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INACTIVATION OF *ALICYCLOBACILLUS*  
*ACIDOTERRESTRIS* USING HIGH PRESSURE  
HOMOGENIZATION AND DIMETHYL  
DICARBONATE

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To the Graduate Council:

I am submitting herewith a thesis written by Wei Chen entitled "INACTIVATION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* USING HIGH PRESSURE HOMOGENIZATION AND DIMETHYL DICARBONATE." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

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**INACTIVATION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* USING HIGH PRESSURE  
HOMOGENIZATION AND DIMETHYL DICARBONATE**

A Thesis Presented for the  
Master of Science  
Degree  
The University of Tennessee, Knoxville

Wei Chen  
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## ABSTRACT

*Alicyclobacillus acidoterrestris* is a spore-forming food spoilage bacterium. Its spore is problematic to the juice industry because of its ability to grow in low pH environments and survive pasteurization processes. The purpose of this study was to investigate the effect of the non-thermal technology, high pressure homogenization (HPH) and the antimicrobial compound, dimethyl dicarbonate (DMDC), on inactivation of *A. acidoterrestris* in a broth system. Vegetative cells and spores of five strains of *A. acidoterrestris* (N-1100, N-1108, N-1096, SAC and OS-CAJ) were screened for their sensitivity to HPH (0, 100, 200 and 300 MPa) in *Bacillus acidoterrestris* thermophilic (BAT) broth. Strain SAC (most resistant) and OS-CAJ (least resistant) were further tested for their sensitivity to 250 ppm DMDC. This was followed by evaluation of combined effects of HPH and DMDC against strain SAC. Effects of HPH and DMDC treatment combinations (no DMDC, 250 ppm DMDC added 12 h before, 2 h before, immediately before, and immediately after 300 MPa HPH treatment) on spores of SAC over a 24-h period were evaluated. After all treatments, samples were serially diluted and surface plated onto BAT agar, and populations were determined after incubation at 44°C for 48 h. All HPH and DMDC treatments significantly ( $P < 0.05$ ) inhibited growth of vegetative cells; spores were less affected by these treatments. HPH caused a 1- to 2-log reduction in vegetative cell populations at 300 MPa for four strains, but only about 0.5-log reduction of the SAC strain. Spores of all five strains were not significantly reduced by HPH. DMDC also slowed growth of vegetative cells significantly. For vegetative cells of SAC and OS-CAJ, 250 ppm DMDC reduced the population by about 2 log whereas spore population was reduced by less than 0.5 log. The addition of DMDC together with HPH slightly enhanced the inactivation effect over a 24-h

period as compared with treatment with HPH alone. These results demonstrate that HPH and DMDC show promise for aiding in control of growth of vegetative cells of *A. acidoterrestris*. However, neither treatment, alone or in combination, is very effective against spores.

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## **CHAPTER I**

### **Introduction and Literature Review**

## INTRODUCTION

*Alicyclobacillus acidoterrestris* is a spore-forming, spoilage bacterium. It requires low pH (3.5-4.0) for optimal growth, which makes it a good candidate of fruit juice and beverage spoilage. *A. acidoterrestris* has been isolated from a number of juice products, and incidents of juice spoilage have been documented since 1984 (Cerny et al., 1984). Spoilage presents as off flavors and off odors that are often described as “phenolic, smoky, medicinal” owing to the metabolites guaiacol and halophenols (2, 6-dibromophenol and 2, 6-dichlorophenol). The spores are very heat resistant and can survive heat pasteurization processes used for juices. D-values of *A. acidoterrestris* in juices at 95°C are reported to range from 0.06 to 5.3 minutes (Silva and Gibbs, 2001).

High pressure homogenization (HPH) is a non-thermal processing method that is primarily used for preparation or stabilization of dispersions and emulsions or for creating physical changes in products in the chemical, pharmaceutical, and food (dairy) industry (Wuytack et al., 2002). Another application of HPH is disruption of microbial cells (Kelemen and Sharp, 1979). Effectiveness of HPH inactivation has been evaluated in many studies on different foodborne pathogens and spoilage microorganisms that have shown promising results, implying potential use of HPH as an alternative to heat pasteurization (Pathanibul et al., 2009; Wuytack et al., 2002; Brookman, 1974; Kelemen and Sharpe, 1979, Vachon et al., 2002). Several microbial inactivation mechanisms of HPH have been proposed: turbulence (Doulah et al., 1975), impingement (Engler and Robinson, 1981) and cavitation (Save et al., 1994).

Dimethyl dicarbonate (DMDC) is an antimicrobial compound approved for use as a direct additive in certain beverages in which the microbial population is no more than 500

CFU/ml (FDA, 2001). DMDC is very reactive, and when added to a substrate, rapidly hydrolyzes to methanol and CO<sub>2</sub>; these hydrolysis products are not antimicrobial. Thus, the antimicrobial activity of DMDC highly depends on how rapidly it hydrolyzes. The inactivation mechanism of DMDC is related to enzyme destruction (Golden, 2005). It is an effective fungicide, especially against wine yeast (Genth, 1979). Bactericide effectiveness has also been reported against a number of bacteria (Genth, 1979, 1980; Fisher and Golden, 1998).

The objectives of this research were:

- (1) to investigate the inactivation effect of HPH against vegetative cells and spores of five strains of *A. acidoterrestris* in *Bacillus acidoterrestris* thermophilic (BAT) broth;
- (2) to investigate the inactivation effect of DMDC (250 ppm and 0 ppm) against vegetative cells and spores of the most and least resistant strain of HPH in BAT broth over a 48-h incubation period at 44°C and;
- (3) To investigate the inactivation effect of four combinations of HPH and DMDC [250 ppm DMDC added 12 h before (DMDC+12), 2 h before (DMDC+2), immediately before (DMDC+0), and immediately after (DMDC-1) 300 MPa HPH treatments) against spores of the most HPH-resistant strain in BAT broth over a 24-h incubation period at 44°C.

## LITERATURE REVIEW

### Characteristics of *Alicyclobacillus acidoterrestris*

#### Historical background

In 1967, Uchino and Doi isolated a spore-forming bacterium from an acidic hot spring in Japan and reported that the bacterium had similar characteristics to *Bacillus coagulans* (Uchino and Doi, 1967). Darland and Brock (1971) isolated a similar bacterium from thermal and acidic environments in Yellowstone National Park and Hawaii's Volcano National Park. Taxonomical tests revealed that this bacterium possessed  $\omega$ -cyclohexyl fatty acids as its main cellular fatty acid. It was then named *Bacillus acidocaldarius*. Deinhard et al. (1987) studied a thermo-acidophilic spore-forming bacterium isolated from soils by Hippchen et al. (1981) and proposed this microorganism as a new species: *Bacillus acidoterrestris*, with acidoterrestris meaning "in favor of acid and isolated from soil." Further taxonomic investigation revealed that *B. acidoterrestris*, along with two other species, *B. acidocaldarius* and *B. cycloheptanicus*, were related but remarkably different from other *Bacillus* species because their main membrane fatty acid component is  $\omega$ -alicyclic fatty acid (Wisotzkey et al., 1992). They were then reclassified into a new genus, *Alicyclobacillus*. Yamazaki et al. (1996) isolated a thermophilic bacterium from spoiled acidic beverages and identified it as *Alicyclobacillus acidoterrestris* based on DNA-DNA homology and 16S ribosomal DNA sequence similarity.

## ***A. acidoterrestris***

The morphology of *Alicyclobacillus* is described as rod shaped vegetative cells with terminal/subterminal endospores. In the early stage of growth, cells are Gram-positive but become Gram-negative or Gram-variable when the culture ages.

*Alicyclobacillus* spp. have been isolated from soil, spoiled fruit juices, and several thermal environments. The bacteria grow at temperatures from 20 to 70°C with the optimum between 40 and 60°C. The pH range for growth is 2.0 to 6.0 with optimum pH from 3.5 to 4.5. The species in the *Alicyclobacillus* genus include, but are not limited to: *A. acidocaldarius*, *A. acidocaldarius* ssp. *rittmanii*, *A. acidiphilus*, *A. acidoterrestris*, *A. cycloheptanicus*, *A. disulfidooxidans*, *A. hesperidum*, *A. herbarius*, *A. pomorum*, *A. sendaiensis*, *A. tolerans*, and *A. vulcanalis* (Walker and Phillips, 2008). All species metabolize sugars with acid production but no gas production (Sawaki, 2007). Water activity greater than 0.9 is required for growth, and some species have been reported to grow in fruit juice with up to 18.2 °Brix (Sprittstoesser et al., 1994). With the exception of *A. pomorum*, the main cellular fatty acids of all species are composed of one of the two types of  $\omega$ -alicyclic fatty acid:  $\omega$ -cyclohexyl and  $\omega$ -cycloheptyl. Of all species in the genus, *A. acidiphilus*, *A. acidoterrestris*, *A. herbarius* and *A. pomorum* have been reported to be associated with spoilage of fruit juices and beverages (Matsubara et al., 2002; Cerny et al., 1984; Goto et al., 2002; Goto et al., 2003).

Krischke and Poralla (1990) found that survival and growth of *A. acidoterrestris* at low pH and high temperature is due to its unique cellular membrane composition of  $\omega$ -cyclohexyl fatty acids. These lipids stabilize the cell membrane, which reduces its permeability

(Kannenberget al., 1984). The percentage of  $\omega$ -alicyclic acids increases when the growth temperature increases (Krischke and Poralla, 1990).

## **Sources**

Soil is considered to be the major sources of *A. acidoterrestris* and also the most important source of contamination of acidic products. Studies have suggested that contamination of fruit juices is most likely caused by fruit contaminated by soil during harvest or by unwashed or poorly washed raw fruit used in processing facilities (Chang and Kang, 2004). Another possibility is that soil can be carried into the manufacturing facilities by employees.

Water has also been proposed to be another important source of contamination. McIntyre et al. (1995) isolated a strain of *Alicyclobacillus* from spoiled juice product and found the same strain in a sample of ingredient water from the processing facility.

## **Heat resistance of *A. acidoterrestris***

Spores of *A. acidoterrestris* can germinate and grow at pH <4 and exhibit high heat resistance. D-values of *A. acidoterrestris* in juices at 95°C are reported to range from 0.06 to 5.3 minutes and a z-value range from 7.2 to 12.9°C (Silva and Gibbs, 2001). Targets microorganisms in the fruit products industry are generally much less heat resistant than spores of *A. acidoterrestris*. The standard juice pasteurization treatment is 80 to 95°C for 45 to 15 seconds, which is not sufficient to inactivate spores of *A. acidoterrestris*. This fact demonstrates the vulnerability of fruit juices and beverages to spoilage by *A. acidoterrestris*. Use of *A.*



*acidoterrestris* spores as the target of pasteurization processes in high acid fruit products has been suggested (Silva et al., 1999).

A summary of *A. acidoterrestris* D-values in different fruit juices are presented in Table 1.1. As shown in Table 1.1, D-value decreased with an increase in temperature, which indicates decreased heat resistance. D-values decreased dramatically when temperature increased from 85°C to 90°C, and the highest D-values were recorded in black currant concentrate (24.1 min at 91°C) and lemon juice concentrate (12.63 min at 95°C). For most of the juices evaluated, D-values were reduced to less than 4 min at 95°C. The higher the sugar content (°Brix), the greater the heat resistance recorded. This indicates that it is more difficult to destroy spores present in concentrated juices than in single strength juices. pH also has an effect on the heat resistance of spores, generally with lower heat sensitivity at higher pH. Other properties of juices may play a greater role in heat resistance than pH. As seen in the table, heat resistance in orange juice is greater than in grape juice, even though grape juice had a slightly higher pH. It was reported that pH affects heat resistance of the spores at lower temperatures. Moreover, the effects of soluble solids and pH were diminished when the temperature reached about 97°C (Silva et al., 1999).

Studies also have shown that the type of organic acid (malic, citric, and tartaric acid) did not significantly affect D-value in the temperature range of 91-100°C (Pontius et al., 1998). It was also found that bacterial spores were more heat resistant as water activity decreased (Härnultv and Snygg, 1972; Murrel and Scott, 1966). Silva et al. (1999) suggested that water activity may be considered instead of soluble solids in future studies because different sugars

generate different water activities at the same concentrations and could have different effects on D-value.

Various methods have been used for controlling juice spoilage in order to extend its shelf life. Heat treatment such as pasteurization is one of the most common methods. Pasteurization refers to mild heat treatment designed to kill vegetative cells of microorganisms and inactivate spoilage enzymes. FDA has not defined pasteurization in terms of juice and juice products and has not specified the parameters for juice pasteurization due to unique characteristics of the various types of juice and juice products (FDA, 2001). Current pasteurization conditions vary by processor. Juice processors choose appropriate pasteurization conditions for their particular types of juices based on literature that provides data on pasteurization times and temperatures. The nature of the product container and the physical properties of the fruit juice often dictate the processes that should be used (Worobo and Splittstoesser, 2005). Pasteurization temperatures between 80 and 100°C are normally used (Silva and Gibbs, 2004). The process is much less severe than those used for low-acid foods and if refrigerated storage is intended, a milder pasteurization would be used to obtain the same shelf-life.

### **Pathogenicity of *A. acidoterrestris***

Wall and Chuyate (2000) tested the pathogenicity of *A. acidoterrestris* by injecting spores directly into mice or by feeding spore-inoculated juice to guinea pigs. No death or illness symptoms were found in mice or guinea pigs, indicating that *A. acidoterrestris* lacks pathogenicity at the level tested. Also, no cases of human illness attributed to consumption of

juice spoiled by *A. acidoterrestris* have been reported. Therefore, although *A. acidoterrestris* can cause serious spoilage problems, it is not a safety concern.

### **Isolation and enumeration techniques**

*A. acidoterrestris* cannot grow in many media, including nutrient agar, trypticase soy agar, brain heart infusion, and veal infusion agar, that support growth of fastidious bacteria. No growth was observed even when these media were acidified to pH 3.5 with tartaric acid (Splittstoesser et al., 1994). However, a number of other media have been suggested to support growth of *A. acidoterrestris*, and new media have been developed to enumerate *A. acidoterrestris*. These include orange serum agar (OSA), K agar, *Bacillus acidocaldarius* (BAM) medium, acidified potato dextrose (aPDA) agar, yeast extract starch glucose (YESG) agar, *Alicyclobacillus* (ALI) agar, *Bacillus acidoterrestris* thermophilic (BAT) agar.

Substantial research has been done to compare different isolation media. Pettipher et al., (1997) found that BAM, OSA and PDA all performed well at recovering *A. acidoterrestris* from orange juice, with OSA recovering the highest numbers. Orr and Beuchat (1999) reported that, compared to OSA (pH 5.0) and PDA (pH 3.5), K agar (pH 3.7) showed the best recovery of chemically treated spores. Murray et al. (2007) evaluated ten test media for their suitability to support *A. acidoterrestris* growth. Their results showed that K agar (pH 3.7), ALI agar (pH 4.0) and BAT agar (pH 4.0) recovered the highest number of spores while OSA (pH 3.5) and Hiraishi glucose yeast extract (pH 3.0) agar recovered the lowest. They also found that surface plating is superior to pour plating and that incubation temperatures of 43 to 50°C did not yield significantly different recovery.

However, some researchers suggest that direct plating is not sufficient to detect very low numbers of *A. acidoterrestris*. Membrane filtration was proposed as a more sensitive method because it can test large sample volumes while traditional plating methods are limited by the volume that can be plated on an agar plate (Chang and Kang, 2004). Pettipher and Osmundson (2000) successfully detected *A. acidoterrestris* from a spoiled juice drink by filtering the juice and incubating the filter itself on OSA, while standard plating methods were unsuccessful. Lee et al. (2007) evaluated five membrane filters for detecting *Alicyclobacillus* spp. spores in apple juice. The recovery rate differed among filters with an average recovery rate of 126.2 % compared to conventional K agar plating.

### **Spoilage caused by *A. acidoterrestris***

Juice is defined as “the aqueous liquid expressed or extracted from one or more fruits or vegetables, purees of the edible portions of one or more fruits or vegetables, or any concentrates of such liquid or puree” (FDA, 2001). Fruit juices are consumed widely around the world, mainly because they are considered as healthy natural sources of nutrients and their protective effects of health.

However, the high water activities and high carbohydrate contents along with other nutrients of fruit juices favor microbial growth. Microbial contamination of fruit juice and juice products has caused several cases of foodborne illnesses and spoilage incidents, which not only results in threats to human health, but also leads to huge economic loss.

## **Microbial contamination of fruit juice**

Outbreaks of illness associated with consumption of fruit juices have been an increasing public health problem since the early 1900s. It was originally thought that the pH of fruit juice is normally below that which supports growth and even survival of pathogenic bacteria. However, in 1991, contamination of fresh pressed apple cider with *Escherichia coli* O157:H7 resulted in foodborne illnesses and subsequent cases of hemolytic uremic syndrome (Besser et al., 1993). From 1995 to 2005, according to the CDC's Foodborne Outbreak Reporting System, there were 21 juice-associated outbreaks that caused 1366 cases of illnesses. Of the reported outbreaks, 13 were of known etiology, among which *E. coli* O157:H7, *Salmonella*, *Cryptosporidium*, and shiga toxin-producing *E. coli* O111 were confirmed as causative agents of illness (Vodjdani, et al., 2008).

Because of pH typically less than 4, fruit juice spoilage is caused by yeasts, mold, and acid tolerant bacteria, such as lactic acid bacteria. Spoilage may result in the formation of haze and sediment in the juice and the presence of gas and/or undesirable flavors or odors (Worobo and Splittstoesser, 2005). Contamination of juices with an initially small number of organisms has the potential to cause wide-spread spoilage that can result in significant economic loss.

### **Spoilage incidents caused by *A. acidoterrestris***

The ability of spores of *A. acidoterrestris* to grow under highly acidic conditions makes it a good candidate for spoilage of shelf stable fruit juices and beverages. Following the first spoilage incident caused by *A. acidoterrestris* reported in 1982, when aseptically packaged apple juice in Germany was contaminated (Cerny et al., 1984), several spoilage incidents have

been reported over the past two decades. Several spoilage cases were reported in Japan, Europe, and the USA in the 1990s (Suzuki, 1989; Splittstoesser et al., 1994; Jensen, 2000). In addition, more food products have been reported spoiled by *A. acidoterrestris*, including isotonic water and lemonade (Yamaziki et al., 1996), carbonated fruit juice drinks (Pettipher and Osmundson, 2000), fruit pulps (Gouws et al., 2005), shelf stable iced tea containing berry juice (Duong and Jensen, 2000), and canned diced tomatoes (Walls and Chuyate, 1998).

### **Off flavors caused by spoilage**

Spoilage of *A. acidoterrestris* is primarily manifested as off flavor or off odor. Visual detection of spoilage is very difficult since no gas is produced during growth and swelling of containers does not occur. The major compounds associated with off-flavors caused by *A. acidoterrestris* are guaiacol and halophenols, including 2, 6-dibromophenol (2, 6-DBP) and 2, 6-dichlorophenol (2, 6-DCP).

### **Guaiacol**

Guaiacol (2-methoxyphenol) is a phenolic compound with the formula  $C_6H_4(OH)(OCH_3)$  (Figure 1.1). Guaiacol is accepted to be the major metabolite associated with off odors in fruit juices and is detected in juices at concentrations about 1000 times higher than halophenols (Jensen, 2000).

Guaiacol is usually derived from wood creosote or guaiacum and can be biosynthesized by a variety of organisms, such as *Bacillus magaterium*, *Pseudomonas acidovorans*, and *A. acidoterrestris*. It is recognized as a flavor compound and has been used as synthetic flavoring

in processed foods with a description of “sweet”, “smoky”, “phenolic”, and “medicinal” (Burduck, 2005; Wasserman, 1966). The characteristic odor of some roasted foods such as Arabica coffee (Mayer et al., 1999) and barley malt (Fickert and Schieberle, 1998) is attributed to the presence of guaiacol. However, it is better known as an off flavor/odor compound in many other foods such as wine (Simpson et al., 1986), fruit juices (Yamazaki et al., 1996), chocolate ice cream (Saxby, 1993), chocolate milk (Jensen et al., 2001) and vanilla yogurt (Whitfield, 1998).

In fruit juices, guaiacol is formed from ferulic acid via vanillin (Bahçeci et al., 2005). Ferulic acid is a major component in lignin and can be found abundantly in plant cell walls. It can be metabolized by bacteria and fungi (Rosazza et al., 1995) and converted to vanillin, vanillic acid, and protocatechuic acid. Vanillic acid can be further converted to guaiacol. Crawford and Olson (1978) demonstrated that several strains of *B. megaterium* and a strain of *Streptomyces* convert vanillic acid to guaiacol and CO<sub>2</sub> by a non-oxidative decarboxylation mechanism. They also suggested that the ability to decarboxylate vanillic acid to guaiacol is quite common among soil bacilli.

The human sensory threshold for guaiacol is low, so it is easily detected. Wasserman (1966) reported that the threshold concentration of guaiacol in water is 0.021 ppm for odor and 0.013 ppm for taste; the odor threshold in oil is 0.07 ppm. The threshold for smelling guaiacol in 12% aqueous ethanol is reported as 0.03 ppm (Chang and Kang, 2004). Pettipher et al. (1997) used a GC-MS method and found that the odor threshold for guaiacol in orange, apple juice, and a non-carbonated fruit juice drink was about 2 ppb. Another study using a sensory panel and the forced-choice ascending concentration method of limits conducted by Orr et al. (2000)

also showed similar results. They reported the best estimate threshold of guaiacol in apple juice is 2.23 ppb.

In the case of *A. acidoterrestris* spoilage, guaiacol is produced when cell numbers reach a critical level. Komitopoulous et al. (1999) reported that guaiacol was detected in apple juice, orange juice, and grape juice stored at 30°C when the population of *A. acidoterrestris* reached 10<sup>5</sup> CFU/ml; at 25°C, the same population was required to detect guaiacol in apple and orange juices, but only 10<sup>4</sup> CFU/ml were necessary to detect it in grape juice. Similarly, Pettipher et al. (1997) reported that 10<sup>5</sup> CFU/ml *A. acidoterrestris* was required before guaiacol was detectable level in orange juice and apple juice stored at 25, 35, and 44°C. Generally, it is proposed that the higher the incubation temperature, the faster guaiacol is produced.

### **Halophenols**

Although guaiacol is considered the predominant off-odor compound, researchers also detect halophenols, 2, 6-DBP (Borlinghaus and Engel, 1997) and 2, 6-DCP (Jensen and Whitfield, 2003) (Figure 1.2 and 1.3) produced by *A. acidoterrestris* in spoiled juices. The odor/flavor is often described as “medicinal” and “disinfectant” (Whitfield, 1998). Halophenols are well known for causing off-flavors in foods. Their occurrence in food can be either from chemical contamination or microbial synthesis (Chang and Kang, 2004). The taste threshold in water of 2, 6-DCP is 6.2 ppt (Young et al., 1996) and 0.5 ppt for 2, 6-DBP (Whitfield et al., 1988). In juices, the taste threshold is reported to be 0.5 ppt for 2, 6-DBP and 30 ppt for 2, 6-DCP (Jensen, 1999).



Halophenols can be formed when weak halogen solutions are used in cleaning raw materials and food processing lines and are inadequately rinsed away. If they are not completely removed, they can be present in the final product and cause off odors and flavors (Chang and Kang, 2004). The biosynthetic pathway has been demonstrated in marine algae, in which a phenolic precursor, hydrogen peroxide, halide ions, and haloperoxidase are key reactants (Flodin and Whitfield, 1999). Jensen and Whitfield (1993) proposed that some strains of *A. acidoterrestris* may contain certain enzymes that are capable of halogenation, which leads to the production of 2, 6-DBP and 2, 6-DCP.

### **Spoilage control methods**

With the emergence of juice-associated outbreaks, the US Food and Drug Administration (FDA) published the juice hazards analysis and critical control point (HACCP) regulation in 2001. This regulation requires that juice processors include in their HACCP plan measures to provide at least a 5-log reduction in the pertinent pathogens most likely to occur (FDA, 2001). The juice HACCP regulation only applies to pathogens, and there is no regulation for controlling juice spoilage. It is necessary for the juice and beverage industries to take measures to ensure the quality of their products.

### **Disinfectants**

Since *A. acidoterrestris* most likely enters processing facilities on fruits that come in contact with contaminated soil, raw materials must first be washed thoroughly to prevent *A. acidoterrestris* contamination. However, bacteria cannot be reduced sufficiently by washing

with just cold or warm water, so detergents or bactericides need to be added to the wash water.

Orr and Beuchat (2000) studied the effectiveness of selected disinfectants for inactivating *A. acidoterrestris* spores; their results showed that when spores of a five-strain mixture were suspended in 200 ppm chlorine, 500 ppm acidified sodium chlorite, or 0.2 % H<sub>2</sub>O<sub>2</sub> for 10 minutes at 23°C, spore populations were reduced by 2.2-, 0.4- and 0.1- logs, respectively. More than a 5-log reduction of spores was observed after treatment with 1000 ppm chlorine or 4% H<sub>2</sub>O<sub>2</sub>. The disinfectants were less effective at killing spores on the surface of apples. Both 500 ppm chlorine and 1200 ppm acidified sodium chlorite reduced viable spores by less than 1 log. Chlorine dioxide was reported to be more effective at killing *A. acidoterrestris* spores. More than a 4-log reduction of spores was observed after treatment with 40 ppm chlorine dioxide for five minutes in aqueous suspension. Treatment with 80 and 120 ppm chlorine dioxide for 5 min reduced spores to undetectable levels. When chlorine dioxide was applied to apple surfaces, greater than a 4.8-log reduction of spores was observed after treatment with 40 ppm free chlorine dioxide for four minutes (Lee et al., 2004). Cortezzo et al. (2004) suggested that treatment oxidizing agents (such as chlorine) results in damage to the spore inner membrane, which sensitizes spores to subsequent stress. The targets of oxidizing agents can be inner membrane proteins or fatty acids, or both.

## **Non-thermal methods**

Traditional thermal pasteurization is effective for inactivating vegetative cells of bacterial foodborne pathogens, but, as stated before, the current juice pasteurization

treatment is not adequate to destroy spores of *A. acidoterrestris*. Moreover, heat treatment could have negative effects of the quality of juices, including loss of nutrients, and change in flavor, color, and texture. Non-thermal treatments can be attractive alternatives to traditional heat treatments for producing high quality, convenient and safe food products. A number of non-thermal methods have been studied for their effectiveness at controlling *A. acidoterrestris*.

### **High hydrostatic pressure (HHP)**

It is suggested that HPP can preserve certain foods better than heat by extending shelf life and inactivating microorganisms while retaining the inherent color, flavor, nutrients, and texture of the food. Pressure is transmitted instantaneously and is independent of mass, so the treatment throughout the food is uniform (Zimmerman and Bergman, 1993). The application of HPP in ensuring food safety and quality has been widely studied. HPP inactivate vegetative cells of microorganisms by breaking non-covalent bonds and causing damage to the cell membrane (Morris et al., 2007). The mechanism of inactivation of bacterial spores through high pressure was suggested to have two steps: high pressure will first induce spore germination and then inactivate the germinated spores (Gould and Sale, 1970).

The application of HHP for inactivating *A. acidoterrestris* has been studied by many researchers over a wide range of pressure treatments. Alpas and Bozoglu (2003) studied the effect of HHP on inactivation of *A. acidoterrestris* vegetative cells in BAM broth and in orange, apple, and tomato juices. After treating with 350 MPa at 50°C for 20 min, a 4.7-log reduction of cells was achieved in BAM broth while in all juices, over a 4-log reduction was achieved. Spore inactivation was reported by Lee et al. (2006). Apple juices (17.5, 35 and 70 °Brix) inoculated

with *A. acidoterrestris* spores were subjected to three pressure treatments (207, 414 and 621 MPa) at 22, 45, 71 and 90°C. Results showed that the effectiveness of treatment increased as pressure and temperature increased. At room temperature, there was no significant reduction of spores in all juice samples for all three pressures, which indicates that in order to use high pressure for spore inactivation, other treatments such as mild heat are required. They also found that the effectiveness of HHP was affected by soluble solids content, with reduction in inhibition observed when the concentration of juice was increased. Over 5- and 4-log reduction was found in juice of 17.5 and 35 °Brix, respectively at 90°C; however, there was no significant reduction of spores at the highest concentration (70 °Brix).

## **Irradiation**

Irradiation of food is the process by which food is exposed to sufficient radiation energy to cause ionization, thereby leading to microbial death due to genetic damage (FDA, 2001). Irradiation is regulated as a food additive in the U.S and requires approval by the FDA for each new application. Dose ranges of <1 to 3 kGy have proven effective for reducing or eliminating populations of foodborne pathogens and postharvest spoilage microorganisms on produce (Marcotte, 1992, Farkas, 1997). Radiation is suitable for inactivation of spores in foods with low moisture content such as powders (Schmidt, 1961). The application of radiation to destroy bacterial spores has been widely studied. It was suggested that ionizing radiation is an effective means to destroy bacterial spores, especially when combined with heat, and has been applied to the sterilization of several kinds of foods that are contaminated primarily with bacterial spores (Nakauma et al., 2004). Pretreatment of *Clostridium sporogenes* spores with gamma rays

enhanced their thermal sensitivity, and combined treatment can efficiently inactivate the spores (Shamsuzzaman, 1988). Fisher and Pflug (1976) reported that *Bacillus subtilis* was better inactivated with a combination of heat and radiation treatment than each with treatment independently.

Nakauma et al. (2004) studied the influence of radiation (electron-beam and gamma rays) combined with heat to inactivate *A. acidoterrestris* spores. Their results showed that the dose of electron-beam and gamma-ray irradiation required for 90% reduction of spores in dextrin was 1.72 and 1.79 kGy, respectively. The required dose was lowered by using irradiation in combination with thermal treatment. Also, it was shown that radiation accelerated the effect of subsequent thermal treatment: 4 log CFU/ml spores were completely inactivated by heating at 95°C for 188 min, and the heating time was reduced to 23 min when combined with a 2.0 kGy electron beam or gamma-ray treatment.

Although irradiation is approved for various foods and appears to be a promising alternative food preservation method, it may have negative effects of food quality. Irradiation disrupts the chemical composition of everything in food - not only harmful microorganisms. For example, fruits and vegetables treated with irradiation can become soft and eggs can lose their color and become runny (Wong et al., 1999). It is worthy to note, however, that heating, while also a highly effective antimicrobial treatment, also disrupts the chemical and physical composition of foods. Moreover, although irradiated food is scientifically proven to be safe, it is not widely accepted by consumers. Many consumers still have concerns regarding the safety of irradiated foods.

## Ultrasound

Ultrasound refers to sound pressure with a frequency of greater than 20 kHz (upper limit of human hearing). It has been applied in the food industry in a wide range of applications including measurement of chocolate layers, fat lean tissues in meat, detection of contaminants like metal, glass or wood, and measurement of particle size. Ultrasound as a potential antimicrobial treatment has been studied for about two decades. Research of its inactivation effect on several foodborne pathogens, *Listeria monocytogenes*, *Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, and others, has been conducted. The mechanism of microbial inhibition is suggested to be due to thinning of cell membranes, localized heating, and production of free radicals (Butz and Tauscher, 2002). The advantages of ultrasound over heat pasteurization are minimizing flavor loss, greater homogeneity, and significant energy savings (Piyasena et al., 2003). It is also suggested that the combination of heat and ultrasound is more efficient at microbial inactivation than either treatment alone (Ordóñez et al., 1984).

Yuan et al. (2009) studied the effect of ultrasound treatment on *A. acidoterrestris* in apple juice and observed that 60% of cells were inactivated after 23 kHz ultrasound treatment at 300 W for 30 min. The inactivation rate increased as treatment time increased, with more than 80% reduction reported after 60 min of treatment. However, the loss of quality of apple juice was observed after treatment. Total sugar content, transmittance, and color (juice browning) were reduced with increased ultrasound processing time and power. Although many studies of ultrasound in food preservation have been conducted, it is still in its infancy and future research is in need to explore its applicability in food preservation.

## **High pressure homogenization (HPH)**

Homogenization was invented by August Gaulin and was presented at the World's Fair in Paris in 1900 (Paquin, 1999). It was developed for emulsion stabilization and has then been used by the food industry in a wide variety of areas, mostly known for use in milk processing. Homogenization improved the texture, taste, flavor, and shelf-life characteristics for food emulsions and dairy products (Dickinson and Stainsby, 1988). A new generation of homogenization - high pressure homogenization (HPH) was developed in the early 1990s due to the demand of higher quality food. HPH can reach pressures as high as 300-500 MPa while the traditional homogenizer reaches less than 50 MPa. HPH enables a more stable food emulsion production with droplets small enough to ensure a longer shelf-life (Burgaud et al., 1990). HPH is now being used in the pharmaceutical, cosmetic, and food industries for the preparation or stabilization of emulsions and suspensions (Pandolf, 1998; Paquin, 1999). It has also used to disrupt microbial cells, leading to the release of intracellular material (Hetherington et al. 1971, Kelemen and Sharp, 1979; Kleinig and Moddelberg, 1996).

A homogenizer consists of a positive displacement pump which forces cell suspensions through the center of a valve seat. The suspension exits the valve assembly and flows to either a second valve or to discharge (Middelberg, 1995). Effluent from the homogenizer is normally chilled in order to minimize damage to heat sensitive products. To increase the efficiency of cell disruption, cell suspensions can either be processed at higher pressure or by multiple runs through the homogenizer (Engler, 1990).

Inactivation of bacteria using HPH has been investigated by a number of researchers. Gram-positive bacteria are more resistant to HPH than Gram-negative bacteria (Kelemen and

Sharpe, 1979, Vachon et al., 2002). It is suggested that the composition in cell wall determines resistance to HPH. The cell wall is necessary for maintaining the structure and shape of the cell and protecting it from osmotic forces. Gram-positive bacteria have a thicker peptidoglycan layer in the cell wall than Gram-negative bacteria, which contributes to their greater resistance to HPH (Kelemen and Sharpe, 1979). It is also suggested that the growth rate of cells affects resistance to HPH. Cells are more easily disrupted when they are growing rapidly (e.g., exponential phase) than cells growing at a slower rate on the same medium (Engler, 1990). Wuytack et al. (2002) compared bacterial inactivation by HPH and HHP. Of all five Gram-positive and six Gram-negative bacteria they studied, large differences in resistance to HHP but not HPH were observed. They concluded that the inactivation mechanisms for both techniques are different due to the different response of test bacteria. High pressure level is not a major factor during HPH, since bacteria are only exposed to high pressure for a second or less while the exposure time for HHP is much longer. Yeasts are generally easier to be destroyed than bacteria. Brookman (1974) reported 100% disruption of *Saccharomyces cerevisiae* cells at 170 MPa with one single pass through the HPH. Bacterial spores are very resistant to physical and chemical stress. Popper and Knorr (1990) stated that spores of *Bacillus* spp. and *Clostridium* spp. are resistant to any treatment including HPH.

The microbial inactivation mechanisms of HPH were widely discussed but still remain as an area of debate. Turbulence (Doulah et al., 1975), stress, shear, or turbulence caused by impingement of a high velocity jet of suspended cells on a stationary surface (Engler and Robinson, 1981), and cavitation (Save et al., 1994), which is the process of gas cavity growth



and collapse in a liquid when the liquid is subjected to rapid pressure change, have all been suggested as responsible for microbial inactivation by HPH.

Inactivation of spores of three *A. acidoterrestris* strains in laboratory media by HPH in a pressure range 500-1700 bar was investigated by Bevilacqua et al. (2007). Their results revealed that up to a 2-log and 0.8-log reduction of vegetative cells and spores, respectively, was achieved, and that resistance of *A. acidoterrestris* to HPH is strain dependent.

### **Food preservatives**

Food additives are defined by the Food Protection Committee of Food and Nutrition Board as “a substance or mixture of substances, other than a basic food stuff, which is present in a food as a result of any aspect of production, processing, storage, or packaging. The term does not include contaminants”. The additives used to prevent biological deterioration are termed “antimicrobials” (Davidson and Branen, 2005). Antimicrobials can help to extend the shelf-life of numerous foods, and the use of antimicrobials has increased in recent years as microbial food safety concerns have become a major issue in the food industry. In order to select the appropriate antimicrobial for a food item, the target microorganisms must be identified first (Leistner, 2000). Then, spectrum of antimicrobial activity, chemical and physicochemical properties of the antimicrobial, composition of the food product, and type of preservation or processing and storage systems used must be taken into consideration (Davidson and Branen, 2005).

A number of antimicrobials such as benzoate, bacteriocins, essential oils, and lysozyme have been studied for their ability to control *A. acidoterrestris*.

## Benzoic acid/benzoate

Benzoic acid is one of the oldest and most widely used antimicrobial in cosmetics, drugs, and foods because of its low cost, lack of color, relatively low toxicity, and ease of incorporation into products. It occurs naturally in several foods and has been identified as a major component in extracts of blackberries (Humpf and Schreier, 1991) and fresh tomatoes (Marlatt et al., 1992). Its salt form, sodium benzoate, is more soluble in water than benzoic acid and was the first chemical preservative approved for use in foods by the FDA (Jay et al., 2005). The maximum tolerance in foods for benzoate or benzoic acid is 0.1%. The mechanism of its antimicrobial activity is most likely the same as other organic acids. The pH greatly affects its antimicrobial activity since it is the un-dissociated form of benzoic acid that can be taken up by cells and reduce the intracellular pH. The pKa of benzoic acid is around 4.19, and the quantity of un-dissociated acid decreases with increasing pH, so the use of benzoic acid or sodium benzoate as food preservative are mostly effective in high acid foods.

Benzoic acid/benzoate is primarily used as an antifungal agent, and most fungi are inhibited by 0.05 to 0.1% of the un-dissociated acid. Bacteria are generally inhibited by 0.01 to 0.02% un-dissociated acid (Chichester and Tanner, 1972; Baird-Parker, 1980). However, there are certain microorganisms have been found resistant to benzoic acid, including *Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii*. The minimum inhibitory concentrations (MICs) of benzoic acid for *Z. bailii* were reported as 1000-1400 mg/l at pH 3.5 and were little affected by type or concentration of sugar in the range 0-20% (w/v) (Warth, 1986). Several studies of the effects of benzoic acid against *A. acidoterrestris* have been conducted. Walker and Philips (2008) reported that 100 mg/l sodium benzoate can inhibit 1 log CFU/ml *A.*

*acidoterrestris* growth in apple juice at 30°C while 500 mg/l can inhibit growth of 4 log CFU/ml cell. Bevilacqua et al. (2008) reported 51-62% inhibition of *A. acidoterrestris* in malt extract broth with 100 ppm sodium benzoate after 13 days. However, the inhibition index decreased as the time increased.

## **Bacteriocins**

Bacteriocins are compounds produced by bacteria to inhibit growth of closely related bacterial strains. About 99% of all bacteria may make at least one bacteriocin, and many of them have not been discovered or studied (Klaenhammer, 1988).

Nisin is a low molecular weight polypeptide produced by the dairy starter culture *Lactococcus lactis* subsp. *lactis*. It is the first bacteriocin approved by FDA as a food additive in pasteurized processed cheese spreads. It has a much broader spectrum than most other bacteriocins and is effective against a wide range of Gram-positive bacteria but not significantly effective against Gram-negative bacteria or fungi (Thomas et al. 2005). However, nisin has been shown effective for reducing populations of Gram-negative bacteria when combined with chelating agents (Stevens et al., 1991). Davies et al. (1998) reported that nisin is stable at pH 3 and maintains more than 70% antibacterial activity at pH 4 when autoclaved at 115°C. It is active against bacterial cells and spores, enabling it to be used as a preservative in pasteurized or heat-treated foods that are not fully sterilized. *Bacillus* and *Clostridium* are very sensitive to nisin, and their spores are more sensitive than vegetative cells (Thomas et al. 2005). It was suggested that nisin did not prevent the germination of spores but prevent post-germination swelling and subsequent outgrowth of spores (Hitchins et al., 1963; Gould, 1964).

The inhibitory effect of nisin against *A. acidoterrestris* was investigated by Yamazaki et al. (2000). They reported MICs of nisin against spores from less than 0.78 to 12.5 IU/ml and from 25 to 100 IU/ml on laboratory media at pH 3.4 and 4.2, respectively. The MICs for vegetative cells were higher than spores: 1.56 to 25 IU/ml and 25-100 IU/ml at pH 3.4 and 4.2, respectively. Furthermore, 25-50 IU/ml nisin inhibits growth of spores in orange and fruit-mixed drinks, but even 600 IU/ml did not inhibit spores in clear apple drink.

Another bacteriocin, AS-48, has been studied against *A. acidoterrestris*. AS-48 is produced by *Enterococcus faecalis* A-48-32. It has been suggested to be an alternative to nisin for food preservation. Grande et al. (2005) studied the inactivation effect of AS-48 against *A. acidoterrestris* in different juices incubated at different temperatures. Their results revealed that a concentration of 2.5 µg/ml is sufficient to render *A. acidoterrestris* undetectable in fruit juices tested for prolonged periods of time. They also performed electron microscopy examination of vegetative cells and spores treated with enterocin AS-48, and noted that substantial cell damage and bacterial lysis were found.

## **Lysozyme**

Lysozyme was discovered by Alexander Fleming in 1921. Fleming found lysozyme present in nasal mucus and tears and hen egg white that had a high level of protein (Johnson and Larson, 2005). It was then found in many other sources such as plants and fungi, and it is also present in high concentration in the human immune system, which makes it an ideal preservative since it is present naturally in the human body, it is expected to have low toxicity. Antimicrobial activity of lysozyme is due to its ability to hydrolyze  $\beta$ -1-4 glycosidic linkages

between N-acetyl glucosamine and N-acetylmuramic acid in bacteria cell walls, which results in cell lysis. It is currently used in foods such as cheese, cooked meat, poultry products, and frankfurters (Davidson and Harrison, 2002). The dose used is usually 20 to 400 ppm (Gould, 2002).

Lysozyme is more effective against Gram-positive bacteria but ineffective against bacteria spores and poorly effective against Gram-negative bacteria. Bevilaqua et al. (2007b) reported lysozyme (0.25-2.0 g/l) was effective at inactivating *A. acidoterrestris*. Spores were more sensitive than cells and were undetectable just after the addition of the compound. Conte et al. (2006) studied the antimicrobial effectiveness of lysozyme immobilized on a polyvinylalcohol based polymeric film in apple juice and in laboratory media against *A. acidoterrestris*. They showed that *A. acidoterrestris* germinated and grew in the samples without film and with lysozyme-free film but populations decreased (up to 2 log CFU/ml) in the presence of film with lysozyme.

### **Essential oils**

The increasing demand for reduced additives, “natural” food products, and pathogen-free foods have promoted the search for natural antimicrobial compounds. Essential oils (EOs) are aromatic oily liquids obtained from plant materials that contain substances that can inhibit various metabolic activities of microorganisms. These compounds may be lethal to microbial cells or simply inhibit production of a metabolite (Beuchat, 1994; Davidson, 2001). Cinnamon and clove are known to exhibit strong inhibitory effect, with eugenol and cinnamaldehyde being the major constituents of the volatile oils of these spices (Vigil et al., 2005). Many factors,

such as the evaluation method and food components, affect the antimicrobial activity of spices, extracts, and EOs (Zaika, 1988).

The antimicrobial activities of leaf extracts of 26 species of eucalyptus against various microorganisms were measured (Takahashi et al., 2004). The MICs of *A. acidoterrestris* for most of the eucalyptus species were over 250 mg/l, while for several of them, 7.8 mg/l inhibited visible growth. Bevilacqua et al. (2008) conducted a study to evaluate the antimicrobial effectiveness of cinnamaldehyde, eugenol, and limonene against spores of two strains of *A. acidoterrestris* in malt extract broth by measuring absorbance at 420 nm. Their results revealed that cinnamaldehyde exhibited the strongest antimicrobial activity, with 100 ppm of cinnamaldehyde inhibiting 96-97% spore germination of both strains after eight days while the same concentration of eugenol only inhibited about 50% of the spore population for one strain and did not affect the other strain. Limonene did not effectively inhibit spore germination for either strain.

### **Dimethyl dicarbonate**

Dimethyl dicarbonate (DMDC), also known as dimethyl pyrocarbonate (Figure 1.4), is a colorless, fruity-smelling liquid. It has a boiling point of 123 to 149°C and a melting point of 15.2°C. Its solubility in water is very low, and it is more soluble in organic solvents (Fisher & Golden, 1998). Diethyl dicarbonate (DEDC) is a related dicarbonate compound similar to DMDC. It was introduced into the market for trial tests in 1959 (Bernhard et al., 1959). The effectiveness of DEDC as a fungicide in wine has been reported, and it did not cause off-odor or off-flavor (Henning, 1959). However, DEDC was banned by the FDA in 1972 because of the

formation of urethane. DMDC was first approved as a yeast inhibitor at a level no more than 200 ppm in wines in 1988 (FDA, 1988). DMDC is now approved as a direct food additive to be used as a microbial control agent in certain beverages in which the microbial population has been reduced to 500 CFU/ml or less by current good manufacturing practices (FDA, 2001). The concentration limit is 250 ppm for carbonated or noncarbonated, nonjuice-containing, flavored or unflavored beverages containing added electrolytes and in carbonated, dilute beverages containing juice, fruit flavor, or both, in which the juice content does not exceed 50%. For wine products, the limit is 200 ppm (FDA, 2001). It is marketed and sold for application to foods under the registered trademark, Velcorin®. The advantage of using DMDC is that no reactions occur with sugar, sugar alcohols, or artificial sweeteners such as saccharin or cyclamate (Golden et al., 2005).

DMDC was synthesized by Kovalenko in 1952 (Golden et al., 2005). The reaction was:  $\text{CH}_3\text{OCOCl} + \text{NaOCOCH}_3 \rightarrow \text{CH}_3\text{OCOOCOCH}_3 + \text{NaCl}$ . He also reported that DMDC is extremely reactive and rapidly hydrolyzes to methanol and  $\text{CO}_2$ . So in order to destroy target microorganisms, the inhibition process needs to be complete before hydrolysis takes place. It is not suitable for long term protection against recontamination or later outgrowth of surviving organisms (Golden et al., 2005). Intrinsic and extrinsic parameters of foods can affect the activity of DMDC. The hydrolysis rate increases as temperature increases, while increasing the ethanol concentration can decrease the hydrolysis rate, thereby enhancing the effectiveness as an antimicrobial. It was found that ethanol increases the effectiveness of DMDC and 20°C is the optimum use temperature (Porter and Ough, 1982).

The inactivation mechanisms of DMDC and DEDC are similar and most likely related to inactivation of microbial enzymes. Enzyme inactivation results from active site blocking and conformational changes. DEDC can completely inactivate lactate dehydrogenase by reaction with the histidyl groups of the enzyme (Holbrook and Ingram, 1973).

Costa et al. (2008) reported the inhibitory effect of DMDC against wine microorganisms in dry red wines with 12% (v/v) ethanol and pH 3.5. At an initial inoculum of 500 CFU/ml, the MIC for *Schizosaccharomyces pombe*, *Dekkera bruxellensis*, *S. cerevisiae*, and *Pichia guilliermondii* was 100mg/l, while *Z. bailii*, *Zygoascus hellenicus*, and *Lachancea thermotolerans* were more sensitive with MIC of 25 mg/l. However, when the inoculum was increased to 10<sup>6</sup> CFU/ml, 200 mg/l of DMDC was not effective against most yeast species tested. Lactic acid and acetic acid bacteria tested were not affected by 300 mg/l DMDC. For mold, 200, 100, 100, 500 and 100 mg/l DMDC was required to kill 500 CFU/ml spores of *Penicillium glaucum*, *Byssoschlamys fulva*, *Botrytis cinerea*, *Mucor racemosus*, and *Fusarium oxysporum*, respectively (Genth, 1979). It was reported that *B. fulva* ascospores were resistant to DMDC up to 1000 mg/l in apple juice, although effectiveness was increased at higher treatment temperature (Van der Riet et al., 1989). Fisher and Golden (1998) reported that 250 ppm DMDC reduced the population of *Escherichia coli* O157:H7 from 7 log CFU/ml to undetectable levels within 3 days in apple cider stored at 4°C. The inhibitory activity of DMDC increased at higher temperature, and no *E. coli* O157:H7 was detected within two days storage at 25°C. Moreover, it was shown that DMDC was more efficient than sodium benzoate (0.045%), or sodium bisulfate (0.0046%) at inactivating *E. coli* O157:H7. Mount et al. (1999) discovered that 100 ppm DMDC and 1% potassium sorbate can extend the shelf life of fresh salsa by several weeks. The aerobic plate



count and yeast/mold count of fresh salsa with an initial inoculum of 6 log CFU/g were reduced to undetectable levels within eight days of storage at 4°C. Golden et al. (2005) stated that the combination of DMDC with sodium benzoate or potassium sorbate increases effectiveness and enhanced protection by providing a secondary barrier against surviving spoilage organisms. Soaking cantaloupes for three minutes in a 10,000 ppm solution of DMDC reduced the population of *Salmonella* from 5.01 log CFU/cm<sup>2</sup> to undetectable levels (Rash, 2003).

DMDC hydrolyzes to methanol and carbon dioxide almost immediately after addition to beverages. Methanol is the major hydrolysis product of concern. Human adults can metabolize up to 1500 mg of methanol per hour without adverse effects (Lehman, 1963). The FDA determined that in the worst case scenario, consumption of wine containing 200 ppm DMDC at a rate of 232 g per person per day would result in a daily intake of no more than 22 mg methanol per person, well within safe limits (Golden et al., 2005). No adverse effects were found in fruit juice and alcoholic beverages with 4000 mg/l DMDC after three months. The amount of methanol produced was well below toxicological levels (Pound, 1987).

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## APPENDIX

Table 1.1. Heat resistance of *A. acidoterrestris* spores in fruit beverages.

Product	pH	°Brix	Temp (°C)	D-value (min)	Reference
Fruit-blend beverage	3.7	12.0	89	10.9	Yokota et al.(2007)
			95	2.1	
Concentrated orange juice	3.15	9	85	50.0	Eiroa et al.(1999)
			90	16.9	
			95	2.7	
Apple juice	3.58	3.58	90	8.58	Walls (1999)
			95	1.47	
			100	0.16	
Lemon juice concentrate	3.5	50	86	68.95	Maldonado et al. (1999)
			92	16.87	
			95	12.63	
Lemon juice	3.5	9.8	86	11.23	Maldonado et al. (1999)
			92	10.54	
			95	9.47	
Grape juice	3.42	N/A	80	54.3	Komitopoulou et al.(1999)
			90	10.3	
			95	3.59	
Grape juice	4.0	N/A	80	52.35	Komitopoulou et al.(1999)
			90	9.44	
			95	1.73	
Orange juice	3.90	N/A	80	54.3	Komitopoulou et al.(1999)
			90	10.3	
			95	3.59	
Berry juice	3.5	N/A	88	11	Splittstoesser et al. (1997)
			91	3.8	
			95	1.0	
Light Blackcurrant concentrate	2.5	26.1	91	3.80	Silva et al. (1999)
Blackcurrant concentrate	2.5	58.5	91	24.10	Silva et al. (1999)

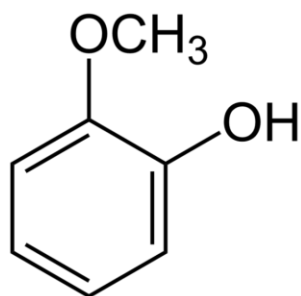


Figure 1.1. Guaiacol

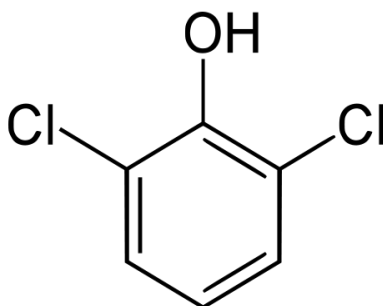


Figure 1.2. 2, 6 – Dichlorophenol

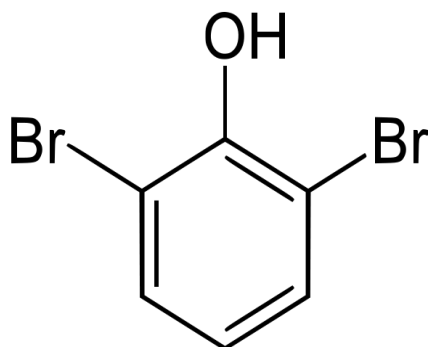


Figure 1.3. 2, 6-Dibromophenol

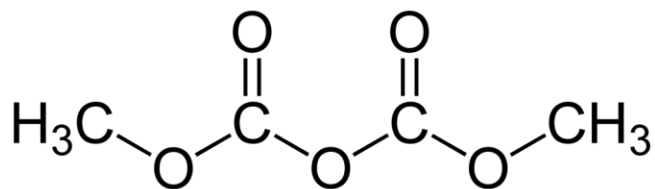


Figure 1.4. Dimethyl dicarbonate

## **Chapter II**

### **Growth study of *Alicyclobacillus acidoterrestris***

## ABSTRACT

*Alicyclobacillus acidoterrestris* is a sporeforming bacterium that causes spoilage of high acid food items, especially fruit juice and juice products. To prevent potential spoilage by *A. acidoterrestris*, its growth characteristics need to be investigated. The objective of this study was to characterize the growth profile of *A. acidoterrestris* in a laboratory broth system and determine the time required for achieve the greatest vegetative cell production, with the fewest spores possible, for future inactivation studies. Five strains of *A. acidoterrestris* (SAC, OS-CAJ, N-1096, N-1108 and N-1100) were separately inoculated into *Alicyclobacillus acidocaldarius* (AAM) broth and incubated for eight days at 43°C for sporulation. After spores were collected using centrifugation, spores of each strain were inoculated into *Bacillus acidoterrestris* thermophilic (BAT) broth and heat shocked at 80°C for 10 minutes to promote germination. Growth and the spore population of each strain over a 24-h period at 44°C were determined by serial dilution and surface plating on BAT agar. Then, the percentage of vegetative cells was calculated. The results showed incubation for 16 h yielded vegetative cell populations of 99.9, 97.9, 99.5, 98.7 and 99.4% for strains N-1100, N-1096, N-1108, SAC and OS-CAJ, respectively. Thus, 16 h incubation after heat shocking at 80°C for 10 min was for vegetative cell production of *A. acidoterrestris*.

## INTRODUCTION

*Alicyclobacillus acidoterrestris* can grow at high temperature (up to 70°C) and low pH (as low as pH 2), with optimum growth between 40 to 50°C and pH 3.5 to 4.0. These distinguishing characteristics are due to the  $\omega$ -cyclohexyl fatty acids in the cell membrane.

*A. acidoterrestris* is a spore-forming bacterium. Endospores (spores) of bacteria are not reproductive structures, but typically are produced in response to nutrient limitation or stress. One vegetative cell transforms into one endospore and, once the spore germinates, it will grow into only one vegetative cell. Spores are the dormant stage of bacteria and do not metabolize. They are also very resistant to harsh environmental stress such as drying, irradiation, toxic chemicals and heating. Spores of *A. acidoterrestris* can germinate and grow at pH <4 and have shown strong heat resistance, which presents a potential threat to the fruit juice and beverage industry. D-values of *A. acidoterrestris* in juices at 95°C have been reported to range from 0.06 to 5.3 minutes, and z-values range from 7.2 to 12.9°C (Silva and Gibbs, 2001). Since 1984, many spoilage incidents in different fruit based products worldwide have been attributed to *A. acidoterrestris* (Cerny et al., 1984; Walls and Chuyate, 1998; Duong and Jensen, 2000). *A. acidoterrestris* spoilage causes off-flavor and off-odor due to the production of guaiacol and halophenols, including 2, 6-dibromophenol and 2, 6-dichlorophenol. Spores of *A. acidoterrestris* have been suggested to be used to validate pasteurization processes in high acid fruit products (Silva et al., 1999). Although *A. acidoterrestris* can cause serious spoilage problems, it does not cause foodborne illness and is not a safety concern (Wall and Chuyate, 2000).

In order to prevent spoilage by *A. acidoterrestris*, methods for detection, enumeration and identification need to be improved. *A. acidoterrestris* is unable to grow on nutrient agar,



tryptone soy agar, brain heart infusion agar, or veal infusion agar, even when they are acidified to pH 3.5 (Splittstoesser et al., 1994). Several media have been reported to support growth of *A. acidoterrestris*, including acidified potato dextrose agar (aPDA), orange serum agar (OSA), *Bacillus acidoterrestris* thermophilic (BAT) agar, K agar, *Alicyclobacillus acidocaldarius* media (AAM), yeast extract starch glucose agar (YSG), and *Alicyclobacillus* (ALI) medium. However, the recommendation of appropriate culture media for *A. acidoterrestris* varies: K agar (pH 3.7) is recommended by the American Public Health Association (Evancho and Walls, 2001) while BAT agar is recommended by the International Federation of Fruit Juice Producers (Internationale Fruchtsaft-Union, 2004), and the Japan Fruit Juice Association (2003) suggests using YSG agar.

The purpose of this study was to evaluate growth characteristics of five strains of *A. acidoterrestris* in *Bacillus acidoterrestris* thermophilic (BAT) broth over time, prepare spore stocks, and to determine the time required to collect the greatest number of vegetative cells for future study.

Media used in this study were AAM for sporulation and BAT for growth. Based on the results of Murray et al. (2007), AAM supported the highest percentage of sporulation, and surface plating samples on BAT agar is the most suitable method to enumerate *A. acidoterrestris*.

## MATERIALS AND METHODS

**Bacterial strains.** Five strains of *A. acidoterrestris* were used. All five strains (N-1096 (ATCC 4905), N-1108, N-1100, OS-CAJ and SAC) were obtained as spore suspensions in sterile deionized water from the Dr. Larry Beuchat, University of Georgia Center of Food Safety.

Sources for these strains are: N-1096, garden soil, Germany; N-1108, apple-cranberry juice; N-1100, OS-CAJ, and SAC, apple juice concentrate.

**Media preparation.** *Alicyclobacillus acidocaldarius* media (AAM): 1.0 g yeast extract, 1.0 g glucose, 0.2 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.6 g  $\text{KH}_2\text{PO}_4$ , 1000 ml deionized water. After autoclaving, pH was adjusted to 4 with 25% sterile malic acid (Yamazaki et al. 1996).

*Bacillus acidoterrestris* thermophilic (BAT) broth: 2.0 g yeast extract, 5.0 g glucose, 0.25 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.6 g  $\text{KH}_2\text{PO}_4$ , 1000 ml deionized water and 1 ml trace minerals solution: 0.066 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.018 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.016 g  $\text{CuSO}_4$ , 0.015 g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.018 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.01 g  $\text{H}_3\text{BO}_3$ , 0.03 g  $\text{Na}_2\text{MoO}_4$ , 100 ml of deionized water. After autoclaving, pH of was adjust to 4 with 1.7 ml 1N  $\text{H}_2\text{SO}_4$ .

BAT agar: BAT broth plus 18 g agar per liter.

**Spore production.** Spore production was conducted according to the method of Murray et al. (2007). Fifteen  $\mu\text{l}$  of a culture of each strain was inoculated into 10 ml AAM. Inoculated broths were heat shocked at 80°C for 10 minutes and then incubated at 43°C for four days. After incubation, each strain was inoculated (0.3 ml) onto each of 15 AAM plates. Plates were incubated at 43°C for 8-10 days.

After at least 85% of the cells had sporulated (determined by phase contrast microscopic examination), plates were flooded with 1-2 ml sterile deionized water and spores were collected by gently rubbing the plate surface using a hockey stick. The spore suspension was then filtered through sterile glass wool. This procedure was repeated several times until most of the spores were collected from the plates. Spore suspensions of each strain collected

from all 15 plates were then centrifuged at 2600 x g for 20 min (Sorvall, RC 5B plus, Kendro Laboratory Products, Asheville, NC). Supernatants were discarded and the pellets were resuspended in five ml sterile deionized water and mixed well. Then 40 ml of sterile deionized water was added to the suspensions which were then centrifuged at 4000 X g for 10 min. Pellets were then washed and centrifuged five times at 2100 X g for 20 min. The final pellets were resuspended in 50 ml of sterile deionized water and divided into 2 ml aliquots in sterile centrifuge tubes. All tubes were stored at -20°C.

Populations of spores in stock suspensions were determined. One ml of spore stock was added to 9 ml sterile 0.1% peptone water (Difco), heat shocked in an 80°C water bath (VWR Scientific, Model 1136, Niles, IL) for 10 minutes, serially diluted in 0.1% peptone water, and surface plated in duplicate onto BAT agar. Colonies were counted after incubation for 48 h at 44°C and the CFU/ml of each strain spore stock was calculated.

**Growth study.** Spore stocks of all five strains were thawed and 45 µl of each stock inoculated into 30 ml BAT broth and heat shocked at 80°C for 10 minutes. Inoculated broths were incubated at 44°C for 24 h. At selected time intervals, samples are taken and divided in two, with one half directly serially diluted in 0.1% peptone water and surface plated in duplicate onto BAT agar and the other half heat shocked before plating to inactivate vegetative cells (at 0 h, samples were plated only after heat shock). After 48-h incubation at 44°C, colonies were counted and CFU/ml calculated. Then percentage of vegetative cells was then calculated using the following equation:

$$\% \text{ vegetative cells} = \frac{\text{Population of non-heat-shocked culture} - \text{population of heat-shocked culture}}{\text{Population of non-heat-shocked culture}} \times 100\%$$

## RESULTS AND DISCUSSION

Figure 2.1 demonstrates growth and spore population of N-1100 over a 24-h period in BAT broth. As shown in the figure, the population of the culture (including vegetative cells and spores) increased for about 12 hours and then reached the stationary phase with a population of about 6.5 log CFU/ml. The population of spores, on the other hand, decreased as more spores germinated into vegetative cells until about 16 h. From 16 h to 18 h, a significant increase in numbers of spores (3.8 log CFU/ml to 4.98 log CFU/ml) was observed and continued until 24 h. At 16h, the vegetative cells of N-1100 accounted for 99.9% of the total population. As seen in Figure 2.1, spore populations began to increase after incubation for 16 h. It was assumed that for all the other strains, spore populations similarly would not increase before 12 h incubation. So 12 h to 24 h was chosen for the growth study of the other four strains used.

The vegetative cell population (population of culture - population of pure spore) of SAC, N-1108, N-1096 and OS-CAJ in BAT broth incubated at 44°C are shown in Figure 2.2. The vegetative growth of the four strains followed a pattern similar to N-1100. For SAC, N-1108 and OS-CAJ, the population of vegetative cells first remained constant, followed by a significant decrease starting at 16 h (i.e., spore numbers began to predominate). The vegetative cell population of N-1096 first increased until 16 h and then gradually decreased. The percentage of vegetative cells at 16 h was 97.9, 99.5, 98.7 and 99.4% for N-1096, N-1108, SAC and OS-CAJ, respectively.

## CONCLUSION

This study provided information of the growth of five strains of *A. acidoterrestris*. As the data suggest, spores heat shocked at 80°C for 10 minutes and grown on BAT media at 44°C for 16 h provided the maximum number of vegetative cells with minimum spores present. Thus, this growth condition will be used in future studies.

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## APPENDIX

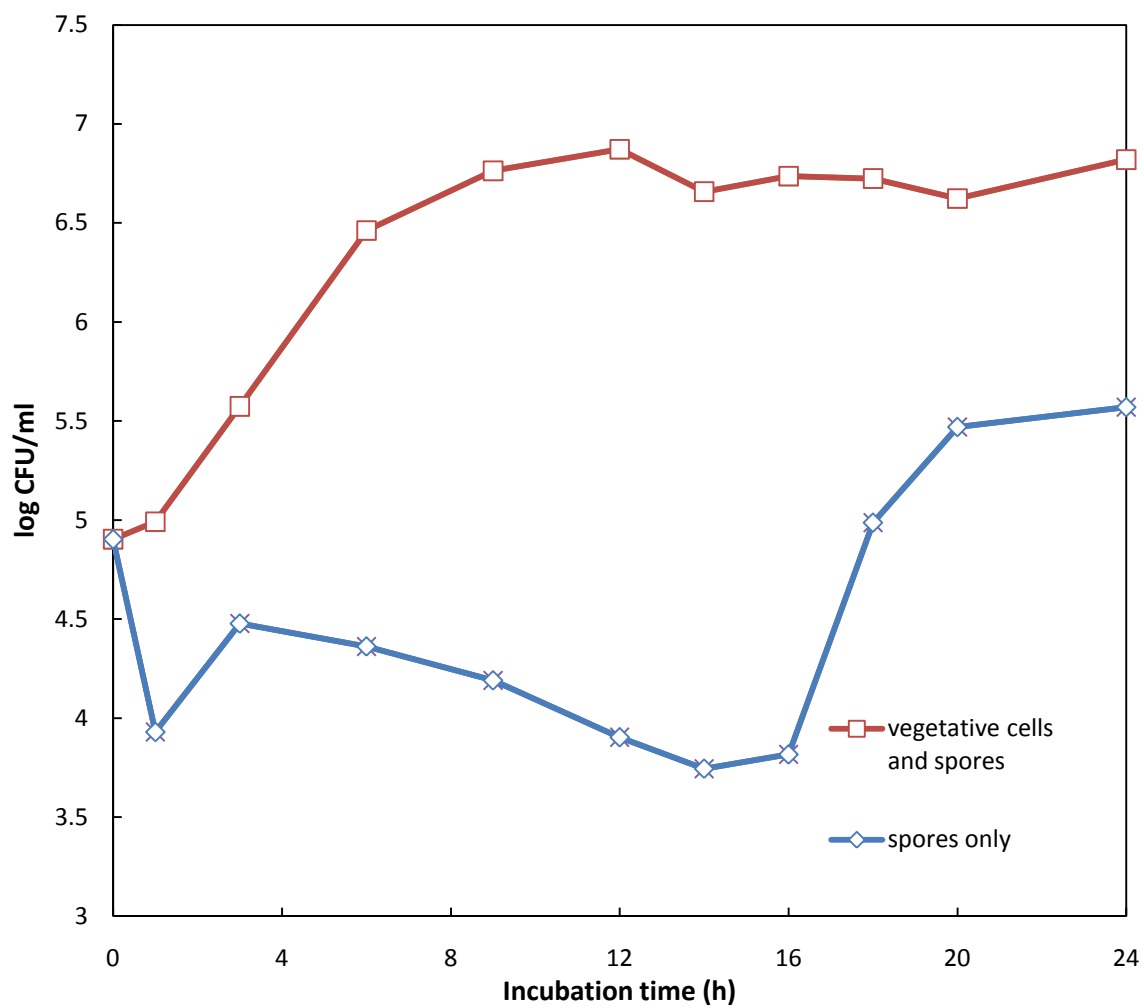


Figure 2.1. Vegetative cell and spore populations of *A. acidoterrestris* N-1100 in BAT broth incubated at 44°C.

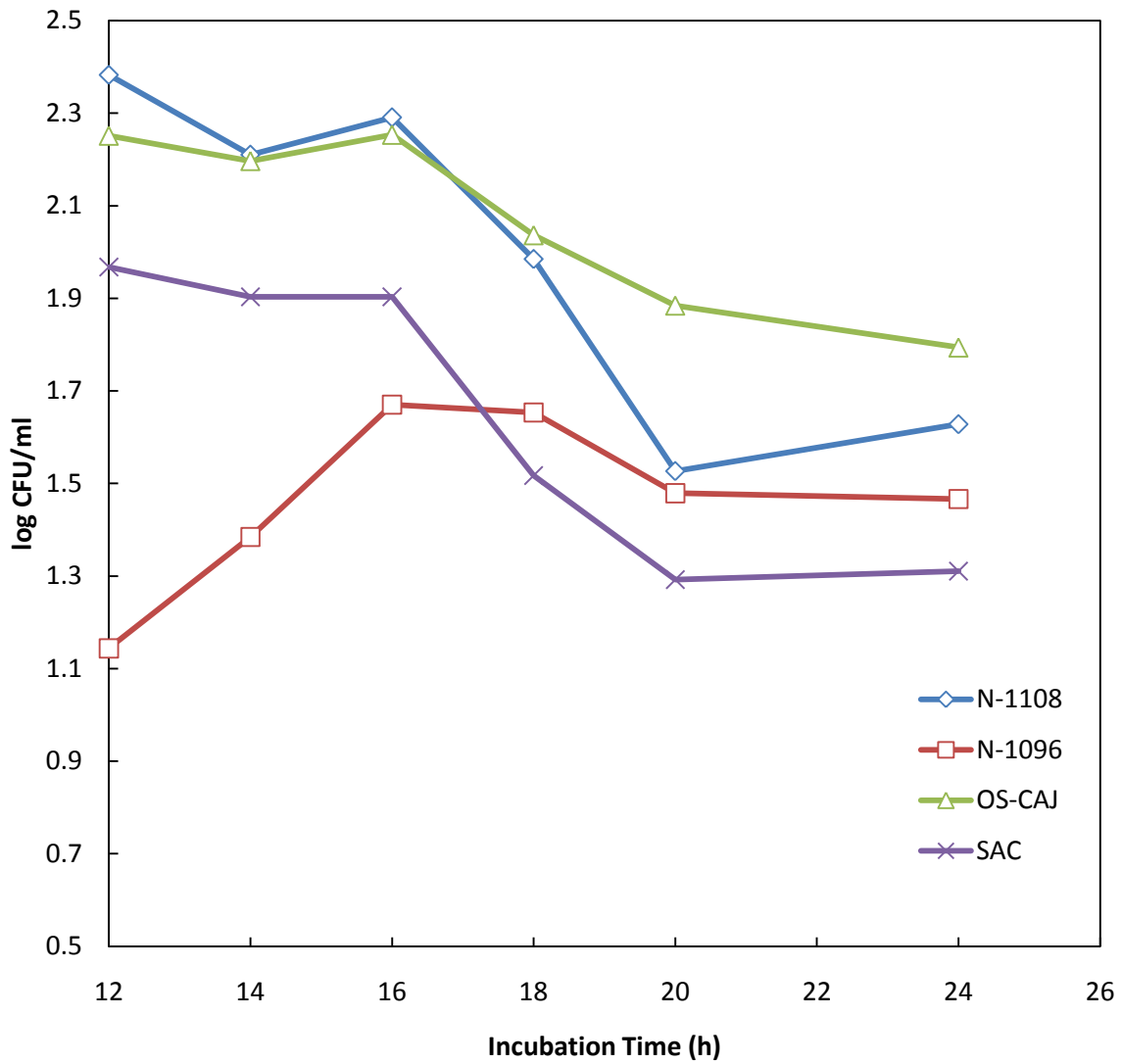


Figure 2.2. Vegetative cell populations of *A. acidoterrestris* N-1108, N-1096, OS-CAJ, and SAC in BAT broth incubated at 44°C.



## **Chapter III**

### **Inactivation of *Alicyclobacillus acidoterrestris* using high pressure homogenization**

## ABSTRACT

High pressure homogenization (HPH), a method commonly used in the dairy industry for emulsion stabilization, has been recognized as a non-thermal, novel technology effective at microbial inactivation and as a possible alternative to heat treatment for food preservation. The aim of this study was to evaluate the inactivation effects of high pressure homogenization on the spoilage bacterium, *Alicyclobacillus acidoterrestris*, in a broth system. Vegetative cells and spores of five strains of *A. acidoterrestris* (SAC, OS-CAJ, N-1008, N-1100 and N-1096) in *Bacillus acidoterrestris* thermophilic (BAT) broth were exposed to high pressure homogenization processing with pressure treatments of 0, 100, 200 and 300 MPa. Survivors after HPH treatment were serially diluted in 0.1% peptone water and surface plated onto BAT agar plates. Survivor populations were determined after incubation for 48 h at 44°C. Results revealed that vegetative cells were more susceptible to HPH than spores. Homogenization at 100 MPa caused significant reduction (over 90%) of vegetative cells of strains OS-CAJ, N-1108, N-1100 and N-1096. However, higher pressures did not significantly enhance the inactivation effect. As the pressure was increased from 100 to 300 MPa, only an addition 0.1- to 0.4-log reduction was achieved. SAC was most resistant to HPH, even at the highest pressure tested (300 MPa), with only a 0.5-log reduction. For spores, no significant reduction of any strain was observed with any of the HPH treatments. HPH can be a promising method for controlling of vegetative cells of *A. acidoterrestris*, but it is not effective for controlling spores.

## INTRODUCTION

Spoilage is a major problem of fruit based products because of the unique physical properties of fruits. Although typically not a health issue, spoilage can cause consumer rejection of products and significant economic loss to the juice and beverage industry. The low pH of fruit products favors growth of yeast, mold, and acid-tolerant bacteria.

Fruit products were not considered a natural reservoir for spore-forming bacteria until 1982 when a large number of pasteurized apple juices in Germany were reportedly spoiled (Cerny et al., 1984). The microorganism responsible for the spoilage was later identified and named *Alicyclobacillus acidoterrestris* – a spore-forming bacterium whose growth is favored by high temperature and low pH. Spoilage by *A. acidoterrestris* occurs primarily when fruits contaminated by soil during production or harvest (Chang and Kang, 2004). Spoilage is difficult to detect since no gas is produced and no unusual appearance of the juice container is observed. Spores of *A. acidoterrestris* are highly heat resistant and can survive current juice pasteurization processes, then later germinate and grow. The D-value of *A. acidoterrestris* at 95°C in orange juice (pH 4.1) was reported to be 5.3 min (Baumgart et al., 1997). During growth in beverages, guaiacol and halophenols (2, 6-dibromophenol and 2, 6-dichlorophenol) are produced, resulting in off-flavors and odors.

The thermal resistance of *A. acidoterrestris* spores has prompted food scientists to search for other non-thermal techniques for its inactivation to increase the shelf-life of juice products. Moreover, non-thermal techniques can help to prevent thermal damage to products. High pressure homogenization (HPH) has been suggested as a substitute for heat pasteurization

in fruit juice products. HPH is a new generation of homogenization and can reach pressures 10 to 15 times greater than traditional homogenizers. HPH is primarily used for preparation or stabilization of dispersions or emulsions to create physical changes in products in the chemical, pharmaceutical, and food (dairy) industries (Wuytack et al., 2002). Researchers have discovered the cell disruption capability of HPH (Hetherington et al., 1971; Kelemen and Sharp, 1979; Engler, 1990), which suggests its potential application in microbial inactivation. The major components of HPH are a displacement pump and a homogenizing valve. Fluids are pumped into the valve where cell disruption occurs.

Much research has been done to study microbial inactivation by HPH, particularly its effect on bacteria. Significant reduction of bacterial populations, such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella* Typhimurium, were reported in juice products (Pathanibul et al., 2009; Wuytack et al., 2002). It was concluded that Gram-negative bacteria are more sensitive than Gram-positive bacteria to HPH because Gram-negatives have a thinner cell wall with less peptidoglycan, thus reducing their resistance to mechanical forces. Yeasts are normally susceptible to HPH, with one pass through the homogenizer at 170 MPa reported to achieve 100% inactivation of *Saccharomyces cerevisiae* (Brookman, 1974).

The object of this study was to investigate the effectiveness of HPH with pressure ranging 0-300 MPa for inactivation of vegetative cells and spores of five strains of *A. acidoterrestris* in a broth system.

## MATERIALS AND METHODS

**Bacterial strains and media preparation.** Five strains of *Alicyclobacillus acidoterrestris* were used. All five strains (N-1096 (ATCC 4905), N-1108, N-1100, OS-CAJ and SAC) were obtained as spore suspensions in sterile deionized water from the Dr. Larry Beuchat, University of Georgia Center of Food Safety. Sources for these strains are: N-1096, garden soil, Germany; N-1108, apple-cranberry juice; N-1100, OS-CAJ, and SAC, apple juice concentrate. New spore stocks were prepared for each strain (Chapter 2) and stored at -20°C.

Media used in this experiment were: *Bacillus acidoterrestris* thermophilic (BAT) broth/agar: 2.0 g yeast extract, 5.0 g glucose, 0.25 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.6 g KH<sub>2</sub>PO<sub>4</sub>, 1000 ml deionized water and 1 ml Trace Minerals Solution: 0.066 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.018 g ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.016 g CuSO<sub>4</sub>, 0.015 g MnSO<sub>4</sub>·H<sub>2</sub>O, 0.018 g CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.01 g H<sub>3</sub>BO<sub>3</sub>, 0.03 g Na<sub>2</sub>MoO<sub>4</sub>, 100 ml of deionized water.

BAT agar: BAT broth plus 18 g agar per liter. After autoclaving, pH was adjust to 4 with 1.7 ml sterile 1N H<sub>2</sub>SO<sub>4</sub>.

**Sample preparation.** Vegetative cell preparation: After thawing spore stocks of all five strains, 30 µl of each stock was inoculated into 20 ml BAT broth and heat shocked at 80°C for 10 minutes in a water bath (VWR Scientific, Model 1136, Niles, IL). Inoculated broths were incubated at 44°C for 16 h (condition required for highest vegetative cell production, see Chapter 2). After incubation, 20 ml of each strain was inoculated into 480 ml BAT broth to obtain a population of approximately 6 log CFU/ml. All samples were stirred for two min before HPH processing.

Spore samples preparation: Three ml of thawed spore stock of each strain was inoculated into 497 ml of BAT broth. The population of spore samples was about 6 log CFU/ml. All samples were stirred for two min before HPH processing.

**High pressure homogenization processing.** Samples were processed through an FPG 12500 bench-top high pressure homogenizer (Stansted Fluid Power, Ltd., Essex, UK). Processed samples were collected into a sterile container from the homogenizer after four different pressure treatments (0 100, 200, and 300 MPa) and immediately placed on ice. The homogenizer consisted of two high pressure pumps and a two-stage homogenization valve. A water bath set at 2°C was connected to the homogenizer to control the outlet temperature. The actual chamber pressure and temperature were monitored and recorded using the supervisory control and data acquisition software package Lookout, version 5.1 and Labview, version 7.1 (National instrument, Austin, TX, USA).

**Enumeration of survivors.** Processed samples were serially diluted in 0.1% peptone (Difco) water and surface plated in duplicate onto BAT agar plates. Following incubation at 44°C for 48 h, colonies were counted and CFU/ml of the samples were calculated.

**Statistical analysis.** Experiments were repeated three times for each strain and each cell type (vegetative cells and spores). Data were fit to a completely randomized experiment design (CRD) with a split-plot treatment. Treatments (cell type and strain) were factorial and applied to the whole experimental unit and four levels of pressure treatment were applied to the sub-experiment unit. Analysis of variance was conducted using Statistical Analysis Software (SAS), version 9.2 (SAS institute, Inc., Cary, NC). Significance of differences was defined at  $P < 0.05$ .

## RESULTS AND DISCUSSION

Figures 3.1 and 3.2 show inactivation of vegetative cells and spores, respectively, of five strains of *A. acidoterrestris* using HPH processing. Data are the average of three replications. As seen in Figure 3.1, HPH significantly reduced the vegetative cell population ( $P < 0.05$ ). A further decrease of vegetative cell population was accompanied by increasing pressure. Low pressure (100 MPa) of HPH caused significant reduction of vegetative cells of N-1100, OS-CAJ, N-1096 and N-1108: 1.22-, 1.28-, 1.23-, and 1.02-log reductions, respectively. However, as the pressure was increased from 100 to 300 MPa, only an additional 0.1- to 0.4-log reduction was achieved. SAC was most resistant to HPH, even at the highest pressure tested (300 MPa), with only a 0.5-log reduction. For spores, no significant reduction of any strain was observed with any of the HPH treatments. Overall, OS-CAJ was the least resistant strain to HPH, with a reduction of 1.7 log CFU/ml at the highest pressure.

Spores were more resistant to HPH processing, with less than a 0.5-log reduction, even at the highest pressure, for all five strains. The inactivation effects of all HPH treatments were not significantly different ( $P > 0.05$ ). For OS-CAJ, the population of spores increased 0.2 log after 200 MPa pressure treatment, suggesting that HPH does not damage spores, but may activate spores into germination.

Results suggest that vegetative cells of *A. acidoterrestris* are much more sensitive to HPH than spores. Samples of 16 h cultures contain about 99.9% vegetative cells, as determined in Chapter 2. At 300 MPa, up to a 1.7-log reduction of vegetative cells of N-1100, OS-CAJ, N-1096 and N-1108 was observed. The effect of HPH treatment can be attributed to damage to

the cell outer membrane or loss of cell wall integrity (Vannini et al., 2004). The fact that vegetative cells of SAC strain showed greater resistance to HPH indicates that the effectiveness of HPH against *A. acidoterrestris* is strain dependent. It is possible that SAC may have a different composition in its cell membrane than other strains. A different distribution of fatty acids in the cell membrane may result in significantly different interactions and exposure of proteins to homogenization (Bevilacqua et al., 2007).

Endospores are the dormant stage of bacteria that are produced in response to nutrient limitations or harsh environmental conditions such as drying, heat, irradiation, high pressure, and toxic chemicals. Bacterial spores have a very different structure than that of vegetative cells, including a double membrane, spore coats, presence of dipicolinic acid, calcium, and DNA-binding proteins. The unique structure of spores contributes to their exceptional resistance to stresses, thereby protecting them from adverse conditions (Bauman, 2007).

The inactivation mechanism and effects of HPH differed substantially from high hydrostatic pressure (HHP) treatments. HHP is capable of altering structural and functional properties of cell components, damaging the cell membrane (Shirgaonkar et al., 1998). Within the same pressure range of HHP and HPH, large differences in bacteria responses have been reported (Wuytack et al., 2002). The microbial inactivation mechanisms of HPH have been widely discussed, but still remain an area of debate. Turbulence, cavitation, shear stress, and impingement have all been proposed as inactivation mechanisms (Doulah et al., 1981; Engler and Robinson, 1981; Save et al., 1994). High pressure itself during HPH may not be a major effect for inactivation since microorganisms are only exposed to the high pressure for a very short period of time (one second or less) (Wuytack et al., 2002).



Results of this study were consistent with those reported by Bevilacqua et al. (2007). These researchers studied the susceptibility of three strains of *A. acidoterrestris* to HPH (5-170 MPa) in acidified malt extract broth (pH 4.5). Their results showed that resistance to HPH was strain-dependent, with 0.25- to 1.68-log reduction of vegetative cell populations and only 0.3- to 0.67-log reduction of spores at 170 MPa.

Although inactivation effects of HPH against various microorganisms have been reported, the susceptibility of bacterial spores by HPH has not been widely studied. Popper and Knorr (1990) stated that spores of *Bacillus* spp. and *Clostridium* spp. are greatly resistant to any treatments, including homogenization. Feijoo (1997) reported only a 0.5-log reduction of *Bacillus licheniformis* spores in an ice cream mix after treatment with 200 MPa HPH.

## CONCLUSION

Results of this study reveal that HPH can be a promising method for controlling of vegetative cells of *A. acidoterrestris*, but it is not effective for controlling spores. In order to improve its effects against spores, future investigations should focus on multiple runs of HPH processing or HPH combined with other physical or chemical treatments.

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## APPENDIX

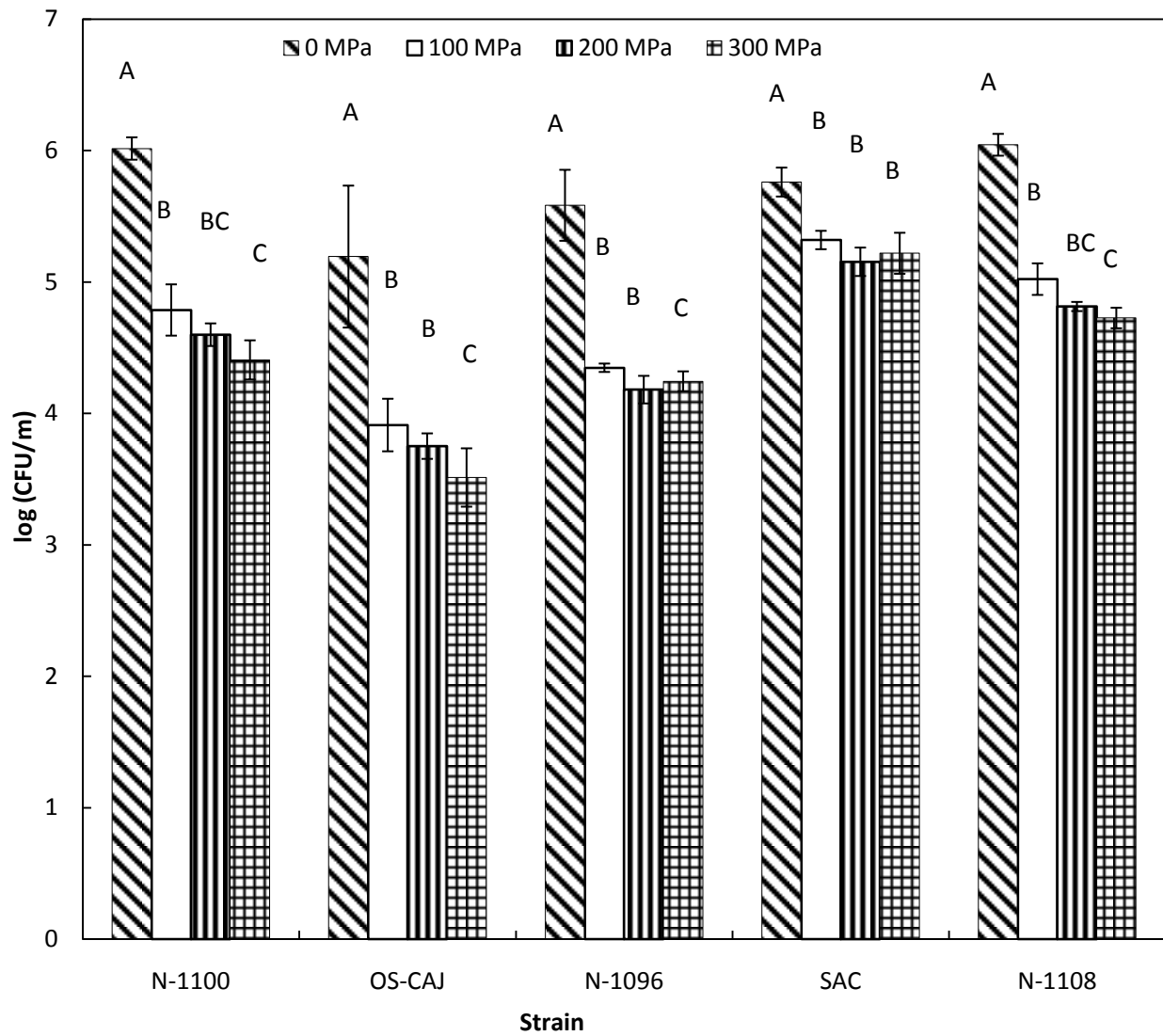


Figure 3.1. Inactivation of vegetative cells of *A. acidoterrestris* in BAT broth using HPH.

Error bars represents the standard deviation of the means.

Treatments with the same letter(s) are not significantly different ( $P > 0.05$ ).

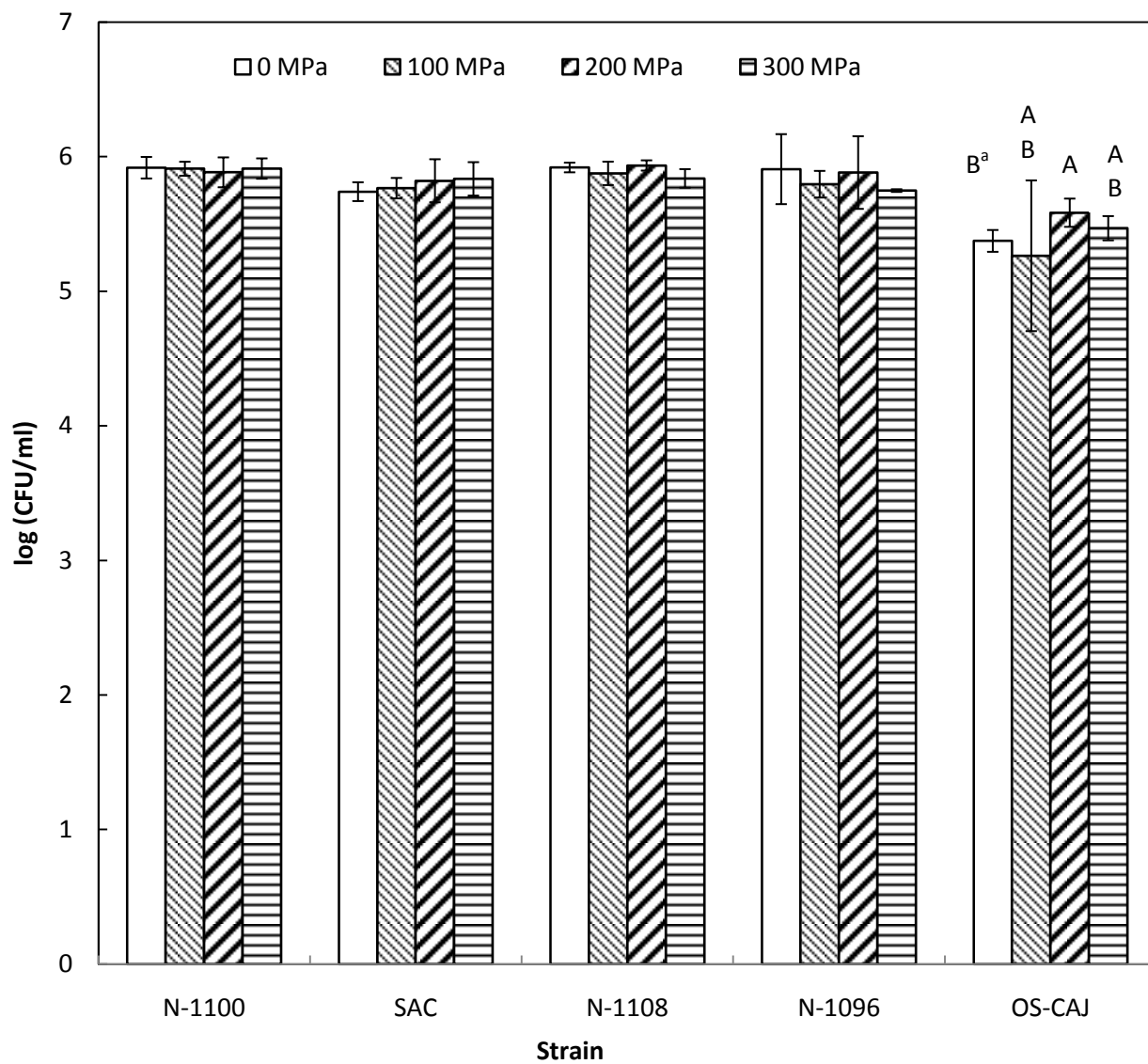


Figure 3.2. Inactivation of spores of *A. acidoterrestris* in BAT broth using HPH.

<sup>a</sup> All treatments for N-1100, SAC, N-1108 and 1096 were not significantly different ( $P > 0.05$ ). For OS-CAJ, treatments with the same letter(s) are not significantly different ( $P > 0.05$ ).

## **Chapter IV**

### **Control of *Alicyclobacillus acidoterrestris* growth using dimethyl dicarbonate**

## ABSTRACT

*Alicyclobacillus acidoterrestris*, a unique spoilage bacterium associated with fruit products, was tested for its susceptibility to the antimicrobial, dimethyl dicarbonate, (DMDC) in a broth system. Vegetative cells and spores of two strains of *A. acidoterrestris* (SAC and OS-CAJ) in *Bacillus acidoterrestris* thermophilic (BAT) broth were each treated with 0 or 250 ppm DMDC and incubated at 44°C. The effect of DMDC on growth of each strain and each cell type (vegetative cells and spores) was determined by serially diluting samples in 0.1% peptone water and surface plating onto BAT agar at selected intervals over a 48-h period. Populations were determined after incubation at 44°C for 48 h. DMDC significantly controlled ( $P < 0.05$ ) both vegetative cells and spores of both strains for up to about 24 h. DMDC effectively slowed growth of both strains by reducing their initial numbers and extending their lag phases. For both strains, spores were more resistant to DMDC than vegetative cells. Populations of vegetative cells of both strains reached 7 log CFU/ml in BAT broth containing 250 ppm DMDC after 24 h incubation, while only 10 h was required to reach the same population in broth without DMDC. Only a 2-h difference in time to reach the same level of growth was required when spores were cultured with (12 h) or without DMDC (10 h). These results demonstrate that DMDC can be a promising aid for controlling growth of *A. acidoterrestris*, especially vegetative cells. However, in order to achieve better control of spores, a higher concentration of DMDC or combining other processing methods may be necessary.

## INTRODUCTION

Food antimicrobials are chemical compounds applied to foods to inhibit growth or kill microorganisms. They are categorized as food preservatives and have been applied in the food industry for years and still play an important role in food preservation. Antimicrobials such as sodium benzoate and potassium sorbate have been widely used in foods. Currently, with increased awareness of foodborne illness and outbreaks, consumers have developed higher expectations towards food safety and quality. In order to provide foods that are free of foodborne pathogens with superior quality and longer shelf-life, many studies have been focused on the development of appropriate and effective antimicrobials for different food products.

Dimethyl dicarbonate (DMDC;  $\text{CH}_3\text{OOCOCOOCH}_3$ ) is a colorless, fruity-smelling liquid that functions as an antimicrobial in juice and beverage products. It was first used as a yeast inhibitor in wines in 1988 as an alternative to diethyl dicarbonate (FDA, 1988). DMDC is now approved as a direct food additive to be used as an antimicrobial control agent in wine, teas, and carbonated and noncarbonated non-juice or juice beverages in which the microbial population has been reduced to 500 CFU/ml or less by current good manufacturing practices (FDA, 2001).

DMDC is commonly used in wine with yeasts as its primary targets. Yeasts, including *Saccharomyces*, *Zygosacchomyces*, *Candida*, and *Pichia* are very sensitive to DMDC (Costa et al., 2008). DMDC has also been shown to be effective in inactivating molds and yeasts in tomato juice (pH 3.7) stored at 5°C (Bizri and Wahem, 1994). Fisher and Golden (1998) found that



DMDC was more effective than sodium benzoate and sodium bisulfate for controlling *Escherichia coli* O157:H7 at 4 and 10°C in apple cider. With the addition of 250 ppm DMDC, the population of *E. coli* O157:H7 was reduced from 7 log CFU/ml to undetectable levels within 3 days at 4°C.

*Alicyclobacillus acidoterrestris* has been recognized since 1967 when it was isolated from an acidic hot spring in Japan (Uchino and Doi, 1967). It was not considered a problem in the food industry until the first reported spoilage incident of *A. acidoterrestris* on a large scale in pasteurized apple juice from Germany in 1982 (Cerny et al., 1984). Since that time, several spoilage incidents reported worldwide have been associated with *A. acidoterrestris* in different food products including fruit juices, isotonic water, lemonade, carbonated fruit drinks, ice tea and even canned diced tomatoes (Duong and Jensen, 2000; Jensen, 2000; Pettipher and Osmundson, 2000; Walls and Chuyate, 1998; Yamaziki et al., 1996).

*A. acidoterrestris* is a spore-forming, thermophilic bacterium that can survive and grow at low pH, allowing it to cause spoilage of shelf-stable, fruit-based products. Its unique thermal-acidophilic characteristics are due to the presence of  $\omega$ -alicyclic fatty acids (Kannenberget al., 1984). *A. acidoterrestris* is not a pathogen and does not cause illness (Walls and Chuyate, 2000). The major source of contamination is soil. Juice products spoiled by *A. acidoterrestris* manifest off-flavors and odors which are often described as “phenolic”, “medicinal,” and “smoky”. The chemical metabolic contributed to the undesirable smell or flavor are guaiacol and halophenols (2, 6-dibromophenol and 2, 6-dichlorophenol). Spores of *A. acidoterrestris* are extremely heat resistant and can survive common thermal treatments used in the juice industry. Silva and

Gibbs (2001) suggested that spores of *A. acidoterrestris* should be used as a reference microorganism to design pasteurization processes for acidic fruit products.

Many antimicrobials including benzoate, bacteriocins, essential oils, and lysozyme have been studied and show promise at controlling *A. acidoterrestris*: 100 ppm cinnamaldehyde inhibited 96-97% *A. acidoterrestris* growth in malt extract broth after eight days storage at 44°C, while a higher concentration of eugenol (500 ppm) was required to completely inhibit growth for 13 days (Bevilacqua, et al., 2008). Nisin effectively inhibited *A. acidoterrestris* growth in laboratory broth media and in commercial juice products with greater effects against spores than vegetative cells (Komitopoulou et al., 1999; Yamazaki, et al., 2000). Another bacteriocin, enterocin AS-48, also showed remarkable inhibitory effects against *A. acidoterrestris*. Concentrations as low as 2.5 ppm can efficiently reduce vegetative cell populations to undetectable levels for prolonged periods of time (Grande et al., 2005). Amalia et al. (2006) developed a polyvinylalcohol film immobilized with lysozyme and achieved a loss of viable spores of *A. acidoterrestris*.

The goal of this study was to investigate the inhibitory effect of 250 ppm DMDC on *A. acidoterrestris* vegetative cells and spores in a broth system over a 48-h period.

## **MATERIALS AND METHODS**

**Bacterial strains and media preparation.** Two strains of *A. acidoterrestris* OS-CAJ and SAC (apple juice concentrate isolates; obtained as spore suspensions in sterile deionized water from Dr. Larry Beuchat, University of Georgia Center of Food Safety; least and most resistant strains, respectively, to high pressure homogenization, as determined in Chapter 3) were used

for this study. New spore stocks were prepared for each strain (see Chapter 2) and stored at -20°C.

Media used in this experiment were: *Bacillus acidoterrestris* thermophilic (BAT) broth/agar: 2.0 g yeast extract, 5.0 g glucose, 0.25 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.6 g KH<sub>2</sub>PO<sub>4</sub>, 1000 ml deionized water and 1 ml Trace Minerals Solution: 0.066 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.018 g ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.016 g CuSO<sub>4</sub>, 0.015 g MnSO<sub>4</sub>·H<sub>2</sub>O, 0.018 g CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.01 g H<sub>3</sub>BO<sub>3</sub>, 0.03 g Na<sub>2</sub>MoO<sub>4</sub>, 100 ml of deionized water.

BAT agar: BAT broth plus 18 g agar per liter. After autoclaving, pH was adjust to 4 with 1.7 ml sterile 1N H<sub>2</sub>SO<sub>4</sub>.

**Sample preparation.** Vegetative cell preparation: After thawing spore stocks of each strain, 15 µl were inoculated into 10 ml BAT broth and heat shocked at 80°C for 10 minutes in a water bath (VWR Scientific, Model 1136, Niles, IL). After samples were chilled, they were incubated at 44°C for 16 h (time required for highest vegetative cell production, see Chapter 2). A 16-h culture (43°C) of each strain was separately inoculated (45 µl) into 30 ml of BAT broth prior to DMDC treatment.

Spore sample preparation: Spore stocks of each strain was thawed and 45 µl were inoculated into 30 ml of BAT broth and heat shocked at 80°C for 10 minutes in a water bath (VWR Scientific, Model 1136, Niles, IL). Samples were chilled prior to DMDC treatment.

**DMDC treatment.** Vegetative cell and spores were each divided into two equal volume portions. DMDC (Sigma, St. Louis, MO) at a concentration of 250 ppm was added to one portion of each sample and vortexed for two minutes, while the other portion contained no DMDC and served as the control. After adding DMDC, broths were incubated at 44°C for 24 h. Samples of

each treated broth were taken at specified time intervals, diluted in 0.1% peptone water (Difco), and surface plated in duplicate onto BAT agar plates. All plates were incubated at 44°C for 48 h after which colonies were enumerated and CFU/ml were determined.

**Statistical analysis.** The experiment was repeated three times for each strain and each cell type. Data were fit to a complete randomized experiment design (CRD) with repeated measures treatment. Analysis of variance was conducted using SAS, version 9.2 (SAS institute, Inc., Cary, NC, USA). Significance of differences was defined at  $P < 0.05$ .

## RESULTS AND DISCUSSION

Comparison of growth of vegetative cells and spores for each strain with or without 250 ppm DMDC treatment are shown in Figures 4.1-4.4. Within each cell type, the inactivation effects of DMDC over 48 h was not significantly different between the two strains ( $P > 0.05$ ). Vegetative cells were more susceptible to DMDC than spores. As seen in Figures 4.2 and 4.4, the population of the control samples of both strains gradually increased and reached 7.14 log CFU/ml after 10 h incubation and did not increase further during the remainder of the 48-h incubation. With the 250 ppm DMDC treatment, cell populations first significantly decreased from 4.84 to 3.13 log CFU/ml (SAC) and 4.14 to 2.26 log CFU/ml (OS-CAJ), but then after 6 h incubation, populations steadily increased until 24 h, when the population reached 7.39 and 7.34 log CFU/ml for SAC and OS-CAJ, respectively.

Spores were less affected by DMDC (Figure 4.1 and 4.3). For OS-CAJ, the population of spores was reduced by DMDC from 5.24 to 4.89 log CFU/ml for 2 h, while a 4-h inhibition was observed for strain SAC, with a decrease in population from 5.18 to 4.92 log CFU/ml. After the

brief periods of inhibition, populations of both strains increased. There was a 2 h difference in the time required for the population of both strains to reach about 6.7 log CFU/ml with (10 h) or without DMDC (12 h). Samples of 16 h cultures contained about 99.9% (3 log) vegetative cells, as determined in chapter 2. DMDC treatment reduced populations of SAC and OS-CAJ to 1.71 and 1.88, respectively.

There are two possible factors associated with the fact that DMDC had less impact on spores. First, the unique structure of spores enables them to survive harsh environmental stress, including toxic chemicals. Thus, spores may have higher tolerance of DMDC. Second, DMDC is an extremely reactive compound that can rapidly hydrolyze to methanol and CO<sub>2</sub>. It can be assumed that the hydrolysis of DMDC is complete before it could affect spores. It also can be concluded that DMDC is not suitable for long term spoilage protection because the antimicrobial activity of DMDC is lost once the compound hydrolyzes into methanol and CO<sub>2</sub> (Golden et al., 2005).

There is little documentation of the inactivation mechanism of DMDC. It has been suggested that the mechanism is similar to diethyl dicarbonate and strongly related to inactivation of microbial enzymes, with the dicarbonate group triggering protein modification (Golden et al., 2005). Moreover, the antimicrobial activity of DMDC can be affected by many physical and chemical characteristics such as temperature, pH, and amount of other reactive substances in the system. Higher temperatures can increase hydrolysis, while the hydrolysis rate can be significantly decreased by increasing ethanol concentration. Higher ethanol concentration (10% vs. 8%) with temperature slightly above 20°C was found to be most effective to control *S. cerevisiae* (Porter and Ough, 1982). In the present study, the incubation

temperature of 44°C is higher than the typical storage temperature of shelf-stable juice products (room temperature), which may increase the hydrolysis rate of DMDC and reduce its effectiveness against *A. acidoterrestris*.

## CONCLUSION

These results demonstrate that DMDC can be a promising aid for controlling growth of *A. acidoterrestris*, especially vegetative cells. However, in order to achieve better control of spores, a higher concentration of DMDC or combining other processing methods may be necessary. Further studies should focus on the inactivation effect of DMDC in juice or beverages while also using hurdle technology to enhance its effectiveness against spores.

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## APPENDIX

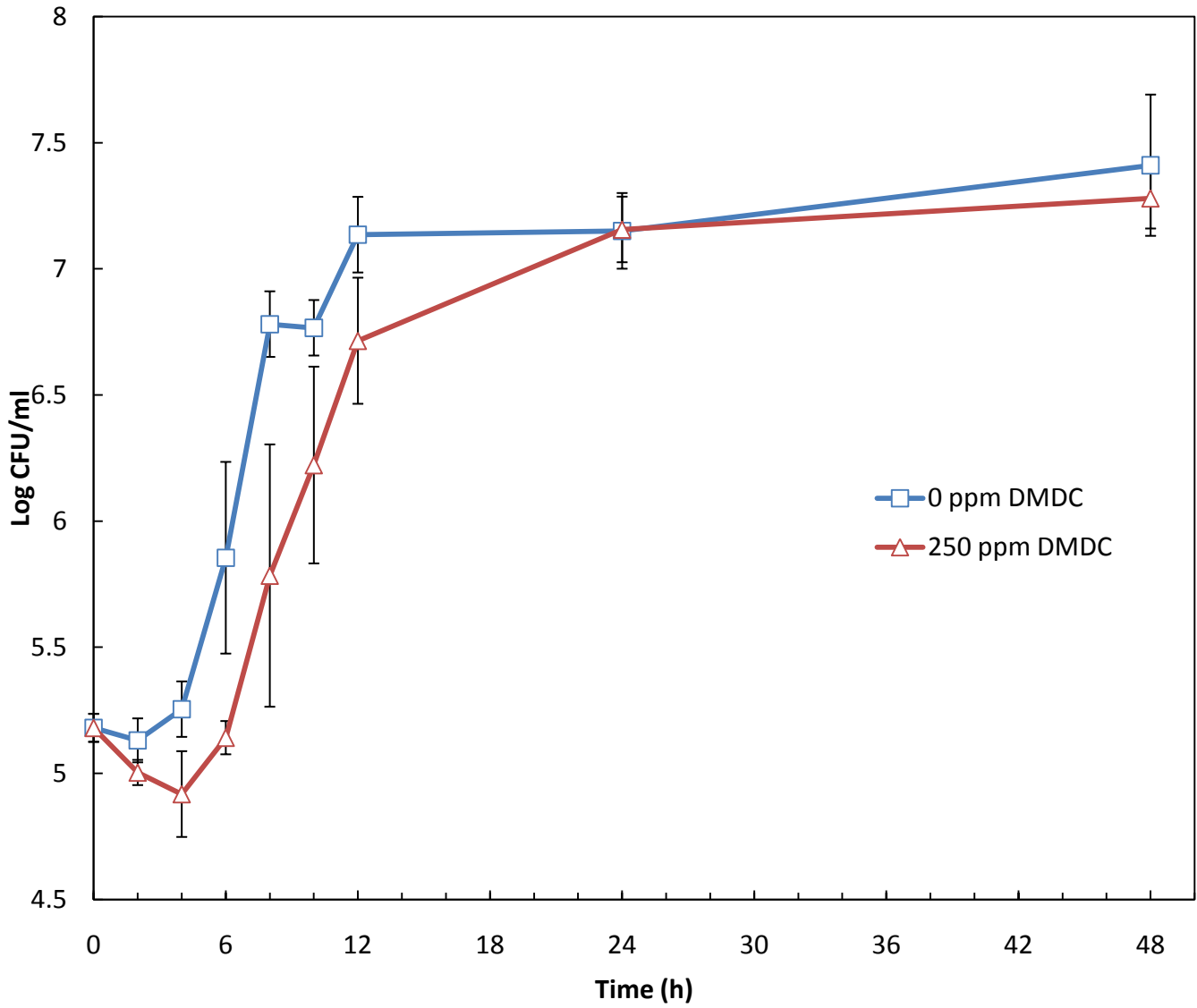


Figure 4.1. Inactivation of *A. acidoterrestris* SAC spores after heat shock (80°C, 10 min) in BAT broth, followed by incubation at 44°C in BAT broth containing 250 ppm DMDC.

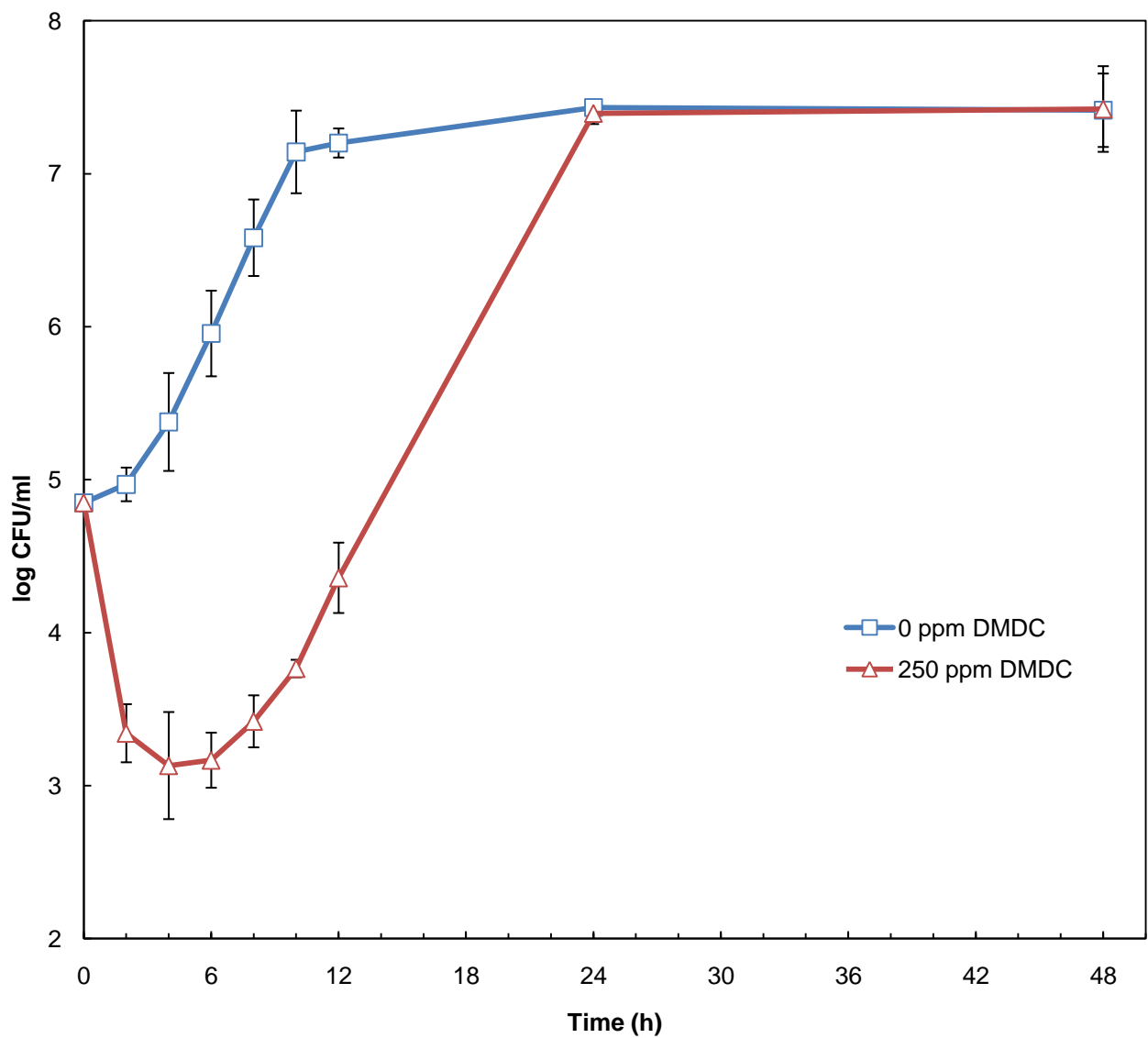


Figure 4.2. Inactivation of *A. acidoterrestris* SAC vegetative cells in BAT broth, followed by incubation at 44°C in BAT broth containing 250 ppm DMDC.

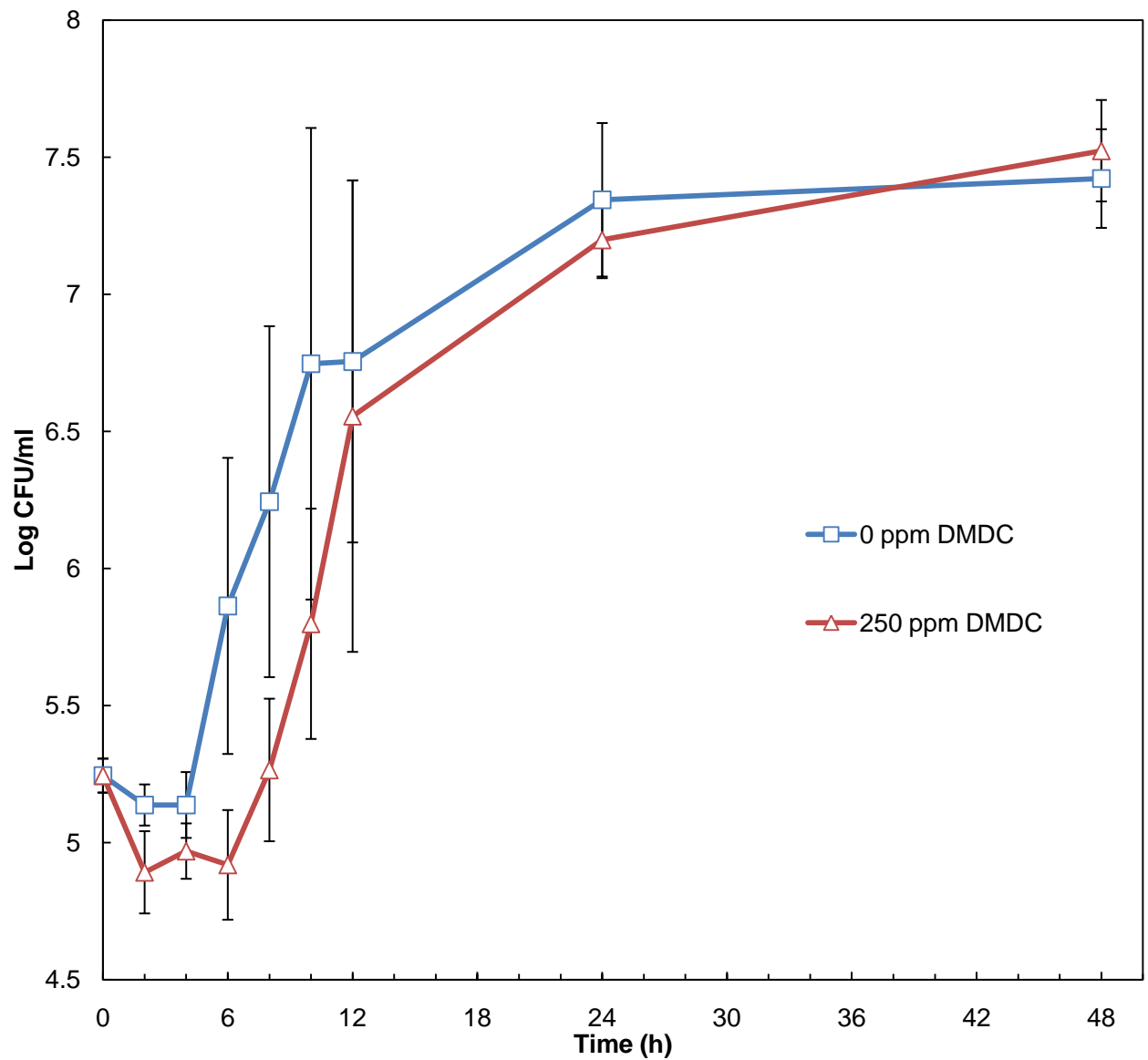


Figure 4.3. Inactivation of *A. acidoterrestris* OS-CAJ spores after heat shock (80°C, 10 min) in BAT broth, followed by incubation at 44°C in BAT broth containing 250 ppm DMDC.

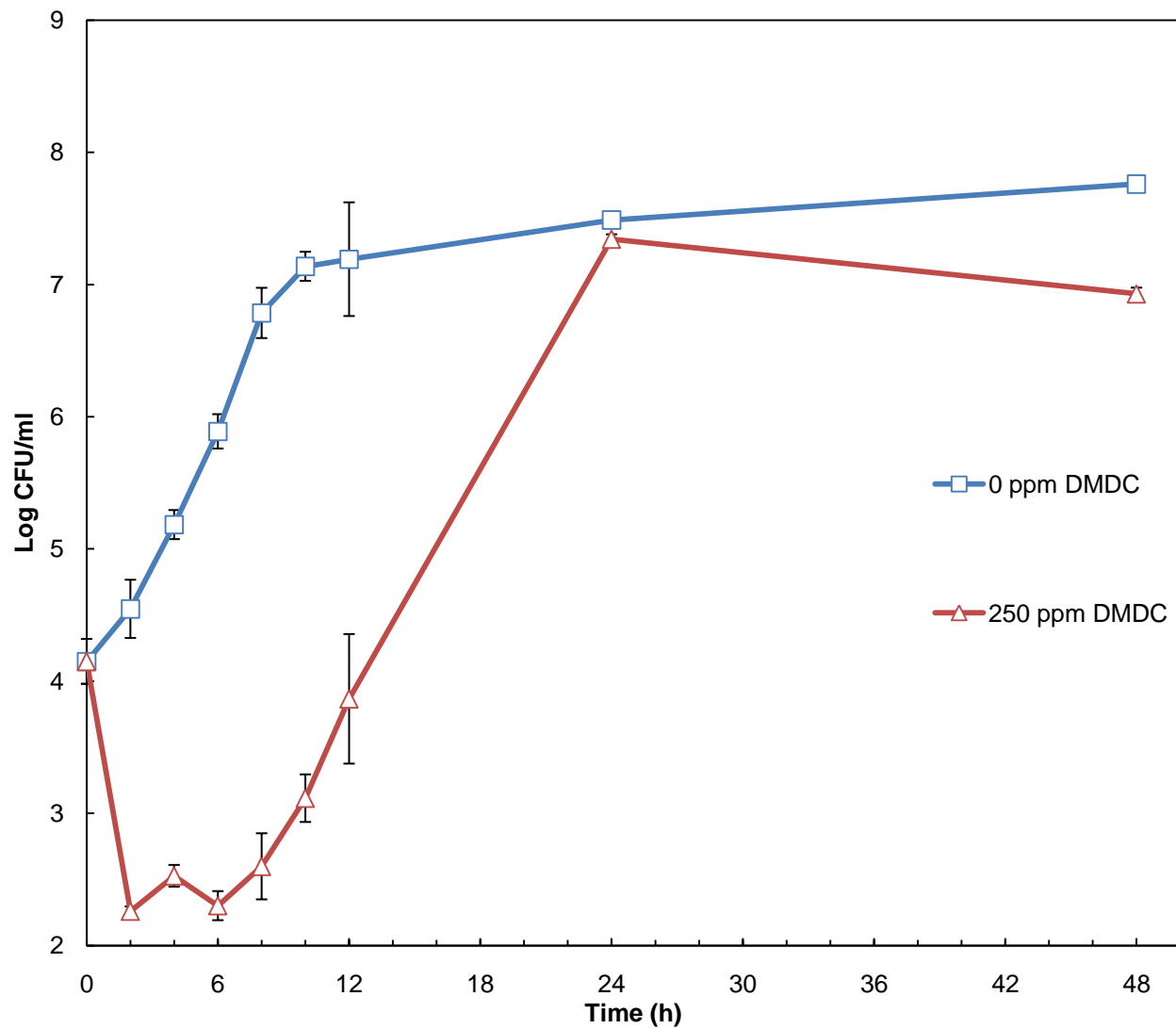


Figure 4.4. Inactivation of *A. acidoterrestris* OS-CAJ vegetative cells in BAT broth, followed by incubation at 44°C in BAT broth containing 250 ppm DMDC.

## **Chapter V**

**Inactivation of *Alicyclobacillus acidoterrestris* spores using  
four combinations of high pressure homogenization and  
dimethyl dicarbonate**

## ABSTRACT

The spoilage bacterium, *Alicyclobacillus acidoterrestris*, has become a major concern in the fruit juice industry because of its acid tolerance and heat resistance. This study aimed to evaluate the combined effect of the non-thermal technology, high pressure homogenization (HPH), and antimicrobial dimethyl dicarbonate (DMDC) against spores of *A. acidoterrestris* in a broth system. Spores of *A. acidoterrestris* strain SAC (from apple juice concentrate) in *Bacillus acidoterrestris* thermophilic (BAT) broth were treated with four different combinations of HPH and DMDC: 250 ppm DMDC added 12 h before, 2 h before, immediately before and immediately after 300 MPa HPH treatments. After all treatments, the population of survivors over a 24 h period at 44°C were determined by serially dilution and surface plating onto BAT agar at selected time intervals. After treatment with only 300 MPa HPH, the spore population was not significantly reduced and steadily increased to 7.88 log CFU/ml after 24 h incubation at 44°C. The four HPH treatments that included addition of 250 ppm DMDC were slightly superior to the post-HPH inactivation over 24 h by reducing the initial spore population (DMDC added 2 h before HPH) and slowing the post-HPH growth rate (DMDC added immediate before and after HPH). Overall, adding DMDC immediately before HPH was most effective compared to other treatments. However, it can be concluded that the combination of 300 MPa and 250 ppm DMDC did not significantly enhance inactivation of *A. acidoterrestris* SAC spores.

## INTRODUCTION

Application of heat treatment to destroy pathogenic and spoilage microorganisms is one of the oldest and most effective food preservation processes that is still widely used today. Since an outbreak of *Escherichia coli* O157:H7 in unpasteurized apple cider in 1991, heat pasteurization is the process most widely used in the fruit juice and beverage industry to ensure safety and extend shelf-life (Vojdani et al., 2008). About 98% of juice processors in the United States pasteurize their juice (FDA, 2001). However, for high-acid foods like fruit based products, no target and criterion for pasteurization is clearly defined because target microorganisms and their heat resistance vary with products. Additionally, the physical components of fruit products can be easily destroyed by heat.

*Alicyclobacillus acidoterrestris*, a spoilage bacterium of shelf-stable high acid foods, especially fruit products, has been suggested as a target of pasteurization (Silva and Gibbs, 2001). Spores of *A. acidoterrestris* are heat resistant, although the heat resistance of spores varies in different juices *A. acidoterrestris* spores can survive current pasteurization process used in the fruit juice industry. In order to control *A. acidoterrestris* and maintain the quality of products, non-thermal methods such as irradiation, high hydrostatic pressure treatment, microwaves, and ultrasound have been suggested.

Homogenization is a novel, non-thermal technology used for stabilization of emulsions and dispersions. It is widely used in food, chemical, pharmaceutical and cosmetic industries. Dairy processing is one of the oldest applications of homogenization. Fat globules in milk are broken down to a smaller size to prevent phase separation. High pressure homogenization

(HPH) was developed in the early 1990s as a new generation of homogenization. HPH can reach a much higher pressure so that a higher degree of dispersion or emulsion of the product can be achieved.

It is also suggested that HPH can cause cell disruption (Kelemen and Sharp, 1979; Engler, 1990). Microbial inactivation can be another application of HPH, potentially replacing standard heat treatments that could lead to deterioration in the quality of products. The microbial inactivation ability of HPH has been studied over the past few years. It has been reported that yeast cells and many Gram-negative bacteria can be successfully inactivated by HPH (Pathanibul et al., 2009; Wuytack et al., 2002; Brookman, 1974). Several different inactivation mechanisms have been proposed. Turbulent flow, stress, turbulence, shear caused by impingement of a high velocity jet on a stationary surface, and cavitation collapse have been suggested as possible mechanisms of cell disruption (Doulah et al., 1975; Engler and Robinson, 1981; Save et al., 1994).

The addition of antimicrobial compounds may enhance the level of microbial inactivation by HPH. Vogels and Kula (1992) reported 98% cell disruption of *Bacillus cereus* treated with 0.5 mg/g cellosyl before a single pass at 70 MPa homogenization, while less than 40% of cells were disrupted by homogenization without cellosyl treatment. For nonpathogenic *Escherichia coli* K12, a 2- to 3-log inactivation difference was found in nisin-treated versus nisin-free samples under about 200 MPa HPH treatments (Taylor et al., 2007).

Dimethyl dicarbonate (DMDC) is an antimicrobial approved for use in wine, juice and other beverages. The main target microorganisms are yeasts such as *Saccharomyces* and *Zygosaccharomyces* (Davidson et al., 2002). It has been reported that 250 ppm DMDC was



more effective than either sodium bisulfate or sodium benzoate against *Escherichia coli* O157:H7 (Fisher and Golden, 1998).

The objective of this study was to explore the effectiveness of different combinations of 300 MPa HPH with 250 ppm DMDC in controlling *A. acidoterrestris* spores in a broth system over a 24-h period.

## MATERIALS AND METHODS

**Bacteria strains and media preparation.** *Alicyclobacillus acidoterrestris* strain SAC (apple juice concentrate isolate; as spore suspension in sterile deionized water obtained from Dr. Larry Beuchat, University of Georgia Center for Food Safety) was used for this study. Spores of this strain were previously demonstrated to be the most resistant strain to high pressure homogenization (among five strains; see Chapter 3). Spore suspensions in sterile deionized water were used; spore stocks were prepared as described in Chapter 2 and stored at -20°C until used.

Media used in this experiment were: *Bacillus acidoterrestris* thermophilic (BAT) broth/agar: 2.0 g yeast extract, 5.0 g glucose, 0.25 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.6 g KH<sub>2</sub>PO<sub>4</sub>, 1000 ml deionized water and 1 ml Trace Minerals Solution: 0.066 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.018 g ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.016 g CuSO<sub>4</sub>, 0.015 g MnSO<sub>4</sub>·H<sub>2</sub>O, 0.018 g CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.01 g H<sub>3</sub>BO<sub>3</sub>, 0.03 g Na<sub>2</sub>MoO<sub>4</sub>, 100 ml of deionized water.

BAT agar: BAT broth plus 18 g agar per liter. After autoclaving, pH was adjust to 4 with 1.7 ml sterile 1N H<sub>2</sub>SO<sub>4</sub>.

**Sample preparation.** Three ml of thawed spore stock of SAC strain was inoculated into 497 ml of BAT broth to obtain a population of about 6 log CFU/ml. Inoculated broth was stirred for two minutes before treatments were applied.

**DMDC and HPH treatments.** Four different treatments were tested: 250 ppm DMDC added immediately before, 2 h before, 12 h before or immediately after 300 MPa HPH treatment. After inoculation, three samples were supplemented with 250 ppm DMDC and then were either treated directly with HPH or incubated at 44°C for 2 h or 12 h prior to HPH treatment. One sample was treated with HPH first, followed by addition 250 ppm DMDC immediately after HPH treatment. Inoculated broth treated with 300 MPa HPH, but without DMDC, served as a control.

Samples were processed through an FPG 12500 bench-top high pressure homogenizer (Stansted Fluid Power, Ltd., Essex, UK). Processed samples were collected into a sterile container from the homogenizer after four different pressure treatments (0 100, 200, and 300 MPa) and immediately placed on ice. The homogenizer consisted of two high pressure pumps and a two-stage homogenization valve. A water bath set at 2°C was connected to the homogenizer to control the outlet temperature. The actual chamber pressure and temperature were monitored and recorded using the supervisory control and data acquisition software package Lookout, version 5.1 and Labview, version 7.1 (National instrument, Austin, TX, USA).

**Enumeration of survivors.** Processed samples were serially diluted in 0.1% peptone (Difco) water and surface plated in duplicate onto BAT agar plates. Following incubation at 44°C for 48 h, colonies were counted and CFU/ml of the samples were calculated.

**Statistical analysis.** The treatment of 250 ppm DMDC added 12 h before 300 MPa HPH was repeated two times, while other treatment combinations were repeated three times. Data were fit to a completely randomized experiment design (CRD) with repeated measures over time treatment. Analysis of variance was done using SAS, version 9.2 (SAS institute, Inc., Cary, NC, USA). Significance of differences was defined at  $P < 0.05$ .

## RESULTS AND DISCUSSION

As seen in Table 5.1, the spore population was not significantly reduced (5.94 to 5.81 log CFU/ml,  $P > 0.05$ ) with a single 300 MPa HPH treatment (no DMDC). Under the post-HPH incubation conditions (44°C), a steady increase in population was observed, from 7.04 log CFU/ml after 6 h incubation to 7.88 log CFU/ml after 24 h. The addition of 250 ppm DMDC immediately before or after HPH enhanced the inactivation effect by slowing growth of *A. acidoterrestris* after HPH treatment. Under both treatments, the population after HPH processing was less than 6 log CFU/ml for 4 h and increased to about 7 log CFU/ml after 8 h. At 24 h, populations of both treatments reached the same level as the control (no DMDC) ( $P > 0.05$ ).

When samples were incubated for 12 h at 44°C with 250 ppm DMDC before HPH processing, the population increased from 5.87 to 6.45 log CFU/ml, followed by a 1-log reduction after 300 MPa HPH treatment (6.45 to 5.49 log CFU/ml). After HPH, the population increased after 4 h and reached 7.30 log CFU/ml at 8 h. The addition of 250 ppm DMDC two h before HPH first decreased the population of *A. acidoterrestris* from 5.85 log to 5.68 log CFU/ml. However, the 300 MPa HPH treatment did not intensify the inactivation effect. The

population after HPH treatment increased for 6 h and did not differ from the treatment without DMDC. These results indicate that 250 ppm DMDC slightly reduced the spore population within 2 h, but when added 12 h before HPH, the antimicrobial effectiveness was lost. DMDC is very reactive and when added to a substrate rapidly hydrolyzes to methanol and CO<sub>2</sub>. The hydrolysis products are not antimicrobial, which explains the short term activity of DMDC (Golden et al., 2005). The significant reduction by HPH of the sample treated with DMDC prior to HPH can be attributed to the fact that after 12 h of incubation, some spores germinated and grew into vegetative cells which are susceptible to HPH treatment (Chapter 3). From 8 h to 12 h after HPH, populations did not significantly increase from 8 h to 12 h after HPH and reached 7.55 log CFU/ml after 24 h.

These results demonstrate that 300 MPa HPH processing alone is not effective against *A. acidoterrestris* spores, which is consistent with the previous study (Chapter 3). However, different combinations of 250 ppm DMDC and 300 MPa HPH are superior to HPH alone over a 24-h period.

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## APPENDIX

Table 5.1. Inactivation of *A. acidoterrestris* SAC spores in BAT broth treated with different combinations of 300 MPa HPH and 250 ppm DMDC and then incubated at 44°C.

Sampling Time	Population (log CFU/ml)				
	No DMDC	DMDC added after HPH	DMDC added before HPH	DMDC added 2 h before HPH	DMDC added 12 h before HPH
2/12 h before HPH processing	ND <sup>a</sup>	ND	ND	5.85	5.87
Right before HPH Processing	5.94e <sup>b</sup> B <sup>c</sup>	5.94e B	5.61e C	5.68f C	6.45c A
Right after HPH processing	5.81e AB	5.81e AB	5.82de AB	5.94e A	5.67de B
2 h	5.94e A	5.82e A	5.84d A	5.94e A	5.49 e B
4 h	6.31d A	5.98 e BC	5.87d C	6.17d AB	5.87d C
6 h	7.04c A	6.47d B	6.44c B	6.97c A	6.51c B
8 h	7.51b A	7.21c BC	7.00b C	7.22b B	7.30ab AB
10 h	7.62b A	7.50b A	7.07b B	7.03bc B	7.12b B
12 h	7.57b A	7.28c B	6.88b C	7.20b B	7.39ab AB
24 h	7.88a AB	8.03a A	7.76a BC	7.57a C	7.54a C

<sup>a</sup> ND, not determined.

<sup>b</sup> Means in the same column that are followed by the same lowercase letter(s) are not statistically different (P>0.05).

<sup>c</sup> Means in the same row that are followed by the same uppercase letter(s) are not statistically different (P>0.05).

## **VITA**

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