SODIUM PHOSPHITE INHIBITION OF THE GROWTH OF SELECTED FOODBORNE SPOILAGE YEASTS

by

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(ABSTRACT)

Sodium phosphite was evaluated for inhibition of growth of spoilage yeasts in laboratory media and in two commercial carbonated beverages. In addition, the effects of pH and atmosphere in combination with sodium phosphite were also examined in laboratory media. Inhibition studies in laboratory media were performed with optimal or near optimal growth conditions for each yeast. Growth was monitored by measuring optical density at 600 nm. A time to significant growth was determined for experiments in laboratory media and was used to evaluate the effect that sodium phosphite and other test variables had on growth. A time to detectable growth was determined for experiments in commercial carbonated beverages and post incubation counts on observations with undetectable growth were used to evaluate the effects of sodium phosphite on yeast growth. Sodium phosphite was most effective in inhibiting growth of Zygosaccharomyces bailii, and less effective against Saccharomyces cerevisiae, and Saccharomyces uvarum respectively. Results from this investigation show the

potentil use of sodium phosphite as an antimicrobial food preservative has potential.

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Introduction

Since ancient times, man has looked for ways to preserve freshly harvested foods. During these early times traditional methods of preservation included the use of heat, cold, drying, and fermenting. In some cases chemicals were used but extensive use did not occur until later years.

The increased use of chemical additives appears to be due to changes in the marketing system for food products (Branen, 1983). Foods are rarely grown and sold locally as was done in years past. Today, foods produced in one area are often shipped to another for processing and to several other areas for immediate or future distribution. To achieve this long term storage that was required by the new marketing system effective means of preservation were utilized. However, because convenience foods, snack foods, diet foods, food substitutes, and specialty foods are demanded by the consumer and the prevalence of preservative-resistant spoilage microorganisms is more apparent, new and more effective means of preservation are sought after constantly.

Recently, sodium phosphite has been reported to be effective against several spoilage yeasts (Rhodehamel and Pierson, 1984). Presently, the potential for sodium phosphite to inhibit growth of other spoilage yeasts has yet to be tested. The following study was designed to determine the

effectiveness of sodium phosphite to inhibit growth of selected foodborne spoilage yeasts in laboratory media and in commercial carbonated beverages previously preserved. In addition, the effects of pH and atmosphere in combination with sodium phosphite were also examined.

History of Soft Drinks

The precursors of carbonated drinks were the mineral waters from the natural springs of Europe. Because mineral water was reputed for its theraputic properties, people travelled great distances to sample it. The knowledge of gas vapors entrapped in mineral waters and attempts to produce this artificially date back to the 16th century.

In the early part of the 17th century the vapor in bubbles was identified by the new words "gas" or "fixed air" (Woodroof and Phillips, 1980). In 1750 Dr. Gabriel Venel first produced an artificial spring water by adding hydrochloric acid to sodium carbonate in a closed vessel (Green, 1978). In 1772 Joseph Priestly published the first paper on how to impregnate water with gas. He studied not only the carbonation of water but also that of wine and beer. Priestly noted that the carbonation of water gave it a delicate flavor which could be drowned by the stronger flavor of other ingredients (Green, 1978). He could not characterize or produce carbon dioxide (CO_2) himself and instead collected it from the top of fermenting vats. Soon after Antoine Lavoisier identified the gas described previously by Priestly as a combination of carbon and oxygen.

During 1775 J.M. Nooth first developed a special apparatus for producing effervescent water, which was subsequently designed to act on a commercial scale. In the

latter part of the 18th century and early 19th century factories were opened in various cities in Europe (Green, 1978). Soon after it was shown that CO_2 was more soluble at low temperatures and that its ability to go into solution was assisted greatly by pressure, agitation, and conducting the water during the carbonation over a great area (Woodroof and Phillips, 1980). By the beginning of the 19th century carbonated water was well accepted in England, especially for the alleged medicinal properties.

At about this time Priestly went to America and encouraged scientists to continue their studies of carbonated water. In 1807 men such as Benjamin Silliman and Josephh Hawkins bottled carbonated water produced artificially and sold it commercially. In 1850 the U.S. Bureau of the Census reported 64 plants producing bottled soda water, with this number doubling in ten years. By 1865, 14 flavors of carbonated fruit beverages were available to the public (Woodroof and Phillips, 1980). It was evident the soft drink industry was on its way. Before the economic crash of 1929, the growth of soda water plants reached an all time high of 8,000 plants. Since then, the number has declined steadily to the present level of approximately 3,000 bottling plants. In 1978 the estimated U.S. per capita consumption of soft drinks was 382.7 twelve oz. containers with 3.5 billion total cases (24 twelve oz. containers) being produced and having a total wholesale

value of 13.3 billion dollars (Woodroof and Phillips, 1980).

The Need For Chemical Preservation in Soft Drinks

Soft drinks are defined as a class of nonalcoholic beverages which contain 86-92% water, 8-14% nutritive sweetners, carbon dioxide, acids and flavorings. Coloring, buffering, emulsifying agents, fruit juice and chemical preservatives may also be added (Berry, 1979). Microbial spoilage of soft drinks has been a problem since the beginning of the industry. In general soft drinks are more susceptible to microbial spoilage than other food products, due to both intrinsic and extrinsic factors (Green, 1978). However, Sand (1969) reported that due to the physico-chemical properties, in particular the low pH value and low nitrogen and oxygen content, soft drinks are a very selective media.

In the United States soft drinks are not pasteurized or given a further heat treatment after packaging. The threat of spoilage caused by the growth of contaminating microorganisms is a constant and significant problem for beverage manufacturers. Soft drinks are an excellent nutritive media for specific types of acidophilic microorganisms. Most microbial spoilage of soft drinks is caused by yeasts, and to a lesser extent molds, and lactic and acetic acid bacteria such as species of Leuconostoc (Splittstoesser, 1987; Sand, 1970). Several investigators have reported that more than 90% of all cases of microbial

spoilage of soft drinks are caused by yeasts (Mossel, 1964; Sharf, 1960).

As viable yeast cells come into contact with a soft drink, they remain static, multiply or die. Spoilage can occur even if the contaminating yeasts are not growing. The rate at which spoilage occurs depends on several aspects including: (a) initial number and type of contaminating yeast cells present at the time when the beverage leaves the bottling or canning line; (b) physical and chemical properties of the beverage including the combined effects of nutrients, pH, redox potential, and water activity may make the beverage more or less susceptible to growth of certain microorganisms; (c) temperature which is an important factor because 21-30^{o}C is the optimal range for growth of most spoilage yeasts; and (d) antimicrobial agents such as carbon dioxide, chemical preservatives, and certain essential oils aid in preventing spoilage (Sand, 1969).

Control of spoilage microorganisms, especially yeasts, in soft drinks is dependent upon the cleanliness of the ingredients, and the plant processing, bottling, and canning equipment. A better understanding of the reasons for spoilage and a more complete knowledge of the epidemiology, ecology, and taxonomy of spoilage strains invariably leads to more effective spoilage prevention (Sand, 1969). The use of good manufacturing practices is not in itself sufficient to assure control of yeast and bacterial contamination in a

carbonated beverage. As a result, the Food and Drug Administration approved preservatives or physical preservation methods necessary to prevent microbial contamination of syrups and beverages (Kelly, 1975).

Properties of Chemical Preservatives

The Code of Federal Regulations, Title 21, Part 101.22 (a) (5) defines a chemical preservative as any chemical which when added to a food, tends to prevent or retard deterioration there of; but does not include common salt, sugars, vinegars, spices, (or oils extracted from spices), or substances added by wood smoke. Before a preservative can be legally added to foods, it must be generally recognized as safe as an additive or given GRAS status under the conditions of intended use.

Preservatives are used to prevent or retard both chemical and biological deterioration of foods. Preservatives used to prevent chemical deterioration include: (a) antioxidants used to prevent autooxidation of pigments, flavors, vitamins, and lipids; (b) antibrowning compounds used to prevent enzymatic and nonenzymatic browning; and (c) antistaling compounds used to prevent texture changes (Splittstoesser, 1987).

The main additives used to prevent biological deterioration are the antimicrobials. Other methods to prevent biological deterioration include physical control processes such as dehydration, low temperature storage, and heat processing. Selection of the proper antimicrobial for a food or beverage is dependent upon several factors, including: (a) the properties and composition of the food or beverage; (b) the type of preservation system other than

chemicals used in the product; (c) the type, characteristics, and number of spoilage microorganisms; (d) the safety of the antimicrobial; and (e) the cost effectiveness of the antimicrobial (Splittstoesser, 1987).

Antimycotic Agents

An antimycotic agent should demonstrate a number of characteriatics before it can be used to control yeasts and molds in foods and beverages. It must be nontoxic in the concentration used and must not have carcinogenic properties. It should prevent the growth of yeasts and molds by inhibitory or antimycotic actions. It should not contribute to undesireable color, odor, or flavor in the concentration used and should be soluble in water (or oil), noncorrosive, and economical. Ideally, it should sustain activity throughout the projected shelf-life of the food (Beuchat, 1979); for example, bottlers of soft drinks want their products to have a shelf-life of at least six months (Woodroof and Phillips, 1980).

Acids, salts of acids, sodium chloride and sugars are most commonly added to foods to control the growth of yeasts and molds. Sorbic, benzoic, and propionic acids, their salts, and the parabens exhibit much higher antimycotic activity at comparable levels to lactic and acetic acids and are among the preservatives most commonly used by the food industry (Beuchat, 1979). Because the latter weak acids show low activity against osmotolerant yeasts and can cause

undesireable organoleptic alterations of the preserved product, especially soft drink beverages, their use is not appropriate (Jermini and Schmidt-Lorenz, 1987). Propionic acid and its salts are highly effective mold inhibitors but at concentrations permitted in foods are virtually without effect in yeasts (Baird-Parker, 1980).

The preservation of foods through the addition of sugars and in some cases sodium chloride is more physical than chemical in nature by lowering the water activity of the food to a level which inhibits or at least delays the metabolic activities of yeasts and molds. However, osmotolerant yeasts, which are the most prevalent spoilage microorganisms in soft drinks, require a high concentration of carbohydrate to be inhibited. Erikson and Fabian (1942) demonstrated yeasts were clearly more tolerant than bacteria to various carbohydrates since 45-60% was required to bring about a preserving action with the yeasts.

Preservatives Used Most Commonly in Soft Drinks

In most countries only benzoic acid, sorbic acid, para-hydroxybenzoic acid (parabens) and its esters, sulfer dioxide and the sulfites, and carbon dioxide are permitted as food preservatives to control microorganisms in soft drinks (Baird-Parker and Kooiman, 1980). The preservation of still soft drink products such as fruit juice by sulfer dioxide and the sulfites is more common in countries outside of the U.S., especially in countries with warm climates and those where frozen storage facilities are limited (Chichester and Tanner, 1975).

Except for the parabens, the most widely used preservatives are the weak organic acids and salts of weak acids including benzoic and sorbic acid. Of these chemical preservatives and in the concentrations used in practice, none of the compounds is lethal to microorganisms in foods and is only inhibitory (Chichester and Tanner, 1975). The choosing of a particular preservative of those above for a soft drink must be based on the anticipated microbial problems, need for selective action, solubility, ease of application, and appropiateness of pH range.

Sodium Benzoate

Benzoic acid, a white, crystalline powder with a sweet or astringent taste, is usually used in the form of sodium benzoate. The sodium salt is preferred because it is more water soluble than the acid form. The solubility of sodium

benzoate in water is 50g per 100ml of water at 25°C. In use the salt is converted to the acid or active form. The pK_a of benzoic acid and its sodium salt is 4.19 (i.e., at a pH of 4.19 50% of the acid is undissociated). Benzoic acid and sodium benzoate are recognized as GRAS substances in the U.S. with a tolerance level of 0.1% (Title 21, CFR, Parts 100-199). Sodium benzoate is relatively inexpensive and probably the most widely used food preservative in the U.S. and other countries (Busta and Foegeding, 1983). The human has a high tolerance to benzoic acid due to a detoxifying mechanism whereby the benzoate is conjugated with glycine or glycuronic acid to produce hippuric acid or benzoyl glucuronide, respectively, and is excreted in that form (Chichester and Tanner, 1975).

Sodium benzoate is generally known to inhibit yeasts more than molds or bacteria. Food poisoning and spore-forming bacteria are generally inhibited by 0.01-0.02% of undissociated acid, while yeasts and molds show greater resistance and are inhibited by 0.05-0.1% of undissociated acid. Kelly (1975) demonstrated that the minimum concentration of sodium benzoate needed to restrict yeast contamination to less than 100 cells per ml of a carbonated grape beverage in the 2.5-4.0 range was 0.1%. At pH 2.5, 3.0, 3.5, and 4.0 the levels of benzoate needed to prevent growth of an unidentified yeast strain were, respectively, 0.015, 0.030, 0.040, and 0.050%. Slightly lower

concentrations of benzoate were needed when the initial population was reduced from 10^4 to 10^2 per ml. Some spoilage bacteria, yeasts, and molds are much more resistant to benzoic acid or sodium benzoate. Therefore, benzoic acid or its salt can not be relied upon solely to effectively preserve foods capable of supporting growth (Baird-Parker, 1980).

The undissociated molecular form of benzoic acid is able to pass through the cell membrane more readily due to its nonpolar, lipophilic nature than the charged form (Freese et al., 1973). Rahn and Conn (1944) first suggested that preservative acids, such as benzoic acid, are effective only when present in the undissociated state, which will prevent ionization. The maximum effective pH of benzoate is 4.5, but is most effective from pH 2.5-4.0 (Creuss and Richert, 1929; Creuss et al., 1931). Higher concentrations are required for preservation when the pH is greater than 4.5. Therefore, sodium benzoate is recommended for preserving food products with a pH below 4.5.

Bosund (1962) found that the inhibitory effect of benzoate increased with a decrease in the pH of the medium. One hypothesis is that in an acidic medium the undissociated form of the benzoic acid enters the cell until the concentrations inside and outside the cell become equal. The neutralization of the undissociated form by the cell buffers cause an acidification of the cells interior, which

subsequently inhibits cell growth (Krebs et al., 1983; Warth, 1985; Bosund, 1962). Benzoic acid or sodium benzoate has also been shown to inhibit specific enzyme systems within cells. Bosund (1962) showed that in many bacteria and yeasts, enzymes involved in acetic acid metabolism and oxidative phosphorylation can be inhibited.

Sodium benzoate has especially wide applicability as a common preservative for acid foods and beverages. It is used in carbonated and still beverages, syrups, fruit salads, icings, jams, jellies, preserves, margerine, mincemeat, pickles, relishes, pie-fillings, and prepared salads. In carbonated beverages sodium benzoate is used generally from 0.05-0.1% (Chichester and Tanner, 1975). Benzoic acid occurs naturally in such foods as cranberries, prunes, greenage plums, cinnamon, and ripe cloves. Among the antimicrobial additives, sodium benzoate has the advantage of low cost. When sodium benzoate is incorporated into some foods, there may be a noticeable taste. When this occurs, lower levels of benzoate are used in combination with another antimicrobial, such as sorbate or the parabens (Chichester and Tanner, 1975).

Esters of Para-hydroxybenzoic Acid

The alkyl esters of p-hydroxybenzoic acid (parabens) make up a group of antimicrobial agents which were first used widely in cosmetic and pharmaceutical products and later recognized as a preservative in foods. Their use in

foods offered a means for extending upward the limiting pH for effective use of benzoic acid. European publications appeared in great numbers during the 1920's describing applications of parabens in pharmaceuticals, cosmetics and foods. Regulations permitting the use of these preservatives in foods were adopted in many European countries between 1932 and 1938 (Chichester and Tanner, 1975).

The methyl and propyl esters of parabens are most commonly used in the U.S., while the ethyl and butyl esters find some usage in European countries. n-Heptyl parabens finds some usage primarily in beer. All of the parabens commonly used in foods are white, free flowing powders. The methyl ester has a faint odor and a taste perception similar to that of sodium benzoate while the propyl ester is virtually odorless (Aalto, 1953; Chichester and Tanner, 1975). Since benzoic acid exerts its antimicrobial activity in the undissociated form, it is likely that the same requiremaent holds for the parabens (Chichester and Tanner, 1975). An advantage of parabens over benzoate and other preservatives is their high pK_a (8.5). By esterifying the carboxyl group of benzoic acid an undissociated molecule can be retained over a much broader pH range, extending the spectrum of activity to foods with a pH near neutrality. Hence, their effectiveness is less pH dependent than that of

benzoic or sorbic acid (Aalto, 1953; Chichester and Tanner, 1975).

The parabens are more active against yeasts and molds than bacteria but are used in foods to inhibit or prevent growth of all three microbial types. Robach and Pierson (1978) demonstrated the ability of methyl or propyl parabens to delay or inhibit Clostrium botulinum growth and toxin production in laboratory media. Pierson et al. (1980) demonstrated the ability of propyl parabens to slow the growth of Salmonella typhimurium and to cause a gradual decline of viable cell numbers of Staphylococcus aureus. As has been demonstrated for bacteria, a lower concentration of parabens was required for inhibition of Saccharomyces cerevisiae when the alkyl chain length of the ester was increased in size (Aalto, 1953; Lueck, 1980).

Although antimicrobial activity of parabens increases with increasing chain length, solubility decreases with increasing chain length. Solubility limits the use of esters larger than three of four carbons in most foods. The antimicrobial activity of branched chain esters is low as well. Methyl and propyl parabens are often combined to achieve good solubility and antimicrobial action (Busta, 1983). The parabens are effective in the pH range of 3-8. However, spoilage problems by yeasts and molds under acid conditions can be controlled by the less expensive sodium benzoate (Chichester and Tanner, 1975). Parabens may be

used to preserve baked goods (nonyeast), soft drinks, beer, fruit products jam jellies, pockles, olives, syrups, creams and pastes (Busta, 1983).

Under U.S. Food and Drug Administration regulations, methyl and propyl parabens are GRAS substances when used as chemical preservatives in foods, with total addition limits of 0.1% (Title 21, CFR, Parts 100-199). They may be used alone or in combination with sorbates, propionates, and benzoates, such that total preservatives do not exceed 0.1% (Chichester and Tanner, 1975). They are stated to be less injurious to health than benzoic or sorbic acids (Aalto, 1953; Chipley, 1983). Soft drinks can be preserved with about 0.03-0.05% of a 2:1 ratio of methyl and propyl parabens.

Sorbic Acid

Sorbic acid, 2,4-hexadienoic acid, is the only unsaturated organic acid currently permitted as a food preservative. It was first manufactured by A.W. Hoffman in 1859 from rowan berry oil (Sofos and Busta, 1981). The ability of sorbic acid to act as an antimicrobial agent was discovered independently by E. Mueller in 1939 and by C M. Gooding in 1940 (Lueck, 1980; Sofos and Busta, 1983). In 1945 Gooding patented the use of sorbic acid as a fungistatic agent in foods and on food wrappers and found that sorbic acid surpassed sodium benzoate in certain applications (Gooding, 1945). Sorbic acid is a white,

crystalline powder with a pK_a of 4.76 and is only slightly soluble in water. The salts of sorbic acid (i.e., sodium, potassium, and calcium) are used most because of thier high water solubility. Potassium sorbate, the most commonly used salt form, is a white, fluffy powder with a water solubility of 139 gm per 100 ml at 20°C, which is much greater than sodium benzoate and the parabens (Chichester and Tanner, 1975).

The antimicrobial effectiveness of sorbates has a wide spectrum of activity against yeasts, molds and bacteria. Extensive research during the 1950's demonstrated the effectiveness of sorbates against a wide range of yeasts and molds and resulted in extensive use of the compounds as fungistatic agents. Effective antimicrobial concentrations of sorbates in most foods are in the range of 0.05-0.3% (Sofos and Busta, 1983). Sorbate is more effective at pH values approaching its pK_a of 4.76. It is able to penetrate the semipermeable microbial cell membrane in the undissociated, uncharged form, and therefore, its activity is higher at lower pH values. According to Sofos and Busta (1981), the more favorable antimicrobial effects at higher pH levels obtained with sorbate as compared to benzoate may be partly explained by considering their pK_a values. The maximum pH for sorbate activity is 6.5, which is higher than that for sodium benzoate, but not as high as that of the parabens. Sorbate is ideal for replacing benzoate in more

acidic foods to avoid off flavors and extend the spectrum of microorganisms inhibited but costs more to use than benzoate.

Sorbic acid and its salts have been shown to inhibit a variety of bacteria. Sorbic acid or potassium sorbate has been found to be effective against Staphylococcus aureus, Salmonella, Vibrio parahaemolyticus, Bacillus sp. and Pseudomonas sp.(Sofos and Busta, 1983). It has also been shown to retard botulinal spore germination in comminuted meat systems where nitrite has not. Studies on sorbate as an antibotulinal agent in meat products, either alone or in combination with low levels of nitrite have demonstrated effectiveness (Sofos et al. 1979; Pierson et al., 1979).

The mechanisms of microbial growth inhibition by sorbate are not well understood. The antimicrobial action of sorbate may be due to inhibition of enzymes and nutrient transport. Many enzymes are reportedly strongly or weakly inhibited by sorbate including fumarase, aspartase, enolase, lactate dehydrogenase, and isocitrate dehydrogenase (York and Vaughn, 1964; Azukas et al., 1961; Lueck, 1980). Whitaker (1959) and Lueck (1980) reported inhibition of sulfhydryl enzymes within microbial cell walls, allowing sorbate to bind covalently with thiol groups.

In the U.S. sorbic acid and sorbates are considered GRAS substances. Since it is a metabolizable fatty acid, the World Health Organization (WHO) has set an acceptable

daily intake of sorbic acid and its salts at 25mg/kg body weight, which is the highest acceptable daily intake allowance of all common food preservatives (Sofos and Busta, 1983). Sorbates are used for mold and yeast inhibition in cheese products, baked goods (nonyeast), fruits, vegetables, soft drinks, wines, fruit juices, jams jellies, syrups, sauerkraut, salads, margarine, meat, and fish products (Chichester and Tanner, 1975). Sorbates may be used alone or in combination with sodium benzoate in carbonated beverages. The sorbate is as effective as benzoate in carbonated beverages and is less likely to alter flavor at effective levels.

Acidulants used in Carbonated Beverages

As an ingredient in beverages, acid is considered third in importance following water and sugar (Phillips and Woodroof, 1981). The functions of an acid in carbonated and noncarbonated beverages include: (a) lend tartness and sourness to taste; (b) enhance palatability; (c) increase thirst quenching effect by stimulating flow of saliva in the mouth; (d) modify the sweetness of the sugar; and (e) act as mild preservative by lowering the pH and by reducing the sterilization time for pasteurized products, especially for fruits and vegetables and their juices which lose their natural structure and nutritive value with prolonged heating. In other foods they are used in products having gelled structures to produce either proper gelation or to modify the rate of set (Chichester and Tanner, 1975). The food acidulants permitted to be used in carbonated beverages in the U.S. include citric, phosphoric, and malic acids.

Citric Acid

Citric acid is a tribasic hydroxy acid, unlike the other hydroxy acids. It is the main acid in citrus fruits and an acidifying agent with unique flavor characteristics. It has been used in foods in the U.S. for more than 100 years (Chichester and Tanner, 1975). Several fresh fruits, such as lemons and limes, owe their tangy taste to the presence of citrate ions. It has a pK_a of 3.1 and a high solubility in water.

Citric acid and its sodium and potassium salts are classified by the FDA as GRAS substances. They are nontoxic and the acceptable daily intake allowance as set by the WHO is not limited (Busta, 1983). Both the acid and its salts are allowed in various fruit juice drinks, diluted juice beverages, and nonalcoholic carbonated beverages. Citric acid is employed extensively in the preparation of carbonated beverages to bring out the flavor and to impart the right mouth feel. It aids in bringing about the required bouquet by modifying some of the sweet flavor components and inactivates trace metals which may cause haze or deterioration of color and flavor (Phillips and Woodroof, 1981; Chichester and Tanner, 1975).

Since many microorganisms can metabolize citrate, this anion rarely has any antimicrobial activity. Borst-Pauwels (1981) reported that buffer containing the citrate anion hardly affects the yeast cell pH. When antimicrobial activity is exerted, citric acid causes leakage of hydrogen ions across the microbial cell membrane, acidifying the cell's interior and inhibiting nutrient transport. It has been reported that 0.3% citric acid lowered the level of Salmonella on poultry carcasses and was effective in reducing the total number of cells (Chichester and Tanner, 1975).

Phosphoric Acid

Phosphoric acid is a strong inorganic acid used extensively in the food industry. It is the second leading acid used in foods, with citric acid being used the most. When antimicrobial action is exerted, phosphoric acid causes an acidification of the cell's interior, which subsequently may retard cell growth. Koburger (1971) was able to show that recovery counts on yeast cells were lowest when laboratory media was acidified with phosphoric acid as compared to other organic and inorganic acidulants. Similarly, Pitt (1974) demonstrated that phosphoric acid was slightly inhibitory to various spoilage yeasts, but he concluded it could not be considered of value as a preservative. The low pH characteristic with its use is usually undesireable in food but is ideal for use as an acidulant in carbonated beverages. Not only is phosphoric acid the strongest acidulant used in foods, but it is the only inorganic acid to be used as an acidifying agent and it is the least costly. The majority of phosphoric acid is used in cola, root beer, sarsparilla, and similar flavored carbonated beverages (Busta, 1983; Chichester and Tanner, 1975).

Malic Acid

Malic acid is the acidulant used the least in carbonated beverages. It is a white, crystalline, odorless powder that gives a smoothly tart taste effect similar to

citric acid. It has been recommended for bringing out the overall taste of fruit flavored carbonated beverages. It has be found in many natural food products as the predominant acid including apples, appricots, bananas, cherries, grapes, orange peels, grapes, peaches, pears, plums, brocoli, carrots, peas, potatoes, and rhubarb (Chichester and Tanner, 1975). Malic acid is recognized by the FDA as a GRAS substance and has been described as a microstatic agent against certain yeasts and molds (Banwart, 1979).

Gases used as Preservatives

It has been known for many years that various naturally occurring and manufactured gases and vapors have the ability to kill or inhibit the growth of microorganisms. The gases used most commonly in commercial practice include carbon dioxide, ethylene oxide, propylene oxide, sulfer dioxide and the sulfites, and ozone. Nitrogen and oxygen are frequently used in packaging and storage of foods but not directly to inhibit the growth of microorganisms. Nitrogen was shown not to be a microbial growth inhibitor to yeasts and bacteria, and in fact, may have actually been stimulatory for growth (Thom and Marquis, 1984). Carbon dioxide, sulfer dioxide and the sulfites, and ozone used in liquid systems while ethylene and propylene oxide have approved usage exclusively in dried food products.

Sulfer Dioxide

Sulfer dioxide has been used for many centuries, especially as a wine preservative. In addition to wines sulfer dioxide, sulfite salts, and bisulfite and metabisulfite salts are used to preserve a variety of foods such as fruit juices, soft fruits, vegetables, meat, and fish.

Sulfites inhibit yeasts, molds, and bacteria with yeasts being the most resistant. The growth inhibiting effects of sulfer dioxide are most intense when sulfurous acid is in the nonionized form. Sulfer dioxide has the

greatest effect on yeasts, bisulfite the lowest activity, and sulfite has no activity (Ough, 1983). Microbial death or inhibition may be due to a combined loss of membrane function and enzyme activity (Clark and Takacs, 1980). Sulfer dioxide is recognized by the FDA as a GRAS substance.

Ozone

Ozone has been used mainly for water sterilization to remove metals, off odor, flavors, and colors in many countries. It is used a maturing agent in ciders and wines and to sterilize beverage bottles. The antimicrobial action of ozone is due to its strong oxidizing effect (Busta and Foegeding, 1983).

Carbon Dioxide

Carbon dioxide (CO_2) is one of the major antimicrobial factors in carbonated beverages. It is a colorless, noncombustible gas with a very faint odor. Since it is approximately one and a half times as heavy as air, it can literally be poured from one vessel to another. Below about 10% CO_2 is not toxic to humans, but above this level unconsciousness occurs with prolonged exposure (Clark and Takacs, 1980).

At room temperature the water solubility of CO_2 is 1 L/L, with solubility increasing with decreasing temperature of the gas and/or of water (Jones and Greenfield, 1982). When dissolved in water or absorbed in foods, carbon dioxide forms carbonic acid, hence lowering the pH in proportion to

the amount of carbonic acid formed. CO_2 is generally used in the form of a liquified gas. CO_2 gas on being compressed and cooled forms a liquid, which on being cooled sufficiently freezes to a solid form or dry ice. These physical properties permit commercial distribution of CO_2 as a liquified gas in a pressurized cylinder handled at air temperature or solidified and handled as dry ice for refrigerant purposes.

Generally, CO_2 concentrations of 5-50% inhibit most yeasts, molds, and bacteria, while lower concentrations are generally without effect. The antimicrobial action of CO_2 is exerted by CO_2 displacing oxygen and intervening in the respiratory metabolism of various microorganisms. Also at high concentrations it lowers the pH of the medium through the formation of carbonic acid. The activity of CO_2 depends upon concentration, the microorganisms under investigation and their stage of growth, the water activity of the system, and the storage temperature. As a result, the effect of CO_2 may be stimulative, no effect, or inhibitory (King and Nagel, 1967; Parekh and Solberg, 1970).

CO_2 generally inhibits growth of microorganisms rather than killing them. The spectra of tolerance to the growth inhibition and toxic effects of CO_2 are varied. Generally, molds are sensitive, yeasts compartively resistant (except for some nonfermentable types), and bacteria are highly varied. Among the bacteria, gram positive are more

resistant than gram negative (Sutherland et al., 1977). The bacteria most affected are the pseudomonads and Acinetobacter-Moraxella group, which grow rapidly and produce off odors in raw meats (Enfors and Molin, 1980). Lactobacilli and clostridia generally are among the most resistant forms. Fortuneately, they do not readily spoil foods and tend to predominate in the microflora of vacuum packaged, cured, and raw meat products (Patterson and Gibbs, 1977). Sporeformers such as Clostridium sp. are fairly CO_2 resistant while Bacillus sp. are strongly inhibited (Parekh and Solberg, 1970; Enfors and Molin, 1978; Clark and Takacs, 1980).

CO_2 is used to control the psychrotrophic spoilage of meat and meat products, poultry, fish, eggs, fruits, and vegetables by addition of dry ice during storage and transfer. The dry ice sublimes to form gaseous CO_2. CO_2 added directly as a gas is used in storage of fruits, vegetables, vacuum packaged meats, and beverages (Busta, 1983).

The carbonation tolerance of various common soft drink spoilage yeasts was investigated by Ison and Gutteridge, (1987). All yeast strains tolerated up to 2 ATM of CO_2 and grew in laboratory growth media. Inhibition of growth occurred with 10 of the 14 strains after exposure to between 2-4 ATM of CO_2, while 4 strains tolerated up to 4.5 ATM. The results were also able to correlate well with the

spoilage microflora of soft drinks poised at specific carbonation levels. The factors relating the resistance or sensitivity to carbonation are not fully understood. In yeasts the membrane disruptive function by CO_2 appears to much more important than its metabolic effects; however, this is not true for all strains (Jones and Greenfield, 1982).

Factors Influencing the Resistance to Preservatives

The ability of an antimicrobial preservative to inhibit a particular microorganism depends on the water activity, pH, acidulant, and humectant of the medium as was previously studied (Jermini and Schmidt-Lorenz, 1987). However, several other limitations to the use of weak organic acids as microbial inhibitors in foods include: (a) when the contamination is too high, chemical preservatives are less effective; (b) the physiological condition of the microorganisms may be altered by exposure to adverse physical or chemical conditions; (c) some microorganisms metabolize organic acids as a carbon source; and (d) resistant species and individual strains with variable resistance have been selected. One of the major concerns of the soft drink industry is the prevalence of preservative-resistant strains of spoilage yeasts.

It has been shown that chemical preservatives were found to be ineffective when the initial level of yeast cells were 10^4-10^5 cells/ml (Jermini and Schmidt-Lorenz, 1987; Baird-Parker, 1980). Therefore, no preservative should be used to cover up the use of spoiled or spoiling foods.

Acquired Resistance to Benzoic Acid, Sorbic Acid, and Other Weak Acid Preservatives by Spoilage Yeasts

The possibility of acquired resistance to benzoic acid by microorganisms has been known for quite some time. Ingram (1960) isolated several benzoate-resistant strains of Zygosaccharomyces bailii from citrus beverages at pH levels below 3.0 that contained 500 mg/l benzoic acid. The resistance was more marked in the presence of 30% sucrose than in 1% sucrose. It has been reported that cells of Z. bailii grew in the presence of high concentrations of benzoic acid (600 mg/l) at pH values below the pK_a of the acid (4.19) (Warth, 1977; Pitt, 1974). Pitt (1974) demonstrated that Z. bailii was resistant to 600 mg/l benzoic acid in yeast nitrogen base media at pH 3.5 containing 10% glucose. Pitt and Richardson (1973) reported on fermentative spoilage of carbonated fruit beverages containing 400 mg/l benzoic acid by Z. bailii and other spoilage organisms and described the ability of Z. bailii to grow at pH 3.2 under 60 p.s.i.g. CO_2 in the presence of 700 mg/l benzoic acid.

Resistance to sorbic acid and other weak acid preservatives by spoilage yeasts, including Z. bailii, has also been reported (Pitt, 1974; Balatsouras and Polymenacos, 1963). Z. bailii was not inhibited by 600 mg/l of sorbic acid in laboratory media with 10% glucose at pH 3.5. Sorbic acid was more effective than benzoic acid against most of

the other yeasts examined (Pitt, 1974). Warth (1985) was able to show that yeast strains that are resistant to one weak acid type of preservative tend also to be resistant to others. There were differences in the responses to individual preservatives, but these differences were small. This suggests that the weak acid preservatives may have a common mechanism of action and that the factors which enable some strains to tolerate high levels of preservative applies to both sorbic and benzoic acid.

It is known that yeast cells rapidly take up benzoic acid from an acid medium and that large concentration gradients in favor of an accumulation of benzoate within the cell can be achieved (Bosund, 1960; Macris, 1975). Warth (1977) proposed that resistance to benzoic acid, sorbic acid, and other weak acid preservatives resulted primarily from an inducible energy requiring system that transported the anion from the cell. Since this system required energy, cells were resistant only when a sufficient energy source was present. Operation of this system, which allows the cell to avoid accumulation of large amounts of preservative, probably is very important in the cells tolerance to a preservative (Warth, 1985).

The effectiveness of benzoic acid and sorbic acid against preservative-resistant yeast cells (i.e., Z. bailii) not previously adapted to the respective preservative are notably the same. However, sorbic acid may be a more

effective preservative in products subject to recontamination where the inoculum is adapted to growth in the preservative. Warth (1985) was able to show that sorbic acid was generally more effective than benzoic acid in inhibiting growth rate and cell yield of Z bailii previously grown in the presence of low levels of each respective preservative.

Studies observing the effects of nutrients and pH on the resistance of Z. bailii and other spoilage yeasts to preservatives have agreed that the effectiveness of benzoic acid decreases with increasing pH. However, reports have been conflicting with regard to the effect of sugar concentration on the resistance of Z. bailii to benzoic acid. Pitt (1974) and Ingram (1960) observed increased resistance by Z. bailii to benzoic acid in the presence of high levels of glucose. However, Warth (1986) observed the lack of major effect of sugar concentration on the resistance of Z. bailii to benzoic acid. Because Warth (1977, 1985) had demonstrated that large amounts of energy were used in eliminating preservative anion from the yeast cell, the current results were unexpected. According to Warth (1986), it appears that high tolerance to certain preservatives can be due to selection and adaptation phenomenon.

Synergistic Effects of Preservatives in Carbonated Soft Drinks

Carbonated soft drink beverages are unique in being among the few packaged foods that do not in general receive any type of physical process to prevent microbial spoilage. Instead, the soft drink industry relies upon the inherent growth inhibiting characteristics of the product and good plant sanitation to prevent spoilage (Witter et al., 1958). Any of the physical or chemical properties of the soft drink may have an effect on the viability of microorganisms, but to determine the pattern of preservative action, factors common to all beverages are of concern. According to Witter et al., (1958), sucrose concentration, acidity, and degree of carbonation are the major characteristics which prevent microbial spoilage in most carbonated beverages.

Over the years two rules of thumb which generally applied to the survival of microorganisms in carbonated soft drink beverages included: (a) spoilage is reduced by increased acidity and carbonation; and (b) the microbial count in a carbonated beverage decreases with increasing storage time. Studies substantiating the effects of carbonation on spoilage yeasts are conflicting. Witter et al. (1958) investigated the viability of an unidentified spoilage yeast isolated from a spoiled carbonated orange drink as a function of pH, sucrose concentration, and carbonation. No lethality was found for the yeast strain

without carbonation regardless of the degree brix or pH. In fact, in the noncarbonated beverage pH had little effect on the growth of the yeasts. However, in the presence of carbonation the lower pH value under these conditions the greater was the lethality. Lethality was independent of the sucrose concentration. In contrast to the previous study, Ison et al. (1987) demonstrated that several common preservative-resistant and nonresistant spoilage yeasts were tolerant to high levels of carbonation at low pH (3.5) and high glucose concentration (5%). These studies may indicate that carbonation tolerance varies among spoilage yeasts and presence of preservative is necessary for mycostatic action to occur.

The separate effects of carbonation, pH, carbohydrate concentration, and preservative under varying conditions has been studied, but only one study has been documented on their combined effects as related to soft drinks under varying conditions. Perigo et al., (1964) developed a turbidostatic continuous culture apparatus which was used to determine the combined effect of variations in carbonation, benzoic acid, and pH on the growth rate of a soft drink spoilage yeast and to derive a model which was believed to be able to predict the spoilage potential of many carbonated soft drinks. They found that in the absence of benzoic acid, the shelf life for any given carbonation was very similar at either pH 2.5 or 3.5. The authors concluded that

the growth rate was not significantly influenced by pH changes over the range of 2.5-3.5. Conversely, in the presence of identical amounts of benzoic acid, the shelf life of any chosen carbonation is longer at pH 2.5 than at 3.5. This observation is in agreement with that of Rahn and Conn (1944) discussed previously. The authors concluded that it was possible that the primary effect of both the carbonation and benzoic acid was mycostatic, reducing the growth rate but not directly increasing the death rate.

An important factor that can not be overlooked in all soft drink spoilage cases is the possibility of a partial inhibition of the preservative by other components of the substrate. Investigations have shown that certain preservatives may be partially neutralized by certain components of the substrate (Sand, 1969). Warth (1986) was not able to demonstrate which components of the minor nutrients from laboratory media were important in reducing preservative resistance.

Control of Yeast Spoilage

It is generally accepted that the main cause of the spoilage problems in bottled and canned soft drinks are caused by yeasts. Yeast growth in carbonated beverages becomes evident through the production of sediment, haze, flocculated clumps, off taste due to metabolic end products, and possibly increased internal pressure of the beverage container from CO_2 production (Berry, 1979). Yeasts are able to gain entry into the plant and susequently into the finished product by several routes including: (a) stockpiles in the plant of returnable bottles and containers awaiting washing; (b) inadequate washing of bottles and cans; (c) air-borne contamination; (d) unsanitary equipment such as tanks, pipelines, pumps, fillers, and can seamers; (e) unsanitary operating conditions; and (f) contaminated sugars and syrups.

The beverage will attain the residual contamination as it flows through the equipment. Normally, the first bottles or cans produced at the beginning of the day's run will have the highest counts and the later ones will have lower counts because of the wash-out effect.

In order to monitor the sanitary quality of the bottling operations microbiological testing must be performed on a regular basis. Key points where samples are taken include water, syrup, product, empty washed bottles, and the bottled or canned product. The number of

contaminating yeasts are then measured by standard measures including plate counting, membrane filtration, and direct microscopic counting.

According to Sand (1969), the yeast strains which spoil soft drinks are mostly vitamin-B autotrophic, and show a good tolerance of the generally low amounts of preservatives allowed in soft drinks in many countries. Zygosaccharomyces bailii can be singled out as one of the most notorious spoilage organisms for soft drinks both canned and bottled (Ingram, 1960; Pitt and Richardson, 1973; Pitt, 1974). Every few years a major spoilage problem occurs in some part of the world involving this organism. Z. bailii is highly resistant to preservatives and is a strong fermenter in the presence of beverages containing fruit juice (Thomas and Davenport, 1985). Spoilage of a product may become evident by exploding containers. Saccharomyces cerevisiae and Saccharomyces uvarum have been responsible for large scale spoilage explosions (Splittstoesser, 1987) and are considered the most dangerous soft drink spoilage yeasts (Sand, 1976; Splittstoesser, 1987; Mossel and Scholts, 1964). Although the inherent antimicrobial factors within a carbonated beverage are the primary inhibitory principles against spoilage yeasts in carbonated beverages, good plant sanitation is the most important factor in preventing spoilage.

Sodium Phosphite

Sodium phosphite (Na_2HPO_3 $5H_2O$) is the acid salt formed from the dibasic acid orthophosphorous acid (H_3PO_3). It is the most soluble of the alkali phosphites with increasing solubility as the temperature increases. Upon heating phosphorous acid and its salts, they undergo decomposition primarily to phosphine and orthophosphates in the absence of water and to hydrogen and orthophosphates in the presence of water. When heated in air above 200°C, sodium phosphite begins to decompose (Corbridge, 1978). Unlike the hypophosphites, the phosphites do not react with concentrated sodium hydroxide solutions to give hydrogen on heating. An important property associated with the phosphite ion is its characterization as a very strong reducing agent. Some of the important physical and chemical properties of sodium phosphite are in table 1.

The dialkyl phosphites are essentially odorless liquids. In water they exhibit no buffering capacity and are neutral in the usual sense of the word, because they do not form salts with bases (Smith, 1971). This property is expected from their accepted structure in which the hydrogen is attached directly to the phosphorous atom and is not an acidic hydrogen.

Observations by Robertson and Boyer (1956) showed that orthophosphites do not interfere in enzymic reactions of phosphate and are relatively nontoxic to intact yeast or

Table 1. Physical and chemical properties of sodium phosphite.

Chemical formula	:	Na_2HPO_3 $5H_2O$
Molecular weight	:	216.04
Physical state	:	crystalline powder
Color	:	white
Odor	:	odorless in solution
Melting point	:	decomposes above 200^oC
Solubility	:	freely soluble in water; insoluble in alcohol and NH_4OH
pH	:	neutral in water
pK values	:	pK_1=1.29; pK_2=6.74

animal cells and young rats. Potassium phosphite had no influence on the rate of glucose fermentation by intact yeast cells in laboratory media at pH 6.0. Rats were found to tolerate without visible distress the intraperitoneal administration of 1750 mg of sodium phosphite, pH 7.3, per kilogram of body weight. Sodium phosphite fed at 250 mg/kg to a small group of rats on alternate days appeared to reduce the growth rate, but 125 mg/kg was without effect. It was then shown that phosphites were not very toxic to weanling rats fed inorganic phosphite and simply could not be utilized as a source of phosphorous. More comprehensive studies are needed on toxicity of inorganic phosphites with respect to humans.

Commercial Uses of Phosphites

Phosphorous acid and the phosphites have been recommended as stabilizers for plastics. Incorporation of phosphites into several vinyl plastics is claimed to stabilize them against heat and ultra-violet light. Acidified aqueous solutions of phosphites have been shown to remove iron impurities from plastics. Similarly, the phosphites have shown the ability to prevent rust formation on metal surfaces.

Sodium Phosphite as an Antimicrobial Agent

To the best of the authors knowledge, there is no known published work on sodium phosphite as an antimycotic agent. However, an unpublished report was written by Rhodehamel and

Pierson (1984) on sodium phosphite inhibition of the growth of selected foodborne pathogenic and spoilage bacteria, yeasts, and molds. The results of the study which are pertinent to this research include the experiments evaluating inhibition of growth of selected yeasts.

The effectiveness of sodium phosphite at four concentrations (0, 1000, 2000, and 3000 micrograms per ml (ug/ml)) was evaluated at three pH levels (4.0, 5.0, and 6.0) with three preservative-nonresistant strains of yeast in laboratory media containing one percent glucose. The yeast strains included Saccharomyces cerevisae, Candida utilis, and Debaryomyces hansenii.

Sodium phosphite was extremely effective against all yeasts tested. Sodium phosphite was fungicidal at 3000 ug/ml at pH 4.0 to D. hansenii. Decreasing the pH of the media increased sodium phosphite inhibition significantly in all yeasts tested. Total inhibition of growth occurred with all three concentrations of sodium phosphite at pH 4.0, as significant growth was not obtained in any variable after 240 hrs incubation. The authors concluded that further research was needed to determine sodium phosphite's potential to inhibit other yeasts and to determine sodium phosphite's efficacy to inhibit yeast growth in food systems.

Materials and Methods

Yeasts

Three ascomycetous strains, Saccharomyces cerevisiae 71-139, Saccharomyces uvarum NRRL Y-379, and Zygosaccharomyces bailii NRRL Y-7256 were obtained from the University of Georgia Department of Food Science. The yeasts cultures were transported in Yeast extract-Malt extract- Peptone-Glucose (YMPG) agar slants.

Preparation of Cultures

Stock cultures were grown on YMPG agar slants (pH=6.7) for 3-5 days at 25°C and then stored at 4°C. Stock cultures were transferred every two weeks.

Inocula for all S. cerevisiae, S. uvarum, and Z. bailii experiments were obtained by inoculating YMPG broth tubes (pH=6.7) from stock cultures and incubating at 25°C for 24 hrs. Three subsequent transfers were made at 24 hr intervals. Approximately 24 hrs after the third transfer, 1 ml of culture was transferred to a Nephalo growth flask containing YM broth (pH=6.7) which was covered with a foam stopper. The growth flasks were incubated and shaken at 25°C and 150 rpm, respectively, in a New Brunswick Psycrotherm Shaker Incubator (Edison, NJ). Growth was monitored with a Bausch and Lomb Spectronic 20 and was allowed to reach late log phase for all three yeast cultures prior to serially diluting them for experiment inoculations.

Late exponential phase was reached in 12 hrs at a level of $2.5x10^7$ cells/ml for S. uvarum and in 23 and 26 hrs at levels of approximately $2.5x10^7$ and $2.5x10^6$ cells/ml of Z. bailii and S. cerevisiae respectively.

YMPG agar micro-spot plates were used to determine initial numbers of of microorganisms per ml. In this technique, 0.1 ml of diluted culture was transferred to a sterile micro-centrifuge tube. A 20 microliter (ul) aliquot of culture was spotted onto a designated zone of the plate. A 10-25x dissecting microscope was used to count colonies of S. uvarum plates incubated for 24 hrs and Z. bailii and S. cerevisiae plates incubated for 36 hrs.

For all experiments, 0.4 ml from each late log phase culture was diluted 1:100, 1:1,000, and 1:10,000 in 0.1% peptone blanks stored previously at 4^oC. The diluted cultures were stored at 4^oC until needed. For preliminary experiments and experiments evaluating the effects of sodium phosphite in a carbon dioxide or nitrogen atmosphere, 0.1 ml aliquots of the diluted culture were dispensed asceptically into sterile screw cap test tubes. Initial inoculum levels for these experiments were approximately $1.4x10^2$, $1.2x10^2$, and $1.2x10^2$ cells per ml of test media for S. cerevisiae, S. uvarum, and Z. bailii respectively. For experiments evaluating the effects of sodium phosphite in commercial carbonated beverages, diluted cultures of Z. bailii were transferred asceptically in 0.1 ml aliquots into sterile

screw cap test tubes. Initial inoculum levels of Z. bailii were targeted at $1x10^3$, $1x10^2$, and $1x10^1$ cells per ml of carbonated beverage.

Sodium Phosphite Solutions

Sodium phosphite was obtained from Pfaltz and Bauer, Inc., (Waterbury, ct), and dissolved in distilled-deionized water to final concentrations of 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0% (wt/v). Stock solutions were filter sterilized through 0.22 micrometers (um) filters (Millipore Corporation, Bedford, Mass) and stored at 4^oC until needed. Appropriate amounts of each sterilized solution were added to the test broths and beverages to give final concentrations of 250, 500, 750, 1000, 1500, and 2000 ug/ml.

Media Preparation

For preliminary experiments, YMPG broth was dispensed in 98 ml aliquots into 500 ml Nephalo growth flasks with foam stoppers before autoclaving at 121^oC and 15 p.s.i. for 15 min. The final pH after autoclaving was pH 6.5. Flasks were then cooled to room temperature before the addition of 1 ml of a 2.5, 5.0, or 10% sterile stock solution of sodium phosphite. Broths were then adjusted to either pH 2.5, 3.5, or 4.5 by addition of sterile 1N hydrochloric acid through the use of replica flasks. Final concentrations in the broths at each pH level tested were 250, 500, and 1000 ug/ml of sodium phosphite.

For experiments designed to evaluate the effects of carbon dioxide and nitrogen atmospheres, media contained Yeast Nitrogen Base (YNB) broth (Difco, Detroit, Michigan) and 0.5% fructose. YNB broth was filter sterilized through 0.22 um filters (Millipore Corporation, Bedford, Mass) before storing at 4°C until needed. YNB broth was buffered with a combination of 0.1M citric acid and 0.2M dibasic potassium phosphate to achieve pH values of 2.5, 3.0, 3.5, 4.0, and 4.5. Adjustments in pH were made with 50% phosphoric acid or 1N NaOH. Solutions of buffered YNB broth were stored at 4°C until needed.

Sodium Phosphite in a Carbon Dioxide Atmosphere

All experiments evaluating the effects of a carbon dioxide (CO_2) atmosphere were performed in a walk-in cooler unit held at 4°C. All media, vessels, and equipment were cooled to 4°C prior to initiation of an experiment to optimize CO_2 dissolution. Solutions of test media at each pH level were transferred asceptically to a sterile pressure tank (Coca Cola Bottling Plant, Dublin, Va) and covered with the tank lid. Modifications were made on the tank which allowed CO_2 to be delivered to the bottom of the tank instead of the head space. Initially, carbon dioxide (Airco Gas Co., Radford, Va) was bubbled into the media at 20 p.s.i. to eliminate air in the head space of the tank. The tank was then sealed and CO_2 pressure increased to 80

p.s.i.. The pressure tank was agitated on a rotary shaker at 175 rpm and left agitating until 2.5 volumes (v/v) of CO_2 could be measured in the media. Carbonation level of the test media was determined by dispensing it into Vacutainer tubes (Beckton Dickinson and Co., Rutherford, NJ) leaving a small headspace and measuring the headspace pressure using an HMB headspace meter (Biotech International, Bellaire, Texas).

Carbonated test media were dispensed asceptically in 9.8 ml aliquots from the pressure tank into sterile screw cap test tubes already containing 0.1 ml of diluted yeast culture in 0.1% peptone. Once the media were dispensed, 0.1 ml of a 5, 10, 15, or 20% sterile stock solution of sodium phosphite was added quickly. Before the cap was screwed onto the tube, a sterile rubber septum was used to seal the tube opening to prevent leakage. The experiment tube was then shaken vigorously and the cap loosened briefly to expel air in the tube's headspace and resealed rapidly. The final volume of CO_2 dissolved in the medium of each tube was 2.0 volumes (v/v). Experiment tubes were incubated at 25°C.

Sodium Phosphite in a Nitrogen Atmosphere

Experiments evaluating the effects of a nitrogen atmosphere were performed at room temperature. Test media were transferred asceptically to a pressure tank (Sartorius, East Granby, Ct) and covered with the tank lid.

Modifications were made on the tank which allowed nitrogen to be delivered directly to the bottom of the tank instead of the headspace. Nitrogen gas (Airco Gas Co., Radford, Va) was bubbled initially at 20 p.s.i. into the medium to expel any air from the headspace. The tank was then sealed and the nitrogen pressure increased to 65 p.s.i.. The pressure tank was agitated on a rotary shaker at 200 rpm and left agitating for 30 minutes. The medium was dispensed asceptically in 9.8 ml aliquots from the pressure tank into sterile screw cap test tubes already containing o.1 ml of diluted yeast culture in 0.1% peptone. Once transfer of the medium was completed, 0.1 ml of a 5, 10, 15, or 20% sterile stock solution of sodium phosphite was added quickly and the tubes capped rapidly. Tubes were vortexed to assure adequate mixing of the contents.

Sodium Phosphite in Commercial Carbonated Beverages

Two types of commercial carbonated beverages, Slice (S) Diet Slice (Diet S), were purchased at a local supermarket. Both beverages claim to contain 10% real fruit juice. High performance liquid chromatography (HPLC) was used to identify and to quantify the carbohydrate present in beverage S. It was found to contain 6.2% fructose, 4.3% glucose, and <1% sucrose and maltose. Diet beverage S contained the nutritive sweetner aspartame (Nutrasweet), which contains the amino acids aspartic acid and phenyl

alanine. The pH of both beverages was approximately 3.2 and remained as such for the experiments. The carbonated beverages were stored at 4°C until needed.

Experiments evaluating the effects of sodium phosphite on yeasts in commercial carbonated beverages were performed in a walk-in cooler unit held at 4°C. Beverages were transferred asceptically to sterile pressure tanks and covered with the tank lid. Initially, CO_2 was bubbled into the beverage at 20 p.s.i. to expel any air in the head space. The tank was then sealed and the CO_2 pressure increased to 65 p.s.i.. The beverages were dispensed asceptically in 9.8 ml aliquots from the pressure tank into sterile screw cap test tubes already containing 0.1 ml of diluted Z. bailii culture in 0.1% peptone. Once the beverage was dispensed, 0.1 ml of a 2.5, 5.0, 7.5, 10.0, 15.0, or 20.0 sterile stock solution of sodium phosphite was added quickly. Before the tube was capped, a sterile rubber septum was used to seal the tube opening to prevent leakage. Each tube was shaken vigorously and the cap loosened briefly to expel the air in the tube's headspace and resealed rapidly. The final volume of CO_2 dissolved in each experiment tube was 2.5. Experiment tubes were incubated at 25°C.

Growth Determination

Growth was monitored by measuring absorbancy at 600 nm with a Bausch and Lomb Spectronic 20 for preliminary

experiments and a Bausch and Lomb Spectronic 21 for all other experiments. Measurements of turbidity were taken every 6 hrs during a 168 hr incubation and every 12 hrs during a 528 hr incubation period for S. cerevisiae, S. uvarum, and Z. bailii for preliminary experiments and all other experiments respectively.

From these measurements, growth curves were plotted and the time to significant growth calculated for each test variable (0.35 in absorbance for all yeasts tested). Absorbance values of 0.35 represented $1.4x10^7$, $4.9x10^6$, and $1.5x10^7$ cells per ml for S. uvarum, S. cerevisiae, and Z. bailii respectively. The time to significant growth was used to evaluate the effect that sodium phosphite and other test variables had on growth for experiments with CO_2 and nitrogen atmospheres.

For experiments designed to evaluate the effects of sodium phosphite in commercial carbonated beverages, post-incubation counts were performed on experiment tubes with undetectable growth using a direct epifluorescent filter counting technique as described by Koch et al., (1986). For each tube inoculated with $1x10^3$, $1x10^2$, or $1x10^1$ Z. bailii per ml, 40, 40, and 400 large eyepiece squares were counted at 40X, respectively, using an Olympus BH2 Epifluorescent Microscope (Olympus Optics, Tokyo, Japan). For each treatment level, post-incubation counts were compared to initial inoculum counts to evaluate the

effects of sodium phosphite in Beverage S and Diet Beverage S.

Time to significant growth (O.D.=0.35) and time to detectable growth (O.D.=0.01) were chosen as such because experiments in respective sections were preliminary.

Observations for each experiment were compared using Duncan's Multiple range test.

RESULTS

Preliminary Experiments

Sodium phosphite (SP) was extremely effective in inhibiting growth of S. cerevisiae, Z. bailii, and S. uvarum at each pH and inoculum level tested. As table 2 indicates, the lowest effective concentration of SP in inhibiting growth of S. cerevisiae and Z. bailii at pH 2.5 and 3.5 was 250 ug/ml for both inocula levels. At pH 4.5, 500 ug/ml was required to inhibit growth in flasks with 10^2 cells/ml and 250 ug/ml inhibited growth in flasks with an inoculum of 10^1 cells/ml for both S. cerevisiae and Z. bailii.

Inhibition of S. uvarum was slightly more varied than the other two yeasts tested. At both inocula levels tested, there was increasing inhibition with decreasing pH. Greatest inhibition was at 2.5 and the least was at 4.5. For pH 2.5, 3.5, and 4.5, the lowest effective sodium phosphite concentrations were 250, 500, and 1000 ug/ml respectively.

Saccharomyces cerevisiae in CO_2 Atmosphere

Inhibition of growth for S. cerevisiae in the CO_2 atmosphere for all pH values combined was highly dependent upon sodium phosphite (SP) concentration ($p<.0001$). There was generally an increase in inhibition with increasing concentrations of SP within each pH value tested (Fig.1).

Table 2. Lowest effective sodium phosphite concentrations in YM Broth.

A. *Saccharomyces cerevisiae* 71-139

pH level	Lowest effective $Na_2HPO_3 \cdot 5H_2O$ concentration (ug/ml) $1x10^2$ cells/ml	$1x10^1$ cells/ml
2.5	250	250
3.5	250	250
4.5	500	250

B. *Zygosaccharomyces bailii*

pH level	Lowest effective $Na_2HPO_3 \cdot 5H_2O$ concentration (ug/ml) $1x10^2$ cells/ml	$1x10^1$ cells/ml
2.5	250	250
3.5	250	250
4.5	500	250

C. *Saccharomyces uvarum*

pH level	Lowest effective $Na_2HPO_3 \cdot 5H_2O$ concentration (ug/ml) $1x10^2$ cells/ml	$1x10^1$ cells/ml
2.5	250	250
3.5	500	500
4.5	1000	1000

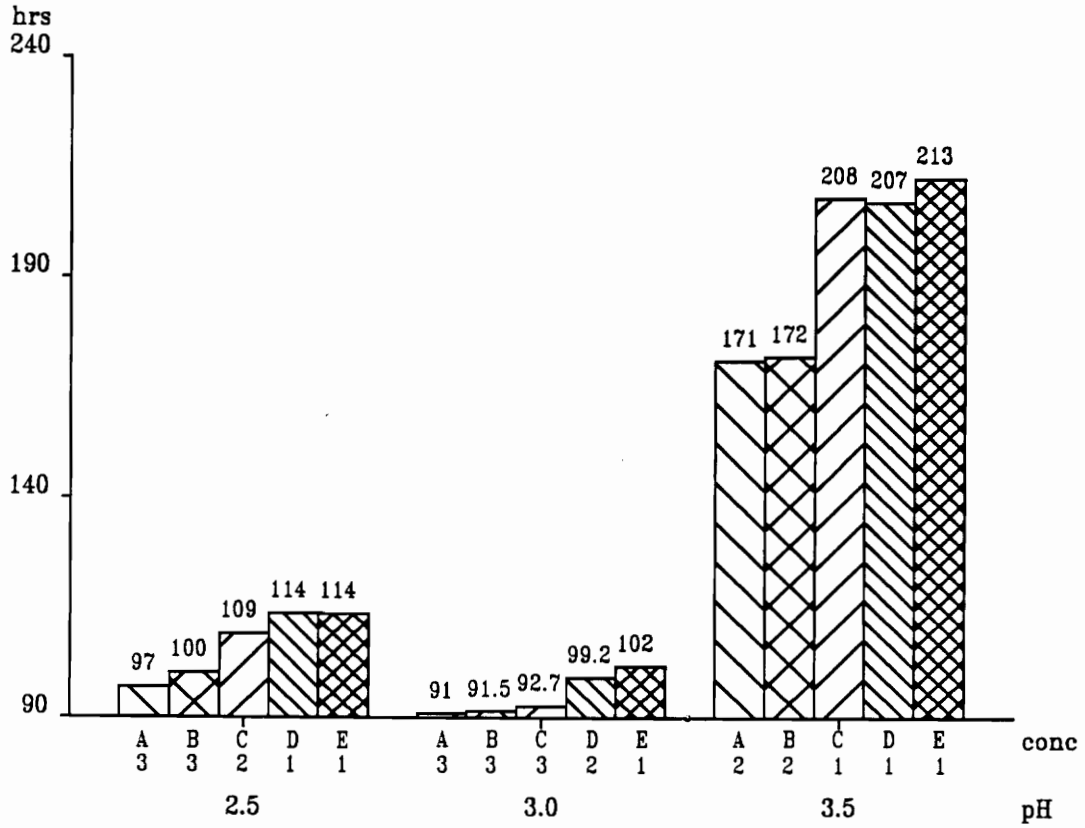

Figure 1. Inhibition of Saccharomyces cerevisiae at 25°C by 0, 500, 1000, 1500, and 2000 ug/ml (Conc. A, B, C, D, and E respectively) sodium phosphite at pH 2.5, 3.0, and 3.5 in Yeast Nitrogen Base with a carbon dioxide atmosphere. Hrs is the time (hours) to reach an O.D. of 0.35 (600 nm). Number at top of each bar is the mean time (hours) to reach an O.D. of 0.35. Concentrations (A-E) wihtin a pH value with different numbers (1, 2, 3, 4, and 5) below them are significantly different ($p \leq .05$).

Except for pH 3.5, there appeared to be increasing inhibition with decreasing pH.

The greatest inhibition of growth at pH 2.5 occurred equally with 1500 and 2000 ug/ml SP. With the addition of 1000 ug/ml to the test media, SP was less effective than the higher concentrations and more effective than the lower concentrations. Although there was a slight increase in inhibition with 500 ug/ml than with the control (0 ug/ml), the difference was not significant.

Inhibition of growth at pH 3.0 was greatest with 2000 ug/ml SP (Fig.1) SP was significantly less effective at 1500 ug/ml but was more effective than the lower concentrations (0, 500, and 1000 ug/ml). There was no difference in the effects of 0, 500, and 1000 ug/ml in inhibiting the growth of S. cerevisiae.

When considering all the pH values tested for S. cerevisiae in the CO_2 atmosphere, the greatest inhibition unexpectedly occurred at pH 3.5 (Fig.1). SP concentrations of 1000, 1500, and 2000 ug/ml were equally and most effective in inhibiting growth at pH 3.5. The addition of 500 ug/ml SP to the test media was not more effective in inhibiting the growth of S. cerevisiae than the control (0 ug/ml).

Saccharomyces cerevisiae in Nitrogen Atmosphere

Overall, inhibition of S. cerevisiae in the nitrogen atmosphere at all pH values combineded was dependent upon the sodium phosphite concentration. However, the ability of SP to inhibit growth was highly variable for each pH value tested. There appeared to be increasing inhibition with decreasing pH values.

The results appear to be in error at pH 2.5 due to an unidentified laboratory accident (Fig.2). Inhibition of growth with the control (0 ug/ml) and 2000 ug/ml SP were equally and most effective. Sodium phosphite concentrations of 500, 1000, and 1500 ug/ml were equally and least effective in inhibiting the growth of S. cerevisiae.

There was an increase in inhibition of S. cerevisiae with increasing concentrations of SP at pH 3.0. Inhibition was significantly greater with 2000 ug/ml sodium phosphite. With the addition of 1500 ug/ml to the test media, SP was less effective than the former concentration but more effective than the lower (0, 500, 1000 ug/ml) levels. SP concentrations of 0, 500, and 1000 ug/ml were equally and least effective in inhibiting the growth of S. cerevisiae. SP was most effective in inhibiting growth of S. cerevisiae at pH 3.5 with 2000 ug/ml. The inhibition of growth with 1500 ug/ml SP was equally and least effective than 0, 500, and 1000 ug/ml. At pH 4.0 and 4.5, inhibition of growth was equally and most effective with 1500 and 2000 ug/ml SP. The

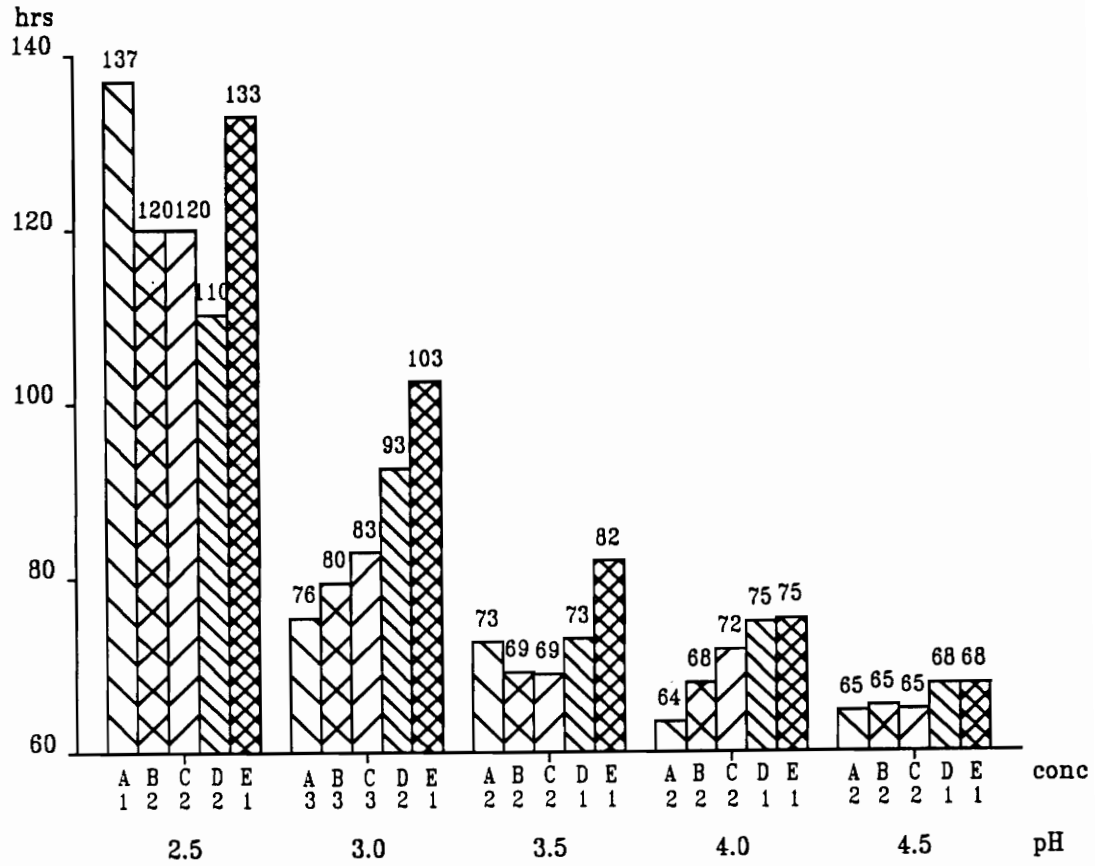

Figure 2. Inhibition of Saccharomyces cerevisiae at 25°C by sodium phosphite at pH 2.5, 3.0, 3.5, 4.0 and 4.5 in Yeast Nitrogen Base with a nitrogen atmosphere. See Figure 1 for explanation of symbols.

lower concentrations (0, 500, and 1000 ug/ml) were equally and least effective in inhibiting the growth of S. cerevisiae.

Zygosaccharomyces bailii in CO_2 Atmosphere

Sodium phosphite was extremely effective in inhibiting the growth of Z. bailii in the CO_2 atmosphere at pH 2.5 and 3.0 ($p<.0001$) but was not effective at pH 3.5 ($p<.2075$). There was an increase in inhibition with increasing concentrations of SP at pH 2.5 and 3.0. Inhibition increased with decreasing pH (Fig.3).

Inhibition of growth with SP at pH 2.5 was greater than inhibition at pH 3.0 and 3.5. Overall, the greatest inhibition occurred with 2000 ug/ml SP at pH 2.5 and 3.0. There was a significant increase in inhibition with increasing concentrations of SP above 500 ug/ml at pH 2.5 but the lower concentrations (0 and 500 ug/ml) were equally and least effective. At pH 3.0, SP was significantly more effective in inhibiting Z. bailii at all levels with increasing concentration.

SP was not more effective in inhibiting growth at pH 3.5. The addition of 2000 ug/ml to the test media was significantly more effective in inhibiting growth than the control (0 ug/ml) but was not more effective than 500, 1000, and 1500 ug/ml SP.

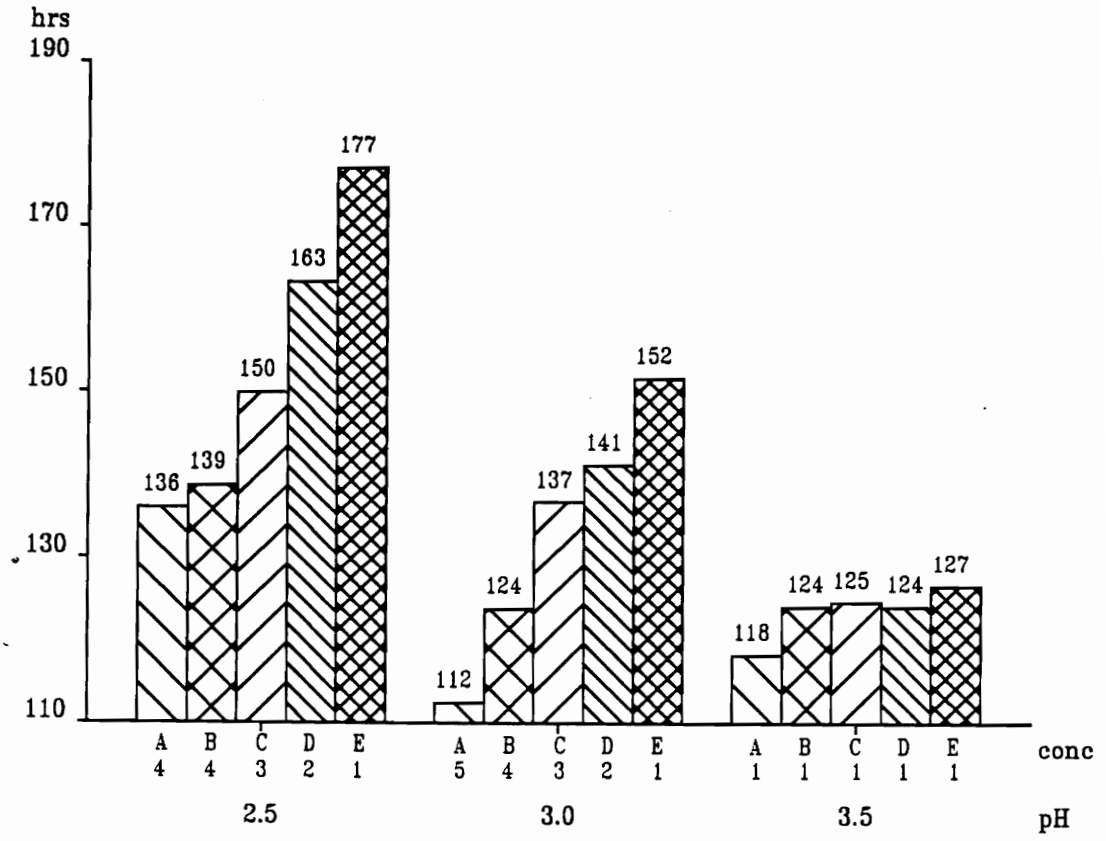

Figure 3. Inhibition of Zygosaccharomyces bailii at 25°C by sodium phosphite at pH 2.5, 3.0, and 3.5 in Yeast Nitrogen Base with a carbon dioxide atmosphere. See Figure 1 for explanation of symbols.

Zygosaccharomyces bailii in Nitrogen Atmosphere

Sodium phosphite was effective in inhibiting the growth of Z. bailii in the nitrogen atmosphere at all pH values combined ($p<.0001$). Decreasing the pH did have an additive effect with SP on growth inhibition. At all pH values except pH 4.5, there was increased inhibition with increasing concentrations of SP (Fig. 4).

Inhibition at pH 2.5 was significantly greater at 2000 ug/ml than at lower concentrations of SP. There was not a significant increase in inhibition with 500, 1000, and 1500 ug/ml SP and thus were equally effective even though the times to significant growth increased as the concentration of SP increased. These concentrations were significantly more effective in inhibiting growth of Z. bailii than the control (0 ug/ml).

Inhibition of growth at pH 3.0, 3.5, and 4.0 was similar. The greatest inhibition occurred with 1500 and 2000 ug/ml SP, but the difference between them was not significant. The addition of 1000 ug/ml SP to the test media was significantly more effective than lower concentrations (0 and 500 ug/ml). The addition of 500 ug/ml SP was not more effective than the control (0 ug/ml) even though the delay in growth was slightly longer with the former concentration.

SP was not effective in inhibiting the growth of Z. bailii at pH 4.5 in the nitrogen atmosphere. The addition

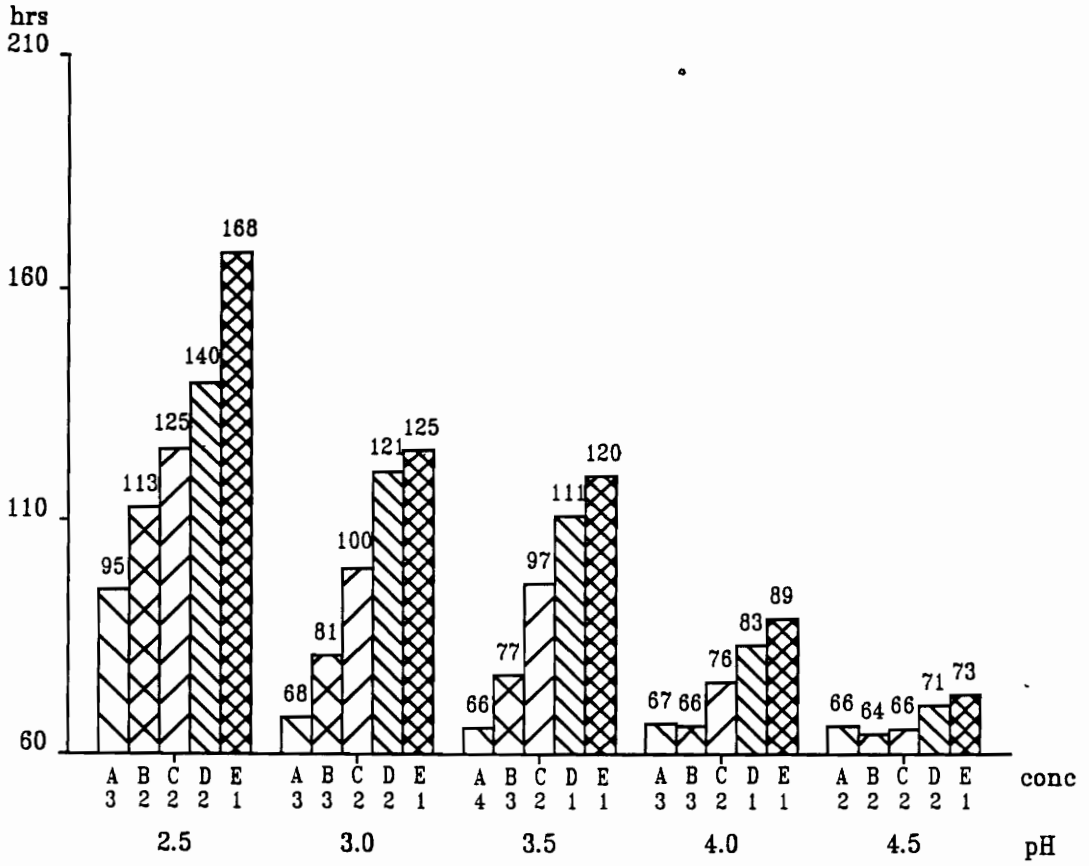

Figure 4. Inhibition of Zygosaccharomyces bailii at 25°C by sodium phosphite at pH 2.5, 3.0, 3.5, 4.0, and 4.5 in Yeast Nitrogen Base with a nitrogen atmosphere. See Figure 1 for explanation of symbols.

of 2000 ug/ml SP to the test media was slightly more effective in inhibiting growth than the control (0 ug/ml) but was not more effective than 500, 1000, and 1500 ug/ml SP.

Saccharomyces uvarum in CO_2 Atmosphere

Inhibition of growth for S uvarum in the CO_2 atmosphere for all pH values combined was not dependent upon SP concentration ($p<.6472$). Generally, there was not an increase in inhibition with increasing concentrations of SP except for pH 3.0. Inhibition did not significantly increase with decreasing pH but was most effective at pH 2.5 (Fig 5).

The greatest inhibition of growth occurred at pH 2.5. However, there was no increase in inhibition with increasing concentrations of SP at pH 2.5 (Fig.5). As table 7 shows, the shortest delay in growth at pH 2.5 was about 14 hrs greater than the longest time at pH 3.0. This fact may help explain why the SP concentration was not statistically shown to influence the inhibition of growth in a greater manner.

There was an increase in inhibition of S. uvarum with increasing concentrations of SP at pH 3.0. Inhibition of growth occurred equally and most effectively with 1500 and 2000 ug/ml SP. With the addition of 1000 ug/ml to the test media, SP was less effective than the former concentrations and more effective than the lower concentrations (0 and 500

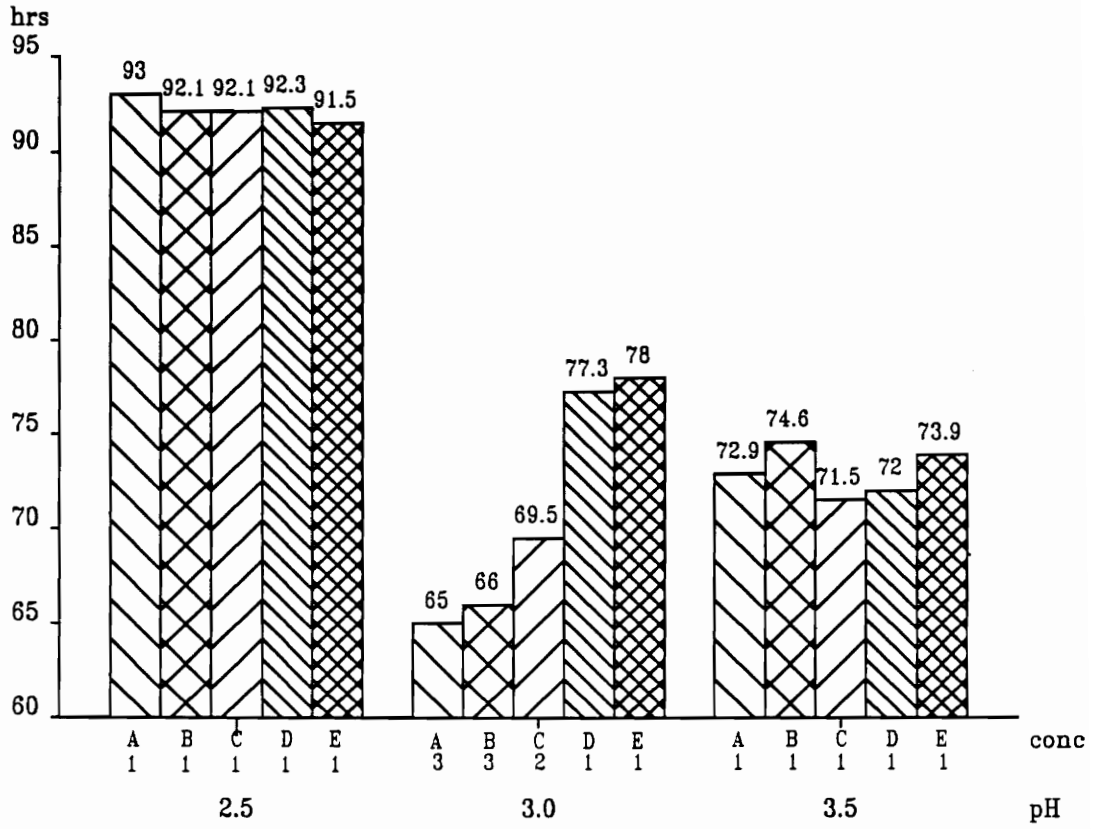

Figure 5. Inhibition of Saccharomyces uvarum at 25°C by sodium phosphite at pH 2.5, 3.0, and 3.5 in Yeast Nitrogen Base with a carbon dioxide atmosphere. See Figure 1 for explanation of symbols.

ug/ml). The addition of 500 ug/ml SP was not more effective in inhibiting the growth of S. uvarum.in inhibiting the growth of S. uvarum.

There was no significant difference in any of the sodium phosphite concentrations tested at pH 3.5.

Saccharomyces uvarum in Nitrogen Atmosphere

Sodium phosphite was effective in inhibiting the growth of S. uvarum in the nitrogen atmosphere for all pH values combined ($p<.0091$). There was an increase in inhibition with increasing concentrations of SP at all pH values tested. Generally, inhibition did not increase with decreasing pH, except for pH 2.5.

Inhibition of growth at pH 2.5 was greater for each concentration of SP than at corresponding concentrations at all other pH values tested (Fig.6). The addition of 1000, 1500, and 2000 ug/ml SP to the test media were equally and more effective than lower concentrations (0 and 500 ug/ml). The addition of 500 ug/ml SP to the test media was sigificantly more effective in inhibiting the growth of S. uvarum than the control 0 ug/ml).

Sodium phosphite was effective in inhibiting the growth of S. uvarum at pH 3.0. Generally, increasing the SP concentration increased the inhibition. Inhibition was significantly more effective with 2000 ug/ml. The addition of 1000 and 1500 ug/ml SP were equally and more effective (0 ug/ml).

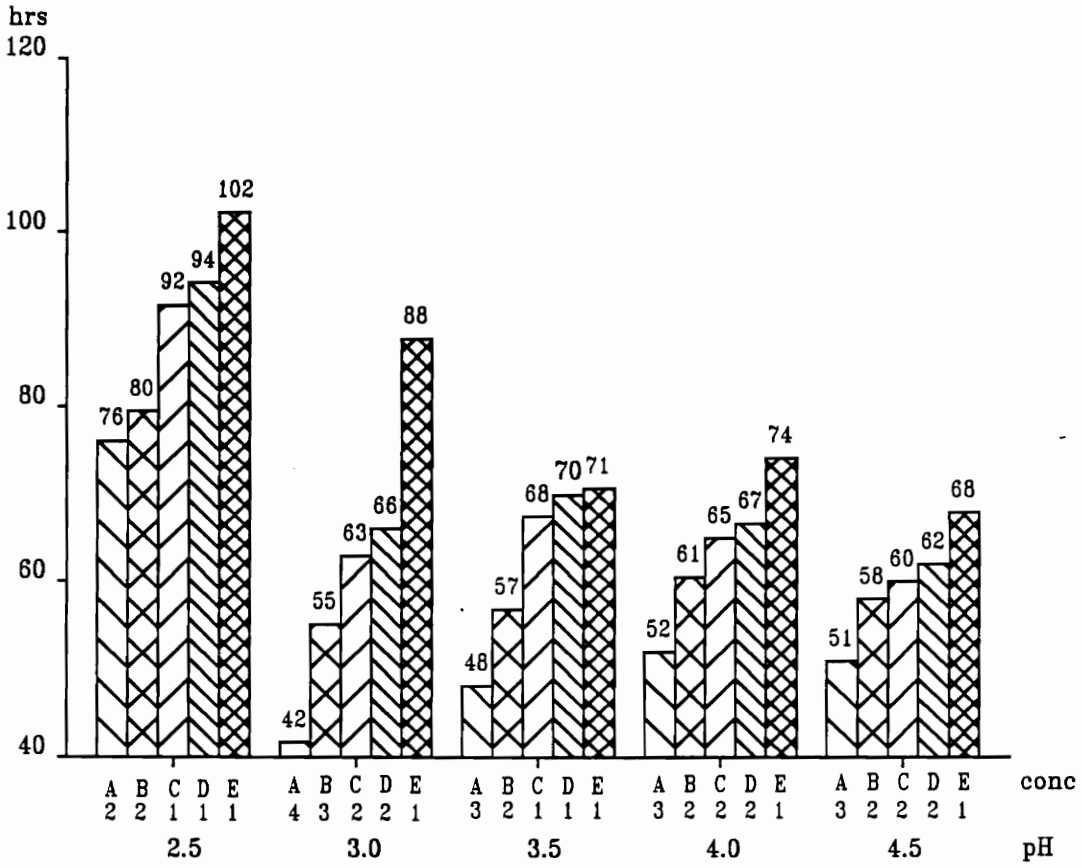

Figure 6. Inhibition of Saccharomyces uvarum at 25°C by sodium phosphite at pH 2.5, 3.0, 3.5, 4.0, and 4.5 in Yeast Nitrogen Base with a nitrogen atmosphere. See Figure 1 for explanation of symbols.

Sodium phosphite was effective in inhibiting the growth of S. uvarum at pH 3.5. There was a slight increase in inhibition with increasing concentration of SP. Inhibition of growth was equally and most effective with 1000, 1500, and 2000 ug/ml SP. With the addition of 500 ug/ml to the test media, SP was significantly more effective in inhibiting growth of S. uvarum than the control(0 ug/ml).

Sodium phosphite was effective in inhibiting growth at pH 4.0 and 4.5. There was a slight increase in inhibition with increasing concentration of SP (Fig.6) The greatest inhibition occurred with 2000 ug/ml SP, which was significantly more effective than the lower concentrations. The addition of 500, 1000, and 1500 ug/ml SP to the test media were equally effective in inhibiting the growth of S. uvarum. These concentrations were significantly more effective than the control (0 ug/ml).

Summary: S. cerevisiae, Z. bailii, and S. uvarum in CO_2 and Nitrogen

Overall, sodium phosphite was more effective in inhibiting the growth of S. cerevisiae, Z. bailii, and S. uvarum in the presence of CO_2 than nitrogen. The delay in growth was more pronounced in CO_2 than in nitrogen.

Sodium phosphite was effective in inhibiting the growth of Z. bailii in the CO_2 and nitrogen atmospheres. In both atmospheres, there was a significant increase in inhibition with decreasing pH. The delay in growth with all concentrations of SP in CO_2 was longer than corresponding concentrations in nitrogen. At pH 2.5 and 3.0, there was a significant increase in inhibition with increasing concentrations of SP in both atmospheres. At pH 3.5, there was a slight but significant increase in inhibition with increasing concentrations of SP in nitrogen but not in CO_2.

SP was significantly more effective in inhibiting the growth of S. cerevisiae than S. uvarum in both CO_2 and nitrogen. In the CO_2 atmosphere, SP demonstrated a slight increase in inhibition of S. uvarum with increasing concentrations at pH 3.0 only. In the nitrogen atmosphere, there was a slight increase in inhibition with increasing concentrations of SP at all pH values tested. Generally, decreasing the pH did not increase the inhibition of S. uvarum. For S. cerevisiae in the CO_2 atmosphere, there was a slight increase in inhibition with increasing

concentrations of SP at all pH values. Decreasing the pH caused an increase in inhibition below pH 3.5. In the nitrogen atmosphere there was a slight increase in inhibition of S. cerevisiae with increasing concentrations of SP at pH 3.0 only. Decreasing the pH caused a slight increase in inhibition, except for pH 2.5 where a laboratory accident was thought to have occurred.

Sodium Phosphite Inhibition of Z. bailii in Beverage S

Sodium phosphite did not completely inhibit the growth of Z. bailii in beverage S during the 528 hr test period with an inoculum size of 10^3 cells/ml. Growth was detectable with all concentrations of SP tested; however, the control (0 ug/ml) had the shortest delay in growth. The times to detectable growth in observations with 250, 500, 750, 1000, 1500, and 2000 ug/ml were highly variable (Table 9). The lowest effective concentration of SP totally inhibiting growth of Z. bailii with a 10^3 inoculum level would be an undetermined concentration greater than 2000 ug/ml (Table 10).

With an inoculum size of 10^2 cells/ml, SP was extremely effective in totally inhibiting the growth of Z. bailii. As table 9 indicates, growth was detectable in the control (0 ug/ml) early in the incubation period. No detectable growth was evident for all test concentrations. Post incubation

counts on observations with undetectable growth were not significantly different from the initial inoculum level counts at SP concentrations of 250-2000 ug/ml (Table 11).

Growth was not detected at any level of SP tested with an inoculum size of 10^1 cells/ml, including the control (0 ug/ml). Post incubation counts on observations with 250, 750, 1500, and 2000 ug/ml SP were not significantly different from the initial inoculum counts (Table 11).

Sodium Phosphite Inhibition of Z. bailii in Diet S

Sodium phosphite was somewhat effective in inhibiting the growth of Z. bailii with an inoculum size of 10^2 and 10^3 cells/ml at all concentratios tested. The times to detectable growth in observations with 250, 500, 750, 1000, 1500, and 2000 ug/ml SP were highly variable at both inoculum levels (Table 9). Growth was detected in the control (0 ug/ml) before any other SP concentration tested.

With an inoculum size of 10^1 cells/ml, SP was slightly effective in inhibiting the growth of Z. bailii. As table indicates the first observation with detectable growth was the control (0 ug/ml). Resistance was demonstrated by

Table 9. Mean time (hours) to detectable growth (O.D.=0.01) for Z. bailii in beverage S and Diet S.

Target Inoculum Size	Beverage Type	Na_2HPO_3 $5H_2O$ (ug/ml) 0	250	500	750	1000	1500	2000
10^3/ml	S	140	402	-	426	438	438	450
10^2/ml	S	222	-	-	-	-	-	-
10^1/ml	S	-	-	-	-	-	-	-
10^3/ml	*DS	64	75	402	316	292	243	147
10^2/ml	*DS	172	268	280	308	308	292	221
10^1/ml	*DS	233	363	-	-	-	-	-

* DS refers to Diet S.
Observations with undetectable growth (-).
For 750 and 1,000 ug/ml, there was 1 observation with significant growth at 411 hrs.

Table 10. Lowest effective sodium phosphite concentrations in commercial carbonated beverages.

Inoculum Size	Commercial Carbonated Beverage Type: Beverage S	Diet Beverage S
10^3/ml	>2000 ug/ml	>2000 ug/ml
10^2/ml	* 250 ug/ml	>2000 ug/ml
10^1/ml	0 ug/ml	2000 ug/ml

* Growth in one experiment tube at the 2000 ug/ml level had detectable growth after 432 hours incubation.

** Lowest effective concentration of sodium phosphite is that concentration capable of totally inhibiting growth of Z. bailii for 528 hrs.

Table 11. Viable log counts on Z. bailii in beverage S and Diet S.

Beverage Type	Target Inoculum Size		Sodium Phosphite Concentration (ug/ml) 0	250	500	750	1000	1500	2000
S	10^2/ml	Initial	2.92	2.92	2.92	2.92	2.86	2.86	2.86
		Inoculum	2.82	2.82	2.82	2.82	2.90	2.90	2.90
		Counts	2.93	2.93	2.93	2.93	2.89	2.89	2.89
		* Post	6.54	3.18	3.72	4.04	2.88	2.88	3.04
		Incubation	6.52	3.18	3.90	2.88	2.88	3.04	3.48
		Counts	LA	2.88	2.88	4.52	3.04	2.88	
	10^1/ml	Initial	1.92	1.92	1.92	1.92	1.89	1.89	1.89
		Inoculum	1.90	1.90	1.90	1.90	1.93	1.93	1.93
		Counts	1.86	1.86	1.86	1.86	1.82	1.82	1.82
		* Post	1.58	1.58	1.58	2.04	2.88	2.04	1.88
		Incubation	1.58	1.88	1.58	1.88	2.18	2.18	2.18
		Counts	1.58	1.88	1.58	2.18	2.88	2.88	1.88
Diet S	10^1/ml	Initial			1.89	1.89	1.93	1.93	1.93
		Inoculum			1.90	1.90	1.92	1.92	1.92
		Counts			1.92	1.92	1.90	1.90	1.90
		* Post			2.48	4.70	3.70	2.18	2.28
		Incubation			2.36	2.78	3.94	2.04	2.04
		Counts			2.28	2.62	4.72	2.28	2.88

* Post incubation counts were listed above if growth was not spectrophotometrically detectable in 2 out of 3 observations for a specific concentration of sodium phosphite and if growth was not observed at a higher concentration of sodium phosphite at that particular inoculum size.

Z. bailii in observations with 250 ug/ml SP and in one observation each for 750 and 1000 ug/ml. Post incubation counts on observations with 2000 ug/ml SP were not significantly different from the initial inoculum counts (Table 11). Counts on observations with 500, 750, 1000, and 1500 ug/ml were significantly different from the initial counts ($p<.0235$).

Discussion

Sodium phosphite was most effective in inhibiting growth of Z. bailii, and to a lesser extent S. cerevisiae and S. uvarum, respectively, in both CO_2 and nitrogen atmospheres. However, SP in test media never totally inhibited (prevented) growth of any of the yeasts tested. Since optimal or nearly optimal growth conditions, healthy inocula, and nonlimiting sterile media were used, these experiments can be considered a difficult test of SP.

SP was effective in inhibiting the growth of Z. bailii in commercial beverage S (containing sugar sweetners) and only moderately effective in Diet S (containing nonsugar sweetner). Z. bailii is the leading and most troublesome spoilage yeast of carbonated beverages in the world due to its preservative-resistant nature. Since the commercial beverages already contained antimicrobial agents and acidulants acting as preservatives, these experiments are an assessment of SP's ability to act in combination with these inherent antimicrobial agents.

The ability of sodium phosphite to inhibit spoilage yeasts, especially preservative resistant types, at low pH values is important especially since the pH of most carbonated beverages containing real fruit juice is between 3.0 and 3.5. High acid food preservatives, such as the benzoates, parabens, and sorbates are used primarily to

inhibit yeasts and molds. The acidity alone is usually inhibitory to most bacteria.

Sodium benzoate is used most commonly as a preservative in carbonated beverages, alone or in combination with sorbate, due to its overall effectiveness and low cost. The parabens have antimicrobial activity over a wide pH range (3-8) and thus have the potential to be used as a preservative in carbonated beverages. However, spoilage problems by yeasts and molds can be controlled by the less expensive sodium benzoate. The sorbates are effective against many yeasts and molds, but have drawn the most attention to their ability to inhibit foodborne pathogens including C. botulinum, S. aureus, and Salmonella (Robach, 1980). Z. bailii has shown resistance to each of these antimicrobial agents when used at their permissable levels. Thus, sodium phosphite has potential use in carbonated beverages as an antimicrobial agent.

SP in the presence of CO_2 was more effective in inhibiting all three yeasts than in the nitrogen atmosphere. The inhibitory effect was observed even though the volume of CO_2 used in these experiments is usually stimulatory to Z. bailii, S. cerevisiae, and, S. uvarum (Ison and Gutteridge, 1987).

The ability of SP to totally inhibit growth in laboratory media with CO_2 and nitrogen atmospheres at low pH values was not demonstrated. Rhodehamel and Pierson (1984)

and current preliminary experiments were able to show total inhibition of three yeast species in laboratory media. However, the incubation times used in these studies were below the known lag times for spoilage yeasts in the presence of inhibitor. Warth (1985) demonstrated maximum lag times to be in excess of 400 hrs for spoilage yeasts in the presence of sorbic acid. Pitt (1974) showed that Z. bailii and S. cerevisiae could be totally inhibited with benzoic and sorbic acid under aerobic incubation in laboratory media and at pH values similar to those used in these studies.

The antimicrobial effectiveness of SP increased with decreasing pH for Z. bailii and S. cerevisiae but not for S. uvarum. Organic acids used as preservatives, such as benzoic acid, are generally used in high acid foods. The antimicrobial effectiveness of these acids is dependent upon the ability to remain in their undissociated fraction, and not the acid anion, to enter the microbial cell (Krebs, 1983; Warth, 1985). As a result, organic acids are most effective at pH values approaching their pK_a values.

Orthophosphorous acid has two dissociating hydrogens and one other hydrogen which stays covalently bonded directly to the phosphorous atom and is not an acidic hydrogen. The pK_a values for orthophosphorous acid are 1.29 and 6.7. The lowest pK_a value, pK_{a1}, is much lower than benzoic or sorbic acid with pk_a values of 4.2 and 4.76

respectively. Apparently, it must be the monodissociated form of the acid of SP which is exerting antimicrobial activity, since over the range of pH values tested, SP existed primarily in the monodissociated fraction. This may help explain why SP inhibition was enhanced by lowering the pH closer to its pk_{a1} value, causing more of the acid to be present in the undissociated form.

Microorganisms are generally inhibited by preservatives through several mechanisms. These include: (a) reaction with the cell membrane causing increased permeability and loss of cellular contents; (b) inactivation of essential enzymes; (c) destruction or functional inactivation of genetic material (Branen and Davidson, 1983). The mode of action of SP is unknown.

The ability of Z. bailii to resist the effects of sodium phosphite in the two carbonated beverages was dependent upon the cell numbers present in the system. This is characteristic of most antimicrobial agents. Resistance was most pronounced at an inoculum level of 10^3 cells/ml and least with an inoculum level of 10^1 cells/ml in both beverages. SP was able to inhibit the growth of Z. bailii in beverage S with an inoculum size of 10^2 cells/ml and concentrations greater than or equal to 250 ug/ml. Any beverage inoculated with 10^2 yeast cells/ml more nearly approximates the yeast contamination that would occur under normal plant conditions if yeast contamination occurred.

Inhibition of of growth of Z. bailii was greater in beverage S than in Diet S. Beverage S contains about 10.5% sugar (6.2% fructose and 4.3 % glucose) and Diet S contains only low levels of carbohydrates from the natural fruit juices and contains aspartame as a nutritive sweetner. Under the conditions in which these experiments were performed, it seems that the carbohydrate concentration had an effect on the ability of SP to inhibit the growth of Z. bailii. Pitt (1974) and Ingram (1960) found that increasing carbohydrate concentration from 0.5 to 10% increased Z. bailii resistance. Warth (1986) observed the lack of major effect of sugar concentration on the resistance of Z. bailii to benzoic acid. From these experiments, increasing the carbohydrate concentration seemed to decrease the resistance of Z. bailii to SP.

As an acid, orthophosphorous acid is stronger than phosphoric acid. An important property associated with it and the phosphite ion is their characterization as strong reducing agents. Due to its chemical nature o-phosphorous acid and the phosphite ion may be altering the cell wall or cytoplasmic membrane and/or inactivating essential transport enzymes. If damage was done to essential transport enzymes located in the cytoplasmic membrane, the cell could no longer transport the benzoate anion out of the cell, which is one of the suggested mechanisms for resistance to benzoate by Z. bailii. Inhibition of Z. bailii may have

occurred in this manner for beverage S, with the 10% sugar concentration aiding in the inhibitory mechanism. Futher studies need to be performed on the effect of SP on the yeast cell structure and its mechanism of action in combination with benzoate.

Further studies need to be performed to determine the potential for SP to inhibit growth of other common spoilage yeasts. Other areas of research include the efficacy of SP in other low pH beverage systems.

Summary and Conclusions

The potential for inhibition of growth by sodium phosphite (SP) in laboratory media at various pH levels and atmospheres and in commercial carbonated beverages in combination with inherent preservatives against selected foodborne yeasts was studied. Experiments were performed in laboratory media to assess the ability of SP to inhibit yeast growth as was demonstrated for other organic acid preservatives such as benzoic and sorbic acid. Because SP could not totally inhibit growth, its effects on growth in a beverage system already preserved was studied to determine if SP has the potential to totally inhibit yeast growth in combination with other preservatives in a beverage system.

Yeast growth was monitored by measuring absorbancy at 600 nm. For studies evaluating the effect of sodium phosphite in different atmospheres in laboratory media, growth curves were plotted and a time to significant growth was calculated for each test treatment. Time to significant growth was used to evaluate the effect that SP and other test variables had on growth. For experiments designed to evaluate the effects of SP in commercial carbonated beverages, post incubation counts were performed on observations with undetectable growth using a direct epifluorescent filter counting technique differentiating viable from nonviable cells. To evaluate the effects of SP on the growth of *Z. bailii*, initial inoculum counts on

viable cells were compared to post-incubation viable cell counts.

From these studies, one can conclude that the use of SP as an antimicrobial preservative has potential. Results show SP was most effective at inhibiting growth of Z. bailii, and less effective against S. cerevisiae and S. uvarum ,respectively, in both CO_2 and nitrogen atmospheres. Inhibition of growth with SP was more effective in the presence of CO_2 than in the presence of nitrogen. Generally, decreasing the pH caused an increase in inhibition for S. cerevisiae and Z. bailii. Although this effect was not exerted on S uvarum, there was greater inhibition with SP at pH 2.5 in both atmospheres.

SP was effective in inhibiting the growth of Z. bailii with 10^2 cells/ml and 10^1 cells/ml in beverage S and less effectively with 10^1 cells/ml in Diet S. There was a decrease in inhibition with increasing cell number for both beverages, but was more pronounced in Diet S. In beverage S, post incubation counts on observations with undetectable growth containing 10^2 and 10^1 cells/ml were not significantly different from initial inoculum counts at all concentrations counted, except for 500 and 1000ug/ml SP at 10^1 cells/ml. In Diet beverage S, post incubation counts on observations with undetectable growth containing 10^1 cells/ml were significantly different from the initial inoculum counts at 2000 ug/ml SP only.

Further research is needed to elucidate sodium phosphite's effect on the yeast cell components and its exact mechanism of action. Another area of research is to observe the combined effects of SP with other organic acid preservatives in laboratory media and in beverage systems with varying concentratios of sugar and salt.

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APPENDIX A

Table 3. Time (hours) to significant growth (O.D.=0.35) for Saccharomyces cerevisiae in carbon dioxide.

	Na_2HPO_3 $5H_2O$ (ug/ml)				
pH=2.5	0	500	1000	1500	2000
	96	98	99	114	114
	98	100	103.5	113.6	114
	99	98	113.5	113.6	114
	95	103	113.5	114	113
	97	103	113	114	114
	97	100	113	114	114
x	97+1.41	100.3+2.25	109.2+6.36	113.8+0.21	113.6+0.49
pH=3.0					
	90	93	91	102	101
	91	91	99	97	100
	90.5	91	91	99	102
	91	91	91	100	103
	91	93	93	98	102
	91	90	91	99	102.5
x	91+0.42	91.5+1.22	92.7+3.20	99.2+1.72	101.8+1.08
pH=3.5					
	171	172	208	206	207
	171	172	207	207	218
x	171+0	172+0	208+0.71	207+0.71	212.5+7.78

pH 4.0 and 4.5 not measured
Values within each pH value for each SP concentration represent replicate tubes.

Table 4: Time (hours) to significant growth (O.D.=0.35) for Saccharomyces cerevisiae in nitrogen.

	Na_2HPO_3 $5H_2O$ (ug/ml)				
pH=2.5					
	0	500	1000	1500	2000
	137	127	120	102.5	130
	138	113	120	107	139
	136	120	120	114	130
	137	120	102.5	118	
x	137+0.82	120+5.72	120+8.75	110.3+6.94	133+5.20
pH=3.0					
	75.5	84	89.5	89.5	89.5
	74	83	84	96	108
	77	75	79	92	
	75	76	79	93	110
x	75.5+1.25	79.5+4.65	83+5.01	92.6+2.69	102.5+11.30
pH=3.5					
	70	71	70	77	80
	76	68	72	71	80
	73	70	71.5	80	80
	72	68	72	85	88
x	72.7+2.50	69.2+1.50	69+0.95	73.1+5.85	82+4.0
pH=4.0					
	70	68	76	74	76
	60.5	65	70	74	75.5
	60.5	70	68	72	74
	63	69	73	80	76
x	63.5+4.49	68+2.16	71.8+3.50	75+3.46	75.4+0.95
pH=4.5					
	62	64	64	67	66
	68	63	64	68	69
	66	64	67	67	68
	63	65	64	69	68
x	64.7+2.75	65.3+0.82	64.8+1.50	67.8+0.96	67.8+1.26

Values within each pH value for each SP concentration represent replicate tubes.

Table 5. Time (hours) to significant growth (O.D.=0.35) for Zygosaccharomyces bailii in carbon dioxide.

	Na_2HPO_3 $5H_2O$ (ug/ml)				
pH=2.5					
	0	500	1000	1500	2000
	136	136.5	148	171	170.7
	138	137.5	146	160	171
	135	147	146.2	170.5	180.3
	135	136	150	160	184
	135	136	149	160	171
	136	137.5	149	160	171
x	136+1.17	138.7+4.13	149.8+4.82	163.2+5.70	177.1+6.82
pH=3.0					
	112	122.3	136	140	152
	112	125	137	140	150
	112.5	124	137	139	152
	112.5	124	137	144	152
	113	124	137	139	151.5
	112.5	123	136	145	152
x	112.4+0.38	123.7+0.94	136.7+0.52	141.2+2.64	151.6+0.80
pH=3.5					
	121	123.8	130	124	136
	123.8	124	124	132	125
	112.7	124	124	120	122
	115	124	120	120	123
x	118.1+5.15	124+0.10	124.5+4.12	124+5.65	126.5+6.45

pH 4.0 and 4.5 not measured
Values within each pH value for each SP concentration represent replicate tubes.

Table 6: Time (hours) to significant growth (O.D.=0.35) for Zygosaccharomyces bailii in nitrogen.

	Na_2HPO_3 $5H_2O$ (ug/ml)				
pH=2.5	0	500	1000	1500	2000
	104	102.4	125	131	185
	94	137	125	145.5	159
	91	105	137	137	157
	91	107	114	145	170
x	95+6.16	112.8+16.21	125.3+9.39	139.6+6.94	167.8+12.84
pH=3.0					
	67.5	88	106	136	121
	68.5	78	93	125	138.5
	68.5	77.5	94	106	116
	67	81	106	115	
x	67.9+0.75	81.12+4.84	99.8+7.23	120.5+12.9	125.2+11.81
pH=3.5					
	63.5	82	107	119.5	118.5
	67	71	90	100	118
	68	71	97	116	125
	64	84	92	108.5	117
x	65.6+2.21	77+6.98	96.5+7.59	111+8.65	119.6+3.64
pH=4.0					
	66	66	74	81	91
	74	67	73	90	93
	66	66	75	79	83
	60.5	65	80	83	89
x	66.6+5.56	66+0.82	75.5+3.11	83.3+4.79	89+4.32
pH=4.5					
	66	65	66	66.5	78
	67	64.5	65	67	74
	66	64.5	65	80.5	66
	65	63	66	68	
x	66+0.82	64.3+0.87	65.5+0.58	70.5+6.70	72.7+6.11

Values within each pH value for each SP concentration represent replicate tubes.

Table 7. Time (hours) to significant growth (O.D.=0.35) for Saccharomyces uvarum in carbon dioxide.

	Na_2HPO_3 $5H_2O$ (ug/ml)				
pH=2.5	0	500	1000	1500	2000
	96	91	91	92	90
	90	91.5	91	91.5	93
	97	91	91.5	93	91.5
	89	93	93	95	90
	89	93	93	95	91
	97	93	93	93	94
x	93+4.05	92.1+1.02	92.1+1.02	92.3+1.47	91.5+1.62
pH=3.0					
	65	65	68.5	78.5	78
	65	65	67	75	78
	65	65.5	68.5	75	78
	65	66	69	78.5	78
	65	66.5	72	78.5	78
	66	67.5	72	78	78
x	65+0.41	66+0.97	69.5+2.05	77.3+1.75	78+0
pH=3.5					
	75.5	80	70.5	77	77.5
	73	74	71.5	70.5	72
	73	70.5	70	70	74
	70	74	74	70.5	74
x	72.9+2.25	74.6+3.94	71.5+1.78	72+3.34	73.9+1.44

pH 4.0 and 4.5 not measured
Values within each pH value for each SP concentration represent replicate tubes.

Table 8. Time (hours) to significant growth (O.D.=0.35) for Saccharomyces uvarum in nitrogen.

	Na_2HPO_3 $5H_2O$ (ug/ml)				
pH=2.5					
	0	500	1000	1500	2000
	76	82	87.5	94	94
	76.5	82	84	92	108
	75	77	98.8	93	88
	77	77	96	98	119
x	76.1+0.85	79.5+2.89	91.6+6.97	94.2+2.63	102.3+13.96
pH=3.0					
	42.5	53	62	63	77.5
	41.5	52	61.5	68	89
	42	55.5	64.5	66	97
	41	60	63.5	67.5	
x	41.7+0.65	55.1+3.57	62.9+1.38	66.1+2.25	87.8+9.80
pH=3.5					
	48	60	69	71	71
	46	56	67	70	69
	52	56	69	72	72
	47	55	65	67	
x	48.2+2.63	56.8+2.22	67.5+1.91	70+2.16	70.7+1.53
pH=4.0					
	53.5	60	63	67	77
	55	60.5	63	66	69.5
	49	60.5	65	67	76
	51	61	69	67	
x	52+2.66	60.5+0.41	65+2.83	66.7+0.50	74.2+4.07
pH=4.5					
	50	57	61	59	68
	51	59	59	62	69
	52	57	61	65	64
	49	57	59	60	69
x	51+1.29	58+1.00	60+1.15	62+2.65	68+2.38

Values within each pH value for each SP concentration represent replicate tubes.

VITA

Steven M. Roth was born April 23, 1964 in Pittsburgh, Pennsylvania. He completed his secondary education at Oakton High School, Vienna, Virginia in 1982 and entered Virginia Polytechnic Institute and State University the same year. He was awarded a Bachelor of Science degree in Biology in 1986. In the fall of the same year, Mr. Roth entered the Graduate School of Virginia Polytechnic Institute and State University to pursue the Master of Science degree in Food Science and Technology.

Mr. Roth is a member of the following honorary and professional societies: Alpha Epsilon Delta, Gamma Sigma Delta, American Society for Microbiology (ASM), and the Institute of Food Technologists.