragmentation patterns (Figures 8 and 9) when subjected to mass spectrometric analysis. These fragmentation patterns are typical of 2, 4-hexadienol (sorbyl alcohol) (Kurogochi et al., 1975). One minor metabolite, however, was detected by injecting two µl of the final neutral extract of a 36 hour culture into the GC/MS system and scanned for the mass 96. The mass spectrometric fragmentation pattern of this minor metabolite was similar to that of 2, 4-hexadienal (sorbaldehyde) (Figure 10).

A possible explanation for having two gas chromatographic peaks for both 4-hexenol and sorbyl alcohol is that sorbic acid could have originally existed in two or more forms, i.e., the sorbic acid used in this study was not 100 percent trans-2-trans-4-hexadienoic acid. Instead, it may be a mixture of two or more isomers. However, it is also possible that microbial enzymes produce isomers of sorbic acid as a means of detoxification.

Tahara et al. (1975) reported that four species of Rhizopus accumulated sorbyl alcohol in their broth after 17 to 18 days of cultivation. Data from the present study indicate that selected species of Rhizopus did produce appreciable amounts of sorbyl alcohol in the early stages of growth. However, it was very difficult to detect any trace of sorbyl alcohol after five days of growth as judged by successive GC analyses. The apparent discrepancy between the two studies may have been the result of the differences in the mode of incubation, i.e, shaked versus still cultures.

Sorbaldehyde, on the other hand, was detected only in the early stages of growth but disappeared quickly. From preliminary studies with

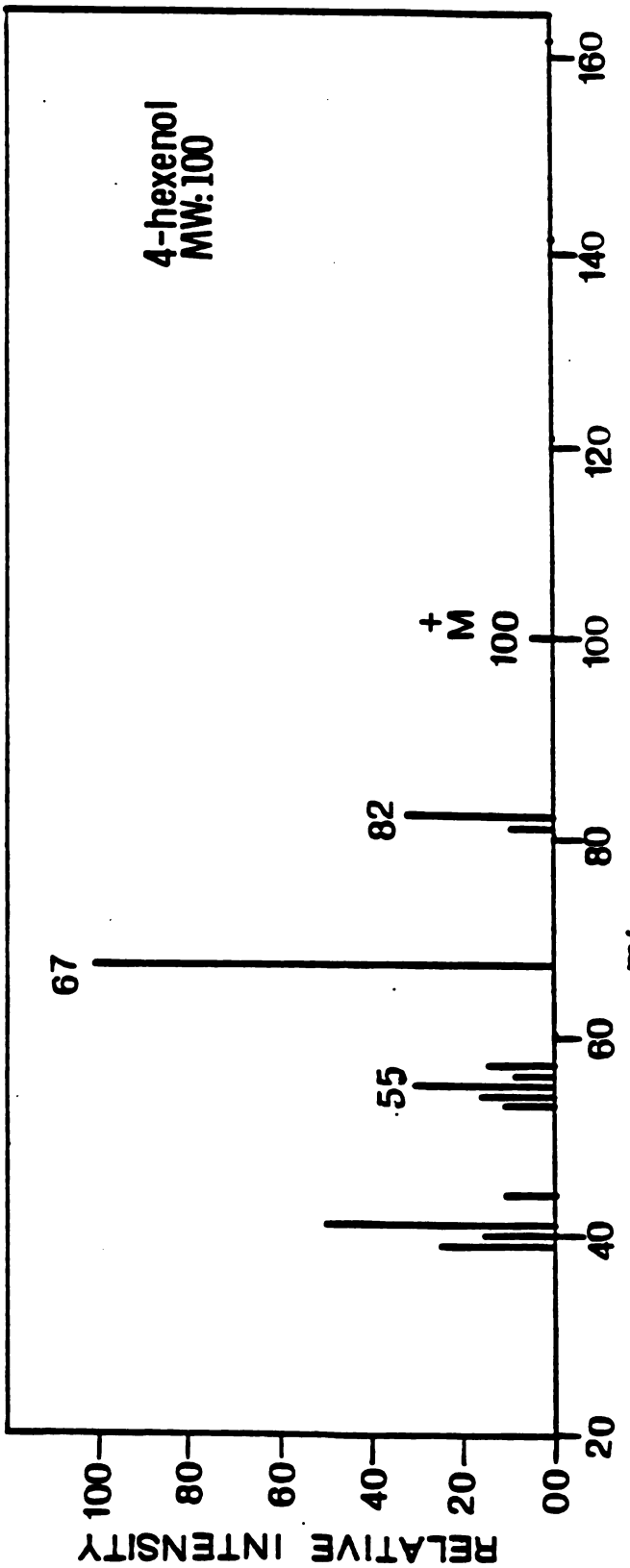


Figure 6. Mass spectrum of 4-hexenol (retention time, 13.0 minutes) isolated from a medium containing sorbate and inoculated with Rhizopus oligosporus

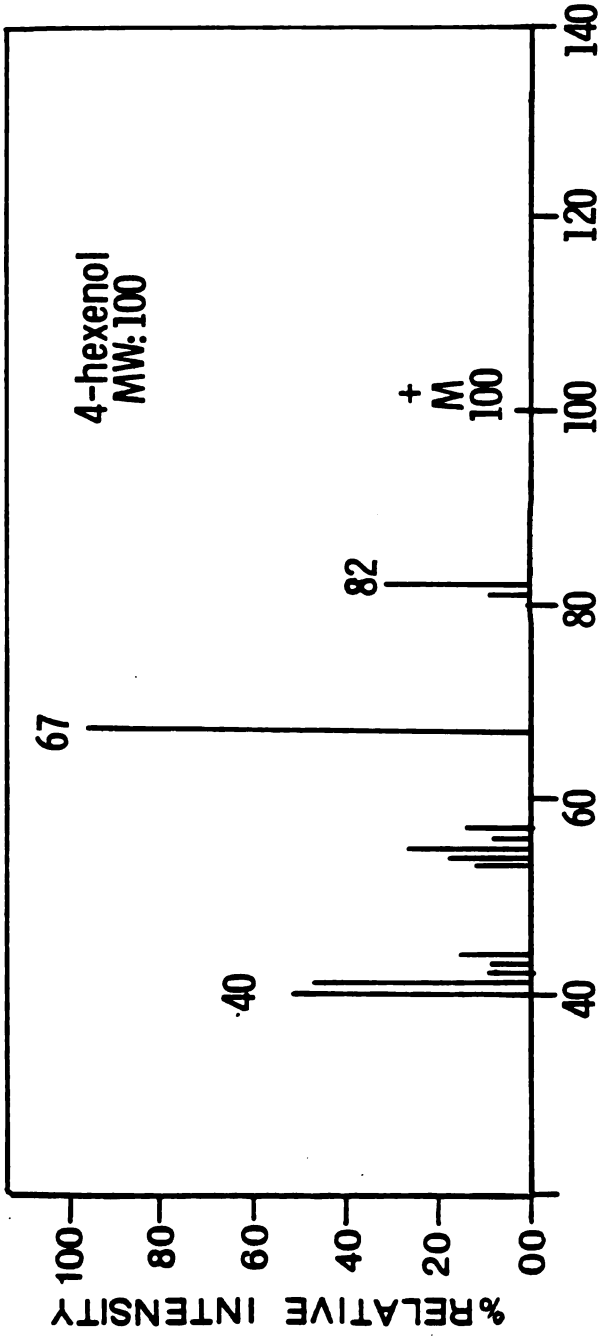


Figure 7. Mass spectrum of 4-hexenol (retention time, 13.2 minutes) isolated from a medium containing sorbate and inoculated with Rhizopus oligosporus

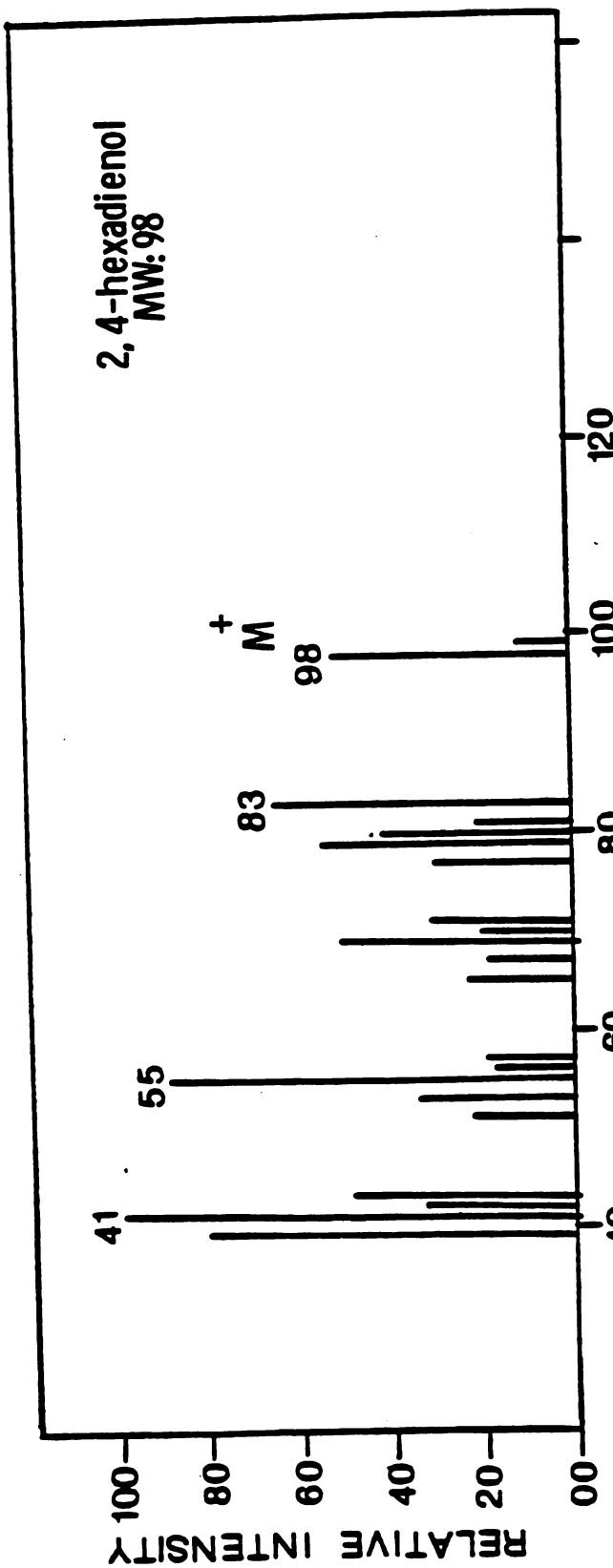


Figure 8. Mass spectrum of 2,4-hexadienol (retention time, 17.5 minutes) isolated from a medium containing sorbate and inoculated with Rhizopus oligosporus.

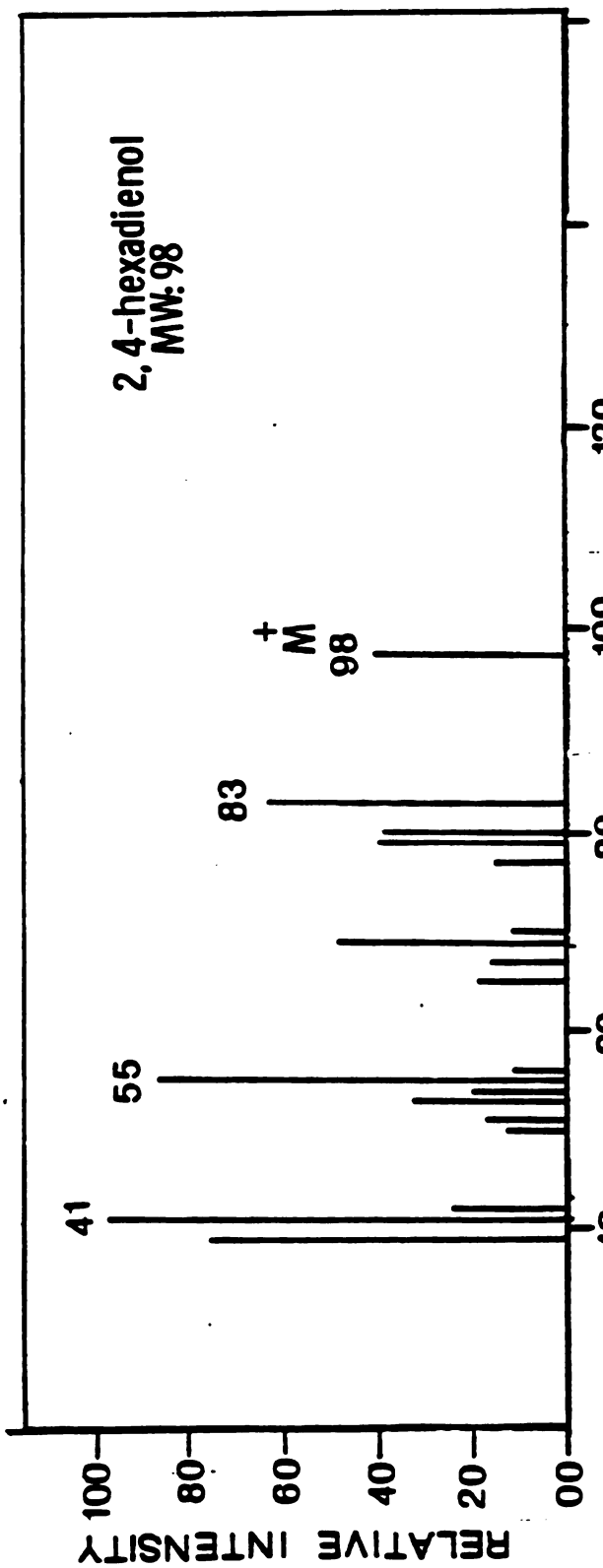


Figure 9. Mass spectrum of 2,4-hexadienol (retention time, 18.1 minutes) isolated from a medium containing sorbate and inoculated with Rhizopus oligosporus

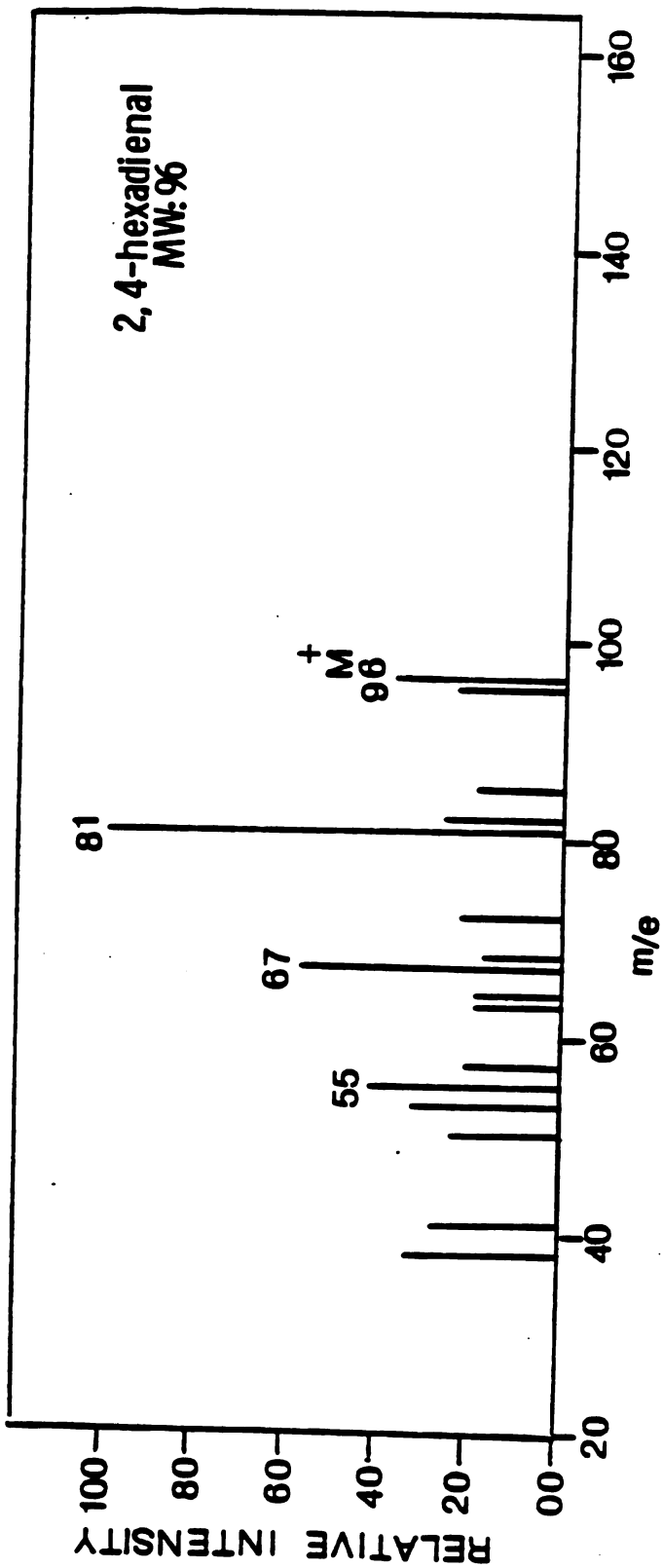


Figure 10. Mass spectrum of 2,4-hexadienal isolated from a medium containing sorbate and inoculated with Rhizopus oligosporus

the same mold, it appears that sorbaldehyde is present as long as sorbic acid is in the reaction medium, after which it disappears.

Similarly, species of Mucor produced sorbyl alcohol in appreciable amounts. The production of this alcohol started in the early stages of growth and it continued to be present until the fifth day of growth, after which it disappeared. During the later stages of growth, 4-hexenol was detected. The production of this alcohol was associated with a strong aroma which was reminiscent of an over-ripe banana. It appears that these molds produce relatively very high amounts of 4-hexenol. Sorbaldehyde, also, has been detected as a minor metabolite in the early stages of growth.

Tahara et al. (1977) tested 15 species of Mucor for their behavior on media containing sorbic acid. They reported that all but one of them grew in the presence of 500 mg/kg of potassium sorbate and produced 4-hexenol in appreciable amounts. One species, however, failed to grow under the same conditions. Small amounts of 4-hexenol were also detected by Tahara et al. (1977) in the cultures of Penicillium crysogenum and Trichoderma viride. In spite of an intensive search for such metabolites in other Penicillium species and Trichoderma viride, no other traces of this compound have been detected.

In an attempt to follow the fate of sorbic acid qualitatively in the presence of Rhizopus and Mucor species, sorbyl alcohol and sorbaldehyde were introduced into the media, individually, instead of sorbic acid. Sorbaldehyde was found to be very toxic to molds, even in small amounts and for this reason, only 100 mg/kg of this compound was added to the media. Sorbyl alcohol was not as toxic as sorbaldehyde since both Mucor sp. and Rhizopus oligosporus grew well in the presence of

750 mg/kg of the alcohol. This may explain why sorbyl alcohol was produced in appreciable amounts by the species of this genus. Troller and Olsen (1967) examined the antimicrobial activities of both sorbaldehyde and sorbyl alcohol and reported that sorbaldehyde possesses a fungistatic activity much less than that of sorbic acid. They also reported that sorbyl alcohol had a fungistatic activity less than that of sorbic acid, especially at low pH. Tahara et al. (1977) reported tht sorbyl alcohol was more toxic than sorbic acid to the cells of Mucor sp. A73.

When sorbyl alcohol was introduced into the medium, a very high amount of 4-hexenol was produced, while only traces of sorbyl alcohol could be detected after two days of growth. A minor acidic metabolite was also shown to be present. The mass spectral fragmentation pattern of this compound was similar to that of the methyl ester of 4-hexenoic acid (Figure 11). Tahara et al. (1975) did not detect this compound in a medium containing either sorbic acid or sorbyl alcohol and inoculated with Mucor sp.

When sorbaldehyde, instead of sorbic acid, was added to a medium inoculated with either Mucor sp. or Rhizopus oligosporus, two compounds, a major and a minor metabolite, were present in the acidic fraction. Two other metabolites were present in the neutral fraction in addition to residual sorbaldehyde (Figure 12). The major acidic metabolite produced a mass spectral fragmentation pattern similar to that of the methyl ester of sorbic acid (Figure 13); hence, the original compound was thought to be sorbic acid. The minor acidic metabolite was identified as 4-hexenoic acid. The major neutral metabolite was identified as sorbyl alcohol, while the minor neutral metabolite was identified as 4-hexenol.

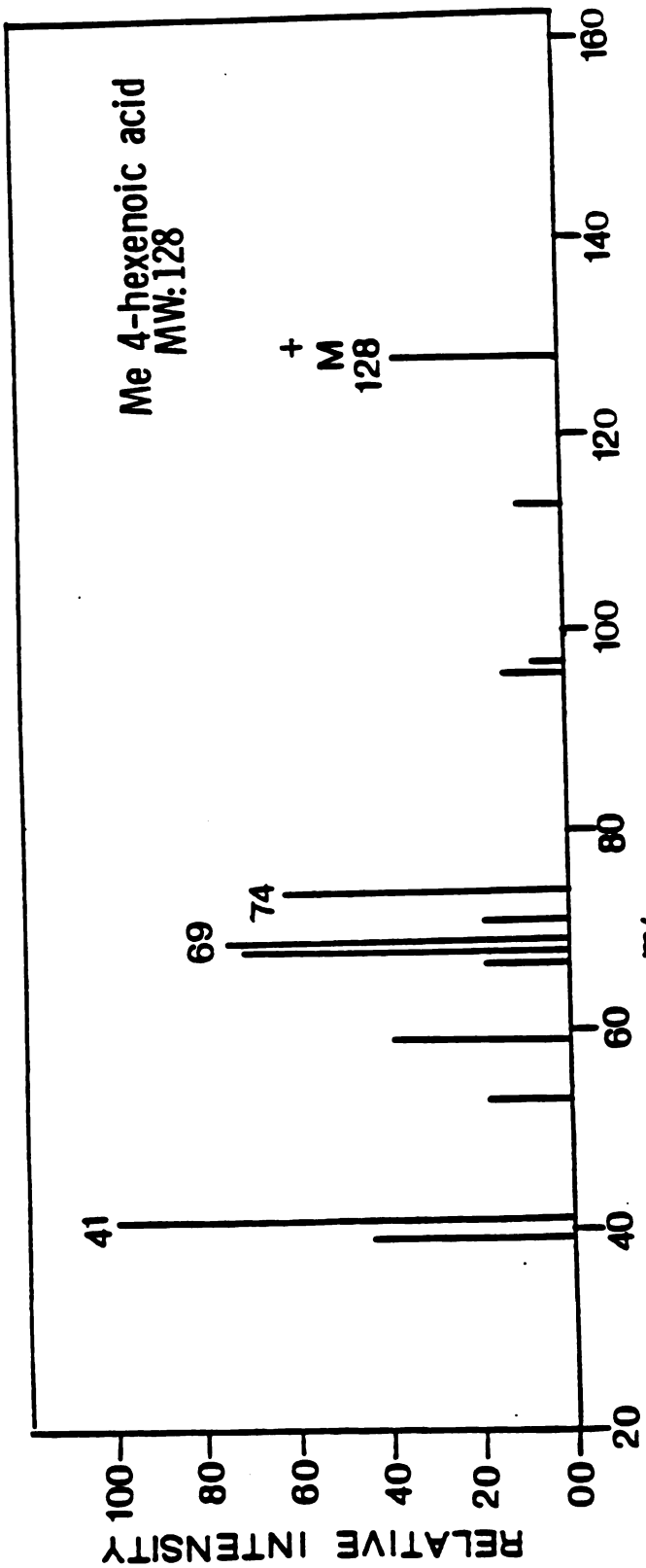


Figure 11. Mass spectrum of methyl ester of 4-hexenoic acid isolated from a medium containing sorbyl alcohol and inoculated with Rhizopus oligosporus

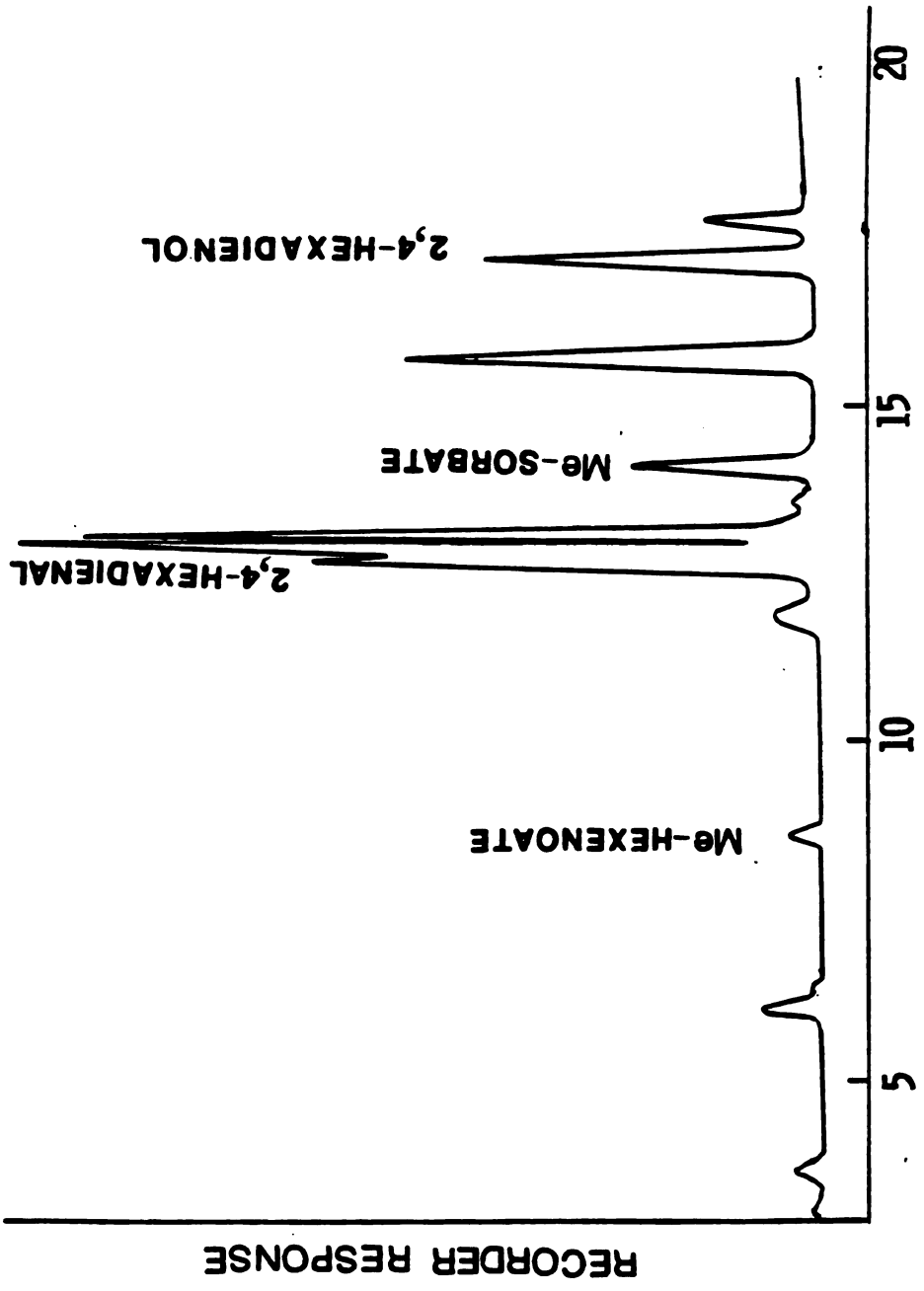


Figure 12. A partial gas chromatogram of both neutral and acidic metabolites of sorbaldehyde-grown Mucor sp.

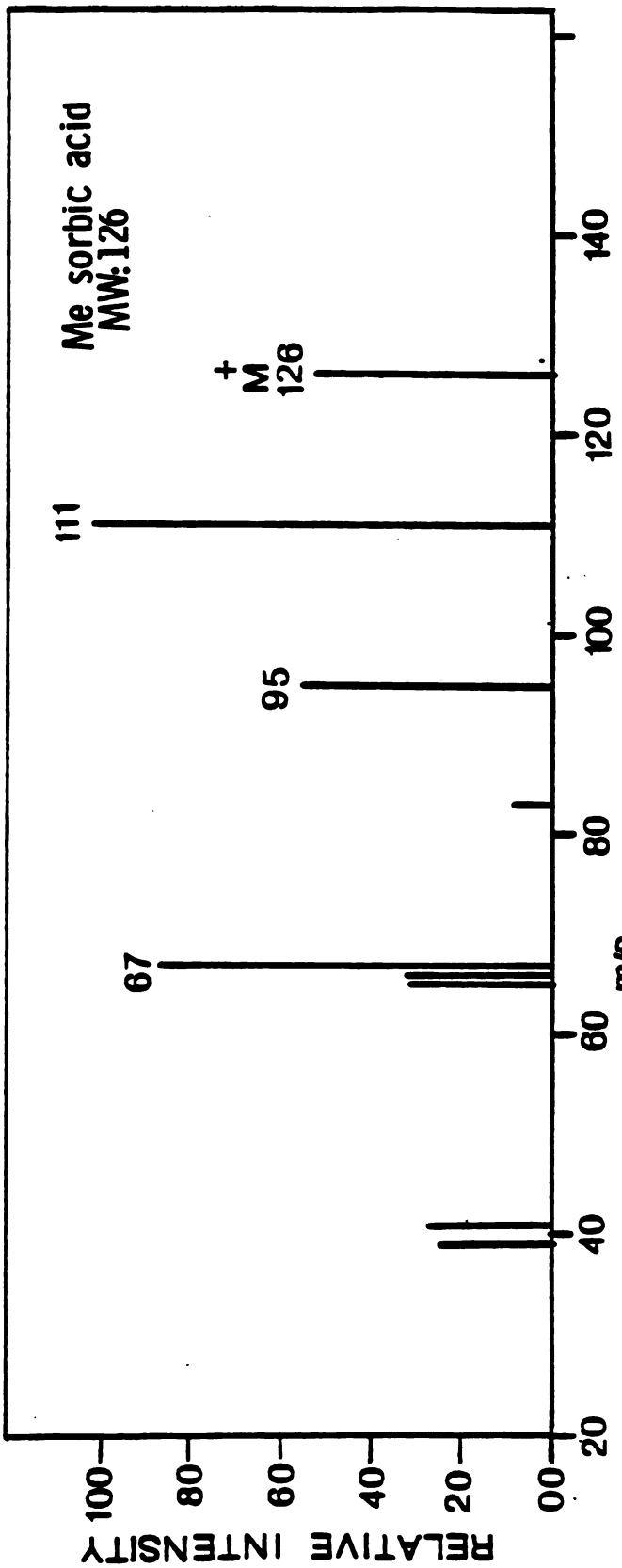
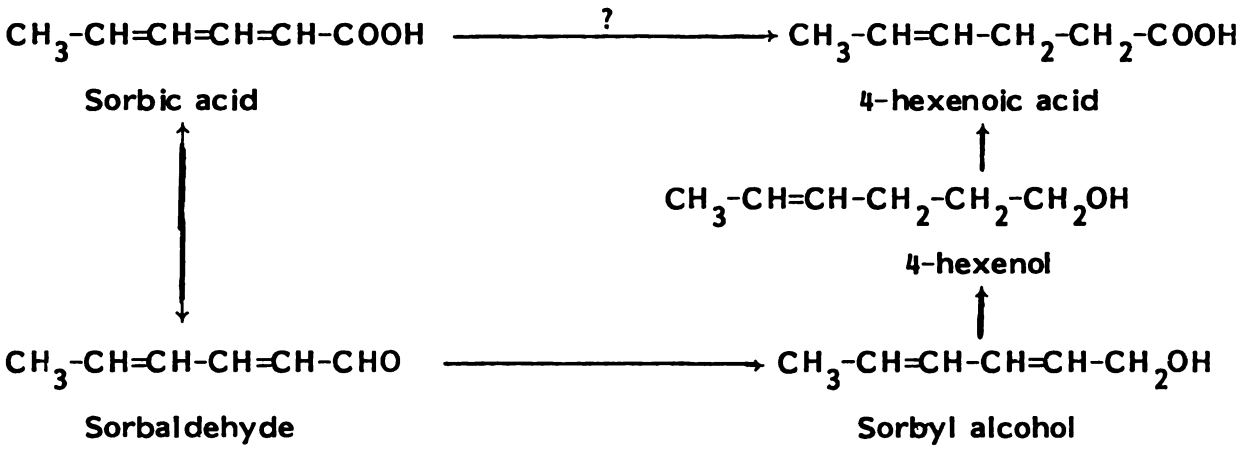


Figure 13. Mass spectrum of methyl ester of sorbic acid isolated from a medium containing sorbaldehyde and inoculated with Mucor sp.

Based on the data gathered in this study on the degradation of sorbic acid by some species of Mucor and Rhizopus and from the data presented by Tahara et al. (1975), the following scheme is suggested as a possible mechanism for the degradation of sorbic acid.



This scheme can be explained as follows:

1. The carboxyl group of sorbic acid is reduced to aldehyde which functions as a transitory compound, i.e., it is reduced further to alcohol as soon as it is formed.
2. Sorbyl alcohol is reduced further to 4-hexenol.
3. It is believed that, at least part of the 4-hexenol is oxidized to the corresponding acid.
4. It is not known at the present if sorbic acid, in part, is reduced to 4-hexenoic acid.

The reduction of the α, β unsaturated double bond of sorbyl alcohol appears to be a slow process which indicates that the enzymes responsible for this process are inducible. Tahara et al. (1977) concluded from a study on resting cells of a Mucor sp. previously grown in the presence of several compounds, such as sorbic acid and hexanoic acid, that the enzymes responsible for this conversion tend to be inducible ones.

It is not known, however, whether 4-hexenoic acid can be formed directly from sorbic acid. Therefore, the possibility exists that this acid is an intermediate in the reduction of sorbic acid to 4-hexenol, or it is formed only upon the oxidation of 4-hexenol as was shown when sorbyl alcohol was introduced into the media. Tahara et al. (1977) investigated the metabolic pathway of the conversion of sorbic acid to 4-hexenol by Mucor sp. and concluded that the reduction process proceeded via two independent reactions:

$$CH_3\text{-}CH{=}CH\text{-}CH{=}CH\text{-}COOH \longrightarrow CH_3\text{-}CH{=}CH\text{-}CH{=}CH\text{-}CH_2OH$$

Sorbic acid — Sorbyl alcohol

$$CH_3\text{-}CH{=}CH\text{-}CH{=}CH\text{-}CH_2OH \longrightarrow CH_3\text{-}CH{=}CH\text{-}CH_2\text{-}CH_2\text{-}CH_2OH$$

Sorbyl alcohol — 4-hexenol

They also suggested that sorbic acid is first converted to sorbaldehyde, which in turn is converted to the corresponding alcohol. The data in the present study indicate that sorbaldehyde is a transitory metabolite since it is reduced as soon as it is formed. Sorbaldehyde, however, was not produced when sorbyl alcohol was used as a substrate, supporting the contention that this compound is an intermediate in the conversion of sorbic acid to sorbyl alcohol.

Philip et al. (1963) have demonstrated the reduction of n-butyric acid to n-butanol by cultures of Aspergillus niger growing in a medium containing glucose and butyric acid. Similarly, Walker et al. (1963), reported the reduction of isobutyric and isovaleric acids to the corresponding alcohols. In both cases, the presence of glucose was necessary for the reduction process. More recently, the reduction of sorbic acid by resting cells of Mucor sp. was

investigated by Tahara et al. (1977). Their results indicated that the reduction process required an energy source such as glucose. Results in the present study on growing cells of Rhizopus oligosporus and Mucor sp. indicate that both a carbon and energy source are necessary for the reduction of sorbic acid, because both molds do not grow at all in the absence of a carbon source other than sorbic acid.

Penicillium sp., Penicillium roqueforti, Aspergillus sp., and Trichoderma viride were found to effectively degrade sorbic acid in culture media. It was surprising initially when the chromatograms of both the neutral and the acidic fractions showed no signs of any metabolite. Hence, it was thought that sorbic acid was rapidly metabolized to CO_2 and H_2O. The hydrocarbon, 1,3-pentadiene, however, has been shown to result from the decarboxylation of sorbic acid by Penicillium roqueforti (Marth et al., 1966). The above mold was observed to produce a strong aroma in the presence of sorbic acid. It was assumed that this aroma may have resulted from the production of 1,3-pentadiene. For this reason, a special procedure to entrap 1,3-pentadiene was developed in order to confirm the identity of compound(s) responsible for the strong aroma.

This substance, when analyzed by mass spectrometry produced a fragmentation pattern similar to that of 1,3-pentadiene (Figure 14). It appears that the reason for the failure to detect the 1,3-pentadiene in the early attempts is that all or most of it is lost during the evaporation of the organic solvents, since 1,3-pentadiene has a boiling point of 42°C. It was not determined whether all of the metabolized sorbic acid was decarboxylated to 1,3-pentadiene, or whether part of it was metabolized through a different pathway, such as β-oxidation, to CO_2 and H_2O. It is

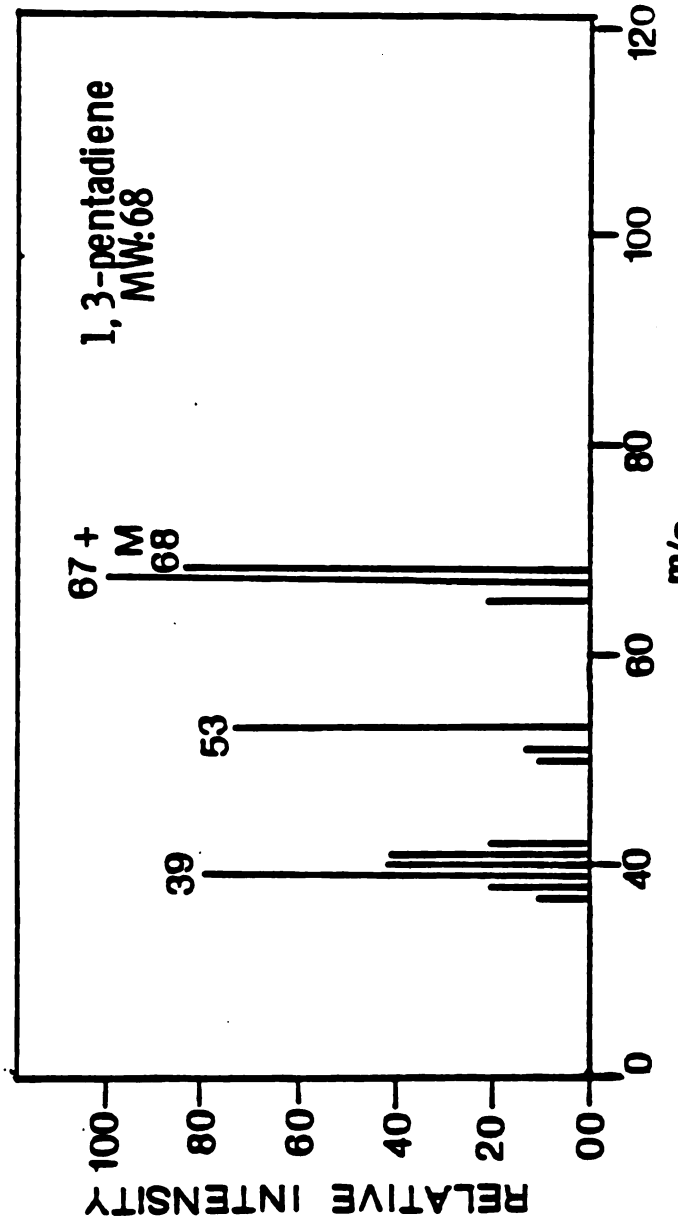


Figure 14. Mass spectrum of 1,3-pentadiene isolated from a medium containing sorbate and inoculated with Penicillium sp.

also not known whether 1,3-pentadiene itself is metabolized by the molds or whether it is lost to the atmosphere.

In an attempt to gain more insight into the decarboxylation process, sorbic acid was used as the sole source of carbon. This system supported the growth of the previously mentioned molds and 1,3-pentadiene was stil produced.

Geotrichum candidum was found to grow vigorously on the YPG medium containing sorbic acid. Sorbic acid slowly disappeared from the medium with the simultaneous formation of the 4-hexenoic acid as a major metabolite (Figure 15). However, it is not known whether 4-hexenoic acid is metabolized by the mold or not. The first possibility is supported by the fact that this acid was no longer detected after four to five days of growth. A precise search for sorbaldehyde, sorbyl alcohol, 4-hexenol and 1,3-pentadiene revealed that such compounds are not produced by Geotrichum candidum. It is also not known whether all or just part of the depleted sorbic acid is reduced to 4-hexenoic acid. A minor compound was detected and was shown by mass spectrometry to be the ethyl ester of sorbic acid (Figures 15 and 16). Kurogochi et al. (1974), reported that Geotrichum sp. partially converted sorbic acid into ethyl sorbate and 4-hexenoic acid. Crowell and Guymon (1975) isolated and characterized ethyl sorbate as one of the compounds derived from sorbic acid which had been added to a red table wine subsequently spoiled by lactic acid bacteria. They believed that it was formed by simple ethyl esterification of the sorbic acid in the wine. An acid medium with a pH less than 4.0 accounts for ethyl sorbate formation. It is not known whether this hypothesis would be applicable in our case, especially since the final pH of the media was

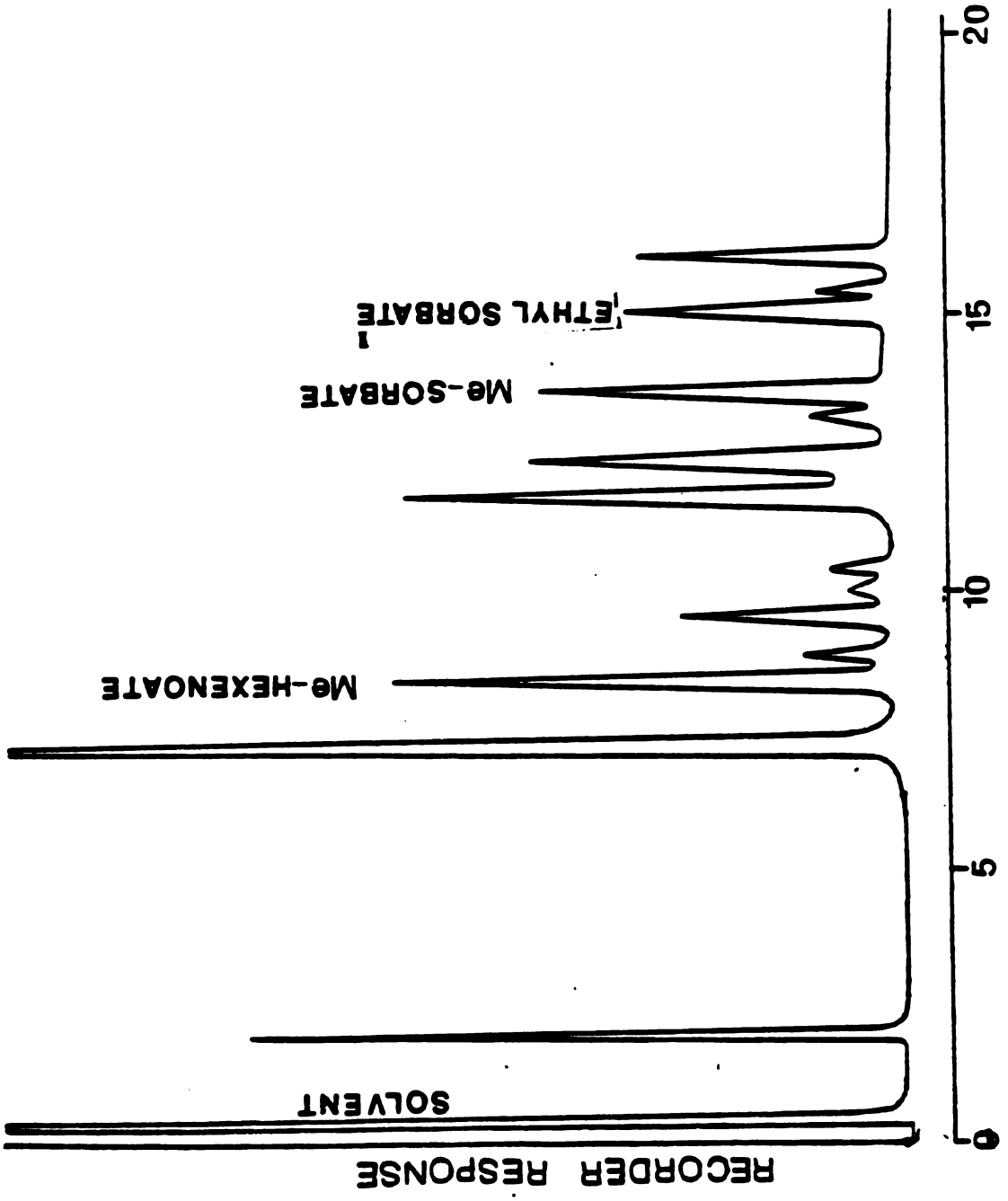


Figure 15. GC chromatogram of both neutral and acidic metabolites of sorbate-grown Geotrichum candidum

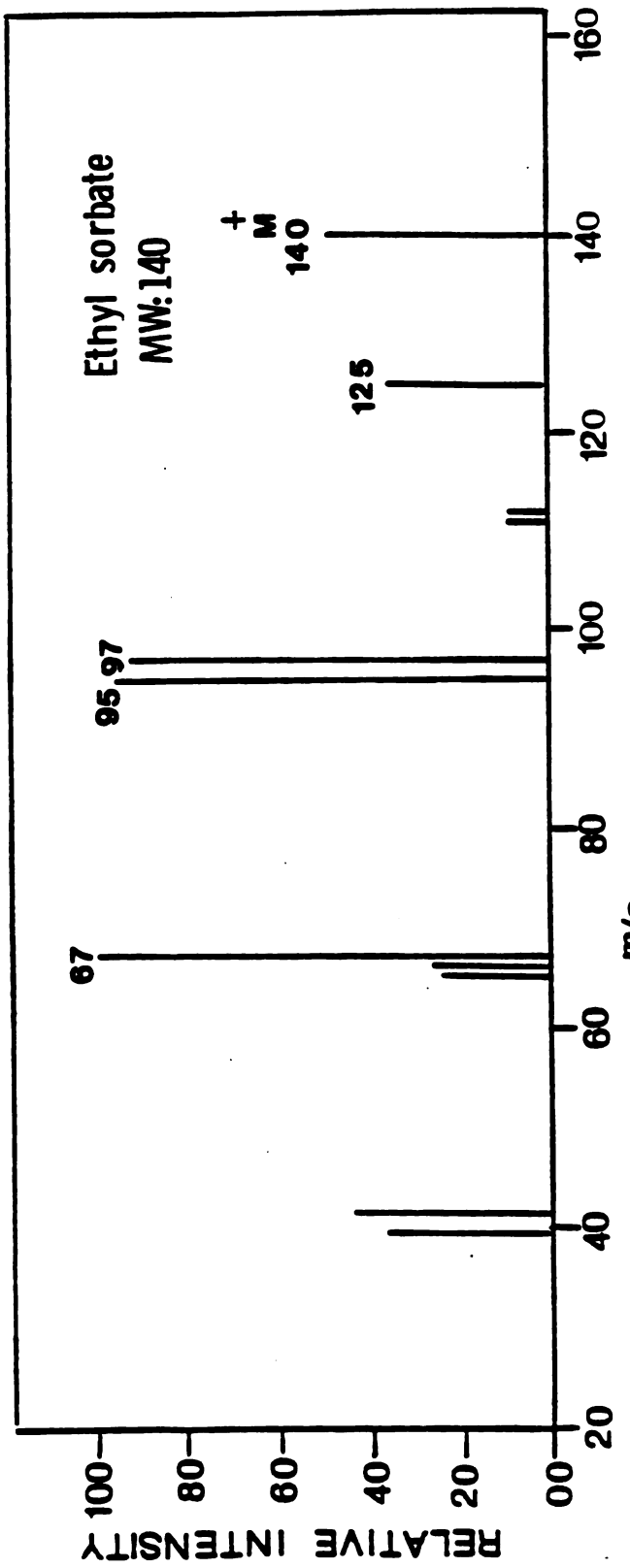


Figure 16. Mass spectrum of ethyl sorbate isolated from a medium containing sorbate and inoculated with Geotrichum candidum

above five. It is worth mentioning that some of the yeasts investigated in this study also produced ethyl sorbate in trace amounts. It is believed that other metabolites may have enhanced the esterification of sorbic acid in the presence of ethyl alcohol, which may have been produced from glucose.

Sorbic acid metabolism by yeasts

Preliminary studies with Candida lipolytica, Debaryomyces membranaefaciens, and Saccharomyces occidentalis indicate that some yeast species of different genera reduce the double bond of sorbic acid in the α, β position producing 4-hexenoic acid. Preliminary quantitative data indicate, however, that this acid does not account for all of the consumed sorbic acid. It is possible that part of sorbic acid is oxidized through a β-oxidization pathway to CO_2 and H_2O.

In an attempt to understand more about the fate of sorbic acid, some of the commercially available metabolites were also used as substitutes for sorbic acid. Sorbyl alcohol was found to be more toxic to yeast cells (Candida lipolytica) than sorbic acid. When used at a concentration of 1000 mg/kg, sorbyl alcohol completely inhibited the growth of Candida lipolytica, even when a heavy inoculum of the microorganism was used. For this reason, the sorbyl alcohol concentration was reduced to 300 mg/kg in subsequent experiments. GC analysis of the metabolites of sorbyl alcohol indicated the formation of sorbic acid, 4-hexenol, 4-hexenoic acid, and sorbaldehyde (Figure 17).

Sorbaldehyde was also used as a substrate, but in small amounts (100 mg/kg) since it was found to be very toxic to the yeast, Candida lipolytica. Troller and Olsen (1967) who examined a number of sorbic

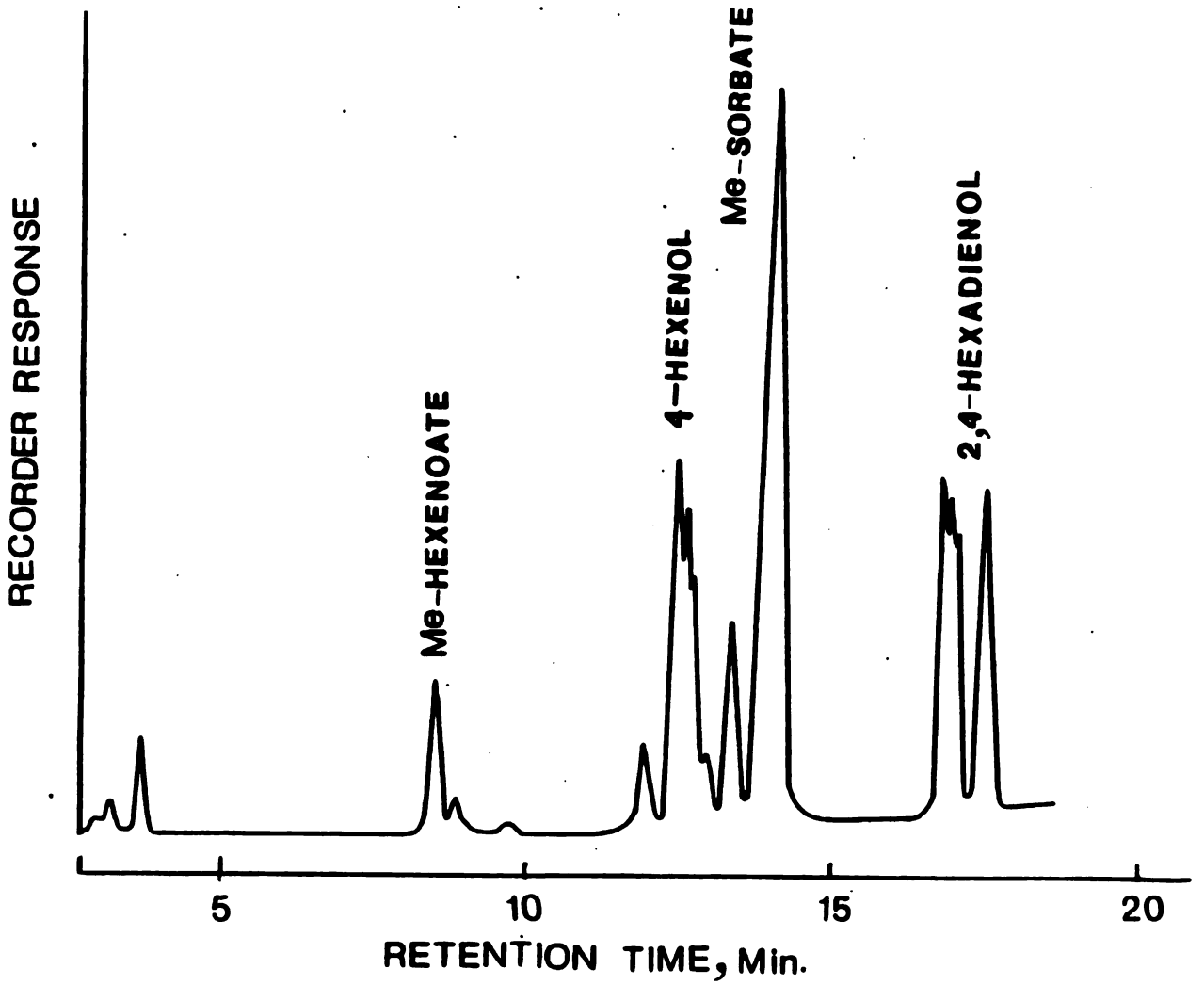
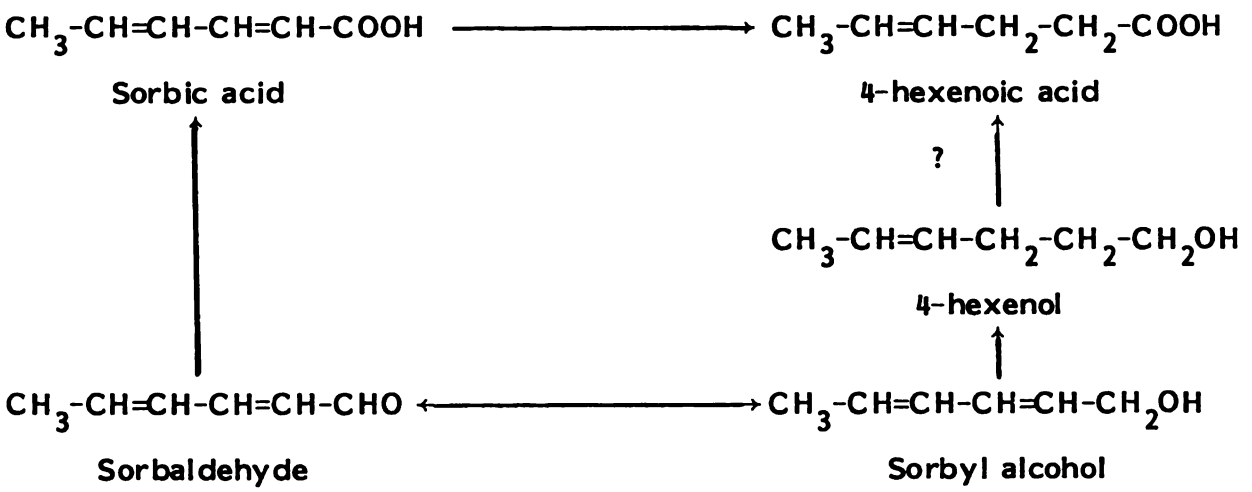


Figure 17. A partial gas chromatogram of both neutral and acidic metabolites of sorbyl alcohol-grown Candida lipolytica

acid derivatives for their antimicrobial activities reported that sorbaldehyde possessed a fungistatic activity much superior to that of sorbic acid itself. On the other hand, sorbyl alcohol possessed a fungistatic activity much less than that of sorbic acid, especially when used at low pH. On the other hand, Tahara et al. (1977) reported that sorbyl alcohol was more toxic to Mucor sp. than was sorbic acid. The preliminary findings in the present study seem to support the conclusions of Troller and Olsen (1967) regarding sorbaldehyde, while agreeing with those of Tahara et al. (1977) with regards to sorbyl alcohol.

Qualititatively speaking, the compounds produced upon the metabolism of sorbyl alcohol were also detected when sorbaldehyde was introduced into the medium.

The results of these studies on the growth of some yeasts in media containing sorbic acid, sorbyl alcohol or sorbaldehyde are summarized as follows:



1. Sorbic acid is reduced to 4-hexenoic acid and it is believed that this is only a partial reduction.
2. Sorbyl alcohol is partially oxidized to sorbaldehyde and the latter is in turn oxidized to sorbic acid.
3. Sorbaldehyde is partially reduced to sorbyl alcohol and the latter is further reduced to 4-hexenol.
4. The possibility exists that 4-hexenol is oxidized to 4-hexenoic acid.

From preliminary growth studies with Debaryomyces membranaefaciens, Saccharomyces occidentalis and Saccharomyces roxii, in addition to Candida lipolytica, it was found that all of these yeasts shared the same pathway for the degradation of sorbic acid, i.e., they reduce sorbic acid to 4-hexenoic acid. Trace amounts of ethyl sorbate were detected in some instances. This indicates a similarity between the yeast-like mold, Geotrichum candidum, and the fore-mentioned yeasts. It is not known why these yeasts and Geotrichum candidum are unable to reduce sorbic acid to alcohols or aldehydes while possessing the ability to oxidize both the alcohol and aldehyde to sorbic acid. The reduction of aldehydes and ketones to the corresponding alcohols by yeasts in a sugar medium was described many years ago by Nord (1919) and Newberg and Nord (1919), as cited by Walker et al. (1963). Farmer et al. (1959) have reported the conversion of certain aromatic acids to aldehydes and alcohols by the wood fungus, Polystictus versicolor. Walker et al. (1963) and Philip et al. (1963) have also demonstrated the ability of Aspergillus niger to reduce lower fatty acids to the corresponding alcohols in the presence of glucose.

Studies with two different cultures of Hansenula wengei indicate that this species produces 1,3-pentadiene from sorbic acid. It is also interesting

to note that this yeast is very effective in degrading sorbic acid when present in sublethal levels. It is not known whether all or part of sorbic acid is decarboxylated to 1,3-pentadiene. It is also not known if the production of 1,3-pentadiene from sorbic acid is common to other yeasts.

Sorbic acid metabolism by bacteria

Studies with aerobic bacteria were limited to Lactobacillus bulgaricus, Lactobacillus plantarum, Pseudomonas fluorescens and Pediococcus cerevisiae. These cultures were grown on trypticase soy broth (pH 6.0) fortified with 1000 mg/kg of potassium sorbate. All of the above cultures grew well, but no significant loss of sorbic acid was observed, except with Pseudomonas fluorescens. In this case, up to 28 percent of the sorbate was lost in three days.

In another series of experiments, different concentrations of potassium sorbate were used with both growing and resting cells of Lactobacillus bulgaricus, Lactobacillus plantarum, Pediococcus cerevisiae and Pseudomonas fluorescens. GC analysis failed to detect any metabolites in the media under the conditions used in the experiment. These findings agree with those reported by Reha et al. (1964) who reported that two lactic acid bacteria showed no signs of utilization of sorbic acid. These authors also reported that Pseudomonas fluorescens metabolized sorbic acid through respiration. Costilow et al. (1955) reported similar conclusions with respect to the lactic acid bacteria.

Three cultures of Clostridia, Clostridia perfringens, Clostridia sporogenes and Clostridia tertium were grown on sorbate-containing media. Clostridia sporogenes and Clostridia tertium were able to convert sorbic acid to 4-hexenoic acid in very high amounts as indicated by GC analysis (Figure 18). Sorbyl alcohol and 4-hexenol were also produced in moderate

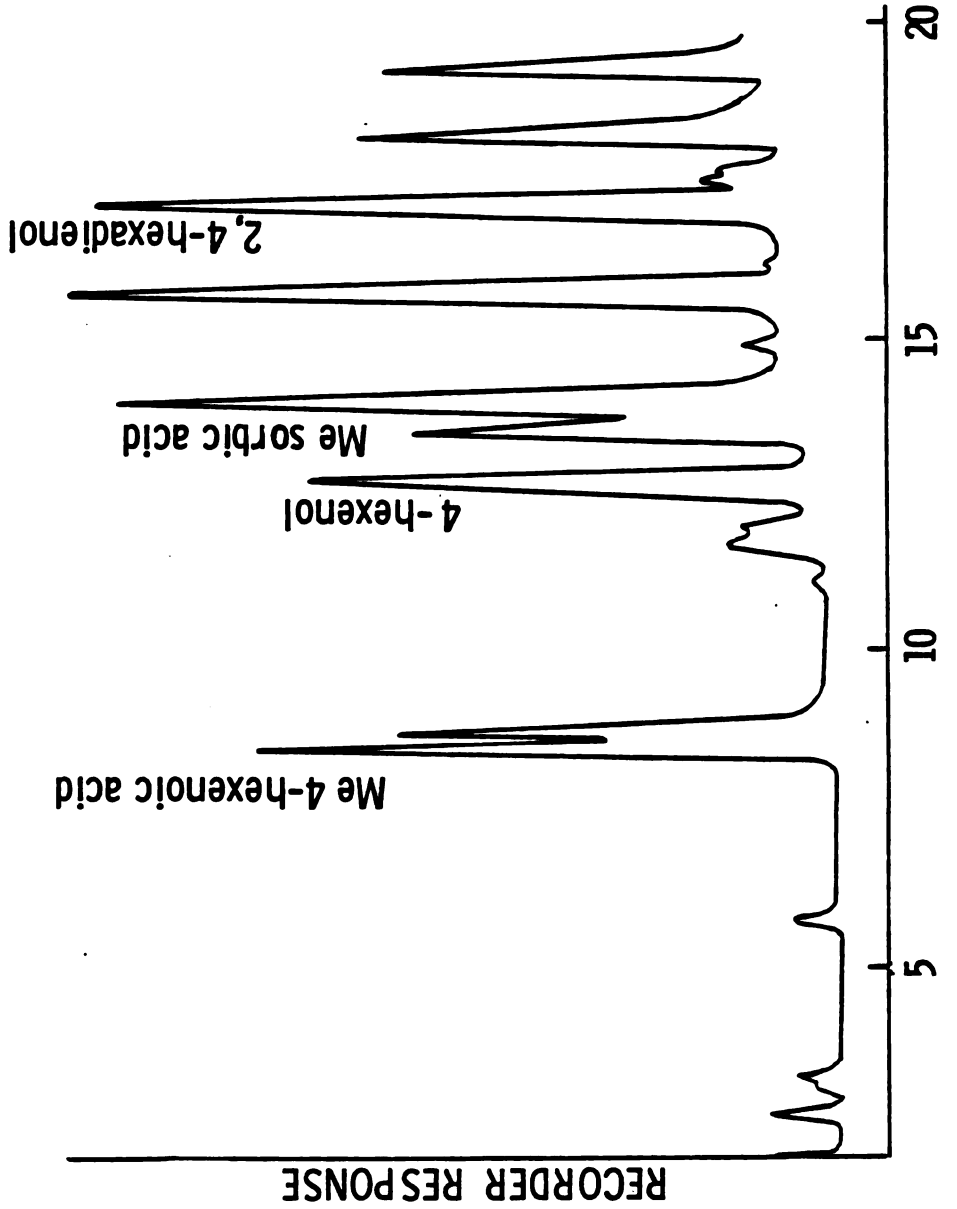
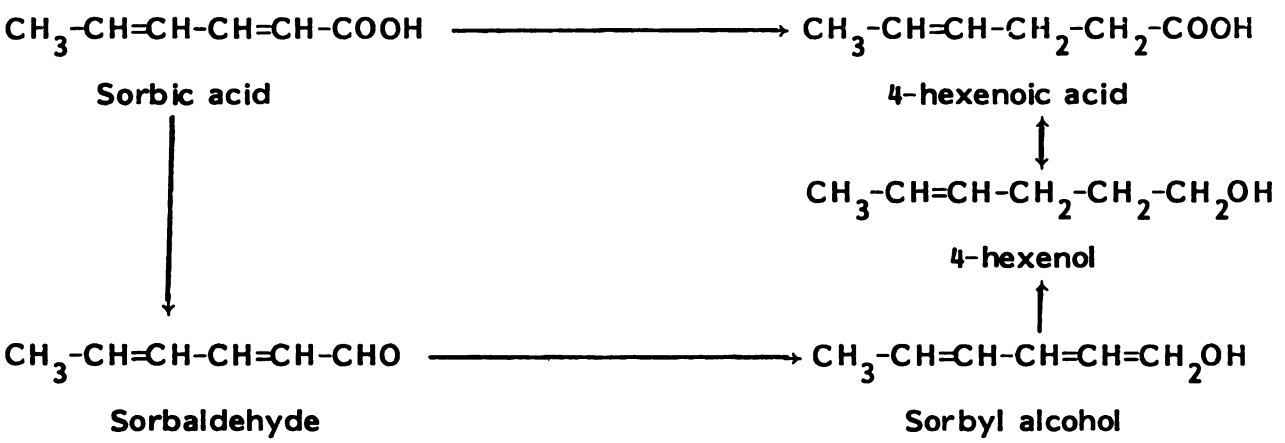


Figure 18. A partial gas chromatogram of both neutral and acidic metabolites of sorbate-grown Clostridium sporogenes

amounts, especially when glucose was added. Trace amounts of sorbaldehyde was also detected. The exact route(s) through which these metabolites were produced is/are not known, but it is believed that two pathways do exist; one is the reduction of the α, β-double bond of sorbic acid producing 4-hexenoic acid which in turn is reduced to 4-hexenol. Thauer et al. (1968) reported that Clostridia kluyveri when grown on media containing crotonate as the sole source of carbon produced butyrate as the major product. Butanol, however, was produced in only negligible amounts. The production of high amounts of 4-hexenol in the present study may be ascribed to the presence of glucose.

The other pathway involves the reduction of the carboxyl group of sorbic acid to sorbyl alcohol. Davis (1942) reported the reduction of n-butyrate to 1-butanol by Clostridium acetobutylicum in the presence of glucose. Barker (1956) postulated that butyraldehyde is formed through the action of aldehyde dehydrogenase and is subsequently reduced to butanol under the influence of alcohol dehydrogenase.



Clostridium perfringens, although it grew well on sorbate-containing media, did not produce any of the above metabolites in measurable amounts.

Fate of Labelled Sorbic Acid

In order to confirm that the metabolites which have been isolated from the media and identified by mass spectrometric analysis were indeed produced from sorbic acid, labelled sorbic acid ([U-^{14}C]-sorbic acid) was introduced into a medium containing cells of Rhizopus stolonifer in order to monitor the radioactivity in the metabolites. However, due to the limited amount of [U-^{14}C]-sorbic acid available, only one metabolite, sorbyl alcohol, was chosen for the study. Thin layer chromatography plates were used to isolate this metabolite from the solvent extract of the medium. Sorbyl alcohol was monitored for the presence of radioactivity and at least 300 counts per minute (cpm) were recorded.

Further confirmation was also achieved by extracting this metabolite from the silica gel on the TLC plate and analyzing the extract by GC. This metabolite had a retention time comparable to that of a standard sorbyl alcohol sample, thus confirming that the precursor of sorbyl alcohol was sorbic acid. It was assumed that the other metabolites also originated from sorbic acid.

SUMMARY AND CONCLUSIONS

Results of the screening tests on three microbial groups, molds, yeasts, and bacteria, confirm the previous reports regarding the wide spectrum antimicrobial activity of sorbic acid. The microbes which have been tested, however, exhibited a wide variation within species of a single genus and, in some cases such variations were observed within cultures of the same species.

Of the molds tested, most of the cultures belonging to the two genera, Aspergillus and Penicillium, were generally found to be more resistant than the other cultures. Of these cultures, Aspergillus flavus, Aspergillus niger, Penicillium sp., Fusarium sp., Geotrichum candidum and Trichoderma viride were also found to be resistant to sorbic acid. All of the above mold cultures grew well in the presence of 3000 mg/kg of potassium sorbate and apparently utilized sorbate as a carbon source. Unexpectedly, the presence of glucose increased the inhibitory action of sorbate against most of the molds which utilized sorbate as a carbon source. This phenomenon was designated as the glucose inhibitory effect.

Of the yeasts tested, Candida lipolytica, Hansenula saturnus, Brettanomyces claussenii and Saccharomyces occidentalis exhibited relatively high resistance to sorbate. The above yeasts, especially Candida lipolytica grew vigorously on a medium containing 3000 mg/kg of sorbate at pH 5.5. Some of the yeasts utilized sorbate as a carbon source. Candida lipolytica and Hansenula californica grew well in the

presence of 3000 mg/kg of potassium sorbate at a pH of 6.0. Once again the "glucose inhibitory effect" was observed.

Most of the bacteria tested were found to be resistant to at least 500 mg/kg of potassium sorbate at pH 5.5. The majority of them were inhibited by potassium sorbate concentrations ranging from 1000 to 2000 mg/kg.

Some lactic acid bacteria and a strain of Pseudomonas fluorescens were found to require more than 3000 mg/kg of potassium sorbate in highly nutritious media. Salmonella senftenberg, Pseudomonas vulgaris and Staphylococcus aureus grew well in media containing sorbate as the only carbon source.

Results of the studies on metabolites indicate that sorbic acid is converted to 4-hexenol by the species of the two genera, Rhizopus and Mucor and some species of Fusarium. Observations on these molds suggest that sorbic acid, when present in sublethal concentrations, is reduced to sorbaldehyde which is immediately reduced to sorbyl alcohol; sorbyl alcohol is reduced further to 4-hexenol. Our observations, also, suggest that the first and second steps are catalyzed by constitutive enzymes while the latter step is catalyzed by inducible enzyme(s).

Penicillium and Aspergillus species and Trichoderma viride produced 1,3-pentadiene.

Some yeasts and a yeast-like mold, Geotrichum candidum, reduced sorbic acid to 4-hexenoic acid. Ethyl sorbate was also detected but in relatively small amounts.

Studies on aerobic bacteria revealed that the metabolism of sorbic acid by aerobic bacteria is not as common as it is the case with molds.

Only Pseudomonas fluorescens showed some loss of sorbic acid, but no signs of metabolites were observed.

Clostridium sporogenes and Clostridium tertium metabolized sorbate mainly to 4-hexenoic acid, 4-hexenol and sorbyl alcohol. Apparently, metabolism of sorbate did not occur when Clostridium perfringens was used, although they grew well in the presence of sorbate.

The use of labelled sorbic acid enabled the confirmation of the assumption that the metabolites come from sorbic acid.

RECOMMENDATIONS FOR FURTHER STUDIES

The metabolism of sorbic acid by various microorganisms has been studied by GC and GC/MS techniques. However, many questions pertaining to the overall metabolism of sorbic acid remain unanswered. Some aspects requiring further study can be classified under the following headings:

1. Toxicology of sorbic acid and metabolites

Since a number of metabolites has been shown to be produced from sorbic acid by certain microorganisms under certain conditions, some attention should be directed toward the toxicological aspects of these metabolites.

2. Metabolism of sorbic acid in human body

Early reports indicate that sorbic acid is metabolized in a similar way to that of the fatty acids, i.e., to H_2O and CO_2 or to ketone bodies. This study indicates that a wide diversity among microorganisms does exist as far as sorbic acid metabolism is concerned. It would be of interest to see if mammalian enzymes such as liver dehydrogenases would convert sorbic acid to sorbyl alcohol or sorbaldehyde.

3. Enzymatic studies

Some efforts should be directed toward enzymes which are responsible for the degradation of sorbic acid in the different microbial groups.

4. The use of food systems

This study was conducted in what might be considered model systems (cultural media). Some work should be done in food systems or in a model

system which simulates the food system of concern, and inoculating this system with microbe(s) of potential hazard.

5. The fate of the metabolites

It would be interesting to follow the fate of the metabolites by using them as substrates to see whether they are metabolized by the microbes, whether they accumulate in the media or whether they are lost to the atmosphere.

6. Glucose inhibitory effect

This phenomenon needs to be further investigated in order to ascertain what concentrations of glucose or other carbohydrate substance (e.g., fructose, sucrose) brings about this effect.

7. Sorbate-nitrite interaction

It would be of interest to study what effect the presence of nitrite may have on the decomposition of sorbic acid in terms of the rate of decomposition and the type of metabolites which result from this decomposition.

8. Sorbate balance experiments

The extraction procedure used for the isolation of sorbate metabolites from the media needs to be assessed in terms of extraction selectivity. The use of labelled sorbic acid would facilitate this study and provide information as to whether the metabolites identified in the present study were the major decomposition products of sorbate.

APPENDICES

APPENDIX 1

Table A.1. Mold cultures used for screening tests

	Mold	Code[1]
1.	Aspergillus flavus NRRL 2999	1
2.	Aspergillus flavus NRRL 6550	
3.	Aspergillus niger (Beneke)	25
4.	Aspergillus oryzae ATCC 11601	26
5.	Aspergillus sp.	7
6.	Alternaria sp.	14
7.	Alternaria tenuis NRRL 2169	16
8.	Calvatia gigantica	21
9.	Cladosporium cladosporiodes QM 489	8
10.	Cladosporium cladosporiodes QM 9485	10
11.	Fusarium sp.	2
12.	Fusarium roseum	9
13.	Fusarium oxysporum NRRL 1943	15
14.	Fusarium sp.	20
15.	Geotrichum candidum (Beneke)	29
16.	Geotrichum candidum	23
17.	Mucor humiculus	28
18.	Mucor pusillus	27
19.	Mucor sp.	24
20.	Penicillium janthinellum NRRL 2016	13
21.	Penicillium oxalicum NRRL 790	12
22.	Penicillium roqueforti	
23.	Penicillium digitatum (Beneke)	
24.	Penicillium italicum (Beneke)	
25.	Penicillium sp.	3
26.	Rhizopus oligosporus ATCC 22959	4
27.	Rhizopus oligosporus NRRL 2710	5
28.	Rhizopus stolonifer	6
29.	Trichoderma viride	11

[1]Code designated by the Food Microbiology Laboratory at Michigan State University.

APPENDIX 2

Table A.2. Yeast cultures used for screening tests

	Yeast	Code[1]
1.	Brettanomyces claussenii NRRL-Y-1414	y-29
2.	Candida krusei	y-2
3.	Candida lipolytica	y-1
4.	Candida steatolytica	y-31
5.	Candida utilis NRRL-Y-900	y-39
6.	Debaryomyces membranaefaciens NRRL-Y-989	y-4
7.	Endomycopsis bispora	y-16
8.	Endomycopsis bispora ATCC 14628	y-18
9.	Endomycopsis selenospora NRRL Y-1357	y-17
10.	Hanseniaspora valbyensis var. guilliermondii ATCC 106	y-30
11.	Hansenula anomala	y-6
12.	Hansenula anomala	y-6D
13.	Hansenula californica NRRL Y-1425	y-19
14.	Hansenula saturnus	y-7
15.	Hansenula wingei ATCC 14355	y-8
16.	Hansenula wingei ATCC 14356	y-9
17.	Nadsonia fulvescens NRRL Y-991	y-20
18.	Pichia fermentans	y-5
19.	Rhodotorula glutinis var glutinis	y-36
20.	Rhodotorula rubra	y-23
21.	Rhodotorula spp. (Beneke Y-9)	y-22
22.	Saccharomyces carlsbergensis ATCC 9080	y-35
23.	Saccharomyces cerevisiae (dry yeast)	y-24
24.	Saccharomyces cerevisiae	y-25
25.	Saccharomyces cerevisiae var. ellipsoideus	y-14
26.	Saccharomyces kluyveri	y-12
27.	Saccharomyces kluyveri (C-26)	y-15
28.	Saccharomyces oleaginosus	y-15
29.	Saccharomyces rouxii ATCC 2619	y-26
30.	Saccharomyces sp.	y-40

Table A.2 (continued)

Yeast	Code[1]
31. Saccharomyces occidentalis ATCC 2320	y-28
32. Schizosaccharomyces japonicus var versatilis	y-38
33. Schizosaccharomyces octosporus	y-10
34. Schizosaccharomyces pombe	y-11
35. Sborobolomyces coralliformis ATCC 16039	y-34
36. Torulopsis sake NRRL Y-1622	y-21
37. Torulopsis sphaerica	y-3
38. Trichosporon cutaneum ATCC 13445	y-33
39. Trichosporon fermentans NRRL Y-1492	y-32
40. Trigonopsis variabilis ATCC 10679	y-27

[1]Code designated by the Food Microbiology Laboratory at Michigan State University.

APPENDIX 3

Table A.3. Bacterial cultures used for screening tests

	Bacteria
1.	Aerobacter aerogenes
2.	Acetobacter aceti
3.	Bacillus cereus
4.	Bacillus coagulans
5.	Bacillus polymyxa
6.	Bacillus subtilis
7.	Escherichia coli
8.	Lactobacillus brevis
9.	Lactobacillus bulgaricus
10.	Lactobacillus casei
11.	Lactobacillus plantarum
12.	Leuconostoc mesenteroides
13.	Micrococcus flavus
14.	Pediococcus cerevisiae
15.	Pseudomonas fluorescens
16.	Pseudomonas fluorescens
17.	Pseudomonas putida ATCC 15070
18.	Pseudomonas putida ATCC 27212
19.	Pseudomonas schuylkilliensis ATCC 15916
20.	Pseudomonas schuylkilliensis ATCC 15917
21.	Pseudomonas vulgaris
22.	Pseudomonas fragi
23.	Salmonella typhimurium
24.	Salmonella senftenberg
25.	Salmonella paratyphi
26.	Salmonella pollorum
27.	Sarcina lutea
28.	Serratia marcescens
29.	Staphylococcus aureus
30.	Staphylococcus epidermidis

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تكسير حرجن السوربيك بواسطة الميكروبات

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١٤٠١هـ/١٩٨١م

ملخص

تكسير حمض السوربيك بواسطة الميكروبات

اعداد: ابراهيم سعد المزيع

يعتبر حمض السوربيك من اهم الاحماض المستخدمة لحفظ الأغذية وذلك لما يتميز به على بقية المواد الحافظة الأخرى من ان سميته منخفضة، حيث انه يهضم في الجسم بالطريقة كما في الاحماض الدهنية الغير مشبعة. ولكن له القدرة على العمل وحتى عند اس هيدروجيني مرتفع، وفعاليته ضد عدد كبير من الميكروبات التي تنتمي الى المجاميع الثلاث (العفن، الخمائر، البكتريا).

بالرغم من فعالية هذه المركبات الا انه توجد ميكروبات لها القدرة على النمو ولو عند وجوده. أجري هذا البحث لدراسة هذه الميكروبات والتي لها القدرة على تكسير هذا الحمض وذلك من زاويتين: أولاهما كمية وتعنى بالكمية المستهلكة في وقت معين وذلك باستخدام الكروماتوجرافيا السائلة (اتش بي ال سي) وثانيهما كيفية وتعنى بنوعية المركبات الناتجة عن اثر التكسير وذلك باستخدام الكروماتوجرافيا الغازية (جي ال سي) وجهاز تحليل الكتلة (ام اس).

أجريت دراسات مبدئية على عزلات ثلاثة ميكروب تنتمي للمجاميع الميكروبية الثلاثة. من هذه الدراسة المبدئية جرى تحديد التراكيز المثبطة وبناء عليه جرى تصنيف الميكروبات الى مجاميع. نتائج هذه الدراسة دلت على ان الميكروبات تختلف في استجابتها تجاه هذا الحمض حتى داخل النوع الواحد، ناهيك عن الجنس الواحد أو الأجناس المختلفة، كما دلت على ان هذا الحمض يملك طيفا واسعا.

بعد هذه الدراسة المبدئية تم اختيار بعض الميكروبات المثلى لاجراء دراسات اخرى، وذلك باستخدام كل من الخلايا النامية والغير نامية في بيئة كاملة وفي محلول منظم على التوالي. فنتائج الدراسة تدل على أن الأجناس رايزوبس، فيوزاريوم، و ميوكر عندما تنمو على تراكيز غير مثبطة تكسر الحمض الى عدد من المركبات وهي كحول والدهايد السوربيك، و ٤-هيكسينول. ومن الجدير بالذكر ان كحول والدهايد السوربيك ولاسيما الالدهايد اثبتت الدراسة انها سامة جدا للميكروبات بالقياس لحمض السوربيك. الأجناس اسبرجلس، بنسيليوم، وترايكودرما كانت فعالة في تكسيره وانتاج مادة ١، ٣-بنتاديين. جيوتريكوم كانديدام، وبعض الخمائر انتجت حمض ٤-هيكسينويك وقليل من سوربات الايثيل.

البكتريا الهوائية ولاسيما بكتريا حمض اللبن فبالرغم من انها تنمو على تراكيز عالية نسبيا الا انها تفتقر على تكسيره. أما اللاهوائية، كلوستريديوم سبوروجينس وشبيهة بالـ كلوستريديوم بوتشولاينوم والمعروفة بأنها تسبب تسمم غذائي مميت، فقد نمت بنجاح على هذا الحمض وتكسيره الى عديد من المركبات الآنفة الذكر. أما كلوستريديوم بيرفرنجنس فقد سلكت سلوك بكتريا حمض اللبن.

باستخدام حمض السوربيك المشع اثبت ان حمض السوربيك هو مصدر المركبات الناتجة.