GUAIACOL PRODUCING *ALICYCLOBACILLUS* SPP.

- DIFFERENTIATION, DETECTION, AND CONTROL

By

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A dissertation submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

WASHINGTON STATE UNIVERSITY
School of Food Science

DECEMBER 2008

To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of SU-SEN CHANG find it satisfactory and recommend that it be accepted.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my utmost gratitude to Dr. Dong-Hyun Kang, my advisor, for giving me the privilege to study here in the United States and his unwavering support and guidance throughout the course of my studies. In addition, his unique "hands-off" approach, though the reason for many struggles, has proved to be a blessing in disguise and ultimately enabled me to improve not only scientifically but as a person in general.

I am also greatly indebted my committee members, Dr. Dale Hancock, Dr. Joseph Powers, Dr. Barbara Rasco, and Dr. Barry Swanson for tolerating many delays and providing invaluable suggestions for my research and writing. I consider myself extremely lucky to have such an experienced committee with diverse expertise to assist me along the way.

Throughout my time here at Washington State University, I have had the good fortune of meeting many individuals that have greatly influenced me through their strive for excellence. In addition to my aforementioned committee members, I would like to express my gratitude to Dr. Charles Edwards, who has shown me that humor and dedication towards students can co-exist with seriousness and the demand for high quality. I am also very grateful to my lab members, Dr. Min-Suk Rhee, Dr. Se-Wook Oh, Dr. Joong-Han Shin, Dr. Sun-Young Lee and Mr. Peter Gray, whose devotion to research, either through long hours spent in the lab, creativity, meticulousness, or unrelenting drive for a solution, have inspired me to challenge my own abilities and do my very best.

Needless to say, it would have been impossible for me to complete my studies without my friends and "family" here in Pullman. I am especially thankful for the friendship and endless encouragement of Ms. Chia-Cheng Chung and my fellow lab member, Ms. A-Reum Han, during the most depressing time of my studies. Without them to jerk me out of the slump, I could not have come this far. I would also like to acknowledge our wonderful department secretaries, Ms. Jodi Anderson and Ms. Marsha Appel, who not only have the answers to everything but are always there to check up on our well-being, give advise, or simply a well-needed hug.

Last but not the least, I owe the successful completion of my graduate studies to my mom, dad, brother, and Butter. In retrospect, I realize that though I was the one studying, my family has experienced every accomplishment, disappointment, struggle, and stress with me. Thank you for being supportive of my decision to pursue an advanced degree and for always being there for me.

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Abstract

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Washington State University
December 2008

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Alicyclobacillus spp. are thermoacidophilic sporeformers that have significant impact on the pasteurized juice industry. Better detection and control methods are needed to minimize *Alicyclobacillus* related spoilage. The objective of this research is to improve existing detection and control methods by (1) the investigation of basic characteristics of guaiacol producing *Alicyclobacillus* and development of a novel detection method; and (2) the evaluation of *Alicyclobacillus* control method during storage and processing.

Differences in growth characteristics were observed but not significant between guaiacol producing and non-guaiacol producing *Alicyclobacillus*. Enzyme and carbohydrate utilization profiles were variable depending on test isolates. Guaiacol formation was detectable within 12 h at 43°C and further accelerated under microaerophilic conditions. The newly developed SK2 agar provides selective identification of guaiacol producing *Alicyclobacillus* with a 95% correct identification rate. SK2 agar is the first known media providing selective isolation of guaiacol producing *Alicyclobacillus*. The effectiveness of aqueous and gas chlorine dioxide against *Alicyclobacillus* spores has been demonstrated. Greater than five log reductions were observed following 1 min and 1 h exposures to 120 ppm $ClO_{2(aq)}$ and 4.32 ppm $ClO_{2(g)}$, respectively.

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Dedication

This dissertation is dedicated to my mother, father, brother,

and in remembrance of my grandmother and cousin.

"It was the best of times, it was the worst of times."

CHAPTER ONE

LITERATURE REVIEW

ABSTRACT

Since the isolation of *Alicyclobacillus acidocaldarius* in 1971, the genus *Alicyclobacillus* has received attention due to its unique thermoacidophilic properties and association with the spoilage of pasteurized fruit juices. This review is intended to provide an overview on the evolution and general characterisitics of the genus *Alicyclobacillus* with particular focus on the significance of *Alicyclobacillus* to the fruit juice/ beverage industry. Challenges and difficulties in quality control that *Alicyclobacillus* pose to the industry will be addressed. Novel technologies for *Alicyclobacillus* detection/control, recent trends in spoilage-related *Alicyclobacillus* research, and possible directions for future research are also discussed.

1. Thirty-five years of *Alicyclobacillus*– history and evolution

The history of the genus *Alicyclobacillus* began with the isolation of *Bacillus acidocaldarius* (later reclassified as *A. acidocaldarius*) from thermoacidic environments in Yellowstone National Park and Hawaiian Volcano National Park (Darland and Brock, 1971). The existence of thermophilic species within the genus *Bacillus* is well documented, but they are primarily adapted to alkaline or neutral habitats (Smith et al., 1952). One noted exception is *B. coagulans* which can grow at temperatures up to 55°C and at pH values as low as 4 (Becker and Pederson, 1950; Smith et al., 1952). *B. acidocaldarius* presented a second and more extreme deviation from the majority of *Bacillus* species due to the fact that *B. acidocaldarius* is strictly thermophilic and acidophilic, and could not be isolated through thermal and alkaline to neutral pH (pH 5-7) enrichments (Darland and Brock, 1971). DNA base content analysis further supported *B. acidocaldarius* as a novel species. Compared with the DNA guanine+cytosine content (mol% G+C) of known *Bacillus* species (36-53 mol% G+C) (Priest, 1981), including *Bacillus* thermophiles such as the obligate thermophile *B. stearothermophilus* (53 mol% G+C, Stenesh et al., 1968) and the facultative thermophile *B. coagulans* (47 mol% G+C; Manachini et al., 1968), the DNA base content of *B. acidocaldarius* was significantly larger at 61.2-62.2 mol% G+C. However, the most distinctive characteristic of *B. acidocaldarius* is its membrane composition of unique lipids (Figure 1-1), including menaquinone MK-9, ω-cyclohexyl fatty acids, hopanoids, and phosphorous-free complex polar lipids (De Rosa et al., 1971b, 1972, 1973; Langworthy et al., 1976; Minnikin et al., 1977; Minnikin and Goodfellow, 1981).

B. *acidocaldarius* remains the only known obligate thermoacidophilic *Bacillus* species with ω-cyclohexane fatty acids and hopanoids (De Rosa et al., 1971a, 1973; Langworthy and Mayberry, 1976) until 1981. *B*. *acidocaldarius* were without exception isolated from hot and acidic habitats (Darland and Brock 1971; De Rosa et al., 1971a, 1971-1973, 1973; Oshima et al., 1977; Loginova et al., 1978). Hippchen et al. (1981) and Cerny et al. (1984) isolated thermoacidophilic bacilli from soil of nongeothermal origins and spoiled apple juice, respectively, that closely resembled *B*. *acidocaldarius*. The isolates contained the unique ω-cyclohexane fatty acids and hopanoids characteristic of *B*. *acidocaldarius* but exhibited distinctively smaller DNA G+C content (51.0-53.3 mol%), optimum growth temperatures and different carbon utilization patterns from *B*. *acidocaldarius* (Deinhard et al., 1987a). In addition to physiological differences, the overall difference in DNA base composition was 7 G+C %mol between *B*. *acidocaldarius* and the new isolates from nongeothermal origins. Since the general rule of DNA base composition suggests establishment of a new species with over 5% G+C differences (Jones and Sneath, 1970; Bradley and Mordarski, 1976), Deinhard et al. (1987a) proposed the classification of a new species, *B. acidoterrestris* (later reclassified as *A*. *acidoterrestris*), to these soil borne thermoacidophilic isolates. In addition, the establishment of *B. cycloheptanicus* (later reclassified as *A*. *cycloheptanicus*) was proposed for soil-isolated thermoacidophilic bacilli containing unique ω-cycloheptane fatty acids and G+C contents of 54.0-56.9 mol%. Unlike *B*. *acidocaldarius* and *B*. *acidoterrestris*, *B*. *cycloheptanicus* have absolute requirements for isoleucine, methionine or Vitamin B_{12}, and pantothenate (Deinhard et al., 1987b).

The broad criteria of Gram-positive, aerobic, endospore-forming rods used to identify microorganisms as *Bacillus* results in a heterogeneous collection of species with differnt phenotypes (Gordon, 1981; Logan and Berkeley, 1981; Priest, 1981; Claus and Fritze, 1989) and not necessarily related at the genus level (32-69 mol% G+C) (Stackebrandt et al., 1987; Ash et al., 1991b; Rössler et al., 1991). The diversity observed among species is great enough that the genus should be subdivided into several genera (Stackebrandt et al., 1987; Ash et al., 1991b; Rössler et al., 1991). As confirmed by16S rRNA catalog and subsequent 16S rRNA and rDNA sequence analyses, at least three main clusters exist in the genus *Bacillus* (Ash et al., 1991b; Rössler et al., 1991). Among the outlying *Bacillus* species, *B. acidocaldarius*, *B. acidoterrestris* and *B. cycloheptanicus* are one of the taxa that branches most deeply (Stackebrandt et al., 1987). The phylogenetic relationship of *B. acidocaldarius*, *B. acidoterrestris*, *B. cycloheptanicus* with other species of the genus *Bacillus* was investigated using 16S rRNA sequence and structure comparision (Wisotzkey et al., 1992). As confirmed by 16S rRNA sequence similarity comparisons, *B. acidocaldarius*, *B. acidoterrestris*, and *B. cycloheptanicus* are closely related and more distant from main *Bacillus* clusters than non-*Bacillus* bacterium *Streptococcus cecorum* (Williams et al., 1989) and *Lactobacillus lactis* (Yang and Woese, 1989). Structural variations of 16S rRNAs further support the divergance of *B. acidocaldarius*, *B. acidoterrestris*, and *B. cycloheptanicus* from the genus *Bacillus*. The most significant structural difference is the deletion of a 12-14 nucleotide apex helical element illustrated in Figure 1-2. Since closely related microorganisms exhibit little differences in secondary structure, variation suggests considerable phylogenetic diversity.

Based on the findings, Wisotzkey et al. (1992) proposed the reclassification of *B. acidocaldarius*, *B. acidoterrestris*, and *B. cycloheptanicus* to the genus *Alicyclobacillus*.

The unique thermoacidophilic characteristic and unusual membrane lipid composition in *Alicyclobacillus* initiated interest in basic biochemical and evolutionary studies (Brock, 1986; Woese, 1987; Stetter, 1993, 1996), the role of ω-alicyclic fatty acids and hopanoids on membrane function (Poralla et al., 1980; Kannenberg et al., 1984), and biotechnological applications of thermoacidostable enzymes derived from *A. acidocaldarius*-like strains (Buonocore et al, 1976; Brock, 1986; Sharp and Munster, 1986; Inagaki, 1997; Albuquerque et al., 2000). Also of increasing concern was the presence of *A. acidoterrestris* in fruit juice spoilage incidences reported worldwide (Cerny et al., 1984; Splittstoesser et al., 1994; Yamazaki et al., 1996a; Pettipher et al., 1997; Jensen, 1999). As a result, many attempts were made to isolate *Alicyclobacillus*. In addition to discovering new isolates belonging to the three initial *Alicyclobacillus* species (De Rosa et al., 1971a; Splittstoesser et al., 1994), novel species and genomic species were continuously reported, including *A. contaminans*, *A. hesperidum*, *A. kakegawensis*, *A. macrosporangiidus*, *A. sendaiensis*, *A. shizuokensis*, *A. vulcanalis*, *Alicyclobacillus* genomic species 1, *Alicyclobacillus* genomic species 2 from soil (Albuquerque et al., 2000; Goto et al., 2002b; Simbahan et al., 2004; Goto et al., 2007), *A. herbarius* from dried hibiscus flower (Goto et al. 2000a), *A. acidiphilus* and *A. pomorum* from spoiled fruit juice (Matsubara et al., 2002; Goto et al., 2003), *A. fastidious* from unspoiled apple juice (Goto et al., 2007), *A. sacchari* from liquid sugar (Goto et al., 2007), and *A. tolerans* from oxidizable lead-zinc ores (Karavaiko et al., 2005). Evidence from in depth chemotaxonomic analysis and 16S rRNA gene sequencing resulted in the reclassification

of *Sulfobacillus disulfidooxidans* (Dufresne et al., 1996) to *A. disulfidooxidans* (Karavaiko et al. 2000, 2005). Figure 1-3 illustrates the relationship of currently known *Alicyclobacillus* species based on 16S rRNA gene sequence comparisons (Goto et al., 2007).

As *Alicyclobacillus* species increase, the original description of the genus *Alicyclobacillus* proposed by Wisotzkey et al. (1992) was outdated as it excluded some species classified to the *Alicyclobacillus* genus according to 16S rRNA, the most appropriate method for determining taxanomic relationships (Fox et al., 1980; Woese, 1987). The description was since emended twice (Goto et al., 2003; Karavaiko et al., 2005) and is summarized as following:

Alicyclobacillus (aliphos, fat; kyklo, circle; alicyclo, referring to circular fatty acids; bacillus, small rod; *Alicyclobacillus*, small rods containing ω-alicyclic fatty acids). Rod shaped cells that are straight or nearly straight (0.3-0.8 × 2.0-4.5 μm). Aerobic or facultatively anaerobic. Gram positive or gram variable. Endospores are formed under broad environmental or nutritional conditions. One endospore per cell. Sporulation is tolerant of oxygen. Growth is obligately acidophilic and occurs at pH 1.5 to 6.5 and at temperatures of <20-70°C. The optimum growth temperature range is 35-60°C. Growth factors may or may not be required. Membrane fatty acid profiles consist of straight- and branched-chain saturated fatty acids and may or may not include ω-alicyclic fatty acids. The main isoprenoid quinone is menaquinone with seven isoprene units (MK-7). Hopanoids may be present; sulfonolipids are present. The 16S rRNA molecules of the species in this genus exhibit more than 92% sequence homology to each other. The G+C content of DNA ranges from 48.7 to 62.7 mol%.

2. Characteristics of *Alicyclobacillus*

Alicyclobacillus are aerobic to facultatively anaerobic, Gram positive to variable, rod shaped, thermoacidophilic sporeforming bacteria. Depending on different species, optimum growth occurs from 35-65°C, with extremes as low as 4°C and as high as 70°C. (Table 1-1). Growth of *Alicyclobacillus* species is reported between pH 0.5-6.0. *A. acidocaldarius* is the most thermophilic member and can grow in environments from pH 2 to 6 and at temperatures close to 70°C. *A. disulfidooxidans* is not as thermotolerant as the other alicyclobacilli and can grow only at mesophilic temperatures (4-40°C), but is extremely acidotolerant and can grow in environments with pH values as low as pH 0.5. Spore formation in *Alicyclobacillus* is terminal or subterminal and the sporangium may or may not be swollen. Colonies on growth medium are creamy white, non-pigmented, flat and circular. *Alicyclobacillus* species are isolated from various sources, including geothermal springs, soil, spoiled juice beverages, water, wastewater sludge, and oxidizable lead-zinc ores.

Unique membrane lipid profiles consisting of hopanoids and ω-alicyclic fatty acids are the most distinctive characteristic of the genus *Alicyclobacillus* (De Rosa et al., 1971a, 1973; Kaneda, 1977; Wisotzkey et al., 1992). With the exceptions of *A. pomorum*, *A. macrosporangiidus*, and *A. contaminans* (Goto et al., 2003, 2007), relatively large proportions of ω-cyclohexyl fatty acids are reported in *A. acidocaldarius*, *A. acidoterrestris*, *A. hesperidum*, *Alicyclobacillus* genomic species 1, *A. acidiphilus*, *A. sendaiensis*, *Alicyclobacillus* genomic species 2, *A. vulcanalis*, *A. tolerans*, *A. disulfooxidans*, *A. sacchari*, *A. fastidious*, *A. kakegawensis*, and *A. shizuokensis* (De Rosa et al. 1971a; Oshima and Ariga, 1975; Hippchen et al., 1981; Deinhard et al.,1987a;

Dufresne et al., 1996; Albuquerque et al., 2000; Matsubara et al., 2002; Tsuruoka et al., 2003; Simbahan et al., 2004; Karavaiko et al., 2005; Goto et al., 2007), whereas ω-cycloheptyl fatty acids are the predominant fatty acids of *A. cycloheptanicus* and *A. herbarius* (Poralla and König, 1983; Allgaier et al., 1985; Deinhard et al., 1987b; Goto et al., 2002). Though ω-cyclohexane fatty acids were also observed in non-sporeforming bacteria *Curtobacterium pusillum* (Suzuki et al., 1981) and *Propionibacterium cyclohexanicum* (Kusano et al., 1997), *Alicyclobacillus* remain the only thermoacidophilic sporeformers with the unique ω-alicyclic fatty acids.

The presence of ω-alicyclic fatty acids in *Alicyclobacillus* is speculated to be a feature of thermoacidophilic adaptation (Oshima and Argia, 1975; Suzuki et al., 1981). In general, the fatty acid composition of bacteria reflects the acyl-primer specificity of their fatty acid synthetase systems and consequently their amino acid metabolism (Lennarz, 1966). However, De Rosa et al. (1972) demonstrated that ω-cyclohexyl fatty acids were synthesized from glucose through shikimate and cyclohexyl carboxylate, and are related to the metabolism of glucose rather than amino acids. A positive correlation between glucose concentration and ω-cyclohexyl fatty acids was also reported (Oshima and Ariga, 1975). The synthesis of ω-cyclohexyl fatty acids through glucose metabolism may represent an adaptation to live in hot springs where amino acid resources are limited.

Not only are ω-alicyclic fatty acids a consequence of thermoacidophilic adaptation but they also function as a component to further stabilize *Alicyclobacillus* under thermoacidophilic conditions. The ability of *Alicyclobacillus*, with an intracellular pH of approximately 6.0 (Krulwich et al., 1978), to adapt and survive in acidic and thermal environments is attributed to the high membrane stability/integrity due to its

unique membrane lipid composition and structure (Brock, 1967; De Rosa et al., 1972; Poralla et al., 1980; Suzuki et al., 1981; Kannenberg et al., 1984). Hopanoids resemble cholesterol in ring structure (Figure 1-1d, Demel and De Kruyff, 1976), however, as its four hydroxy groups are located in the side chain, the orientation of the ring system within a membrane bilayer is inside out compared to cholesterol (Blume et al., 1978). Similarly, the presence of ω-alicyclic fatty acids within the membrane also leads to denser packing within the membrane core (Kannenberg et al., 1984). The bulky ring structures from hopanoids and ω-alicyclic fatty acids decrease acyl tail trans-isomerization and the denser packing translates to a lower membrane fluidity at physiological temperatures (Figure 1-4). As there is a well-established relationship between membrane fluidity and the permeability of small non-electrolyte molecules (Demel and De Kruyff, 1976), the reduction of membrane fluidity by bulky alicyclic fatty acids is of considerable advantage for survival at low pH, since passive diffusion of protons and solutes through the membrane will be diminished, making it easier for the organism to maintain its neutral cytoplasmic pH (Poralla et al., 1980). Further evidence for the role of ω-cyclohexane lipids as an aspect of thermoacidophilic adaptation is the finding that ω-cyclohexane lipids increase with increasing temperatures and decreasing pH (Kannenberg et al., 1984). The increase of ω-cyclohexane fatty acids in response to elevated temperatures was also observed in *Curtobacterium* (Suzuki et al. 1981). Kannenberg et al. (1984) demonstrated that ω-alicyclic fatty acids containing lipids pack densely, resulting in low diffusion at high temperatures. It is of considerable importance to stress that the exceptional membrane stability of *Alicyclobacillus* is due to the presence of both hopanoids and ω-alicyclic fatty acids. Dreher et al. (1976) demonstrated in *B*.

subtilis that the alteration of its fatty acid composition to resemble the large concentration of ω-alicyclic fatty acids in *A*. *acidocaldarius* does not enable the growth of *B*. *subtilis* at pH 5 and 47°C.

Following the isolation of *A*. *acidoterrestris* from pasteurized apple juice by Cerny et al. (1984), spoilage incidences attributed to *Alicyclobacillus* were continuously reported (Splittstoesser et al., 1994; Yamazaki et al. 1996a; Pettipher et al., 1997; Komitopoulou et al., 1999). The presence of *Alicyclobacillus* in pasteurized apple juice and its ability to survive pasteurization processes initiated concern of this microorganism as a potential pathogen. Two studies were undertaken by Walls and Chuyate (2000) to determine the pathogenicity of *Alicyclobacillus*: one through intraperitoneal injection into mice and the other through feeding guinea pigs *Alicyclobacillus* at concentrations of 5×10^6 cells/ml. No deaths or adverse symptoms were observed in either the mice or guinea pigs. Combined with anecdotal reports of individuals consuming spoiled juice and suffering no ill effects, it was confirmed that while the presence of *Alicyclobacillus* is a serious economic/quality issue for the juice industry, it is not a safety concern (Walls and Chuyate, 2000).

Though general characteristics are discussed in this section, considerable cross-species and within-species variations of *Alicyclobacillus* exist. Detailed species-specific characteristics in physiology, morphology/chemotaxonomy, and carbon utilization are summarized in Table 1-1, Table 1-2, and Table 1-3, respectively.

3. Biotechnological/industrial significance of *Alicyclobacillus*

Alicyclobacillus-related research is divided into three major directions: the isolation and characterization of novel species, biotechnological/industrial applications,

and spoilage-related studies. This section will discuss the contributions of *Alicyclobacillus* to advancements in biotechnological and industrial applications.

3.1 Basic physiological reactions in thermophilic and/or acidophilic microorganisms

3.1.1 (ADP-Ribosyl)ation

Mono(ADP-ribosyl)ation is a reversible post-translational modification of proteins that transfers a single ADP-ribose unit from NAD^+ to specific acceptor proteins through mono(ADP-ribose) transferases (ADPRTs; ARTs; EC 2.4.2.31) (Moss and Vaughan, 1990; Corda and Di Girolamo, 2003). It is generally accepted that phylogenetically, mono(ADP-ribosyl)ation is an ancient mechanism, and although the related enzymes often fail to show amino acid sequence similarity, prokaryotic and eukaryotic ADPRTs are thought to be evolutionarily related (Pallen et al., 2001).

An (ADP-ribosyl)ating system was also confirmed in *Alicyclobacillus* in addition to other thermophilic microorganisms. The ADPRT system of *Alicyclobacillus* has an enzyme molecular mass with the range of 45-50 kD, was endogenous, and mono(ADP-ribose) was the sole synthesized compound (Faraone-Mennella et al., 2000, 2006). Protein activity/expression was generally independent of growth conditions. The main feature distinguishing the thermophilic ADPRT from heat-labile eukaryotic ADPRTs is the high working temperature of 65°C (Amé et al., 2004; Faraone-Mennella et al., 2006). Though the role of ADP-ribosylating within thermophilic bacteria remains to be explored, the finding of ADPRTs in *Alicyclobacillus* confirms the wide occurrence of the reaction in living organisms.

3.2 Novel thermophilic/acidophilic/thermoacidophilic biotechnological applications

3.2.1 α-Amylase Family Enzymes

3.2.1.1 α-Amylase

The first enzyme isolated from *Alicyclobacillus* species was α-amylase from *A. acidocaldarius* Agnano 101 (Buonocore et al., 1976). The inducible α-amylase was optimally active at pH 3.5 and 75°C. Other α-amylases from different strains of *Alicyclobacillus* are also reported and listed in Table 1-4 (Boyer et al., 1979; Uchino, 1982; Kanno, 1986).

The *A. acidocaldarius* ATCC 27009 α-amylase was sequenced by Koivula et al. (1993) and exhibited a molecular mass of 160 kDa larger than other *Alicyclobacillus* α-amylases. Insights into enzyme acid stability was provided by comparing the sequence of ATCC 27009 α-amylase with other closely related neutrophilic enzymes. Schwermann et al. (1994) determined that approximately 30% of the charged residues at the surface of ATCC 27009 α-amylase were replaced by neutral polar residues. The reduction in charged residues prevents the unfolding and denaturation of proteins resulting from electrostatic repulsion at low pH, and was proposed as a mechanism for the acidostability of ATCC 27009 α-amylase.

3.2.1.2 Cyclomaltodextrinase-neopullulanases-maltogenic α-amylase

A gene termed *cda*A that translates into a cytoplasmic protein CdaA belonging to the α-amylase family of cyclomaltodextrinases-neopullulanases-maltogenic α-amylase was identified while studying genes involved in starch degradation by *A. acidocaldarius* ATCC 27009 (Matzke et al., 2000). Degradation of cyclomaltodextrins and pullulan to panose was observed, with pH and temperature optima of 5.5 and 55°C, respectively. The

temperature optimum of CdaA was the second highest within the cyclomaltodextrinases-neopullulanases-maltogenic α-amylase group, and only slightly lower than 60°C reported for a *Thermus* strain (Kim et al., 1999b). Matzke et al. (2000) concluded that the classification of CdaA was indeterminate because its primary structure aligned equally well with representatives of neopullulanase, cyclomaltodextrinase, and maltogenic α-amylase, and thus may belong to a new subgroup of neopullulanase, cyclomaltodextrinase, and maltogenic α-amylase. Alternatively, other researchers determined that neopullulanase, cyclomaltodextrinase, and maltogenic α-amylase do not exhibit characteristical nor structural differences to warrant different classifications (Lee et al., 2002a). Thus CdaA may be included in the general neopullulanase, cyclomaltodextrinase, and maltogenic α-amylase group.

3.2.2 L-Arabinose isomerase

L-Arabinose isomerase (EC 5.3.1.4) is a dual function intracellular enzyme that catalyzes (1) isomerization of L-arabinose to L-ribulose involved in the pentose phosphate or the phosphoketolase pathway (Patrick and Lee, 1968; Izumori et al., 1978), and (2) bioconversion of D-galactose into D-tagatose (Cheetham and Wootton, 1993; Roh et al., 2000; Kim et al., 2002; Lee et al., 2004; Lee et al., 2005b). In recent years, the latter isomerization reaction generated great interest due to the reported properties, health benefits, and GRAS status of D-tagatose (Zehner, 1988; Zehner and Lee, 1988; Seri et al., 1993; Levin et al., 1995; Buemann et al., 1999a, b; Donner et al., 1999; Lærke and Jensen, 1999; Buemann et al., 2000; Levin 2002; Nabors, 2002; Kim, 2004), and the industrial feasibility of utilizing D-galactose and L-arabinose isomerase (Kim et al., 2003a; Oh, 2007).

It is currently established that isomerization performed at higher temperatures (>70°C) offers advantages including higher conversion yield, faster reaction rate, and decreased viscosity of the substrate in the product stream (Lee et al., 2005a). However, L-arabinose isomerases that are thermal stable usually have an alkaline optimum pH (Kim et al., 2003; Kim and Oh, 2005). Bioconversion of D-galactose to D-tagatose under high temperature and neutral conditions introduce undesirable effects like browning and unwanted by-product formation (Liu et al., 1996; Kim, 2004). To overcome these problems, a thermostable L-arabinose isomerase with an acidic pH optimum would be ideal for industrial applications (Kim, 2004; Lee et al., 2005a). A breakthrough to the limitations of current L-arabinose isomerase was found when Lee et al. (2005a) reported the first acidic thermostable L-arabinose isomerase isolated from *A. acidocaldarius*. The L-arabinose isomerase from *A. acidocaldarius* converted D-galactose to D-tagatose optimally at 60°C and pH 6.0. Comparative analysis of the pH activity profiles was also conducted and the authors proposed that the Lys-269 residue of *A. acidocaldarius* L-arabinose isomerase could be responsible for the ability of the enzyme to act at low pH. Through site-directed mutagenesis, Lys-269 was replaced by a Glu residue to mimic the Glu-268 residue in *Bacillus halodurans* L-arabinose isomerase and resulted in a 1 pH unit change of optimum pH from 6.0 to 7.0. When the *B. haloduran* L-arabinose isomerase was mutated through the change from Glu-268 to Lys-269, a lowering of optimum pH from 8.0 to 7.0 was observed. The results from this study not only illustrate the potential application of *A. acidocaldarius* in the industrial production of D-tagatose, but also provide valuable insights for possible site-directed mutagenesis to alter the optimal pH of known L-arabinose isomerase to reduce unwanted browning and by-product formation.

3.2.3 Cello-oligosaccharide transporter system

Alicyclobacillus was previously described as noncellulolytic (Bergquist et al., 1999). However, recent studies documenting the successful isolation of β-1,4-glucanases CelA (Eckert et al., 2002) and CelB (Eckert and Schneider, 2003), and β-glucosidase Aaβ-gly (Di Lauro et al., 2006) from *A. acidocaldarius* ATCC 27009 provide evidence that not only is *Alicyclobacillus* cellulolytic, but suggest that the genus may have a specialized transporter system responsible for the degradation of cellulose.

CelA and CelB share several similarities, both being endo enzymes and capable of hydrolyzing carboxy methyl cellulose and oat spelt xylan. However, the pH and temperature stability of CelA and CelB differ greatly and are reflective of their relative localization within the bacterial cell. The optimum temperature and pH for CelA is 70°C and pH 5.5. At pH 3.5, the optimum growth pH for *A. acidocaldarius*, CelA is unstable and does not exhibit any catalytic activity. Taken together with the lack of a signal peptide for transportation across the cytoplasmic membrane, it is assumed that CelA is intracellularly localized since bacteria cytoplasmic pH is near neutral (Bakker, 1990; Eckert et al., 2002). CelB is similarly thermophilic to CelA with an optimum temperature of 80°C, but is active between pH 3-5, with optimum activity at pH 4. The optimal working conditions of CelB, in addition to nucleotide sequence analysis, indicate that CelB is of extracellular localization (Eckert and Schneider, 2003).

Di Lauro et al. (2006) successfully isolated and characterized β-glycosidase (Aaβ-gly) from *A. acidocaldarius* ATCC 27009. Aaβ-gly is an intracellular enzyme and optimally active at 65°C and pH 5.5. A wide variety of linear oligosaccharides (up to four glucose units) are recognized by Aaβ-gly as its substrates, including β-D-gluco-, galacto-

and -fucosides and glucose disaccharides such as cellobiose and gentiobiose. Lactose was also efficiently hydrolyzed by Aaβ-gly. Longer oligosaccharides, such as cellopentaose, are hydrolyzed less efficiently (Di Lauro et al., 2006).

A cello-oligosaccharide transporter system was suggested for the degradation of cellulose by *Alicyclobacillus* based on the location, substrate specificity, and hydrolysis products from CelA, CelB, and Aaβ-gly (Eckert et al., 2002; Eckert and Schneider, 2003; Di Lauro et al., 2006). It is speculated that the enzymatic cascade for the degradation of β-glucans to monosaccharides is initiated by CelB which hydrolyzes xylan and CMC to smaller oligosaccharides. The resulting oligosaccharides are imported into the cytoplasm and further hydrolyzed to monosaccharides by intracellular CelA or Aaβ-gly.

3.2.4 Collagenase

Enzymatic degradation of collagen and gelatin by collagen-specific Zn^{2+}-dependent metalloproteinases (collagenases) produces collagen peptides that are of wide commercial use, including as an immunotherapeutic agent (Ku et al., 1993; Khare et al., 1995), a moisturizer for cosmetics, a preservative (Gaffney et al., 1996), and seasoning and dietary materials (Honda, 1998).

Tsuruoka et al. (2003) isolated a carboxyl proteinase, ScpA, from *A. sendaiensis* strain NTAP-1 that had an optimum pH of 3.9 and retained more than 80% of its original activity after incubation at pH 4.0 and 60°C for 1 h. Though bacterial collagenases were previously identified (Harrington, 1996), industrial production of collagen peptide by microorganisms remains limited due to the instability of the enzymes. On the other hand, ScpA posses several advantages over other existing bacterial collagenases (Tsuruoka et al., 2003). Not only does the thermostability of ScpA enable industrial production of

collagen peptides, but the thermoacidophilic conditions at which ScpA is most active (pH 4, 60°C) effectively eliminates the possibility of microbial contamination of the reaction system. It is also suggested that ScpA is capable of utilizing low-grade collagenous materials such as hide powder in addition to purified collagen and gelatin. The instability of ScpA at 60°C under neutral pH conditions indicates that a simple shift of the reaction system to neutral pH can easily terminate the degradation reaction.

3.2.5 Esterase

Esterases EST1 (esterase 1) and EST2 (esterase 2) were isolated from *A. acidocaldarius* by Manco et al. (1994, 1997). Despite the common isolation source and several shared characteristics, EST2 was more extensively studied for biotechnological purposes due to its stability over a wide temperature range (10-75°C) and activity over a broad pH (pH 5-8) (Manco et al., 1998, 1999, 2001; Del Vecchio et al., 2002a, b; Mandrich et al., 2004; Agafonov et al., 2005).

Currently, the most attractive EST2 biotechnological application involves creating fusion proteins with EST2 for the detection, quantification, and purification of target polypeptides (Agafonov et al. 2005; Huang et al., 2007; Wang et al., 2007). Since the N- and C-terminal ends are exposed on the surface of EST2 and not involved in catalysis or structure stabilization, the enzyme can be linked with other polypeptides without affecting its native fold and enzymatic activity (Huang et al., 2007). The attached EST2 on the C-terminus of the target polypeptide permits quantitative determination by measuring esterase activity spectrophotometrically. As EST2 is capable of fast folding into a stable, active single domain structure (De Simone et al., 2000), esterase activity can also be detected in polyacrylamide gels after SDS electrophoresis and removal of the

SDS. Agafonov et al. (2005) reported that photometric assays allow the detection of 10^{-12} mol of esterase per 100 µl assay volume, a sensitivity that can be further increased to be comparable to radioisotope labeling through the use of microplates. The exceptional sensitivity of EST2 was also reported by Wang et al. (2007) who reported the detection of 3 pM oligodeoxynucleotide/0.5 µl when EST2 was used as a reporter enzyme conjugate for oligodeoxynucleotide. In addition to being a sensitive reporter enzyme, EST2 can also be used as an affinity tag for purification. Trifluoromethyl-alkyl-ketone Sepharose CL-6B was previously reported to be a suitable matrix for the purification of esterase as trifluoromethyl-alkyl-ketone derivatives inhibit esterases through the formation of a hemiketal linkage with the putative serine at the esterase's active site (Hanztik and Hammock, 1987). Agafonov et al. (2005) and Huang et al. (2007) utilized TFK-Sepharose CL-6B for one step affinity purification of Green Fluorescence Protein-EST2 and NADH Oxidase-EST2, respectively. In both studies, the reported binding efficiencies of the EST2 fusion proteins to TFK-Sepharose were high and harsh conditions were required to release EST2 from the affinity matrix. Use of Factor Xa protease released GFP and NADH oxidase from the affinity matrix into the supernatant while EST2 remained attached to the matrix. In addition to its high sensitivity and affinity binding properties, another advantage of EST2 is that it surpasses the limitations of current affinity fusion systems. Most commonly used affinity fusion systems such as polyhistidines, glutathione S-transferase streptavidin and cellulose binding domain can only be detected by immunological methods, making the real time detection of fusion proteins difficult (Smith and Johnson, 1988; Greenwood et al. 1989; Hochuli, 1990; Brown et al., 1998). EST2, on the other hand, allows near real-time detection for the

expression of fusion proteins. Currently, EST2 is used for monitoring the extent of DNA hybridization (Wang et al., 2007), the detection and isolation of Green Fluorescence Protein (Agafnov et al., 2005) and NADH oxidase (Huang et al., 2007).

The esterase activity of EST2 is not only of use as a dual function reporter enzyme/affinity marker, but also of interest to the cheese industry. One of the problems currently of concern to the cheese making industry is overcoming the destructive effect of pasteurization on the flavor formation of cheese. Alternatives using thermoresistant strains or strains (natural or mutant) with high levels of endogenous lipolytic and/or proteolytic activities as starters or adjuncts for fermentation have been reported (McSweeney et al., 1993; Smit et al., 1995; Mendìa et al., 1999; Ayad et al., 2001; Smit et al., 2005). Manrich et al. (2006) compared the esterase activity of EST2 against EstA, the enzyme from *Lactococcus lactis* mainly responsible for the development of flavor in cheese (Nardi et al., 2002), and determined that EST2 is more active than EstA in both esterase and thioesterase activity and the synthesis of esters and thioesters. In addition, characteristics of EST2 such as thermostability, resistance to organic solvents, and ease of expression/purification reinforces its applicability for the development of characteristic flavors and/or to accelerate cheese ripening. It was also suggested that the overexpression of EST2 in *L. lactis* cells used as a starter or adjunct culture may result in better cheese curd distribution and a more balanced enzymatic action (Mandrich et al., 2006).

3.2.6 Squalene:hopene cyclase (SHC)

The squalene:hopene cyclase (SHC) from *A. acidocaldarius* ATCC 27009 was isolated and characterized as an enzyme composed of two identical 75 kDa subunits

(Neumann and Simon, 1986) with optimum conversion at 60°C and pH 6 (Ochs et al., 1990).

Since the products of SHC cyclization include biosynthethic precursors of various hopanoids and are of importance to bacterial membranes, the highly complex reaction is widely studied (Ourisson and Rohmer, 1992; Abe et al., 1993). The mechanism of *A. acidocaldarius* ATCC 27009 SHC was successfully elucidated by Wendt et al. (1997) and is similar to that of 2,3-oxidosequalene cyclases which catalyze the cyclization step in cholesterol biosynthesis. The cationic cyclization cascade of *A. acidocaldarius* ATCC 27009 SHC is initiated by an acidic group within the enzyme cavity and converts linear triterpene squalene to fused ring compounds such as hopene or diplopterol. The cyclization cascade is terminated by a base (Wendt et al, 1999). The most remarkable aspect of *A. acidocaldarius* ATCC 27009 SHC is its capability to accept various nonphysiological substrate analogues (C15-C35) and efficiently produce unnatural cyclic polyprenoids (Abe et al., 2002; Tanaka et al., 2004, 2005). Chemical structures of unnatural novel products produced as a result of SHC cyclization are illustrated in Figure 1-5.

3.2.7 Thioredoxin

Thioredoxin (Trx) is a protein disulphide oxidoreductase typically with a molecular mass of 10-12 kDa and an acidic pH, and is present in eukaryotic and prokaryotic cells (Eklund et al., 1991; Loferer and Hennecke, 1994; Holmgren and Björnstedt, 1995). Trx is a key enzyme in various fundamental cellular processes such as deoxyribonucleotide biosynthesis, regulation of photosynthetic events, regeneration of oxidative damage, and activation of transcription factors (Buchanaa et al., 1994). Trx is

also of importance as a solution to food allergenicity, inflammatory diseases, and progression of viral diseases (Buchanan et al., 1994). Moreover, Trx is useful as a purification tag for the overexpression of economically valuable proteins and peptides (LaVallie et al., 1993; Yasukawa et al., 1995).

The Trx of *A. acidocaldarius* (BacTrx) is the first Trx so far described from a thermophilic source (Bartolucci et al., 1997). Compared to *E. coli* Trx, BacTrx exhibits higher conformational heat stability, less bound water, and a transition temperature approximately 10°C higher than that of *E. coli* Trx. Significant structural differences between BacTrx and *E. coli* Trx, including shorter loop regions, introduction of proline residues, and an increase in the number of ion-pairs, may contribute to the thermostable characteristics of BacTrx. The structural differences between *E. coli* and *A. acidocaldarius* Trx recapitulates existing studies where thermophilic enzymes contained reduced loops with 'rigidifying' proline residues whereas such structural characteristics were absent from their mesophilic counterparts (Bartolucci et al., 1997). Studies comparing thermophilic and mesophilic proteins also indicated that the increased number of ion-pairs serves as a stabilizing factor for thermophilic enzymes (Vieille et al., 1996). It was also suggested that the formation of new hydrogen-bonds and salt-bridge interactions between side chains also contributes to the thermostability of BacTrx by linking different secondary elements and increasing the unfolding resistance of BacTrx (Pedone et al., 1998, 1999, 2003).

4. *Alicyclobacillus* as a spoilage microorganism

4.1. Significance and impact to the beverage industry

Sporeformers are not originally associated with spoilage incidents in juices as acidic pH prevents the outgrowth of spores and/or toxin production (Blocher and Busta, 1983). Yeasts, molds, and some acid tolerant non-sporeforming bacteria are thus the major microbial concerns for fruit juices and were successfully controlled through a hot-fill-and-hold pasteurization procedure which includes a heating period of 90-95°C for 15-20 s, a cooling period to 82-84°C prior to product filling, and a hot hold period of approximately 2 min before cooling in a cooling tunnel (Castberg et al., 1995).

Since the isolation of *A. acidoterrestris* by Cerny et al. (1984), *A. acidoterrestris* has become a major issue in the pasteurized fruit juice industry. Not only is conventional hot-fill-and-hold pasteurization insufficient to destroy *A. acidoterrestris* spores, but the acidic pH of fruit juices provides excellent conditions for spores to germinate and proliferate (Darland and Brock, 1971; Hippchen et al., 1981; Cerny et al., 1984; Deinhard et al., 1987b; Splittstoesser et al., 1994;Brown, 1995; Pettipher et al., 1997; Splittstoesser et al., 1998; Walls and Chuyate, 1998; Wisse and Parish, 1998; Eiroa, et al., 1999).

Despite being reported as non-pathogenic (Walls and Chuyate, 2000), *A. acidoterrestris* is still of great concern due to its ability to cause spoilage. *A. acidoterrestris*-related spoilage is characterized by the formation of medicinal or antiseptic off-odors (Walls and Chuyate, 1998). However, spoilage is often inconspicuous without obvious pH change, packaging deformation, product discoloration, or the definite formation of sediment (Brown, 1995; Walls and Chuyate, 1998). Usually, spoilage remains undetected until consumer complaints are received

(Walls and Chuyate, 1998). Resistance to low pH, pasteurization procedures, and its ability to produce off-odors has led many researchers to acknowledge *Alicyclobacillus* as a spoilage agent and an important quality control target for pasteurized fruit juices/beverages (Splittstoesser et al., 1994; Baumgart et al., 1997; Splittstoesser et al., 1998; Walls and Chuyate, 1998; Eiroa et al., 1999; Orr and Beuchat, 2000; Siegmund and Pöllinger-Zierler, 2006).

4.2 Off-odor formation

Off-odors associated with *Alicyclobacillus*-related spoilage are typically described as "medicinal", "smokey", or "antiseptic". The chemical compounds responsible for the distinctive off-odors are identified as guaiacol (Yamazaki et al., 1996a; Pettipher et al., 1997; Splittstoesser et al., 1998) and the halophenols 2,6-dibromophenol, and 2,6-dichlorphenol (Baumgart et al., 1997; Borlinghaus and Engel, 1997). The characteristics, sensory thresholds, and possible synthesis pathways of the off-odors are extensively reviewed elsewhere and shall not be reiterated in this section (Chang and Kang, 2004).

Though complete pathways for the formation of guaiacol by *Alicyclobacillus* are yet to be determined, the relationship between guaiacol formation and *Alicyclobacillus* is well established (Yamazaki et al., 1996; Pettipher et al. 1997; Jensen, 2000). In contrast, the link between *Alicyclobacillus* and halophenols is more ambiguous. 2,6-dibromophenol and 2,6-dichlorophenol can be produced chemically through contact between halogen-containing disinfectants and phenol-containing water (van Pée, 1996). The biosynthesis of halophenols by *A. acidoterrestris* remained a speculation until a causal relationship was established by Jensen and Whitfield (2003). In addition to the production of each compound by *A. acidoterrestris*, a possible link between guaiacol and

halophenols should also be considered (Michalowicz et al., 2007). As guaiacol is a phenolic compound and the water used in juice plants may contain oxidative chlorination chemical residues such as gaseous chlorine and sodium hypochlorite (Kowal et al., 1986; Glaze, 1990), it is likely that the presence of guaiacol may contribute to the chemical production of the halophenols. In a study investigating the transformation of phenolic compounds exposed to sodium hypochlorite, 99.7% of the guaiacol transformed to various chlorine derivatives under acidic pH (pH 4.0), including 2-chlorophenol, 2,4-dichlorophenol, tetrachlorophenol, pentachlorophenol, 4,5,6-trichlorophenol, and tetrachloroguaiacol (Michalowicz et al., 2007). The possibility of guaiacol contributing to the formation of other taint compounds further complicates off-odor production and quality control issues.

4.3 Widespread distribution of *Alicyclobacillus*

The number of spoilage reports accumulated worldwide imply that rather than isolated incidences, *Alicyclobacillus* is an industry-wide issue, abundant in the environment, can grow or exist in a multitude of different media, and has a great potential for economic impact (Cerny et al., 1984; Walls, 1994; Yamazaki et al., 1996; Pettipher et al., 1997; Walls and Chuyate, 1998). The widespread distribution of *Alicyclobacillus* and products susceptible to *Alicyclobacillus* contamination is reviewed in this section and provides an outlook on the potential impact *Alicyclobacillus* has on the juice and beverage industry.

4.3.1 Ready-to-drink beverages susceptible to *Alicyclobacillus* contamination

Pasteurized apple juice is a desirable growth medium for *Alicyclobacillus* (Splittstoesser et al., 1998; Siegmund and Pöllinger-Zierler, 2006) and the most

commonly reported spoiled juice product associated with *Alicyclobacillus* (Walls and Chuyate, 1998; Orr and Beuchat, 2000). According to Baumgart and Menje (2000), 9 out of 25 apple juice samples (34%) were contaminated. A similar contamination rate was reported by Chang and Kang (2005) where *Alicyclobacillus* was isolated from 13 of 44 (29.55%) randomly selected pasteurized apple juice.

Beverage contamination and subsequent spoilage by *Alicyclobacillus* is not limited to apple juice. Apple-grape-raspberry juice, apple-cranberry juice, apple-pear juice blend, fruit-carrot juice blend, lemonade, orange juice, and pear juice are also reportedly contaminated with *Alicyclobacillus* (Yamazaki et al., 1996a; Pinatti et al., 1997; Baumgart and Menje, 2000; Walls and Chuyate 2000). As demonstrated in an artificially inoculated experiment, grapefruit juice, white grape juice, and tomato juice can also support growth of *Alicyclobacillus* (Walls and Chuyate, 2000). There are also reports of juice-free beverages such as iced tea and isotonic water spoiled by *Alicyclobacillus* (Yamazaki et al., 1996a; Duong and Jensen, 2000).

Factors that affected the ability of *Alicyclobacillus* to cause spoilage in beverages were investigated by Splittstoesser et al. (1998). Growth of *Alicyclobacillus* was inhibited when the sugar content exceeded 18°Brix or at ethanol concentrations above 6%. Growth differences between white and red grape juices also suggested that phenolic compounds may be an important factor. The inhibitory effect of red grape juice was eliminated when neutral phenolics were removed. When various neutral phenolics were tested by adding them to apple juice, catechingallate was found to be inhibitory to *Alicyclobacillus*. It is also suggested that other phenolic compounds such as anthocyanin pigments may also be inhibitory and exert an additive or synergistic inhibitory effect on *Alicyclobacillus*.

4.3.2 Concentrates susceptible to *Alicyclobacillus* contamination

According to the United States Department of Agriculture, the soluble solids concentration for most juice concentrates is at least 35°Brix (AMS-USDA, 2008). Growth media with high soluble solids concentrations do not support the growth of *Alicyclobacillus* (Splittstoesser et al., 1994). However, the contamination of juice concentrates is important as *Alicyclobacillus* spores can germinate during processing and cause large-scale spoilage once the concentrate is diluted. *Alicyclobacillus* contamination in juice concentrates is not uncommon. Pettipher et al. (1997) isolated the sporeformers from 14.7% of the 75 orange juice concentrate samples obtained from 11 suppliers. In another study, 86.33% commercial apple juice concentrates harbored *Alicyclobacillus* (Chang and Kang 2005). Other juice concentrates contaminated with *Alicyclobacillus* include apricot, banana-apricot, mango, peach, pineapple, pink grapefruit, strawberry, white grape, and white peach concentrates (Pinatti et al., 1997; Baumgart and Menje, 2000; Walls and Chuyate 2000).

4.3.3 Raw material susceptible to *Alicyclobacillus* contamination

Fruits used for juicing are a substantial means for *Alicyclobacillus* to enter into the juice manufacturing process. In the United States, limited amounts of fruit are grown specifically for juicing (FAS-USDA, 2007). Most juice fruit are culled fruit that do not meet the standards of fresh packing lines (Lea, 1995). The substandard fruits may have been in contact with contaminated soil, and as a result harbor *Alicyclobacillus* within its grooves, lenticels, and crevices (Orr and Beuchat, 2000). Parish and Goodrich (2005) studied the prevalence of presumptive *Alicyclobacillus* on oranges entering juice-processing facilities. Among the 1575 fruits sampled, 591 (38%) were positive for

Alicyclobacillus. The contamination rate of the two facilities differed according to the products produced. Facility A produced an unpasteurized product, thereby requiring a harvest method that would prevent ground contact, reflected in the lower contamination rate of 414/1350 (31%). On the other hand, the heat concentrated juice product produced at facility B necessitated a less stringent standard on the incoming fruits, and exhibited a higher contamination rate of 177/225 (79%). *Alicyclobacillus* most likely gains access to fruit juice through contact of the fruit with contaminated soil (Brown, 1995; Deinhard et al., 1987b; Orr and Beuchat, 2000), however, variations in contamination rates exist depending on different harvesting methods and in-plant cleaning procedures (Parish and Goodrich, 2005).

4.3.4 Environmental sources of *Alicyclobacillus*

Alicyclobacillus are soilborne microorganisms isolated from a variety of different soils as listed in Table 1-1 (Wisse and Parish, 1998). The ubiquitous nature of soil may provide an explanation of why contaminant *Alicyclobacillus* was found not only on the surfaces of incoming fruits, but also on equipment surfaces and the air (Walls and Chuyate, 1998).

Water used in fruit juice processing facilities is also identified as containing *Alicyclobacillus* (McIntyre et al., 1995; Wisse and Parish, 1998). There are two likely sources for this. First, the contaminating bacteria may have entered into the water by the washing of soil into water. Another could be through cross-contamination by fruits containing *Alicyclobacillus*. It is common at citrus concentrate facilities for “condensate” water, recovered from the juice during the concentration process, to be used for fruit

washing (Parish and Goodrich, 2005). This water harbors *Alicyclobacillus* (Wisse and Parish, 1998) and could serve as a source of contamination in the wash step.

4.4 Possible contamination route of *Alicyclobacillus*

A simplified contamination route of *Alicyclobacillus* is illustrated in Figure 1-6. In general, soilborne *Alicyclobacillus* can come in contact with intact tree fruit through dust or animal transmission, and onto fallen fruit through direct contact. Once the fruits enter into the manufacturing facility, inadequate washing or washing with contaminated water allows *Alicyclobacillus* to enter into the juice. Soilborne *Alicyclobacillus* can also enter directly into the manufacturing plant or onto processing equipment through dust or breaches in the manufacturing facility's good manufacturing procedures.

Another major route of contamination is through water. *Alicyclobacillus* can be washed from soil into surface water, and circulate onto fruit surfaces or into the manufacturing process through irrigation and the use of tap water. As previously mentioned, condensate water is often recycled as washing water, creating more possibilities of *Alicyclobacillus* to contaminate fruit, juice, equipment, and potentially lead to large scale spoilage.

Cross contamination is also very likely due to the ever-present nature of soil and the use of water within the manufacturing process. In summary, the entrance of a small number of *Alicyclobacillus* has the potential to contaminate large amounts of juice, equipment, and result in major spoilage issues with significant financial losses.

4.5. Novel challenges *Alicyclobacillus* presents to the beverage industry

The only report to date documenting the extent of *Alicyclobacillus* spoilage was conducted by National Food Processors Association in 1998 (Walls and Chuyate, 1998).

Among the respondents, 35% (12/34) experienced spoilage characteristic of acidophilic sporeformers. Spoilage percentage per company was approximately 5% per lot with 1-2 incidences within the last 5 years. Despite the lack of more recent information, there is reason to believe that *Alicyclobacillus* spoilage issues have not subsided. The increasing number of *Alicyclobacillus* research published each year illustrates the lack of a universally effective control method. From a quality control standpoint, due to the heat resistance of the *Alicyclobacillus* spores, there is little possibility to prevent spoilage (Baumgart and Menje, 2000). The unusual challenge *Alicyclobacillus* poses to the juice industry is discussed in the following section.

Soil is ubiquitous in nature, and being a soilborne microorganism, *Alicyclobacillus* can potentially contaminate any surface by way of wind and animal vectors. As a result, it is impossible to render raw materials entirely free of *Alicyclobacillus* contamination. One of the methods used to reduce debris and microbial load of incoming fruits is through washing. For apples, the water filled canals used to float the apples to the mills doubles as fruit washers (Downes, 1995). In the instance of citrus, a brush washer is used to clean the fruit prior to the final grading (Rebeck, 1995). In both types of fruit juice processing, washing water is usually recycled many times, thus the efficacy of the fruit wash would be highly dependent on the quality of the fruits entering into the process. The washing water may serve as a vector for a wide scale contamination rather than a preventive measure if caution is not exercised. Even with the use of sanitizers, the effectiveness of the fruit wash is highly variable depending on the nature and location of the microorganism and the type of produce entering the juicing process (Burnett and Beuchat, 2001). While studying the prevalence of presumptive

Alicyclobacillus on orange surfaces, Parish and Goodrich (2005) observed significant differences between two processing plants. In plant A which produced unpastuerized juice, the number of contaminated oranges was reduced from 48% to <5% after washing. In striking contrast, 73.3-84% of washed oranges were positive with *Alicyclobacillus* contamination at plant B which produced a heat-concentrated juice product. The type of orange used in each juicing plant may contribute to the pronounced difference in *Alicyclobacillus* contamination rates: oranges used at plant A did not or had minimal contact to soil while those used at plant B most likely had extensive contact with soil (Parish and Goodrich, 2005).

Pasteurization is a critical procedure for maintaining the safety and quality of pasteurized fruit juices. Unfortunately, spores of *Alicyclobacillus* are highly heat resistant and not affected by the pasteurization process (Splittstoesser et al., 1994, 1998). Common juice preservatives such as sulfur dioxide and sorbic acid which reduce the heat resistance of mold ascospores are also unable to sensitize *Alicyclobacillus* spores to heat (Splittstoesser and Churey, 1989, 1991). On the contrary, pasteurization with a heating period of 15-20 s at 90-95°C may actually serve as a heat shock to the dormant spores of contaminant *Alicyclobacillus* and initiate spore germination which leads to subsequent growth and spoilage.

Another juice production practice that is advantageous to *Alicyclobacillus* is the prolonged warming during natural cooling of the hot-filled juice. Pinhatti et al. (1997) report examples in Europe where hot-fill juice in large volumes were allowed to cool naturally during high summer temperatures and subsequent spoilage was detected. A similar incidence was also observed in iced tea (Duong and Jensen, 2000). It is suggested

that the slow cooling of the hot-filled product may contribute to the development of spoilage by allowing the spores sufficient time to germinate and initiate growth (Duong and Jensen, 2000).

In addition to the normal ”hurdles” of juice pasteurization becoming supportive of *Alicyclobacillus* spore germination and subsequent spoilage, the typically undesirable growth environment of acidic juices is also a favorable growth medium for *Alicyclobacillus*. The microaerophilic conditions created by filling juices into PET bottles is also supportive of *Alicyclobacillus* growth (Cerny et al., 2000).

The spoilage characteristics of *Alicyclobacillus* are also major challenges for the juice industry. A small number of *Alicyclobacillus* have the potential to contaminate large volumes of juice, resulting in severe financial losses for the manufacturers (Pettipher and Osmundson, 2000). Under ideal conditions, as little as 1 spore/10 ml juice can result in spoilage. *Alicyclobacillus* growth results in flat-sour type spoilage, and though some incidences may be accompanied by sediment, most are not visibly detectable (Walls and Chuyate, 2000; Eisele and Semon, 2005), making it difficult to detect spoilage before the juice reaches consumers. In addition to the low spoilage requirement and difficulties to visualize spoilage, the chemical compounds contributing to the medicinal off-odor of *Alicyclobacillus*-related spoilage exhibit very low sensory thresholds. The odor threshold for guaiacol in apple juice is within a range of 0.57-2 μg/l apple juice (Pettipher et al., 1997; Orr et al., 2000; Eisele and Semon, 2005; Siegmund and Pöllinger-Zierler, 2006). The reported thresholds for 2,6-DBP and 2,6-DCP are 0.5 ppt and 30 ppt, respectively (Jensen, 1999). The low sensory thresholds indicate the high likelihood of consumers to

detect and reject a spoiled juice should a contaminant *Alicyclobacillus* spore germinate within a beverage product.

Despite temperature studies that indicate the inhibition of *Alicyclobacillus* spore germination and outgrowth at temperatures below 20°C (Jensen and Whitfield, 2003), pastuerized fruit juices are mainly distributed under ambient temperatures, and chilling the juices would dramatically increase production costs. Increasing pasteurization temperature up to the sterilization temperature region is another alternative, but the extreme heat required to inactive the spores would also cause detrimental quality and organoleptic changes in the juice.

In summary, due to the unique characteristics of *Alicyclobacillus* spores, it is generally recognized that once *Alicyclobacillus* enters the juice, there are not many interventions to prevent spoilage. Baumgart and Menje (2000) suggested the use of GMP procedures, effective washing of fruits, and improving cleaning regimes to minimize the entrance of *Alicyclobacillus* into the juicing facility.

5. Current technologies for controlling *Alicyclobacillus* spores

As the potential economic losses and reputation damage resulting from *Alicyclobacillus*-related spoilage are realized, there is constant demand for effective novel methods to control *Alicyclobacillus* spores. This section reviews physical, chemical, and hurdle/combination control methods that may be used as a control measure against *Alicyclobacillus* spores.

5.1 Physical control methods

5.1.1 Heat

One of the most common methods used to control sporeformers is heat (Brown, 2000). However, bacterial spores are much more resistant to heat than their vegetative counterparts due to the number and structure of their outer envelopes (Earnshaw et al., 1995), requiring more extreme temperatures or longer treatment time for successful inactivation. A schematic representation of a spore structure is shown in Figure 1-7. The heat resistance of spores is affected by optimal growth temperatures, spore core water content, total and specific mineral content, sporulation temperature and cortex size (Gerhardt and Marquis, 1989). Spores with lower water contents in the protoplast are more heat resistant than spores with higher water contents (Beaman and Gerhardt, 1986).

Several studies have been conducted to determine the decimal reduction time and *z*-values of *Alicyclobacillus* spores in media of various pH and soluble solids concentrations. As shown in Table 1-5, the *D*-values of the spores are highly variable depending on the spore isolate, treatment medium, and medium pH. In general, the *D*-values obtained for apple juice and orange juice, the two frequently spoiled beverages, were in the range of 50.0 to 65.6 min at 85°C, 7.38 to 23 min at 90°C, and 2.3 to 3.59 min at 95°C. The *z*-values were between 7.7 and 12.9°C. These results not only confirm that conventional hot-fill and hold pasteurization is insufficient against the inactivation of *Alicyclobacillus* spores, but also reflects the extreme heating conditions that must be applied to juices to eliminate the risk of *Alicyclobacillus* spore spoilage. Since nutritional qualities of fruit juices are easily destroyed, it is not feasible to heat fruit juices for prolonged periods at elevated temperatures. The heat resistance of *Alicyclobacillus* spores

and the characteristics of pasteurized fruit juices has lead many researchers to acknowledge that conventional heating alone is not an applicable treatment for controlling *Alicyclobacillus* (Baumgart and Menje, 2000; Cerny et al., 2000). On the other hand, ultra high temperature (UHT) treatment may be an alternative to conventional heating. Among the 74 juices sampled by Pettipher et al. (1997), 5/18 fresh refrigerated juices, 4/4 pasteurized apple juices, and 1/4 apple juice concentrates were positive for *Alicyclobacillus* spores, while all of the 56 commercial UHT fruit juice products were negative for the presence of *Alicyclobacillus*. The sterilization temperature and short heating time of UHT treatments may be a good solution for balancing fruit juice quality and reducing the risk of *Alicyclobacillus*-related spoilage. However, as there are no studies examining the heat resistance of *Alicyclobacillus* spores to UHT treatments, more research must be done to understand the characteristics of *Alicyclobacillus* spores subjected to UHT treatment and to evaluate possible effects on sensory quality.

5.1.2 Ultrasound

The bactericial effect of ultrasound is well known (Earnshaw et al., 1995), and can be attributed to a three-fold mechanism ultrasound has on the target cell. Ultrasonic waves with high amplitude that pass through a liquid will produce bubbles, a phenomenon known as cavitation (Scherba et al., 1991). The formed bubbles, depending on if they are stable or transient, react in two different ways with the target cell to result in damage. When the ultrasound passes through a liquid, the bubbles vibrate which create microcurrents in the liquid (microstreaming) (Hughes and Nyborg, 1962; Scherba et al., 1991). The streaming effect caused by the oscillations of these small bubbles provides a large force, without the bubbles having to burst (Williams et al., 1970). This force

effectively ‘rubs’ the membrane surfaces of cells causing them to shear and the cell membrane breaks down (Kinsloe et al., 1954; Alliger, 1975). On the other hand, transient cavitation damages target cells by the high pressures (up to 100 MPa) and high temperatures (up to 5000K) momentarily produced when bubbles collapse with different intensities (Mason et al., 1994; Sala et al., 1995). The produced pressures and temperatures are strong enough to disrupt cell wall structures or to remove particles from surfaces (Schett-Abraham et al., 1992). In addition to the effects of physical disruption caused by cavitation, application of ultrasound to a liquid can lead to the formation of free radicals. The primary target site of free radicals in the cell is the DNA and sonication sensitizes cell DNA to free radicals by breaking the DNA chain and forming small DNA fragments (Hughes and Nyborg, 1962).

It is suggested that ultrasound alone does not affect spores due to the sturdy structure of spores (Sanz et al., 1985). However, promising results are reported for sonication when used in combination with heat. A 43% reduction in the heat resistance of *Bacillus subtilis* was observed when sonicated in water within the temperature range of 70-95°C (Garcia et al., 1989). Similar reductions in heat resistance are reported for *B. cereus* and *B. licheniformis* after sonication treatment at 20 kHz (Burgos et al., 1972). The combined application of ultrasound with heat was recently termed thermosonication (Hurst et al., 1995).

One disadvantage to using high temperatures with ultrasound is the loss of cavitation effect due to high water tension at temperatures exceeding the boiling point (Garcia et al., 1989). Pressurizing the liquid can help overcome this drawback. The combination treatment of ultrasound/heat/pressure is effectively used against

sporeformers such as *B*. *cereus*, *B*. *stearothermophilus*, and *B*. *coagulans* using pressures of 300 kPa in combination with 112°C (Raso et al., 1994). It is thought that such a combination subjects cells to intense vibration and cavitation (Raso et al., 1994), resulting in the release of DPA and low molecular weight polypeptides from the spores (Palacios et al., 1991). Such a degradation of the cortex leads to rehydration of the protoplast, resulting in loss of heat resistance (Gould and Dring, 1975), making the cells more susceptible to heat and ultrasound treatments.

5.1.3 High pressure

Under the reduced volume conditions which result from high pressure, water triggers strong perturbation of electron cloud distribution (electrostriction) around ionized molecules and affects the function of water (Tauscher, 1995). Active sites of enzymes are altered in terms of ligand solvation (Hui Bon Hoa et al., 1992), substrate ionic status is changed (Tauscher, 1995), and structural entities such as membrane bound proteins may lose stability and function (Earnshaw et al. 1995; Smelt, 1995).

However, bacterial spores are relatively resistant to pressure (Timson and Short, 1965; Gould and Sale, 1970; Sale et al., 1970). It is reported that bacterial spores are resistant to pressures of 981 MPa for 40 min and 588 MPa for 120 min (Nakayama et al., 1996). The resistance of spores was explained by the lack of solvation-derived effects in the relatively dry structure (Sale et al., 1970). Rather, pressure related inactivation is attributed to pressure induced germination and subsequent destruction of the vegetative cells by other treatments (Sale et al., 1970), including heat (Okazaki et al.,1996; Roberts and Hoover, 1996), acidification (Roberts and Hoover, 1996), carbon dioxide (Haas et al., 1989), and antimicrobial substances such as nisin (Roberts and Hoover, 1996) and

sucrose palmitic acid ester (Hayakawa et al., 1994a). A likely mechanism for pressure induced germination is protoplast hydration (Gould and Sale, 1970; Murrell and Wills, 1977). Gould and Jones (1989) also formulated a theory that electrostrictive ionization increases the solubility of immobilized ions in the spore protoplast that subsequently reduced the spore resistance to hostile agents/environments and stimulates germination. Leakage of bacterial spore DPA from pressurized spores, which directly results in reduced heat resistance, is also reported (Sale et al., 1970).

The combined effect of high pressure and temperature enhances antimicrobial efficacy (Hayakawa et al., 1994b; Harwood and Cutting, 1990), and was investigated on the inactivation of *Alicyclobacillus* spores in apple juice by Lee et al. (2002b). As expected, high pressure (up to 621 MPa) treatments alone did not reduce spore viability. However, treatment with 414 or 621 MPa at 71°C for 10 min resulted in a >5.5 log reduction, reducing the number of viable spores to undetectable concentrations. Similar reduction levels were also reported with combined high pressure/heat treatment of 90°C/414 or 621 MPa for 1 min. Spores subjected to the combined high pressure and heat treatment appeared crushed or shattered according to SEM photographs (Lee et al., 2002). Whereas the single effect of high pressure is limited on spores, the combination of high pressure with mild to high heat shows promise as an effective measure for controlling *Alicyclobacilllus* spores.

5.1.4 Irradiation

Treatment with ionizing radiation is an effective means of destroying bacterial spores and is applied to the sterilization of several kinds of foods that are contaminated primarily with bacterial spores (Brown, 2000). It is particularly effective for foods that

require sterilization without an increase in temperature. As listed in Table 1-5, the heat resistance of *Alicyclobacillus* spores make them extremely difficult to inactivate without causing any detrimental effects to juice quality, thus the non-thermal effect of irradiation pose an appealing possibility towards controlling *Alicyclobacillus* spoilage without compromising juice quality.

Nakauma et al. (2004) reported no significant differences between electron-beam and gamma-ray irradiation. The D-values of *A. acidoterrestris* spores inoculated onto filter paper were 1.02±0.12 kGy and 1.1±0.07 kGy for electron beam and gamma ray, respectively. For a 5-log reduction to occur, 5.0 to 5.5 kGy of irradiation was required. A decrease in spore heat resistance was observed when irradiation was combined with heat. Complete inactivation of 10^4 CFU/ml of *A. acidoterrestris* by heating at 95°C required 188 min. By contrast, the heating period was reduced to 23 min when combined with a 2.0-kGy electron beam or gamma ray radiation treatment.

The effect of irradiation was also investigated in dextrin as *Alicyclobacillus* often contaminate not only the juicing fruits but also other food additives/juice ingredients. The reported D-values of the spores for electron-beam and gamma-ray irradiations were 1.72±0.15 kGy and 1.79±0.11 kGy, respectively, slightly higher than the values for the spores irradiated on a filter paper. These phenomena are often seen when materials that exhibit radical elimination ability are in the surroundings of spores (Fuld et al., 1957). A synergistic effect of irradiation and heat was also observed for spores in dextrin. The necessary times for a 4-log reduction of *A. acidoterrestris* was 156 min at 95°C, but the heating period was reduced to 35 min when combined with 1.0 kGy of electron beam treatment. When dextrin contaminated with 10^4CFU/g of *A. acidoterrestris* spores was

irradiated with a 1.0-kGy electron beam and heated for 20 min at 95°C, *A. acidoterrestris* was not observed for the duration of the sampling period (7 days at 45°C).

The combined effect of irradiation and heat on the inactivation of bacterial spores is reported by various researchers (Fuld et al., 1957; Fisher and Pflug, 1976; Pallas and Hamdy, 1976; Shamsuzzaman, 1988). Ionizing radiation changes the permeability of the *B. subtilis* spores. Low permeability plays a major role in the heat resistance of spores (Pontius et al., 1998; Setlow, 1995). Thus, the decrease in the thermal tolerance of *A. acidoterrestris* might also be related to the increase in the permeability of spores. Hyashi et al. (1995) reported that heat treatment retarded the synthesis of protein and RNA. The different effects of irradiation and heating might contribute to the synergistic effect of the two treatments.

5.2 Chemical sanitizers

While physical control methods are more focused on controlling *Alicyclobacillus* spores that have gained access and are suspended in the juice medium, chemical control methods are easy to apply and can reduce the initial contaminant concentrations of *Alicyclobacillus* prior to their entrance into the juice.

Orr and Beuchat (2000) examined the effectiveness of sodium hypochlorite, acidified sodium chlorite, trisodium phosphate, hydrogen peroxide, and Tsunami™ against planktonic *Alicyclobacillus* spores. When exposed to sodium hypochlorite (NaOCl, pH 6.9±0.2), *Alicyclobacillus* spore reductions were 2.35 log, 5.59 log, and >6 log at chlorine concentrations of 200 ppm, 500 ppm, and 1,000 ppm, respectively. Significant decreases in spore concentration was also detected with 2% H_2O_2 (pH 4.4±0.2). $NaClO_2$ (pH 2.5) and Na_3PO_4 (pH 11.5±0.1) slightly decreased *Alicyclobacillus*

spore concentrations but statistical differences could not be detected. Tsunami™ (pH 3.7±0.2) was not effective against *Alicyclobacillus* spores (Orr and Beuchat, 2000). Chlorine at 500 or 1,000 ppm significantly reduced *Alicyclobacillus* spores inoculated on unwaxed apples, but to a lesser extent than when used on planktonic spores. The decreased effectiveness of chlorine is likely due to the inaccessibility of spores located in cracks or natural openings of the apple surface (Lund, 1983; Adams et al., 1989; Seo and Frank, 1999) or to the inactivation of chlorine in the presence of organic matters on the surface of apples (Brown, 2000). The use of surfactants (Adams et al., 1989; Beuchat and Ryu, 1997) or mild heat (Orr and Beuchat, 2000) was suggested to enhance the lethality of the sanitizers.

5.3 Microbiological/natural substances control methods

5.3.1 Nisin

Nisin is an antimicrobial polypeptide produced by certain strains of *Lactococcus lactis* subsp. *lactis.* It is well documented that nisin exhibits antimicrobial activities against a wide range of Gram-positive bacteria, particularly spore-formers (Delves-Broughton, 1990), while showing little or no inhibitory effects against Gram-negative bacteria, yeast or fungi (Yamazaki et al., 2000). Nisin affects the post-germination stages of spore development through sporostatic inhibition of the pre-emergent swelling and thus interfering with subsequent outgrowth and formation of vegetative cells (Ramseier, 1960; Hitchins et al, 1963). Nisin is stable at high temperatures and low pH (Davies et al., 1998), especially at the range of pH 3-4, and the enhanced antimicrobial activities under acidic conditions (Campbell and Sniff, 1959; Becker et al., 1994) make nisin an attractive alternative for the control of thermoacidophilic *Alicyclobacillus*.

The effect of nisin on *Alicyclobacillus* spores in fruit juices were investigated by Komitopoulou et al. (1999) and Yamazaki et al. (2000). Komitopoulou et al. (1999) reported that complete inhibition of spore outgrowth was achieved with 100 IU/ml nisin in apple and orange juices stored at 44°C. A nisin concentration of 5 IU/ml inhibited spore outgrowth in grapefruit juice under identical storage conditions. When the juices were stored at 25°C to simulate the commercial conditions of pasteurized fruit juice drinks, 5 IU/ml nisin was sufficient to prevent the outgrowth of *Alicyclobacillus* spores (Komitopoulou et al., 1999). Similarly, Yamazaki et al. (2000) reported that the outgrowth of *A. acidoterrestris* spores was inhibited by the addition of 25-50 IU/ml nisin in orange and mixed fruit drinks incubated at 40°C. On the other hand, spore outgrowth in clear-apple drink was not inhibited by nisin concentrations of up to 600 IU/ml. The decreased effectiveness was attributed to possible binding of nisin to apple particles (Yamazaki et al., 2000).

In addition to eliciting a direct antimicrobial effect on *Alicyclobacillus* spores, nisin also contributes to a reduction in spore thermal resistance. Reported D-values for untreated verses nisin-treated apple juice (50 IU/ml nisin), respectively, were 41.15±0.24 min and 23.75±0.12 min for 80°C, 7.38±0.85 min and 4.56±0.09 min for 90°C, and 2.30±0.03 min and 1.95±0.02 min for 95°C (Komitopoulou et al., 1999). Yamazaki et al. (2000) also noted a 71-76% reduction in thermal resistance of *Alicyclobacillus* spores when juice drinks were supplemented with 5 ppm active nisin. The benefits of nisin addition prior to the pasteurization process are two fold. By reducing the thermal resistance of spores, the effectiveness of pasteurization would be enhanced. Spores surviving pasteurization remain inhibited by nisin and thus spoilage of the juice through

the germination and outgrowth of *Alicyclobacillus* would not occur (Komitopoulou et al., 1999).

5.3.2 Enterocin AS-48

A relatively new bacteriocin, enterocin AS-48, was also recently evaluated as a potential control agent for *Alicyclobacillus* spoilage (Grande et al., 2005a). Enterocin AS-48 is a broad-spectrum cyclic peptide produced by *Enterococcus faecalis* S-48 (Gálvez et al., 1989) and its mutant strain A-48-32 (Martínez-Bueno et al., 1990), and exhibited antimicrobial activities against *Bacillus cereus* (Muñoz et al., 2004).

Grande et al. (2005a) have reported that the addition of enterocin AS-48 at a concentration of 2.5 μg/ml to commercial fruit juices reduced the viable counts to less than the detection limits within the first 15 min of incubation at 37°C. No viable cells were detected in any of the bacteriocin-treated juices for an incubation period of up to 90 days. Similar inhibition of spore outgrowth by AS-48 was observed in juices stored at 4°C and 15°C. Enterocin AS-48 was equally effective against vegetative cells of the tested *Alicyclobacillus*.

The mode of action of AS-48 on spores and vegetative cells of *Alicyclobacillus* was examined using electron microscopy. Untreated endospores of *A. acidoterrestris* DSMZ 2498 exhibited densely stained spore protoplasts surrounded by a less dense spore cortex and a thick and multilayered spore coat. During the course of germination, the cortex became less dense and larger in size, and the endospore coats became more diffuse and partially degraded. After 15 min of incubation with enterocin AS-48, some endospores exhibited a protoplast surrounded by a cortex less densely stained and also of larger size, and some exhibited localized and partial degradation of the spore coats. After

8 h, spore protoplasts were irregularly shaped and did not stain homogeneously, while degraded endospores were also observed. After 24 h, some endospores still retained intact spore coats and dense protoplasts, while others were surrounded by more diffuse layers and an ill-defined protoplast of very low density. Cell wall damage and loss of cytoplasmic content was observed in vegetative cells after 1 hr of bacteriocin addition. Cell disorganization was more pronounced after 7 h of incubation, yielding abundant cell debris. The short incubation time and small bacteriocin concentration (as small as 2.5 μg/ml) required for endospore inactivation suggests that AS-48 is rapidly adsorbed into the spore.

Despite the effectiveness of enterocin AS-48 to *Alicyclobacillus* as evidenced by the results of electron microscopy analysis, one critical disadvantage of enterocin AS-48 is the gradual loss of antimicrobial activity under ambient storage temperatures (Grande et al., 2005b). When commercial fruit juices were stored at 28°C for 120 days, the activity of AS-48 retained was between 31.5% (apple juice) and 67.71% (peach juice) the initial activity. Compared to nisin, enterocin AS-48 can not be applied prior to pasteurization due to rapid degradation under high temperatures. Based on the results of Grande et al. (2005a), enterocin AS-48 may be more effective when added into the pasteurized juices after the juice is cooled to room temperature.

5.3.3 Warnericin RB4

Warnericin RB4 is a bacteriocin produced by *Staphylococcus warneri* RB4 isolated by Minamikawa (2004) from a rice ball. The antimicrobial activity of warnericin RB4 is stable after heat treatment at 100°C for 15 min and through the pH range of 2 to 6 (Minamikawa et al., 2005). Partial inactivation (50% residual activity) was observed

following autoclaving in pH values of 7 to 10. Among 34 bacterial species tested using the agar diffusion assay, strong antibacterial activity of *S. warneri* RB4 was observed against vegetative cells of *A. acidoterrestris*, *A. acidocaldarius*, and two strains of *Micrococcus luteus*. The preliminary data reported by Minamikawa et al. (2005) indicate the possibility of using warnericin RB4 to control *Alicyclobacillus*. However, more in depth research is necessary to confirm the effectiveness of warnericin RB4 against *Alicyclobacillus* spores and to validate its antimicrobial activities in commercial fruit juices.

5.3.4 Lysozyme immobilized film

A novel lysozyme immobilized film was fabricated and tested against *A. acidoterrestris* in an acidified malt extract broth and in commercial apple juice (Conte et al., 2006). With an initial starting spore concentration of approximately 5 log CFU/ml, a decrease of 1.5 log CFU/ml for malt extract broth and 1 log CFU/ml for apple juice was observed over a storage period of 200 h at 44°C. Though Conte et al. (2006) concluded that the lysozyme film was effective against *Alicyclobacillus* spores, the antimicrobial efficacy and commercial applicability of the film remains in question. The reported 1-1.5 log CFU/ml reduction over 200 h may not be sufficient to prevent the production of guaiacol by *Alicyclobacillus* spores, and the method of application of the antimicrobial film to pasteurized juice packaging remains unclear.

6. Advances in *Alicyclobacillus*-related detection methods

Alicyclobacillus-related spoilage is characterized as a phenolic off-odor due to the production of guaiacol and possibly 2,6-DBP and 2,6-DCP (Walls and Chuyate, 2000). The detection of *Alicyclobacillus* spp. in both raw materials and final products is critical

for industrial quality control purposes (Connor et al., 2005). A schematic diagram of conventional agar plating methods used for the detection of *Alicyclobacillus* is illustrated in Figure 1-8. Conventional methods are time consuming and may result in delays in juice production, thus a more pragmatic approach for the industry would be to utilize rapid methods to detect *Alicyclobacillus*. This section discusses two new rapid detection advances since our previous review in 2004 (Chang and Kang, 2004).

6.1 Real-time PCR

Real-time polymerase chain reaction (PCR) approaches recently emerged as a preferred diagnostic tool in both medical and agricultural fields (Haugland et al., 1999; Danbing et al., 2000; Hein et al., 2001; Kaiser et al., 2001; Makino et al., 2001; Whiley et al., 2002; Khan and Yadav, 2004; McKillip and Drake, 2004). The incorporation of a fluorescent dye into the reaction mixture and the coupling of an optical module with the thermocycler enables the amplification results to be detected near real-time (Luo et al., 2004). Two primer-probe sets targeting squalene-hopene cyclase (SHC) and 16S rRNA of *Alicyclobacillus* were successfully developed for use in real-time PCR by Luo et al. (2004) and Conner et al. (2005), respectively.

Squalene-hopene cyclase (SHC) is a key biosynthesis enzyme for hopanoids, the membrane components of *Alicyclobacillus* involved in maintaining membrane fluidity and stability (Kannenberg and Poralla, 1999). Luo et al. (2004) reported the use of SHC encoding genes (*shc*) for real-time PCR, including the forward primer 5'-ATGCAGAGYTCGAACG-3', the reverse primer 5'-AAGCTGCCGAARCACTC-3', and the probe 5'-TCRGARGACGTCACCGC-3'. Utilizing this primer-probe set, Luo et al. (2004) reported that <10 cells per reaction can be readily detected within 3-5 h without

pre-extraction or enrichment procedures. In a similar approach, Conner et al. (2005) developed a primer-probe set for detecting *Alicyclobacillus* by targeting the 16S rRNA encoding gene sequences. The primer-probe set, including primers 5'-CGTAGTTCGGATTGCAGGC-3' (forward) and 5'-GTGTTGCCGACTCTCGTG-3' (reverse), and the probe 5'-CGGAATTGCTAGTAATCGC-3' enabled the detection of less than 100 *Alicyclobacillus* cells which can be completed within 5 h.

Results from Luo et al. (2004) and Conner et al. (2005) are comparable to data from previous studies reporting real-time PCR detection limits ranging from 1.0×10^1 - 1.0×10^2 to per reaction (Bassler et al., 1995; Nogva et al., 2000; Hein et al., 2001). The efficacy of the real-time PCR method is unaffected by juice ingredients, thus is applicable for use in juice systems. However, both studies were validated using *Alicyclobacillus* cells. For the detection of contaminant spores, additional modifications such as inducing spore germination must be used prior to DNA extraction.

6.2 Fourier transform infraredsSpectroscopy

Fourier transform infrared (FT-IR) is a useful method for classifying and identifying microorganisms and requires minimal sample preparation (Naumann, 2000). It has been successfully applied towards the identification of yeast (Lucia et al., 2001), cyanobacteria (Kansiz et al., 1999), lactic acid bacteria (Oberreuter et al., 2000), *Bacillus* spp. (Beattie, et al., 1998; Lin et al., 1998) *Listeria* spp. (Lefier et al., 1997), *Pseudomonas* spp. (Filip and Hermann, 2001), and coryneform bacteria (Oberreuter et al., 2003), and for monitoring microbial spoilage of meat (Ellis et al., 2002) and investigating microbial colony heterogeneity (Choo-Smith et al., 2001). The FT-IR spectral pattern provides a strain-specific "fingerprint" reflective of the compositions of

the cell wall, membranes, and cellular cytoplasm (Goodacre et al., 1996; Kansiz et al., 1999; Lin et al., 2005). Al-Qadiri et al. (2006) reported three adsorption peaks at approximately 2960 cm^{-1}, 2929 cm^{-1}, and 1740 cm^{-1} which were distinctive to *Alicyclobacillus*. These bands are mainly due to asymmetric stretches of methyl, methylene, and C=O groups (Kummerle et al., 1998; Kansiz et al., 1999), and most likely signifies the unique ω-alicyclic fatty acids in the membrane of *Alicyclobacillus*. In addition to being useful for distinguishing *Alicyclobacillus* from other contaminant microorganisms in apple juice (Al-Qadiri et al., 2006), FT-IR may also be applied in discriminating between guaiacol producing and non-guaiacol producing *Alicyclobacillus* (Lin et al., 2005). However, similar to the disadvantage of the previously discussed real-time PCR, the work by Al-Qadiri et al. (2006) and Lin et al. (2005) were based on vegetative cells of *Alicyclobacillus*. As contaminant *Alicyclobacillus* in pasteurized fruit juices may exist as spores, additional adjustments may be required for a more accurate identification of *Alicyclobacillus*.

7. Trend shifts in *Alicyclobacillus* research

The perceived threat of *Alicyclobacillus* to the pasteurized fruit juice industry has undergone several changes since its first link to a spoiled aseptically packaged apple juice in 1984 (Cerny et al., 1984). The shifts in research focus since are briefly discussed in this section.

The first *Alicyclobacillus* species identified as the causal microorganism in the spoiled aseptically packaged apple juice was *A. acidoterrestris* (Cerny et al., 1984). At that time, *Alicyclobacillus* was a relatively new genus consisting of the species *A. acidocaldarius*, *A. acidoterrestris*, and *A. cycloheptanicus* (Wisotzkey et al., 1992). It

was widely accepted that *A. acidoterrestris* was the only species capable of spoilage and spoilage related studies focused on this species (Nakayama et al., 1996; Baumgart et al., 1997; Splittstoesser et al., 1998; Eiroa et al., 1999; Silva et al., 1999; Baumgart and Menje, 2000; Orr et al., 2000; Silva and Gibbs, 2001; Lee et al., 2002; Jensen and Whitfield, 2003; Conte et al., 2006). Subsequently, new species including *A. acidiphilus*, *A. herbarius*, and *A. pomorum* were isolated from spoiled acidic drinks and it was evident that spoilage incidents were not specific to *A. acidoterrestris* (Matsubara et al., 2002; Goto et al., 2002a, 2003). Studies on contamination incidences, control, and detection of spoilage related *Alicyclobacillus* expanded to include the more recently identified spoilage species (Chang and Kang, 2005). In 2003, Chang provided evidence that *Alicyclobacillus* isolates were not uniform in the ability to produce guaiacol, and reported that even within the species of *A. acidoterrestris*, guaiacol production was limited to certain isolates. Since non-guaiacol producing *Alicyclobacillus* are neither pathogenic nor of significance to the juice industry, the novel approach of focusing on guaiacol producing *Alicyclobacillus* was proposed.

Detection and control procedures also changed with what type of *Alicyclobacillus* was perceived as important to the industry. Initially, it was important to have a sensitive detection procedure for *A. acidoterrestris*. Several recovery media and PCR methods were developed for this purpose (Chang, 2003; Luo et al., 2004; Connor et al., 2005). With the significance of guaiacol-producing *Alicyclobacillus* recognized, methods such as KV (peroxidase) method (Chang, 2003) and FT-IR (Lin et al., 2005) were developed to providing differentiation between guaiacol and non-guaiacol producing *Alicyclobacillus*. In terms of control measures, eliminatation of all contaminant

alicyclobacilli to reduce spoilage incidences was originally desired. However, complete eradication of alicyclobacilli was impractical due to the high heat resistance of *Alicyclobacillus* spores and the degradation of fruit juice quality by extreme measures. (Pontius et al., 1998). Rather than rely solely on destroying *Alicyclobacillus*, more preventive methods are being studied to reduce the concentration of *Alicyclobacillus* entering into the juice (Orr and Beuchat, 2000). Reduction of contaminating alicyclobacilli followed by combinations of mild treatments may prove to be an effective quality control measure against *Alicyclobacillus*.

8. Future research

Research on spoilage-related *Alicyclobacillus* has progressed significantly. Characterisitics of individual *Alicyclobacillus* species are widely studied, and in particular, detailed knowledge of *A. acidoterrestris* provided the fundamental basis for development of various detection and control methods (Brown, 1995; Pettipher et al., 1997; Pontius et al., 1998; Komitopoulou et al., 1999; Cerny et al., 2000; Orr and Beuchat, 2000; Grande et al., 2005a). The new concept of focusing on guaiacol-producing *Alicyclobacillus* is gaining acceptance, but basic characteristics of guaiacol-producing *Alicyclobacillus* remain largely unknown. Specifically, what differentiates guaiacol producing *Alicyclobacillus* from non-guaiacol producing *Alicyclobacillus* is of great interest. Information on the differences in guaiacol and non-guaiacol producing *Alicyclobacillus* may enable specific detection and targeted control of guaiacol producing isolates, and initiate metabolic studies on the mechanism of vanillic acid conversion to guaiacol by guaiacol producing isolates.

Another research direction that needs to be continuously updated involves the control of *Alicyclobacillus* spp. Present control measures are focusing more on reducing the contamination concentration of *Alicyclobacillus* spp. on fruits prior to juicing. The juice manufactuering industry currently relies on fruit washes with or without sanitizers to reduce contaminant concentrations of microorganisms. Chlorine is the most widely applied sanitizer for fruits and vegetables (Brown, 2000), but has limited effectiveness towards *Alicyclobacillus* spores due to loss of activity in the presence of organic matters and the protective effect of apple structure on spores (Orr and Beuchat, 2000). Recently, liquid and gaseous chlorine dioxide was demonstrated to be effective against various microorganisms (Brown and Wardowski, 1986; Foegeding et al., 1986; Roberts and Reymond, 1994; Cutter and Dorsa, 1995; Han et al., 1999; Lindsay et al., 2002; Beuchat et al., 2004; Lee et al., 2004; Sy et al., 2005a,b; Kreske et al., 2006a,b; Popa et al., 2007). Compared to chlorine, chlorine dioxide is not as readily inactivated by organic compounds and the gaseous application may be efficient in inactivating *Alicyclobacillus* spores located within the apple crevises.

Collectively speaking, the major objective of this research is to gather fundamental information on guaiacol-producing *Alicyclobacillus* to improve existing detection and control methods. Specific goals include:

1. To identify growth or physiological characteristics specific to guaiacol producing or non-guaiacol producing *Alicyclobacillus*. Successful identification of differential factors is useful towards developing specific detection and control methods for guaiacol producing *Alicyclobacillus*.

2. To investigate factors that may induce or accelerate the production of guaiacol by guaiacol producing *Alicyclobacillus*. Information gathered can be further utilized in metabolic studies on the conversion of vanillic acid to guaiacol.
3. To develop a highly sensitive selective media for isolating guaiacol producing *Alicyclobacillus*.
4. To study the potential use of different chlorine dioxide forms as control procedures for *Alicyclobacillus*.

Table 1-1. Physiological Characteristics of Known *Alicyclobacillus* Species.

Species	Growth		pH		Temperature (°C)		Growth factor requirement
	Aerobic	Anaerobic	Range	Optimum	Range	Optimum	
A. acidocaldarius	+	-	2.0-6.0	3.0-4.0	45-70	60-65	None
A. acidoterrestris	+	-	2.2-5.8	3.5-4.5	35-55	42-53	None
A. cycloheptanicus	+	ND	3.0-5.5	3.5-4.5	40-53	48	Methionine (or Vit. B_{12}), pantothenate, and isoleucine
A. hesperidum	+	ND	2.5-5.5	3.5-4.0	35-60	50-53	None
Alicyclobacillus genomic species 1	+	ND	2.5-5.5	3.5-4.0	40-70	60-63	None
A. herbarius	+	ND	3.5-6.0	4.5-5.0	35-65	55-60	None
A. acidiphilus	+	ND	2.5-5.5	3	20-55	50	None
A. sendaiensis	+	-	2.5-6.5	5.5	40-65	55	ND
A. pomorum	+	-	3.0-6.0	4.0-4.5	30-60	45-50	None
Alicyclobacillus genomic species 2	+	-	2.0-6.5	3.0-3.5	40-70	60	None
A. vulcanalis	+	ND	2.0-6.0	4.0	35-65	55	ND
A. tolerans	+	ND	1.5-5.0	2.0-2.7	<20-55	38-42	None
A. disulfidooxidans	+	ND	0.5-6.0	1.5-2.5	4-40	35	Yeast extract
A. sacchari	+	-	2.5-5.5	4.0-4.5	30-55	45-50	None
A. fastidious	+	-	2.5-5.0	4.0-4.5	20-55	40-45	None
A. kakegawensis	+	-	3.5-6.0	4.0-4.5	40-60	50-55	None
A. shizuokensis	+	-	3.5-6.0	4.0-4.5	35-60	45-50	None
A. macrosporangiidus	+	-	3.5-6.0	4.0-4.5	35-60	50-55	None
A. contaminans	+	-	3.5-5.5	4.0-4.5	35-60	50-55	None

ND: not described in original reference

Table 1-2. Morphological and Chemotaxonomical Characteristics of Known *Alicyclobacillus* Species.

Species	Type strain	Origin	Gram Stain	Morphology			Cell Wall Components		%G+C
				Colony	Cell	Spore	Wall lipids	Menaquinones	
A. acidocaldarius	104-1A^T =ATCC 27009^T =DSM 446^T	Thermal acid habitats in National Parks	+/v	Flat, unpigmented with irregular margins	2-3 × 0.7-0.8 μm rods, often in short chains of five or six cells	Ellipsoidal and terminal to subtermial endospores	ω-cyclohexyl fatty acids C17:0 (78%) C19:0 (16%)	MK-7	61.2-62.2
A. acidoterrestris	GD3B^T =DSM 3922^T	Soil (garden, oak wood, fir wood, moor, and apple juice)	+	Round, creamy white, translucent to opaque	2.9-4.3 × 0.6-0.8 μm rods	Oval, subterminal to terminal spores with slightly to unswollen sporangia, 1.5-1.8 × 0.9-1.0 μm	ω-cyclohexyl fatty acids C17:0 C19:0	MK-7* MK-6	51.6-53.3
A.cycloheptanicus	SCH^T =DSM 4006^T	Soil (car service station, garden, compost, wood, and from the Botanical Garden)	+	Round, smooth, creamy white and opaque	2.5-4.5 × 0.35-0.55 μm rods	Oval subterminal spores with slightly swollen sporangia; 1× 0.75 μm	ω-cycloheptylundecanoic acid, ω-cycloheptyltridecanoic acid, and ω-cycloheptyl-α-hydroxyundecanoic acid	MK-7* MK-6 MK-9	54.0-56.9
Alicyclobacillus genomic species 1	FR-6^T =DSM 11984^T	Acidic soils from solfataric areas	+	Not pigmented	2.1-4.2 × 0.5-0.8 μm rods	Terminal spores; sporangia not swollen	ω-cyclohexyl fatty acids C17:0 C19:0 branched chain fatty acids	MK-7	60.3

Table 1-2. Morphological and Chemotaxonomical Characteristics of Known *Alicyclobacillus* Species (cont'd).

Species	Type strain	Origin	Gram Stain	Morphology			Cell Wall Components		%G+C
				Colony	Cell	Spore	Wall lipids	Menaquinones	
A. herbarius	CR-1^T =DSM 13609^T =IAM 14883^T =NRIC 0477^T	Dried hibiscus flowers	+	Not pigmented		Oval subterminal endospores with swollen sporangia	ω-cycloheptane fatty acids C18:0 (67.1%) C20:0 (4.5%) ω-cycloheptane (2-OH) C18:0 (3.5%)	MK-7* MK-3 MK-8	56.2
A. acidiphilus	TA-67^T =DSM 14558^T =IAM 14935^T =NRIC 6496^T	Acidic beverage	+	round, smooth, creamy white and opaque	4.8-6.3 × 0.9-1.1 μm rods	Ellipsoidal to oval and terminal to subterminal with swollen sporangia	ω-cyclohexane C17:0 (82.6%) and C19:0 (12.3%)	MK-7	54.1
A. sendaiensis	NTAP-1^T =JCM 11817^T =ATCC BAA-609^T	Soil	-	circular, convex, white, and semi-tansparent	2-3 × 0.8 μm	Ellipsoid endospores	ω-cyclohexane C17:0 (44.1%) and C19:0 (30.2%)	MK-7	62.3
A. pomorum	3A^T =DSM 14955^T =IAM 14988^T	Spoiled mixed fruit juice	+^{o}	flat.smooth, and creamy white	2-4 x 0.8-1.0 μm	Oval and subterminal with swollen sporangia	Consists mainly of iso- and anteiso branched acids, no ω-alicyclic fatty acids	MK-7* MK-3	53.1
Alicyclobacillus genomic species 2	ND	Hot springs	-/v	smooth, opaque, white to cream colonies	2-3 x 0.6-0.8 μm straight rods	No endospores	ω-cyclohexane fatty acids	MK-7* MK-3	62.6-62.9
A. vulcanalis	CsHg2^T =ATCC BAA-915^T =DSM 16176^T	Geothermal pool	+	Semi-transparent to white, convex, up to 1 mm in diameter	1.5-2.5 × 0.4-0.7 μm rods	Terminal spores	ω-cyclohexyl 17:0 (46%) ω-cyclohexyl 19:0 (22.6%)	ND	62

Table 1-2. Morphological and Chemotaxonomical Characteristics of Known *Alicyclobacillus* Species (cont'd).

Species	Type strain	Origin	Gram Stain	Morphology			Cell Wall Components		%G+C
				Colony	Cell	Spore	Wall lipids	Menaquinones	
A. disulfidooxidans	SD-11^T =ATCC 51911^T =DSM 12064^T	Wastewater sludge	+/v		0.9-3.6 × 0.3-0.5 μm straight rods; single, paired, or in short chains	Oval, subterminal or terminal with swollen sporangia, 0.9-1.8 × 0.7-0.9 μm	ω-cyclohexane fatty acids		52-54
A. sacchari	RB 718^T =DSM 17974^T =IAM 15230^T	Liquid sugar	+^{o}	Non-pigmented, circular, opaque, 3-5 mm in diameter	4.0-5.0 × 0.6-0.7 μm straight rods	Ellipsoidal and subterminal with swollen sporangia	ω-cyclohexyl C17:0 and ω-cyclohexyl C19:0	MK-7	56.6
A. fastidious	S-TAB^T =DSM 17978^T =IAM 15229^T	Apple juice	+^{o}	Non-pigmented, circular, opaque, flat, 3-4 mm in diameter	4.0-5.0 × 0.9-1.0 μm straight rods	Ellipsoidal and subterminal with swollen sporangia	ω-cyclohexyl C17:0 and ω-cyclohexyl C19:0	MK-7	53.9
A. kakegawensis	5-A83J^T =DSM 17979^T =IAM 15227^T	Soil	+^{o}	Non-pigmented, circular, opaque, flat, 2-3 mm in diameter	4-5 × 0.6-0.7 μm straight rods	Oval and subterminal with swollen sporangia	ω-cyclohexyl C18:0, ω-cyclohexyl C18:0 2-OH and ω-cyclohexyl C20:0	MK-7	61.3-61.7
A. shizuokensis	4-A336^T =DSM 17981^T =IAM 15226^T	Soil	+^{o}	Non-pigmented, circular, convex, 1-2 mm in diameter	4-5 × 0.7-0.8 μm straight rods	Oval and subterminal with swollen sporangia	ω-cyclohexyl C18:0, ω-cyclohexyl C18:0 2-OH and ω-cyclohexyl C20:0	MK-7	60.5

Table 1-2. Morphological and Chemotaxonomical Characteristics of Known *Alicyclobacillus* Species (cont'd).

Species	Type strain	Origin	Gram Stain	Morphology			Cell Wall Components		%G+C
				Colony	Cell	Spore	Wall lipids	Menaquinone	
A. macrosporangiidus	5-A239 -2O-A^T =DSM 17980^T =IAM 15370^T	Soil	+^{o}	Non-pigmented, circular, opaque, convex, 2-4 mm in diameter	5-6 × 0.7-0.8 μm straight rods	Oval and terminal with swollen sporangia	iso-C16:0 (44.2%), iso-C17:0 (16.7%), and anteiso-C17:0 (25.2%)	MK-7	62.5
A. contaminans	3-A191^T =DSM 17975^T =IAM 15224^T	Soil	+^{o}	Non-pigmented, circular, opaque, 3-5 mm in diameter	4-5 × 0.8-0.9 μm straight rods	Ellipsoidal and subterminal with swollen sporangia	iso-C16:0 (13.7-16.9%), iso-C17:0 (22.8-29.3%), and anteiso-C17:0 (43.8-53.2%)	MK-7	60.1-60.6

^{o}: variable in older cultures
*: major menaquinone
ND: not described in original reference

Table 1-3. Carbon Source Assimilation by Known *Alicyclobacillus* Species.

Carbon Source	*Alicyclobacillus* Species																		
	A. acidocaldarius	*A. acidoterrestris*	*A.cycloheptanicus*	*A. hesperidum*	*Aicyclobacillus* genomic species 1	*A. herbarius*	*A. acidiphilus*	*A. sendaiensis*	*A. pomorum*	*Alicyclobacillus* genomic species 2	*A. vulcanalis*	*A. tolerans*	*A. disulfidooxidans*	*A. sacchari*	*A. fastidious*	*A. kakegawensis*	*A. shizuokensis*	*A. macrosporangiidus*	*A. contaminans*
N-Acetyl-Glucosamine	-	-	ND	-	-	ND	-	-	ND	ND	ND	ND	ND	-	-	-	-	-	-
Adonitol	-	v	ND	-	-	ND	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aesculin	+	+	ND	-	-	+	+	-	+	ND	ND	+	+	-	-	+	+	+	+
Amygdalin	-	v	-	-	-	+	-	-	+	ND	ND	+	+	-	-	+	-	-	-
D-Arabinose	-	-	+	+	-	+	+	-	-	ND	ND	w	+	-	+	+	-	+	-
L-Arabinose	+	+	-	+	+	+	+	+	-	ND	ND	w	+	+	+	+	+	+	+
D-Arabitol	-	+	-	-	-	ND	-	-	ND	ND	ND	ND	ND	-	-	+	-	+	-
L-Arabitol	-	-	ND	-	-	ND	-	-	ND	ND	ND	ND	ND	-	-	-	-	-	-
Arbutin	+	v	ND	-	-	+	+	+	-	ND	w	-	-	+	-	+	+	-	+
Cellobiose	ND	ND	ND	ND	ND	+	+	+	-	+	ND	-	+	+	-	+	+	-	+
Citrate	+	+	ND	-	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dulcitol	-	-	v	-	-	ND	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Erythritol	-	+	ND	-	-	-	-	-	-	ND	-	-	-	-	-	v	-	-	-
Esculin	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ethanol	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
D-Fucose	-	-	ND	-	-	+	-	-	ND	ND	ND	-	+	-	+	-	-	-	-
L-Fucose	-	-	v	-	-	ND	-	-	-	ND	ND	ND	ND	-	+	-	-	-	-
Galactose	+	+	+	+	+	+	+	+	-	ND	ND	+	-	+	+	+	+	+	+
Gelatin[b]	+	+	ND	+	+	-	-	ND	+	ND	ND	ND	ND	+	+	-	-	-	+
β-Gentiobiose	+	+	ND	+	-	+	+	-	-	ND	-	ND	ND	+	-	+	+	+	v

Table 1-3. Carbon Source Assimilation by Known *Alicyclobacillus* Species (cont'd).

Carbon Source	*Alicyclobacillus* Species																		
	A. acidocaldarius	*A. acidoterrestris*	*A.cycloheptanicus*	*A. hesperidum*	*Aicyclobacillus* genomic species 1	*A. herbarius*	*A. acidiphilus*	*A. sendaiensis*	*A. pomorum*	*Alicyclobacillus* genomic species 2	*A. vulcanalis*	*A. tolerans*	*A. disulfidooxidans*	*A. sacchari*	*A. fastidious*	*A. kakegawensis*	*A. shizuokensis*	*A. macrosporangiidus*	*A. contaminans*
Gluconate	-	-	ND	-	-	ND	-	-	ND	ND	ND	ND	ND	-	-	-	-	-	-
Glycerol	+	+	-	+	+	+	-	+	+	ND	ND	w	+	+	-	-	-	+	+
Glycogen	+	v	ND	+	-	-	-	+	-	ND	+	+	+	+	-	-	-	-	-
Hippurate	+	+	ND	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Inositol	v	v	v	-	v	ND	-	-	-	ND	+	-	-	-	+	v	-	-	-
Inulin	-	-	ND	-	-	ND	-	-	ND	ND	ND	ND	ND	+	-	-	-	-	-
2-Keto-Gluconate	-	-	ND	-	-	ND	-	-	ND	ND	ND	ND	ND	-	-	-	-	-	-
5-Keto-Gluconate	-	-	v	-	-	+	-	-	+	ND	w	+	+	-	-	-	-	-	-
Lactose	v	v	ND	+	+	+	+	+	-	ND	-	w	-	+	-	+	-	+	v
D-Lyxose	-	-	-	-	-	-	-	-	-	ND	ND	ND	ND	-	+	+	-	+	-
Malate	+	-	ND	v	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Maltose	+	+	ND	+	+	+	+	+	+	+	ND	w	+	+	-	+	+	+	+
Mannitol	+	+	+	+	+	+	-	+	+	ND	+	+	-	+	+	+	+	-	+
D-Mannose	+	+	+	+	+	+	+	+	+	ND	ND	+	-	+	+	+	+	+	+
Melezitose	v	-	ND	-	-	+	+	-	-	ND	ND	+	+	+	-	v	-	-	-
Melibiose	v	v	ND	v	v	+	-	-	-	ND	+	-	+	+	+	-	+	-	-
α-Methyl-D-glucoside	v	+	ND	-	-	+	+	+	+	ND	w	+	+	+	-	+	-	-	-
α-Methyl-D-mannoside	-	-	ND	-	-	+	-	-	-	ND	ND	ND	ND	-	-	+	-	+	-
β-Methylxyloside	-	-	ND	-	-	ND	-	-	ND	ND	+	ND	ND	+	+	-	-	-	-
Pyruvate	+	+	ND	-	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D-Raffinose	+	-	ND	-	v	+	+	+	-	ND	+	w	+	+	+	-	-	-	-
Rhamnose	v	+	v	-	v	+	-	-	-	ND	-	-	-	+	+	+	-	+	v
Ribitol	-	+	ND	-	-	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-	-	-	-

Table 1-3. Carbon Source Assimilation by Known *Alicyclobacillus* Species (cont'd).

Carbon Source	*Alicyclobacillus* Species																		
	A. acidocaldarius	*A. acidoterrestris*	*A.cycloheptanicus*	*A. hesperidum*	*Aicyclobacillus* genomic species 1	*A. herbarius*	*A. acidiphilus*	*A. sendaiensis*	*A. pomorum*	*Alicyclobacillus* genomic species 2	*A. vulcanalis*	*A. tolerans*	*A. disulfidooxidans*	*A. sacchari*	*A. fastidious*	*A. kakegawensis*	*A. shizuokensis*	*A. macrosporangiidus*	*A. contaminans*
Saccharose	+	v	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Salicin	+	+	ND	-	-	+	+	+	+	ND	-	-	-	+	-	+	+	+	v
Sodium Acetate	-	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sodium citrate	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sorbitol	-	+	+	-	-	-	+	-	-	ND	-	-	+	-	-	+	-	+	-
Sorbose	-	-	v	-	-	ND	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
L-Sorbose	-	-	ND	-	-	-	+	-	+	ND	ND	+	+	-	-	+	-	+	v
Starch	v	v	ND	w	-	-	-	ND	-	ND	ND	+	+	+	-	-	-	-	-
Succinate	+	+	ND	-	+	ND	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
Succinic acid	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sucrose	+	ND	ND	+	+	+	+	+	+	ND	+	+	+	+	-	+	+	+	+
D-Tagatose	-	-	ND	-	-	-	-	-	+	ND	ND	w	+	-	+	v	-	-	v
Trehalose	ND	ND	ND	ND	ND	+	+	+	+	+	+	+	-	+	+	+	+	+	+
D-Turanose	+	v	ND	+	+	+	+	+	+	ND	+	w	-	+	-	+	-	-	-
Tween 40	+	+	ND	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tween 60	+	+	ND	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tween 80	+	+	ND	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Xylitol	-	v	ND	-	-	-	+	-	-	ND	-	ND	ND	-	-	+	-	+	-
D-Xylose	+	+	+	-	-	+	+	+	-	ND	+	+	+	+	+	+	+	+	+
L-Xylose	-	-	ND	-	-	-	-	-	-	ND	ND	+	+	-	-	+	+	+	-

+ : positive reaction –: negative reaction w: weak reaction v: reaction variable based on test isolate
ND: not described in original article

Table 1-4. Function and biotechnological significance of enzymes isolated from *Alicyclobacillus*.

Enzyme	Isolation Source	Function	Biotechnological Significance
ADP-Ribose Transferase	*A. acidocaldarius*	Transfers single ADP-ribose unit from NAD^+ to specific acceptor proteins	High working temperature (65°C) compared to heat-liable eukaryotic ADPRT
α-amylase	*A. acidocaldarius* Agnano 101		First known thermoacidophilic α-amylase with optimums at 75C and pH 3.5
	A. acidocaldarius 11-1S		Thermoacidophilic α-amylase with optima of 70°C and pH 2.0
	A. acidocaldarius A-2		Thermoacidophilic α-amylase with optima of 70°C and pH 3.5
	A. acidocaldarius ATCC 27009	1. Random degradation of starch 2. Some pullulanase activity	1. First reported sequence of α-amylase in *A. acidocaldarius* 2. Provides insight for enzyme acidostability
L-Arabinose Isomerase	*A. acidocaldarius*	1. Isomerize L-arabinose to L-ribulose 2. Convert D-galactose into D-tagatose	Optimal conditions of 60°C and pH 6.0 is advantageous over current limitations in the production of D-tagatose
Collagenase	*A. sendaiensis* NATP-1	Degradation of collagen and gelatin	Optimal conditions of 60°C and pH 4.0 is advantageous over the instability of currently known collagenases

Table 1-4. Function and biotechnological significance of enzymes isolated from *Alicyclobacillus* (cont'd).

Enzyme	Isolation Source	Function	Biotechnological Significance
Cyclomaltodextrinase-neopullulanase (α-amylase subgroup)	*A. acidocaldarius* ATCC 27009	Degrade cyclomaltodextrin and pullulan to panose	Second highest temperature optimum of 55°C within this enzyme group
Esterase 1 (EST1)	*A. acidocaldarius*		
Esterase 2 (EST2)	*A. acidocaldarius*		1. Dual function highly sensitive marker enzyme/affinity tag 2. Alternative to *L. lactis* EstA for cheese flavor development
β-Glycosidase (Aaβ-gly)	*A. acidocaldarius* ATCC 27009	Hydrolysis of various linear oligosaccharides and disaccharides	
Squalene Hopene Cyclase	*A. acidocaldarius* ATCC 27009	Cyclization of linear squalene to fused ring compounds	Produces a wide array of unnatural cyclic polyprenoids
Thioredoxin	*A. acidocaldarius*	Key enzyme in various fundamental cellular processes	Higher heat stability than *E. coli* Trx

Table 1-5. Heat resistance of *Alicyclobacillus acidoterrestris* spores in different treatment matrix.

Treatment Matrix	pH	Soluble Solid	Treatment Temperature (°C)	Spore Isolate	D-value (min)	Z-value (°C)
0.25 mM Citrate Buffer						
	4.0	NR	85	NCIMB 13137	70.5±5.39[a]	8.83±2.24
		NR	90	NCIMB 13137	16.1±0.25[a]	
		NR	95	NCIMB 13137	5.19±0.94[a]	
Malt Extract Broth						
	2.5	5	85	NCIMB 13137	35.5±2.3	NR
	2.5	5	97	NCIMB 13137	0.771±0.037	
	2.5	32.5	91	NCIMB 13137	4.35±0.3	
	2.5	60	85	NCIMB 13137	60.3±2.4	
	2.5	60	97	NCIMB 13137	2.15±0.14	
	4.25	5	91	NCIMB 13137	25.3±1.6	
	4.25	32.5	85	NCIMB 13137	65.9±2.5	
	4.25	32.5	91	NCIMB 13137	10.2±0.1-15.5±0.6	
	4.25	32.5	97	NCIMB 13137	1.39±0.06	
	4.25	60	91	NCIMB 13137	25.3±1.6	
Apple juice						
	3.51	NR	80	Z CRA 7182	41.15±0.24	12.2
		NR	90	Z CRA 7182	7.38±0.85	
		NR	95	Z CRA 7182	2.30±0.03	
	3.5	11.4	85	VF	56.0±14.0	7.7
			90	VF	23.0±7.50	
			95	VF	2.8±0.70	
Berry juice						
	3.5	NR	88	NR	11	7.2
			91	NR	3.8	
			95	NR	1	
Blackcurrant concentrate						
	2.5	58.5	91	NCIMB 13137	24.1±2.7	NR
Light blackcurrant concentrate						
	2.5	26.1	91	NCIMB 13137	3.8±0.5	NR

Table 1-5. Heat resistance of *Alicyclobacillus acidoterrestris* spores in different treatment matrix cont'd).

Treatment Matrix	pH	Soluble Solid	Treatment Temperature (°C)	Spore Isolate	D-value (min)	Z-value (°C)
Cupuaçu extract						
	3.6	11.3	85	NCIMB 13137	17.5±1.1	9.0
		11.3	91	NCIMB 13137	5.35±0.57	
		11.3	95	NCIMB 13137	2.82±0.27	
		11.3	97	NCIMB 13137	0.57±0.03	
Cupuaçu nectar						
	3.2		95	NCIMB 13137	3.82±0.48[b]	29±10
			95	NCIMB 13137	5.5±1.2[c]	31±6
Grape juice						
	3.3	15.8	85	WAC	57.0±13.0	7.2
			90	WAC	16.0±4.10	
			95	WAC	2.4±0.90	
Grapefruit juice						
	3.42	NR	80	Z CRA 7182	37.87±0.20	11.6
		NR	90	Z CRA 7182	5.95±0.32	
		NR	95	Z CRA 7182	1.85±0.05	
Orange juice						
	NR	NR	85	DSM 2498	50.0	7.9
		NR	90	DSM 2498	16.90	
		NR	95	DSM 2498	2.70	
	3.5	11.7	85	NCIMB 13137	65.6±5.5	7.8
			91	NCIMB 13137	11.9±0.6	
	3.90	NR	80	Z CRA 7182	54.30±0.42	12.9
		NR	90	Z CRA 7182	10.30±0.30	
		NR	95	Z CRA 7182	3.59±0.04	

NR: not reported in original article
[a] data presented are those calculated from the first logarithmic phase
[b] results obtained by isothermal method
[c] results obtained by paired equivalent isothermal exposures (PEIE) method
NCIMB 13137=GD3B=DSM 3922=ATCC 49025
(*Source*: Splittstoesser et al., 1994; Walls, 1997; Eiroa et al., 1999; Komitopoulou et al., 1999; Silva et al., 1999; Vieira et al., 2002; Nakauma et al., 2004)

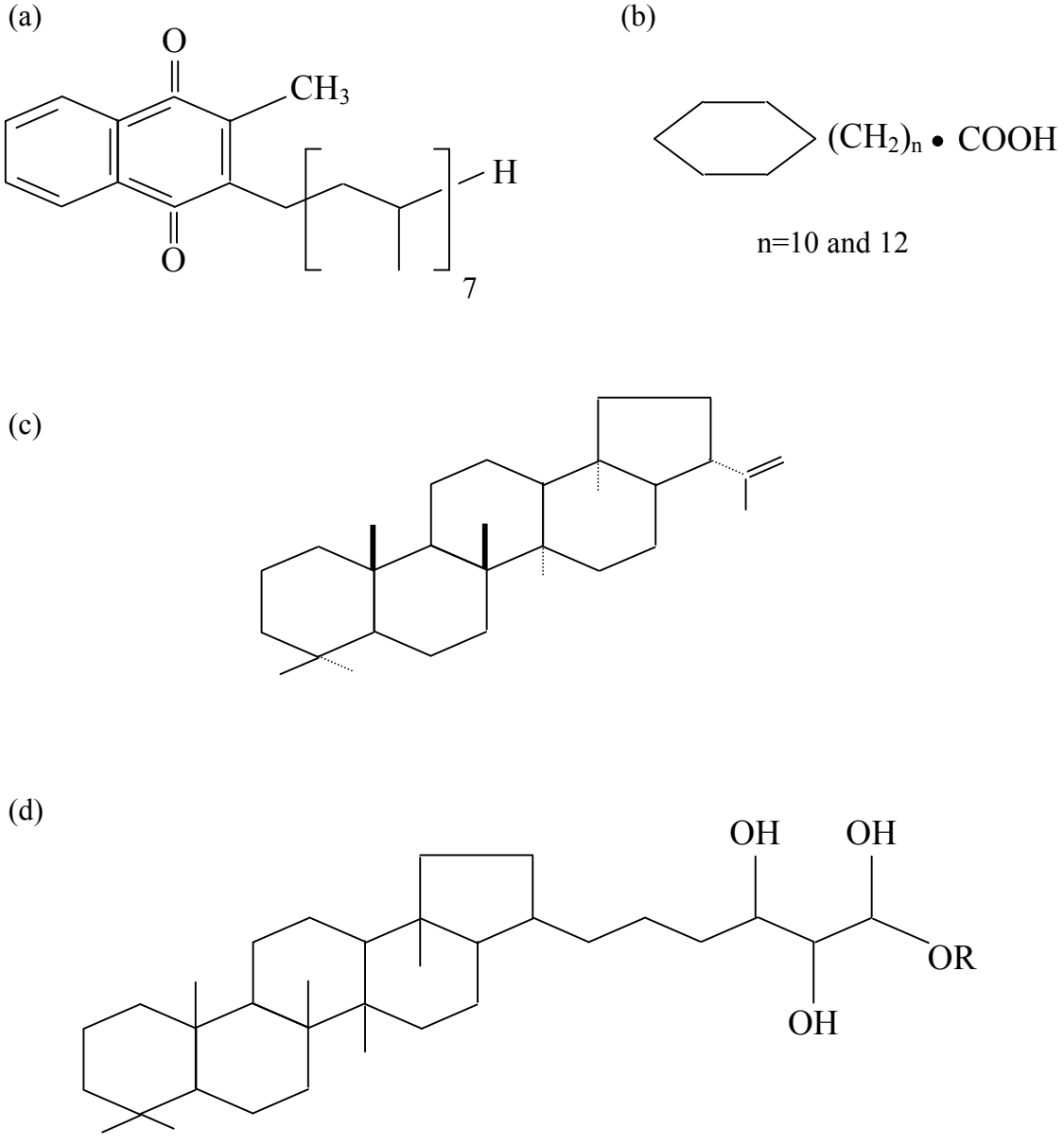
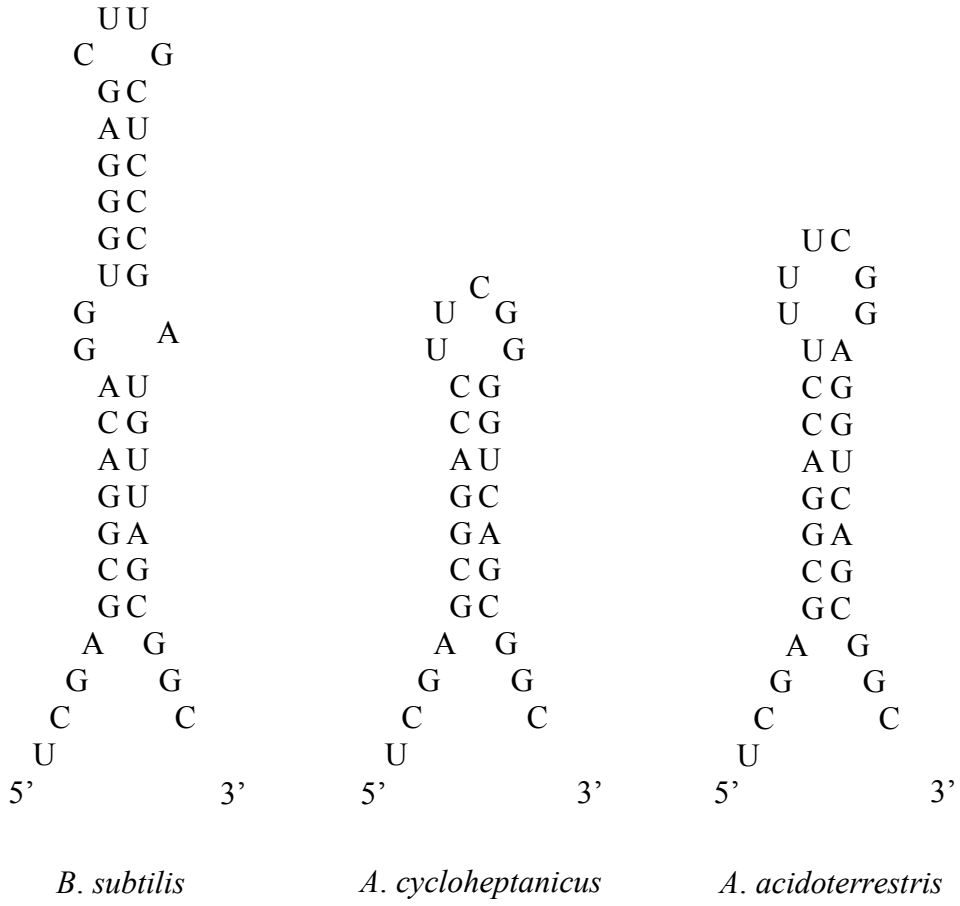


Figure 1-1. Chemical structures of unique lipids found in *Alicyclobacillus acidocaldarius*, including menaquinone MK-7 (a); ω-cyclohexyl fatty acids (b); pentacyclic triterpenes hop-22-(29)-ene (c), tetrol (d, R=H) and N-acylglucosaminyltriterpenetetrol (d, R=N-acylglucosamine). (*Source*: Minnikin and Goodfellow, 1982)



B. subtilis *A. cycloheptanicus* *A. acidoterrestris*

Figure 1-2. Diagram of the helix 6 region (*E. coli* positions 65 to 104) in *B. subtilis*, *A. cycloheptanicus*, and *A. acidoterrestris*. (*Source*: Wisotzkey et al., 1992)

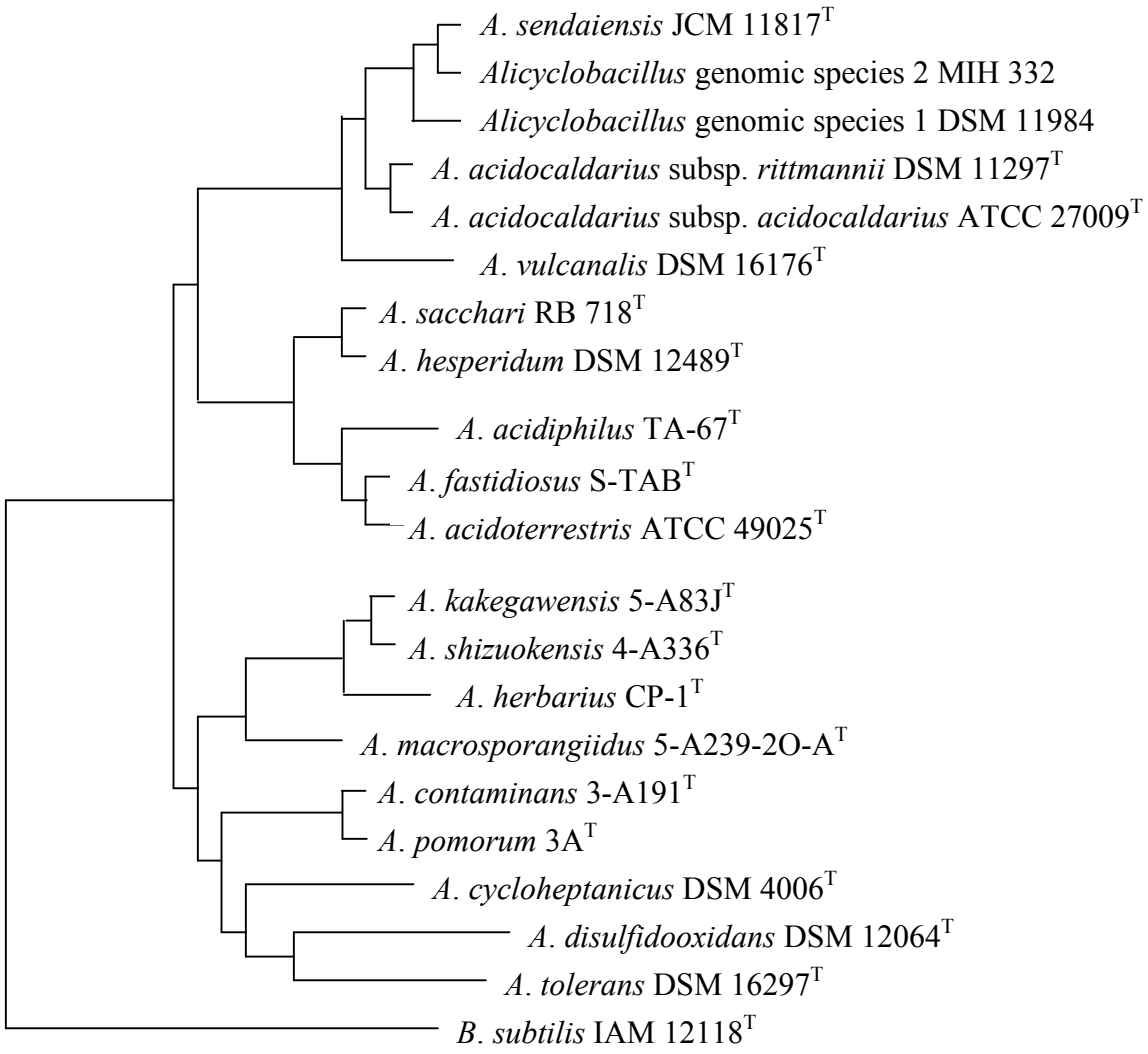
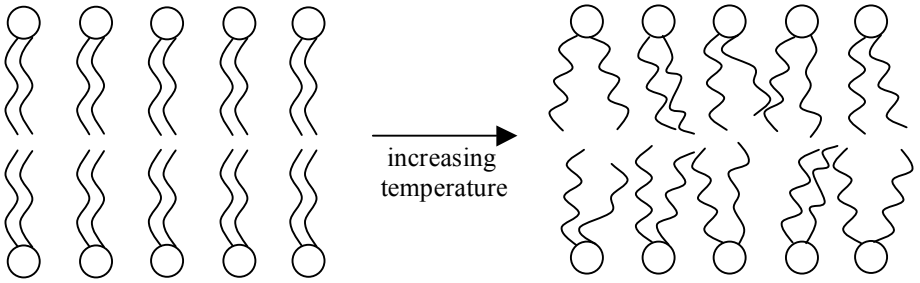


Figure 1-3. Neighbour-joining phylogenetic tree of *Alicyclobacillus* reference species based on 16S rRNA gene sequence comparisons. (*Source*: Goto et al., 2007)

(a) normal bacterial membrane bilayer



(b) membrane bilayer stabilized with hopanoids and alicyclic fatty acid

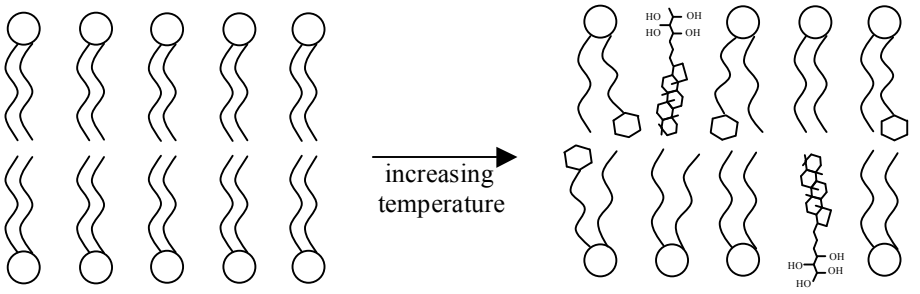
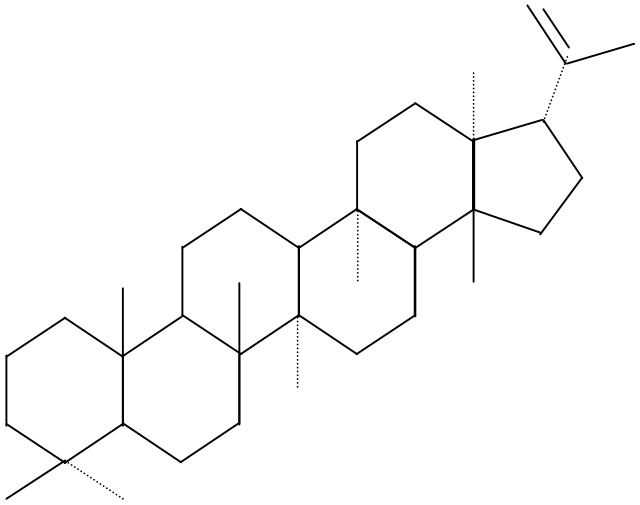
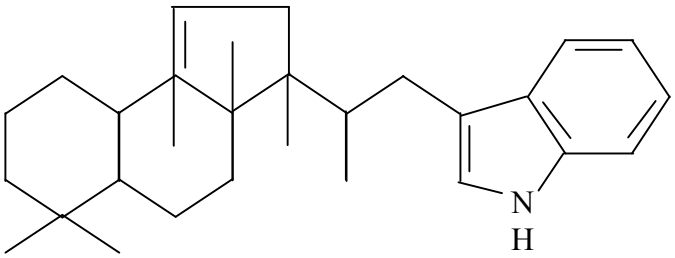


Figure 1-4. Schematic representation of the stabilizing effect of hopanoids and ω-alicyclic fatty acids on membrane bilayer.
(*Source*: Kannenberg and Poralla, 1999)

(a)



(b)



(c)

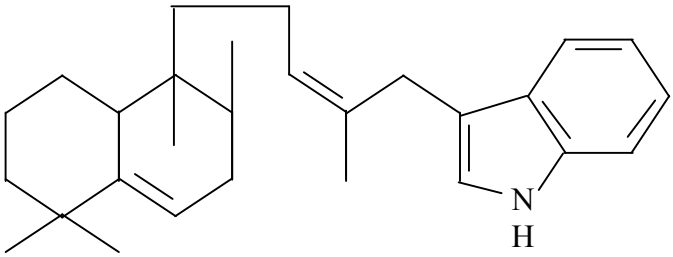


Figure 1-5. Formation of unnatural (a) hexacyclic, (b) pentacyclic, and (c) tetracyclic polyprenoids by *Alicyclobacillus acidocaldarius* ATCC 27009 squalene:hopene cyclase (SHC). (*Source*: Abe et al., 2002; Tanaka et al., 2005)

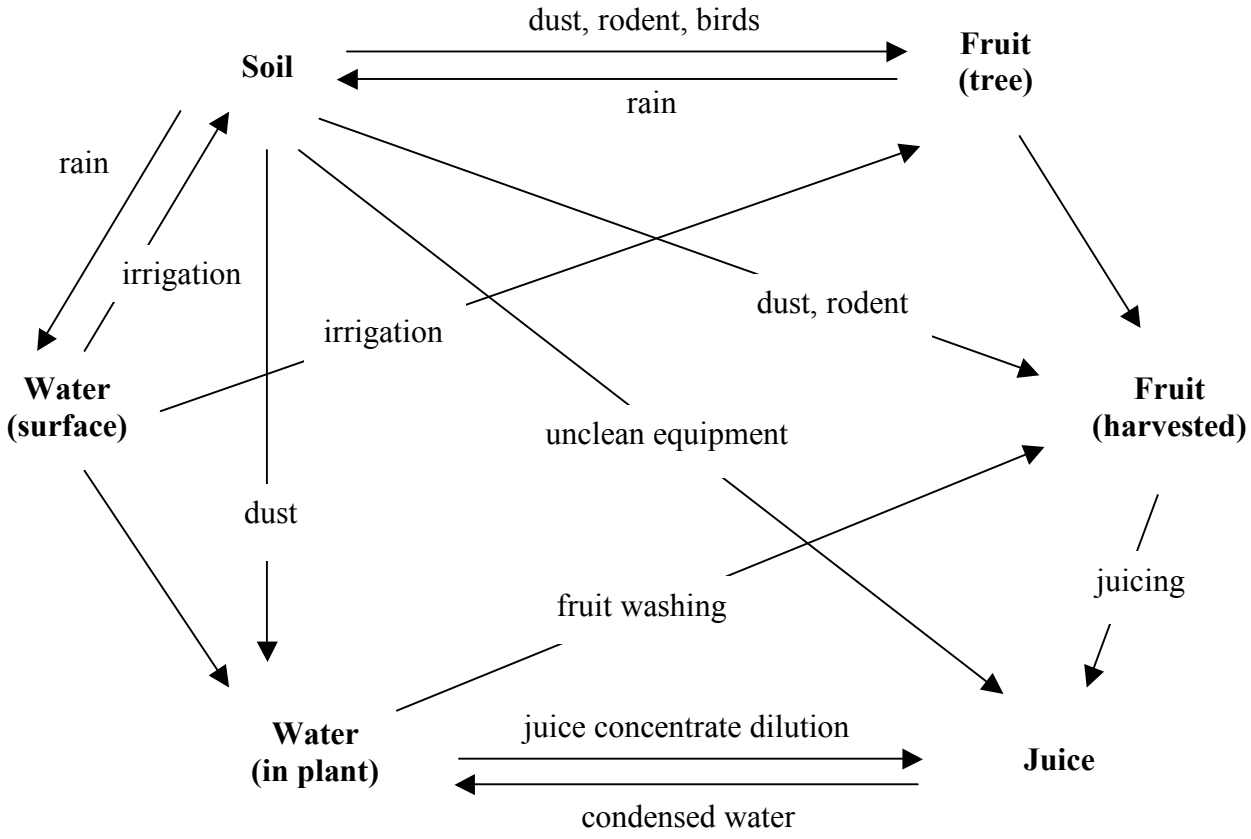


Figure 1-6. Possible contamination route of *Alicyclobacillus*.

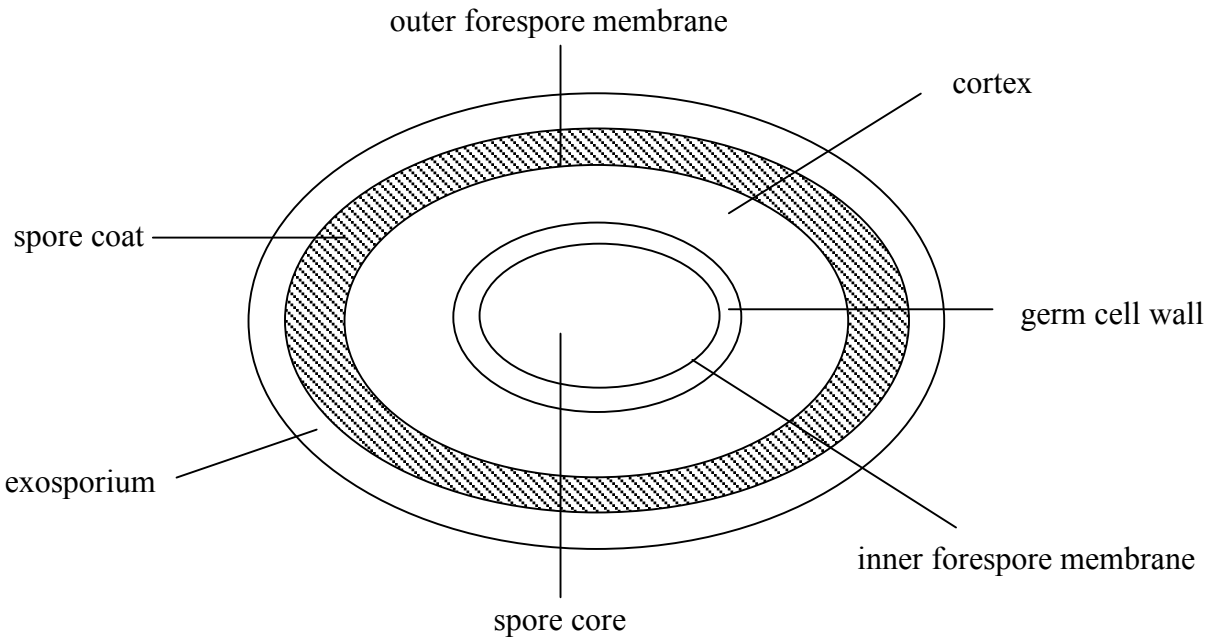


Figure 1-7. Structure of a spore. Structures are not drawn to size.

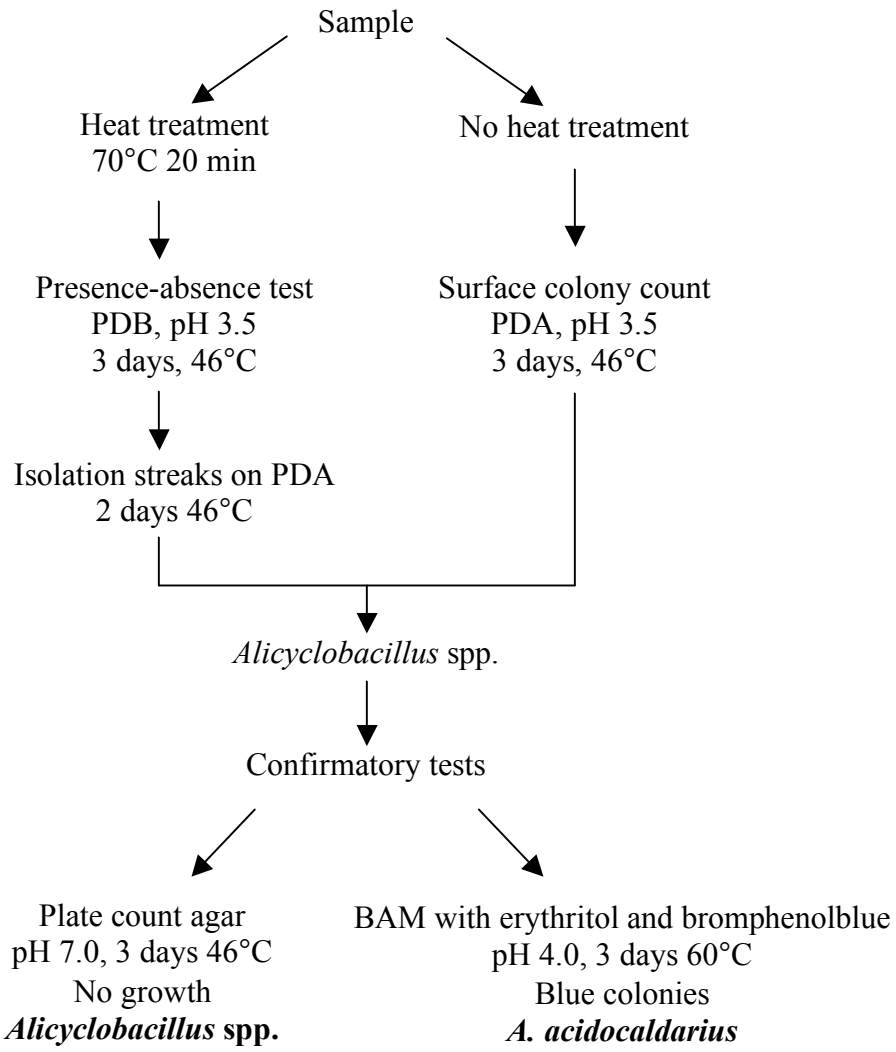


Figure 1-8. Conventional detection of *Alicyclobacillus* (*Source*: Baumgart and Menje, 2000).

CHAPTER TWO

DIFFERENTIATION OF GUAIACOL PRODUCING AND NON-GUAIACOL PRODUCING *ALICYCLOBACILLUS* SPP. BY GROWTH AND PHYSIOLOGICAL CHARACTERISTICS

ABSTRACT

Alicyclobacillus spp. are spoilage bacteria implicated in pasteurized juice products, especially in apple juice. Spoilage is characterized by guaiacol production. Recent studies indicate that not all *Alicyclobacillus* spp. are capable of producing guaiacol. In this study, effect of growth factors, carbohydrate utilization profile, and enzymatic profile differences between guaiacol-producing and non-guaiacol producing *Alicyclobacillus* spp. are investigated for possible differentiation parameters. Non-guaiacol producing isolates were more sensitive to growth factors and exhibited narrower optimum growth ranges compared to the guaiacol producing isolates. Despite growth rate differences, all test isolates grew between 32-55°C, pH 3.0-4.5, and under both aerobic and microaerophilic conditions regardless of the ability to produce guaiacol. Differentiation between the two types of alicyclobacilli was observed under different soluble solids concentrations. Non-guaiacol producing isolates were inhibited in media exceeding 4.9°Brix whereas guaiacol producing isolates grew in media with soluble solid concentrations up to 9.69°Brix. Carbohydrate utilization and enzyme profiles determined using api®CH 50 and api® ZYM varied among isolates. Gentiobiose utilization was specific to non-guaiacol producing *Alicyclobacillus* isolates but could not be used in routine analysis due to its high price. Though differences exist between guaiacol and non-guaiacol producing *Alicyclobacillus*, a simple and economic differentiation cannot be

achieved by adjusting incubation temperature, medium pH, oxygen concentrations, through the utilization of selected carbohydrates, or the presence of selected enzymes. The inhibition of non-guaiacol producing *Alicyclobacillus* by soluble solids concentrations exceeding 4.9°Brix is noteworthy but unsuitable for use in a differential detection media.

INTRODUCTION

Since the initial isolation of *Alicyclobacillus acidoterrestris* from pasteurized apple juice (Cerny et al., 1974), the significance of spoilage-related *Alicyclobacillus* is acknowledged and still remains an important issue in the juice industry (Murakami et al., 1998; Walls and Chuyate, 1998; Jensen and Whitfield, 2003; Chang and Kang, 2004). Research for the isolation and control of *Alicyclobacillus* in pasteurized fruit juices was originally based solely on *A. acidoterrestris* (Komitopoulou et al., 1999; Silva et al., 1999; Yamazaki et al., 2000; Nakauma et al., 2004; Grande et al., 2005a). It is now known that other *Alicyclobacillus* species are also responsible for spoilage in pasteurized drinks (Duong and Jensen, 2002; Matsubara et al., 2002; Goto et al., 2003).

From our previous study, it was concluded that isolates of *A. acidoterrestris* are not universally capable of producing guaiacol (Chang, 2003). The introduction of this concept in addition to reported spoilage incidents by *A. acidiphilus* and *A. pomorum* revolutionized the *Alicyclobacillus*-related quality control focus for the pasteurized fruit juice industry. Previously, all presumptive colonies isolated from juice samples were assumed capable of causing spoilage. Due to high isolation frequencies and the resistance of *Alicyclobacillus* spores to conventional pasteurization, the fruit juice industry resorted to using harsh control methods, including use of high temperatures to sterilize juice despite the loss of desirable juice qualities. Furthermore, new species of *Alicyclobacillus* unassociated with spoilage were identified from juice and raw materials (Goto et al., 2007). As *Alicyclobacillus* are not pathogenic bacteria, emphasis should be focused on spoilage related *Alicyclobacillus*, namely isolates that produce guaiacol.

Many basic studies on *Alicyclobacillus* species are available (Darland and Brock, 1971; Deinhard et al., 1987a,b; Dufresne et al., 1996; Albuquerque et al., 2000; Goto et al., 2002a,b; Matsubara et al., 2002; Goto et al., 2003; Simbahan et al., 2004; Karavaiko et al., 2005; Goto et al., 2007), however, research comparing *Alicylcobacillus* spp. based on the ability to produce guaiacol is limited. The focus of this study is to evaluate *Alicyclobacillus* spp. for potential growth and physiological differences between guaiacol producing and non-guaiacol producing *Alicyclobacillus*. Differential factors can be used to assist in the development of novel detection and control methods specific for guaiacol producing *Alicyclobacillus*.

MATERIALS AND METHODS

1. Preparation of laboratory media and chemical solutions.

Preparation of K agar/K broth.

K agar was prepared from individual components as described by Walls and Chuyate (2000): 2.5 g yeast extract, 5.0 g peptone, 1.0 g glucose, 1.0 g Tween® 80, 15 g agar and 1.0 L deionized water. The mixture was mixed and brought to a slight boil using a stirrer-hot plate to facilitate the solubilization of agar and dispersion of Tween® 80 prior to sterilization at 121°C for 15 min. Unless otherwise noted, the pH of K agar was adjusted to pH 4.0 with 10% (w/v) filter sterilized malic acid (J.T Baker, Phillipsburg, NJ) following sterilization. The agar medium was poured into disposable sterile petri dishes and air dried at room temperature for 48 h prior to use. K broth was prepared as described for K agar with agar omitted from the formula.

Preparation of KV broth.

KV basal broth was prepared by dissolving the components of K broth in 990 ml of deionized water and sterilizing at 121ºC for 15 min. A 1% (w/v) vanillic acid (Sigma®, St. Louis, MO) solution was prepared and filter sterilized using 0.22 μm filter membrane. KV broth was prepared by adding 10 ml of the 1% sterilized vanillic acid solution to the basal broth and adjusting the pH to pH 4.0 with 10% malic acid.

HPLC reagents and solvents.

Acetonitrile and formic acid used for the mobile phase were obtained from J.T. Baker (Phillipsburg, NJ), and water was treated with the MilliQ water purification system (Millipore, Billerica, MA) and sterilized using a 0.22 μm 500 ml filter sterilization unit (VWR International, West Chester, PA) prior to use. Guaiacol and vanillic acid were purchased from Sigma-Aldrich (St. Louis, MO) and used as standards.

HPLC instrumentation and chromatographic conditions.

The identification of vanillic acid and guaiacol was performed on an Agilent 1100 system equipped with CHEMSTATION software, a degasser, a gradient pump, a thermoautosampler, and a column oven (Agilent Technologies, Santa Clara, CA). The column system consisted of a Nova-Pak® 4 μm C18 cartridge (3.9×150 mm) in conjunction with a Nova-Pak® 4 μm C18 guard column (3.9×20 mm) from Waters (Milford, MA). The column was operated at 25°C with a mobile phase of acetonitrile:water:formic acid (20:80:1) at a flow rate of 0.5 ml/min. The ultraviolet wavelength used for the identification of guaiacol was 275 nm.

2. Differentiation of *Alicyclobacillus* isolates based on guaiacol producing ability.

Bacterial cultures and selection of representative isolates.

One hundred and nineteen *Alicyclobacillus* spp. isolates were obtained from the Washington State University Department of Food Science and Human Nutrition Culture Collection (Pullman, WA) and used in this study. For each isolate, 10 µl of the frozen stock spore suspension was inoculated into 9 ml KV broth using sterile 10 µl disposable loops (Copan Diagnostics Inc., Murrieta, CA) and incubated at 43°C. After 48 h of incubation, each cell culture was subjected to verification of guaiacol production with HPLC analysis and KV test. Simultaneously, the growth of the cell cultures in KV broth were confirmed by streaking onto K agar and incubating at 43°C for 48 h. Four isolates, including two guaiacol producing and two non-guaiacol producing isolates were selected to evaluate growth differences.

Verification of guaiacol production by HPLC.

One milliliter aliquots of the 48 h old cell culture was removed and filter sterilized into amber HPLC 1.5 ml vials (Agilent Technologies, Santa Clara, CA) with 0.22 µm Millex®GP filter units (Millipore, Carrigtwohill, CO) and sterile Monoject 3 ml syringes (Tyco, Mansfield, MA). The filtrates were injected into the HPLC at a volume of 50 µl and analyzed for guaiacol using the previously described HPLC conditions. The peaks of each isolate were compared with the standard retention time of vanillic acid and guaiacol standards. Isolates in which a peak correlated to the standard retention time of guaiacol was designated as guaiacol producing.

Verfication of guaiacol production by KV test.

Three milliliter aliquots of the 48 h old cell culture was removed and placed into a separate sterile test tube. For each tube, 75 µl of 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) and 25 µl of peroxidase (Sigma-Aldrich, St. Louis, MO) were added and placed at room temperature for 5 min for the color formation to stabilize. Guaiacol production was positive in tubes with a reddish brown pigmentation.

3. Evaluation of growth differences between guaiacol and non-guaiacol producing *Alicyclobacillus*.

Preparation of diluted spore inoculum.

Frozen spore suspensions of the four representative *Alicyclobacillus* spp. isolates were loop inoculated into sterile deionized water and heat shocked at 80°C for 10 min. After terminating the effect of heat by chilling in an ice bath, the heat shocked spore suspension was 10-fold serially diluted with 9 ml sterile deionized water to yield a spore inoculum of approximately 10^{2}-10^{3} CFU/ml for the following experiments.

Growth differences at different temperatures.

The diluted spore inoculum (100 µl) of each isolate was inoculated into 9.9 ml K broth (pH 4.0) and incubated aerobically at 25°C, 32°C, 37°C, 43°C, and 55°C. Aliquots of 1 ml were removed every 12 h for a duration of 48 h, 10-fold serially diluted with 9 ml sterile deionized water, spread plated onto K agar, and incubated at 43°C for 48 h prior to enumeration. The experiment was conducted in triplicate.

Growth differences at different pH.

The diluted spore inoculum (100 µl) of each isolate was inoculated into 9.9 ml K broth adjusted to pH 3.0, 3.5, 4.0, and 4.5 with 10% (w/v) malic acid and incubated at 43°C. Sample aliquots of 1 ml were removed every 12 h for a duration of 48 h, serially

diluted with 9 ml sterile deionized water, and plated onto K agar. Growth differences were evaluated by enumerating growth on K agar after incubation at 43°C for 48 h. The experiment was conducted in triplicate.

Growth differences at different oxygen concentrations.

Each diluted spore inoculum (100 μl) was inoculated into three sets of 9.9 ml K broth. The anaerobic set was prepared by tightly sealing the inoculated K broth tubes in an anaerobic jar containing a GasPak™ EZ Anaerobe Pouch System (BBL, Franklin Lakes, NJ). Likewise, the microaerophilic set was prepared using a GasPak™ EZ CampyPouch™ System (BBL, Franklin Lakes, NJ). The aerobic set was prepared by directly placing the inoculated K broth tubes into the incubator without additional adjustments. All three sets were incubated at 43°C and sampled every 12 h for a duration of 48 h. The anaerobic and microaerophilic conditions were maintained after each sampling by replacing the used GasPak™ EZ Anaerobe and CampyPouch™ Systems with fresh ones. Sample aliquots were serially diluted with 9 ml sterile deionized water, plated onto K agar (pH 4.0), and enumerated after incubation at 43°C for 48 h. The experiment was conducted in triplicate.

Growth differences at different soluble solids concentrations.

Fructose was obtained from Sigma-Aldrich (St. Louis, MO) and added to K broth at concentrations of 0, 2.5, 5, 7.5, and 10 g fructose/100 ml K broth. K broth was inoculated with the diluted spore inoculum at an initial starting concentration of 10^1-10^2 CFU/ml and incubated at 43°C. Aliquots of 1 ml were removed at 12, 24 38, 60 h, 10-fold serially diluted with 9 ml sterile deionized water, and plated onto K agar. Growth

was enumerated after incubation at 43°C for 48h. The experiment was conducted in triplicate.

Statistical analysis.

Triplicate data from each treatment per isolate were collected and analyzed statistically using Minitab Student Version 9.0 (Minitab Inc., State College, PA). Type I error rate was designated at 5%. The collected data was assessed in two ways, one to determine the difference among different isolates at a given treatment parameter and the other to determine the optimum growth conditions for each test isolate. The effect of each treatment parameter on growth of the selected isolates was analyzed comparing the growth of the four isolates at a given treatment. The optimum growth condition of each individual isolate was determined by comparing the growth of one isolate across various treatment parameters and determining the presence and location of significant difference. For each type of analysis, analysis of variance was first performed to determine significant differences (P<0.05) existed. When significant difference was detected by ANOVA, Tukey's multiple range test was performed to determine the location of where significant differences (P<0.05) existed between treatment means.

4. Determination of physiological differences between guaiacol producing and non-guaiacol producing *Alicyclobacillus*.

In addition to the four isolates used in the previous growth experiment, three each of guaiacol producing and non-guaiacol producing *Alicyclobacillus* spp. isolates were randomly chosen and used to assess physiological differences between the two types of *Alicyclobacillus*.

Differences in carbohydrate utilization.

Frozen spore suspensions of the selected *Alicyclobacillus* spp. isolates were loop inoculated (10 μl) into 9 ml K broth without glucose (K w/o G, pH 4.0). Bromophenol blue (LaMotte Chemical Products Co., Baltimore, MD) was prepared into a 0.1% (w/v) solution by dissolving in deionized water and sterile filtering. One droplet of 0.1% bromophenol blue was added into each inoculated 9 ml K w/o G tube, vortexed vigorously to evenly distribute the dye, and dispensed into api®CH 50 (bioMérieux, Hazelwood, MO) strips according to the manufacturers' instructions. Each api®CH 50 box was wrapped in Saran wrap to reduce moisture loss and incubated at 43°C for 7 d. Carbohydrate utilization was positive with the decrease of pH reflected in the change of color indicator from light blue to green. Reactions were designated as weak when the color changed from light blue to pale blue green.

Enzyme profile differences between guaiacol producing and non-guaiacol producing *Alicyclobacillus*.

Frozen spore suspensions of the selected *Alicyclobacillus* spp. isolates were loop inoculated (10 μl) into 9 ml K broth and incubated at 43°C. After 48 h, the actively growing cell culture was transferred into 50 ml K broth and incubated at 43°C for an additional 48 h to ensure the abundant growth of healthy cells. Following incubation, the cell suspension was centrifuged at 3,800 x g for 30 min using an IEC Centra CL2 benchtop centrifuge (Thermo Fisher Scientific Inc., Waltham, MA) and the supernatant was discarded. The cell pellet was resuspended with 5 ml sterile deionized water, vortexed, and centrifuged as previously described. The washing procedure was repeated for a total of three times. After the final centrifugation, the cell pellet was retained and resuspended with sterile deionized water to a turbidity between McFarland Standard 5

and 6 (bioMérieux, Hazelwood, MO). All cell suspensions were adjusted to the same turbidity using deionized water and 65 µl of the suspension was dispensed into each cupule of the api® ZYM (bioMérieux, Hazelwood, MO) strip. One drop each of ZYM A and ZYM B (bioMérieux, Hazelwood, MO) was added to each cupule following incubation at 43°C for 4 h. The api® ZYM strip was placed at room temperature for 5 min to allow stable color development prior to recording color changes. Results were recorded as recommended by the manufacturer with a numbering of 0-5 based on the intensity of color formation with "0" being no color formation and "5" being the strongest. The results were simplified to the following: no reaction (intensity 0), weak reaction (intensity 1-2), and positive reaction (intensity 3-5).

RESULTS

Differentiation of *Alicyclobacillus* spp. based on guaiacol production ability.

Typical reactions of guaiacol producing and non-guaiacol producing *Alicyclobacillus* as detected by HPLC analysis and KV test are illustrated in Figure 2-1 and Figure 2-2, respectively. The ability of 119 test isolates to produce guaiacol are listed in Table 2-1. Guaiacol was produced by 100 isolates (84.03%) and not produced by 19 isolates (15.97%). Isolates 1016, 1101, 19220, and C-GD 1-1 were selected based on these results to represent guaiacol producing and non-guaiacol producing *Alicyclobacillus* and used in the subsequent growth difference experiments.

Incubation temperature and guaiacol and non-guaiacol producing *Alicyclobacillus* isolates.

The effect of selected incubation temperatures on the growth of *Alicyclobacillus* spp. isolates are illustrated in Figure 2-3. At the incubation temperature of 25°C, no

apparent outgrowth in any isolate was observed after 48 h. At 32°C, outgrowth of isolates 1016 and 1101 was faster and significantly greater cell concentrations were achieved (P<0.05) than isolates 19220 and C-GD 1-1. Similar growth patterns were observed for incubation at 37°C and 43°C. Growth rates for isolates 1016 and 1101 were similar throughout the incubation range of 32°C-43°C, but the growth of isolates 19220 and C-GD 1-1 steadily increased with increasing temperature. Growth of isolate 19220 was significantly higher (P<0.05) than isolates 1016 and C-GD 1-1 at 55°C after 48 h. Isolate 1101 also grew well at the elevated temperature and no difference was observed between isolate 19220 and 1101 after 48 h. Isolates 1016 and C-GD 1-1 grew to different extents at 55°C, with final concentrations after 48 h at 10^5 and 10^3 CFU/ml, respectively.

Figure 2-4 illustrates the effect of the selected incubation temperatures on each individual isolate. For isolate 1016, the optimum incubation range was between 32-43°C. Incubation at 25°C and 55°C resulted in significantly (P<0.05) smaller cell concentrations after 48 h. A growth patterns as a function of temperature for isolate 1101 was similar to isolate 1016. No growth was observed at 25°C within the 48 h incubation period. At all other temperatures, rapid outgrowth was observed. Growth of isolate 1101 was greater at incubation temperatures of 43°C and 55°C than at 32°C and 37°C though no statistical differences could be determined. For isolate 19220, growth increased as the incubation temperature increased. Growth of isolate 19220 after 48 h was significantly higher at 55°C than at the other incubation temperatures. The growth of isolate C-GD 1-1at different incubation temperatures is as follows: 43°C>37°C>55°C>32°C>25°C. Incubation for 48 h at 43°C resulted in significantly higher (P<0.05) cell concentrations and thus was the optimum incubation temperature for isolate C-GD 1-1.

Comparing *Alicyclobacillus* spp. isolates based on their ability to produce guaiacol, temperature effect was more pronounced on non-guaiacol producing isolates (isolates 19220 and C-GD 1-1) than on the guaiacol producing isolates (isolates 1016 and 1101). For both 1016 and 1101, the final cell concentration of each isolate reached after 48 h incubation at 32°C, 37°C, and 43°C were not significantly different (P>0.05) from one another. On the other hand, non-guaiacol producing isolates were more sensitive to temperature change within this temperature range. The growth rate of non-guaiacol producing isolates increased as a function of temperature as evidenced by the increased slope of the growth curve (Figure 2-4). As shown in Figure 2-3, significant (P<0.05) differences existed between guaiacol producing and non-guaiacol producing isolates at 32°C. After 48h at 32°C, guaiacol producing isolates reached approximately 10^6 CFU/ml while non-guaiacol producing isolate populations remained under 10^3CFU/ml. Growth patterns at 37°C and 43°C were similar to those at 32°C except the growth of non-guaiacol producing isolates increased with the elevated temperatures. At 37°C after 48 h, growth of isolates 1016 and 1101 were significantly higher than that of isolate 19220, which in turn, was also significantly higher than isolate C-GD 1-1. At 43°C, no significant differences could be determined between the final concentrations of 1016, 1101, and 19220. On the other hand, growth of isolate C-GD 1-1 still remained significantly lower than the other isolates. When the incubation temperature increased to 55°C, no clear pattern could be distinguished between the two types of *Alicyclobacillus*. No significant differences existed between isolates 1101 and 19220, both reaching concentrations of 10^6 CFU/ml at 48h. Growth of isolates 1016 and C-GD 1-1 were

inhibited to different extents at 55°C, with final concentrations at 10^5 CFU/ml and 10^3 CFU/ml, respectively.

Effect of pH on the growth of guaiacol producing and non-guaiacol producing *Alicyclobacillus* isolates.

The effect of selected pH on the growth of *Alicyclobacillus* isolates at 43°C is shown in Figure 2-5. At pH 3.0, only isolate 1016 was observed to have achieved outgrowth after 48 h and was significantly different ($P<0.05$) from the other three isolates. At pH 3.5, outgrowth of isolates 1016, 1101, and 19220 was observed and reached 10^6 CFU/ml at 48 h. Growth of C-GD 1-1 increased, though final cell concentration remained significantly lower ($P<0.05$) than the other three isolates. The increase of media pH to pH 4.0 and above supported outgrowth of all test isolates. In general, increasing the pH of the media resulted in increased growth of *Alicyclobacillus*.

The effect of media on each individual isolate is shown in Figure 2-6. Isolate 1016 was capable of growing within the pH range of 3.0-4.5, though a slightly delayed log phase was observed for pH 3.0. Isolate 1101 grew well at a pH range of pH 3.5-pH 4.5. Limited growth was observed for isolate 1101 at pH 3.0. Similar to isolate 1101, growth of isolate 19220 was slower at pH 3.0 than at the other tested pH. The optimum growth pH for isolate 19220 was pH 3.5 which resulted in significantly higher cell concentrations than at pH 4.0 and pH 4.5. The effect of pH on growth of C-GD 1-1 was divided into two distinctive trends. Isolate C-GD 1-1 readily grew at pH≥4.0. At pH values of ≤pH 3.5, outgrowth was slow and resulted in only 10^3 CFU/ml after 48 h.

No pH provided distinctive differentiation between guaiacol producing and non-guaiacol producing *Alicyclobacillus*. At pH 3.0, growth of isolate 1016 was significantly

(P<0.05) different from the other isolates, including the guaiacol producing isolate 1101. Growth of isolate C-GD 1-1 was significantly (P<0.05) lower than the other isolates at pH 3.5 and 4.0 while no difference was determined among the other three isolates. No differences in growth among the four tested isolates could be determined at pH 4.5 after 48 h.

Effect of oxygen concentrations on the growth of guaiacol and non-guaiacol producing *Alicyclobacillus* isolates.

As illustrated in Figure 2-7, oxygen concentration affected the growth of *Alicyclobacillus*. All *Alicyclobacillus* isolates grew under aerobic and microaerophilic conditions, but no growth was observed under anaerobic conditions. Little to no differences existed between growth under aerobic and microaerophilic conditions for all isolates (Figure 2-8). The amount of oxygen present did not affect the growth of either type of *Alicyclobacillus* as evidenced by the nearly identical growth curves obtained under aerobic and microaerophilic conditions.

Effect of soluble solids concentration on the growth of guaiacol and non-guaiacol producing *Alicyclobacillus* isolates.

Calculated soluble solids concentrations (°Brix) of each K broth supplemented with fructose are listed in Table 2-2. Outgrowth of guaiacol producing isolates decreased as soluble solids concentrations increased (Figure 2-9). Isolates 1016 and 1101 grew in 4.94°Brix and 9.69°Brix K broth. Isolate 1101 was the only *Alicyclobacillus* isolate to grow at 14.44°Brix, reaching final concentrations of 10^3 CFU/ml after 60 h at 43°C. No growth was observed at a soluble solids concentration of 19.19°Brix. As soluble solids concentrations decreased, faster outgrowth was observed, and less time was required for

the isolates to reach the stationary phase. Growth of isolates 19220 and C-GD 1-1 were observed only in K broth with no supplemented fructose (0.19°Brix).

The effect of different soluble solids concentrations on individual test isolates is illustrated in Figure 2-10. All isolates were affected by soluble solids changes in the incubation media. Isolate 1016 was inhibited in 14.44°Brix and 19.19°Brix K broth. Growth of 1016 was slower at 9.69°Brix than at 0.19°Brix and 4.94°Brix, but the final cell concentration reached after 60 h was equal. Growth of isolate 1101 was more sensitive to the different soluble solids concentrations. The final cell concentrations of 1101 reached after 60 h incubation at 43°C were significantly different as a function of soluble solids concentration. As previously mentioned, growth of isolates 19220 and C-GD 1-1 was observed only in the control K broth. The growth properties of guaiacol producing *Alicyclobacillus* and non-guaiacol producing *Alicyclobacillus* differed greatly with regard to tolerance towards soluble solid concentration changes.

Selection of test isolates for the evaluation of physiological differences.

In order to gain a broad perspective of the *Alicyclobacillus* spp. population, and to reduce error due to a small sampling size, *Alicyclobacillus* isolates 41-2, 97, 113, 849, 6348, and WAC were used in addition to the previously mentioned isolates 1016, 1101, 19220 and C-GD 1-1 for the analysis of physiological differences. Isolates 97, 113, 1016, 1101, and WAC were representatives of the guaiacol producing population while isolates 41-2, 849, 6348, 19220, C-GD 1-1 reflected the non-guaiacol producing populations.

Physiological differences based on carbohydrate utilization.

Table 2-3 lists the ability of the selected *Alicyclobacillus* isolates to utilize 49 different carbohydrates. All carbohydrates could be utilized by at least one

Alicyclobacillus isolate. D-Ribose, D-glucose, arbutin, esculin ferric citrate, and potassium 5-ketogluconate were utilized by all isolates. In general, non-guaiacol producing isolates were capable of using a wider range of carbohydrates than guaiacol producing isolates. In addition to the previously mentioned carbohydrates utilizable by all isolates, the carbohydrates that could be utilized by all non-guaiacol producing isolates includes L-arabinose, L-xylose, D-galactose, dulcitol, inositol, D-mannose, salicin, D-saccharose, D-trehalose, D-melezitose, D-raffinose, xylitol, gentiobiose, and D-lyxose. The only other carbohydrate that was universally used by guaiacol producing isolates was D-fructose. The utilization of gentiobiose was specific to non-guaiacol producing isolates. No carbohydrate utilization was specific to guaiacol producing isolates.

Enzyme profile differences between guaiacol producing and non-guaiacol producing *Alicyclobacillus*.

The enzyme profiles as determined by api® ZYM are listed in Table 2-4. All *Alicyclobacillus* isolates regardless of guaiacol production ability were strongly positive for naphthol-AS-BI-phosphohydrolase, and negative for α-fucosidase. Other enzymes common to the *Alicyclobacillus* isolates in spite of some weaker reactions were esterase, esterase lipase, and acid phosphatase. Enzyme profiles were highly variable according to each individual isolate and no enzymes specific to either the guaiacol producing or non-guaiacol producing *Alicyclobacillus* could be determined.

DISCUSSION

The results presented in Table 2-1 are in agreement with previously reported observations that *Alicyclobacillus* can be differentiated into two types based on the production of guaiacol (Chang, 2003). Within the 119 isolates evaluated, 84.03% were

capable of causing spoilage by the production of guaiacol. The high percentage of guaiacol producing isolates as contaminants on the surface of orchard fruits and in commercial pasteurized juices and concentrates further justifies the juice industry classifying *Alicyclobacillus* as an important target microorganism (McIntyre et al., 1995; Yamazaki et al., 1996a; Wisse and Parish, 1998; Silva and Gibbs, 2001). However, *Alicyclobacillus* isolates of no significance to the juice industry exist as demonstrated by the 15.97% of contaminant *Alicyclobacillus* isolates that are not capable of producing guaiacol. Detection and control procedures must be redirected to focus solely on guaiacol-producing *Alicyclobacillus*. Though new species of *Alicyclobacillus* are continuously being discovered and novel detection/control methods are constantly being reported (Nakauma et al., 1996; Yamazaki et al., 1996; Baumgart et al., 1997; Splittstoesser et al., 1998; Komitopoulou et al., 1999; Orr and Beuchat, 2000; Chang and Kang, 2004; Luo et al., 2004; Grande et al., 2005a; Lin et al., 2005), limited information is available for guaiacol producing *Alicyclobacillus*. This study serves a two-fold purpose: (1) to provide basic knowledge on the characteristics of guaiacol producing *Alicyclobacillus*, and (2) to evaluate possible factors that can be used to easily differentiate guaiacol producing *Alicyclobacillus* from non-guaiacol producing *Alicyclobacillus*.

The effect of various growth parameters on the growth of *Alicyclobacilus* isolates 1016, 1101, 19220, and C-GD 1-1 are summarized in Table 2-5. The observed growth temperature and pH were consistent with previous *Alicyclobacillus* charaterization studies (Walls and Chuyate, 1998; Albuquerque et al., 2000; Goto et al., 2003, 2007). In addition to differences between guaiacol producing and non-guaiacol producing

Alicyclobacillus, individual differences also exist among isolates. The variations in growth characteristics are not unexpected as the categorization of *Alicyclobacillus* based on guaiacol producing ability encompasses several species with different physiological growth characteristics. Currently, *Alicyclobacillus* species that produce guaiacol include *A. acidoterrestris*, *A. herbarius*, *A. acidiphilus*, and *A. pomorum* (Cerny et al., 1974; Goto et al. 2002a; Matsubara et al., 2002; Goto et al., 2003).

The lowest temperature at which growth was observed for the test isolates was 32°C (Figure 2-3). No growth was observed when the isolates were incubated at 25°C. Several *Alicyclobacillus* spp. isolates are reported to grow at temperatures ≤25°C (Splittstoesser et al., 1994; Matsubara et al., 2002; Goto et al., 2007), however, the majority of *Alicyclobacillus* related research report growth starting at 30-35°C (Baumgart et al., 1997; Walls and Chuyate, 1998; Baumgart and Menje, 2000; Jensen, 2000; Walls and Chuyate, 2000; Jensen and Whitfield, 2003). Throughout the temperature range of 32-43°C, growth of guaiacol producing isolates were consistently higher than that of non-guaiacol producing isolates at any given sampling point despite the gradually increasing growth rate of the non-guaiacol producing isolates. At 55°C, within-group differences were more apparent than between-group differences. Growth of isolate 1101 and 19220 were rapid and reached 10^6 CFU/ml at the end of the 48 h. Both 1016 and C-GD 1-1 grew at 55°C but were inhibited compared to their respective growth at 43°C. The inhibition of 1016 and C-GD 1-1 at 55°C may be due to the fact that 55°C exceeded the optimum temperature at which the two isolates could grow. In view of providing a simple differentiation procedure, temperature is not a suitable parameter as both guaiacol producing and non-guaiacol producing isolates can grow between 32-55°C. Despite

growth rate differences illustrated in Figure 2-3, slow growth is not no growth, and thus it is impossible to distinguish which colonies on K agar are guaiacol producing and which are non-guaiacol producing after 48 h incubation.

The inability of non-guaiacol producing isolate C-GD 1-1 to grow at 55°C is of significance with regard to the temperature differentiation method for *Alicyclobacillus* used by the NFPA. Temperature differentiation method assumes that non-guaiacol producing alicyclobacilli are *A. acidocaldarius* which grow at elevated temperatures of 65°C. As demonstrated in this study, non-guaiacol producing *Alicyclobacillus* are not necessarily more thermotolerant than guaiacol producing *Alicyclobacillus*. Other reported guaiacol producing *Alicyclobacillus* species such as *A. herbarius* can grow at elevated temperatures of 65°C (Goto et al., 2002a), further highlighting potential problems with the temperature differentiation method.

Similar to temperature, growth of *Alicyclobacillus* isolates were affect by pH change (Figure 2-5). With the exception of isolate 19220, growth rates increased as pH increased. Optimum growth of isolate 19220 was observed at pH 3.5, and slightly decreased at pH 4.0 and pH 4.5. The ability of all the test isolates to grow at pH 3.0-4.5 and optimally between pH 3.5-pH 4.5 further emphasizes the challenge *Alicyclobacillus* posses to the juice industry. According to the U.S Food and Drug Administration, the approximate pH of several commonly consumed fruit juices are as follows: apple juice (pH 3.35-4.0), grapefruit juice (pH 2.93-3.25), California orange juice (pH 3.3-4.19), Florida orange juice (pH 3.3-4.15), pineapple juice (pH 3.3-3.6), prune juice (pH 3.95-3.97), and tomato juice (pH 4.1-4.6) (CFSAN-FDA, 2007). The pH range of the aforementioned juices coincide with the pH range of *Alicyclobacillus* growth, and thus

are at high risk of spoilage should guaiacol producing *Alicyclobacillus* enter into the production line. More specifically, apple, orange, and tomato juices may be most susceptible to *Alicyclobacillus* spoilage because the juice pH supports optimum growth of the contaminant microorganisms (Splittstoesser et al., 1994, 1998; Siegmund and Pöllinger-Zierler, 2006). Despite the effect of pH on growth rates of guaiacol producing and non-guaiacol producing *Alicyclobacillus*, pH does not inhibit non-guaiacol producing *Alicyclobacillus* and cannot be used as a simple differentiation method.

In contrast to results from temperature and pH, the effect of oxygen on *Alicyclobacillus* is straightfoward and consistent between guaiacol producing and non-guaiacol producing isolates. Test isolates grew at aerobic and microaerophilic conditions, but failed to grow anaerobically (Figure 2-7). The inability of *Alicyclobacillus* to grow anaerobically is conclusive (McIntyre et al., 1995; Cerny et al., 2000). Growth under aerobic and microaerophilic conditions were nearly identical for the four test isolates (Figure 2-8). Our results confirmed the results of Cerny et al. (2000) that *A. acidoterrestris* could grow under microaerophilic conditions in both apple and orange juices. In orange juice, as little as 0.1% oxygen was sufficient for outgrowth of *Alicyclobacillus*. Growth of *Alicyclobacillus* in apple juice required more than 3% residual oxygen. In both juice media, growth of alicyclobacilli was inhibited in anaerobic conditions (Cerny et al., 2000). Compared to guaiacol producing isolates, growth of non-guaiacol producing isolates was slower and required an additional 12 h incubation to reach maximum cell concentrations. Oxygen concentrations could not be used to differentiate between guaiacol and non-guaiacol producing *Alicyclobacillus*.

Since pasteurized juice and juice concentrates are the major products contaminated by *Alicyclobacillus*, the effect of soluble solids concentrations on the outgrowth of *Alicyclobacillus* is naturally of interest. Sugar components in juice differ and are reflective of the composition of the initial fruit (Lea, 1995). The main sugar in apple juice is fructose, with smaller and similar quantities of glucose and sucrose (Lea, 1995; Eisele and Drake, 2005). In orange juice, fructose, glucose, and sucrose are present in approximately equivalent concentrations (Southgate et al., 1995). Being the major sugar present in the juices that are most commonly associated with *Alicyclobacillus* spoilage, fructose was used to adjust the soluble solids concentrations in K broth. Results were expressed as °Brix to facilitate comparison with existing literature.

As illustrated in Figure 2-9 and Figure 2-10, non-guaiacol producing isolates were sensitive to changes in soluble solids concentrations, and did not grow in any media other than the control K broth. Guaiacol producing isolates grew between soluble solids concentrations of 0.2°Brix-9.7°Brix, but growth rates decreased as soluble solids concentrations increased. Outgrowth at 14.5°Brix was limited to isolate 1101 and very slow. The inability of *Alicyclobacillus* isolates to grow at 19.2°Brix was consistent with previous reports (Splittstoesser et al., 1994). Though reported inhibitory soluble solids concentrations vary depending on test isolates and juice type, it is generally accepted that sugar concentrations over 18°Brix are inhibitory towards *Alicyclobacillus*. Splittstoesser et al. (1994) observed that soluble solid concentration in Riesling grape juice had a predominant effect of the ability of the juice to support the growth of *Alicyclobacillus*. Limited growth of *Alicyclobacillus* was observed in 100% Riesling grape juice of 21.6°Brix. When the 100% Riesling grape juice was diluted with water to soluble solids

concentrations of 16.2°Brix and lower, *Alicyclobacillus* test isolates resumed growth, with maximum growth occurring at 16°Brix. Interestingly, *Alicyclobacillus* isolates did not grow if the Riesling grape juice was diluted using glucose solutions to maintain the original soluble solids concentration of 21.6°Brix. Soluble solids concentrations are inhibitory to *Alicyclobacillus* dependening on the type of juice. When Seyval grape juice was used, inhibition of *Alicyclobacillus* isolates was observed at 19.2°Brix (Splittstoesser et al., 1994). The soluble solids concentrations of common commercial juices and juice concentrations are listed in Table 2-6. All single strength fruit juices (including those reconstituted from juice concentrate) were smaller than 13°Brix, thus can readily support growth should *Alicyclobacillus* have the opportunity to enter into the finished product. Juice concentrates, on the other hand, exhibit Brix values of at least 22.9°Brix. It is unlikely that guaiacol producing *Alicyclobacillus* can grow and produce guaiacol directly within the juice concentrate. However, sporeforming bacteria such as *Alicyclobacillus* have the advantage of forming spores under poor nutritional conditions or hostile environments, and remaining dormant for a long period of time. As with its close relative *Bacillus*, dormant spores of *Alicyclobacillus* can rapidly germinate when suspended in a suitable environment (Doi, 1989), thus while guaiacol producing *Alicyclobacillus* are inhibited by the high soluble solid concentrations of juice concentrates, they can rapidly grow and result in spoilage when the concentrate is diluted to single strength. The inability of non-guaiacol producing isolates to grow at any soluble solids concentrations other than the control K broth is consistent with previous sections studying the effect of pH and temperature on growth. Non-guaiacol producing isolates were more sensitive to changes in temperature and pH, and often grew within a smaller range than its guaiacol

producing counterparts. A slightly increased soluble solids concentration of 4.9 °Brix may be a stressful environment for the non-guaiacol producing isolates and result in sporulation rather than outgrowth of the isolates. By far, soluble solids concentrations is the growth factor that provides the most distinction between guaiacol producing and non-guaiacol producing *Alicyclobacillus* isolates.

The general trend observed between guaiacol and non-guaiacol producing *Alicyclobacillus* in the growth characteristics study was that non-guaiacol producing *Alicyclobacillus* were more sensitive to environmental changes and had narrow growth ranges compared to the guaiacol producing *Alicyclobacillus*. Interestingly, the carbohydrate utilization profiles presented in Table 2-3 indicate an opposite trend. Acid was produced by the selected non-guaiacol producing isolates in 20 of the 49 carbohydrates (40.82%). In comparison, only 7 out of 49 carbohydrates (12.24%) could be utilized by the selected guaiacol producing isolates. The majority of the carbohydrates (29/49; 59.18%) were either weakly or not utilized by guaiacol producing isolates. Gentiobiose was specifically utilized by non-guaiacol producing *Alicyclobacillus* isolates, but is not suitable for differentiation due to its high price. Since the categorization of *Alicyclobacillus* according to guaiacol producing ability transcends several species, variation in characteristics within a type (guaiacol producing or non-guaiacol producing) is not unexpected. Differences in carbohydrate utilization among strains of the same *Alicyclobacillus* species were also observed in previous studies (Deinhard et al., 1987a,b; Albuquerque et al., 2000; Goto et al., 2002b).

Despite the different carbohydrate utilization profiles of each isolate, all tested *Alicyclobacillus* isolates could utilize D-glucose, D-ribose, arbutin, esculin ferric citrate,

and potassium 5-ketogluconate. However, when compared with 62 previously characterized *Alicyclobacillus* spp. isolates (Deinhard et al., 1987a,b; Albuquerque et al., 2000; Goto et al., 2002a,b; Matsubara et al., 2002; Goto et al., 2003; Tsuruoka et al., 2003; Simbahan et al., 2004; Karavaiko et al., 2005; Goto et al., 2007), glucose was the only carbohydrate that is universally utilized by *Alicyclobacillus*. Three pathways are associated with glucose metabolism, the Embden-Meyerhof-Parnas (EMP) pathway, the hexose monophosphate (HMP) pathway, and the Entner-Doudoroff (ED) pathway (Doelle, 1969). The EMP pathway produces two moles of ATP and pyruvate, but does not produce ribose 5-phosphate and erythrose 4-phosphate, important precursors for purine and pyrimidine synthesis. On the other hand, the HMP pathway produces the precursors necessary for purine and pyrimidine biosynthesis, but only one mole of ATP. Microorganisms utilizing the HMP pathway must exhibit a partially active EMP pathway to produce cellular energy since pyruate is not produced directly by the HMP pathway. Unlike the EMP and HMP pathways, the ED pathway can produce pyruvate, energy (one mole of ATP), and pentose precursors for pyridine and pyrimidine, and can be the sole glucose metabolic pathway of microorganisms (Cheldelin, 1961). In most facultative aerobes, EMP and HMP pathways work in cooperation to produce ATP and important precursors for purine and pyrimidine synthesis (Doelle, 1969). It is reported that in resting cells of *B*. *cereus*, 1-2% of the glucose was utilized by the HMP and the remainder by the EMP pathway (Blumenthal, 1961). The glucose catabolic pathway of *B*. *subtilis*, the closest *Alicyclobacillus* species relative with extensive metabolic studies, is estimated to contain 74% EMP and 26% HMP (Cheldelin, 1961). The majority of *Alicyclobacillus* species do not have specific growth requirements (Table 1-2). For a cell

to be independent of any purine or pyrimidine requirements, the HMP or the EP pathway must be active within the cell. As any microorganism with an active HMP pathway must also contain an operating EMP pathway, it is possible that *Alicyclobacillus* can utilize glucose either by a combination of EMP and HMP pathways or through the EP pathway. *Salmonella typhimurium* (Fraenkel and Horecker, 1964) and *Haemophilus parainfluenzae* (White, 1966) are reported to utilize glucose via all three glucose catabolic pathways.

The results of the enzyme profile of each *Alicyclobacillus* test isolate as determined by api ZYM® (Table 2-4) was similar to that of the carbohydrate utilization profiles. Enzyme profiles varied between isolates, but no enzyme was exclusive to either of the two types of *Alicyclobacillus*. With regard to functionality, slight differences were observed between esterase/proteinase and sugar hydrolases. Generally speaking, most ester hydrolases and peptide hydrolases were weak to positive in the test isolates, and no clear trend between guaiacol producing and non-guaiacol producing *Alicyclobacillus* was determined. On the other hand, the reactions for most sugar hydrolases were weak to negative with non-guaiacol producing isolates more reactive than guaiacol producing isolates. For example, α-glucosidase was positive in 80% non-guaiacol producing isolates, whereas its activity was undetected in 80% of the guaiacol producing isolates. Among the eight isolates showing positive β-glucosidase activity, non-guaiacol producing isolates exhibited strong activity by intense color formation as opposed to the weak reaction observed in guaiacol producing isolates. The weak enzymatic activities of the sugar hydrolases in guaiacol producing isolates may partially account for its weak ability to utilize disaccharides and polysaccharides and its narrow carbohydrate utilization range compared to the non-guaiacol producing isolates (Table 2-3). The api

ZYM® system is an invaluable tool providing fundamental information for the development of rapid differentiation methods. Differential methods based on api ZYM® findings have been reported for *Neisseria gonorrheae* and *N. meningitidus* (D'Amato et al., 1978), *Enterobacter sakazakii* from other *Enterobacter* species (Muytjens et al., 1984), *Pseudomonas cepacia* from other *Pseudomonas* species (Poh and Loh, 1988), and *Crypotococcus* species (Carcía-Martos et al., 2000). Based on our results, no specific enzyme can differentiate guaiacol producing from non-guaiacol producing *Alicyclobacillus*.

CONCLUSIONS

In summary, guaiacol producing and non-guaiacol producing *Alicyclobacillus* are not distinctively different with regard to growth patterns, carbohydrate utilization or enzyme profiles. The most distinctive characteristics identified between the two types of *Alicyclobacillus* include the inability of non-guaiacol producing isolates to grow in K broth supplemented with fructose and the specific utilization of gentiobiose by the non-guaiacol producing *Alicyclobacillus*. Growing *Alicyclobacillus* in a high soluble solids media is impractical for industrial application and the high price of gentiobiose limits its use in routine analysis. The factors tested within this study could not be used as a simple and economic differentiation among guaiacol and non-guaiacol producing *Alicyclobacillus*.

Table 2-1. Guaiacol Producing Ability[a] of *Alicyclobacillus* spp. Isolates

Isolate	Guaiacol	Isolate	Guaiacol	Isolate	Guaiacol
50	+	6348	-	A-Gala 4-1	+
97	+	6730	+	A-Gala 4-2	+
110	+	6871	+	A-Gala 6-1	-
113	+	7060	+	A-Gala 6-2	+
118	+	9500	-	A-Gala 9-1	+
282	+	9501	-	A-Gala 9-2	-
848	+	19101	-	A-Gala A4	+
849	-	19217	+	B-Gala 9	+
852	+	19218	+	B-GD 10	+
879	+	19219	-	C-Fuji 1	+
1016	+	19220	-	C-Fuji 3	+
1035	+	19297	+	C-Fuji 6	+
1101	+	19298	+	C-Fuji 8	+
1300	+	19432	+	C-GD 1-1	-
1746	+	107-1	-	C-GD 4-1	+
1938	+	112-1	-	C-RD 1	+
1972	+	14-1	+	C-RD 2	+
2056	+	14-2	+	C-RD 3	+
2057	+	16-1	+	C-RD 4	+
2058	+	16-2	+	C-RD 6	+
2329	+	18-1	-	C-RD 8	+
2330	+	18-2	+	C-RD 9	+
2331	+	19221-1	+	D-GD 1-1	+
2332	+	19221-2	+	D-GD 2-2	-
2333	+	19221-3	+	D-GD-4-1	+
2334	+	213937 5074 KC	-	F2-2514	+
2335	+	2304A	+	F2-2534	+
2450	+	2304B	+	F3-2534	+
2473	+	41-1	+	KF	+
2483	+	41-2	-	Oly 21	+
2723	+	51-1	+	Oly 3/29	+
2773	+	51-2	+	Oly 9-1E	+
2854	-	79-1	+	Oly 9-2B	+
3346	+	79-2	+	Pasteur-1	+
4988	+	81-1	+	Raw-1	+
4989	+	81-2	+	Raw-2	+
4999	+	A-Gala 1-1	+	RD-1	-
5301	+	A-Gala 1-2	-	VF	+
5700	+	A-Gala 2-1	+	WAC	+
5701	+	A-Gala 2-2	+		

Total isolates: 119
Guaiacol producing isolates: 100 (84.03%)
Non-guaiacol producing isolates: 19 (15.97%)
[a]: as determined by HPLC and KV method

Table 2-2. Conversion from fructose concentration to °Brix.

Fructose[1] (g)	Glucose[1](g)	Fructose + Glucose Concentration (g)	°Brix[2]
0	0.1	0.1	0.2
2.5	0.1	2.6	4.9
5.0	0.1	5.1	9.7
7.5	0.1	7.6	14.4
10.0	0.1	10.1	19.2

[1]: concentrations listed are grams per 100 ml of K broth
[2]: °Brix= (Fructose + Glucose Concentration)×342.3/180.16

Table 2-3. Carbohydrate utilization of *Alicyclobacillus* spp. isolates by api®50 CH at 43°C for 7 days.

Carbohydrate	*Alicyclobacillus* spp. Isolate									
	Guaiacol producing					Non-guaiacol producing				
	97	113	1101	1016	WAC	41-2	849	6348	19220	C-GD 1-1
Glycerol	w	w	-	-	w	+	+	+	-	+
Erythritol	w	+	-	-	w	-	+	+	-	-
D-Arabinose	w	-	-	-	w	-	+	+	+	-
L-Arabinose	+	-	-	w	-	+	+	+	+	+
D-Ribose	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	-	-	w	+	+	-	+	+
L-Xylose	w	-	-	-	w	+	+	+	+	+
D-Adonitol	w	-	-	-	-	-	+	+	-	-
Methyl-βD-Xylopyranoside	-	-	-	-	-	-	+	-	-	-
D-Galactose	-	-	w	-	w	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	w	-	+	+	w
D-Mannose	+	-	+	-	+	w	+	+	+	w
L-Sorbose	w	-	-	-	w	w	+	+	+	w
L-Rhamnose	+	-	-	-	w	w	+	+	+	w
Dulcitol	w	-	-	-	w	+	+	+	+	+
Inositol	+	-	+	-	-	+	+	+	+	+
D-Mannitol	w	+	-	-	w	+	+	+	+	+
D-Sorbitol	-	-	-	-	-	-	+	+	+	-
Methyl-αD-Mannopyranoside	+	-	-	-	-	-	-	+	-	-
Methyl-αD-Glucopyranoside	w	-	-	-	w	-	+	+	+	-
N-Acetylglucosamine	w	-	-	-	w	-	-	+	+	-
Amygdalin	w	-	-	+	-	-	+	+	+	-
Arbutin	+	+	+	+	+	+	+	+	+	+
Esculin ferric citrate	+	+	+	+	+	+	+	+	+	+
Salicin	w	+	-	+	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	-	+	+	+	+	+
D-Maltose	-	-	-	+	-	-	+	+	+	-
D-Lactose	-	-	-	-	-	-	+	+	+	-
D-Melibiose	-	-	-	-	-	-	-	+	w	-
D-Saccharose (sucrose)	-	-	-	w	-	+	+	+	+	+
D-Trehalose	-	-	-	w	-	+	+	+	+	+
Inulin	-	-	-	-	-	-	+	+	-	-
D-Melezitose	-	-	-	w	-	+	+	+	+	+
D-Raffinose	-	-	-	w	-	+	+	+	+	+

Table 2-3. Carbohydrate utilization of *Alicyclobacillus* spp. isolates by api®50 CH at 43°C for 7 days (cont'd).

Carbohydrate	*Alicyclobacillus* spp. Isolate									
	Guaiacol producing					Non-guaiacol producing				
	97	113	1101	1016	WAC	41-2	849	6348	19220	C-GD 1-1
Amidon (starch)	-	-	-	w	-	+	-	+	+	+
Glycogen	-	-	-	w	-	+	-	+	+	+
Xylitol	-	-	-	w	-	+	+	+	+	+
Gentiobiose	-	-	-	-	-	+	+	+	+	+
D-Turanose	-	-	-	-	-	-	+	+	w	-
D-Lyxose	+	-	-	+	w	+	+	+	+	+
D-Tagatose	w	-	-	-	-	-	+	+	+	-
D-Fucose	w	-	-	-	-	-	+	+	+	-
L-Fucose	w	-	-	-	-	-	+	+	+	-
D-Arabitol	w	-	-	-	-	-	+	+	+	-
L-Arabitol	w	-	-	-	-	-	+	+	+	-
Potassium gluconate	-	-	-	-	-	-	w	+	+	-
Potassium 2-ketogluconate	-	-	+	w	-	-	-	-	+	-
Potassium 5-ketogluconate	+	+	+	+	+	+	+	+	+	+

+: color change from light blue to green
w: color change from light blue to light blue-green

Table 2-4. Enzyme profile of *Alicyclobacillus* spp. isolates by api®ZYM at 43°C.

	Alicyclobacillus spp. Isolate									
	97	113	1016	1101	WAC	41-2	849	6348	19220	C-GD 1-1
Alkaline phosphatase	w	-	-	-	-	-	+	+	+	w
Esterase (C4)	+	w	+	+	w	w	+	+	+	+
Esterase lipase (C8)	+	w	+	+	w	+	+	+	+	+
Lipase (C14)	-	-	+	w	-	-	w	w	+	w
Leucine arylamidase	+	w	+	+	w	+	-	+	+	w
Valine arylamidase	+	w	+	+	w	+	-	+	+	+
Cystine arylamidase	-	-	w	w	-	-	w	-	w	w
Trypsin	+	+	+	+	w	+	w	+	+	-
α-Chymotrypsin	-	w	w	-	w	-	-	+	-	w
Acid phosphatase	+	+	+	+	+	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	-	w	w	-	-	-	w	+	w	w
β-Galactosidase	-	-	-	-	-	-	-	w	w	w
β-Glucuronidase	-	-	-	w	-	-	-	-	-	w
α-Glucosidase	-	-	-	w	-	w	+	+	+	+
β-Glucosidase	-	w	w	w	w	+	+	+	+	-
N-Acetyl-β-glucosaminidase	-	-	-	-	-	-	-	-	-	w
α-Mannosidase	-	w	-	w	-	-	-	-	-	-
α-Fucosidase	-	-	-	-	-	-	-	-	-	-

-: color intensity 0
w: color intensity 1-2
+: color intensity 3-5

Table 2-5. Growth ranges and optimum conditions of *Alicyclobacillus* at selected growth parameters.

Growth Parameters	*Alicyclobacillus* spp. Isolate			
	Guaiacol producing		Non-guaiacol producing	
	1016	1101	19220	C-GD 1-1
Temperature (°C)				
Range	32-55	32-55	32-55	32-55
Optimum	37-45	43-55	55	43
pH				
Range	3.0-4.5	3.0-4.5	3.0-4.5	3.0-4.5
Optimum	3.5-4.5	3.5-4.5	3.5	4.0-4.5
Oxygen Requirement				
Aerobic	+	+	+	+
Microaerophilic	+	+	+	+
Anaerobic	-	-	-	-
Soluble Solids (°Brix)				
Range	0.19-9.69	0.19-14.44	0.19	0.19
Optimum	0.19	0.19	0.19	0.19

Table 2-6. USDA soluble solid standards of various juice products commonly identified in *Alicyclobacillus*-related spoilage

.

Product Type	Grade	Dilution Factor	Minimum Brix (°)	Other
Apple Juice	A		11.0	
	B		10.5	
Apple Juice Concentrate	None	1+1	22.9	
		2+1	33.0	
		3+1	42.2	
		4+1	50.8	
		5+1	58.8	
		6+1	66.3	
		7+1	73.3	
Grape Juice Concentrate		1+1	24.8	
		2+1	35.5	
		3+1	45.4	
		4+1	54.5	
		5+1	62.8	
		6+1	70.7	
Frozen Concentrated Grapefruit Juice	Unsweetened		38.0	Brix after reconstitution: 10.6°
	Sweetened		38.5	Brix after reconstitution: 10.6°
Grapefruit Juice from Concentrate	Unsweetened		10.0	
	Sweetened		11.5	
Orange Juice	A		10.5	
	B; unsweetened		10.0	
	B; sweetened		10.5	
Frozen Concentrated Orange Juice	Sweetened		41.8	Brix after reconstitution: 11.8°
	Unsweetened		42.0	Brix after reconstitution: 11.8°
Pineapple Juice	A; unsweetened		12.0	
	A; sweetened		12.5	
	B; unsweetened		10.5	
	B; sweetened		11.0	
Pineapple Juice from Concentrate	A; unsweetened		12.8	
	A; sweetened		13.0	
	B; unsweetened		12.8	
	B; sweetened		13.0	

(*Source*: Agricultural Marketing Service, USDA)

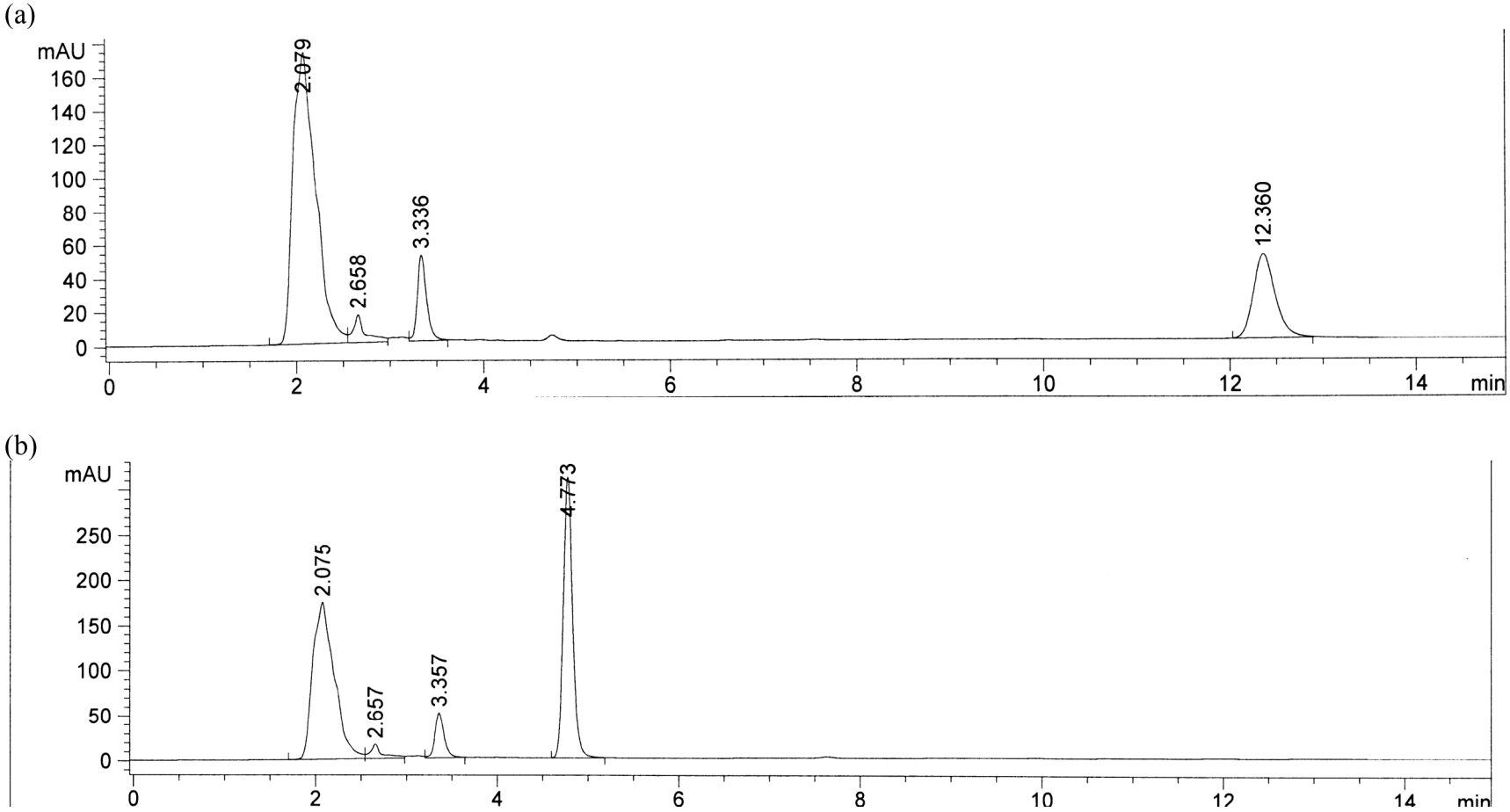
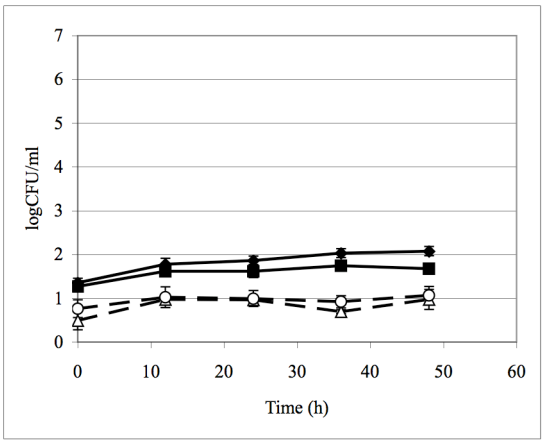


Figure 2-1. Lithograms of (a) guaiacol producing and (b) non-guaiacol producing *Alicyclobacillus* after incubation at 43°C for 48h. Standard retention time vanillic acid and guaiacol are 4.773 min and 12.36 min, respectively.

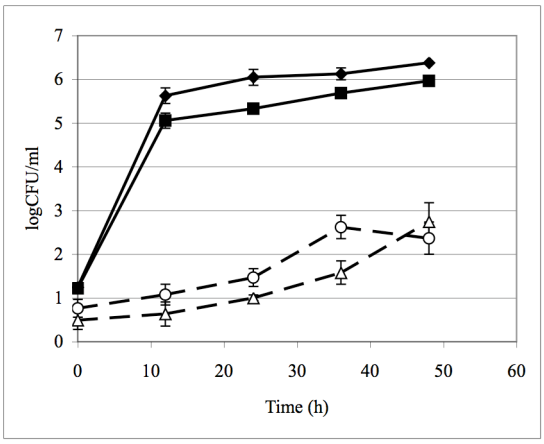
(a) (b)

Figure 2-2. Color differentiation of (a) non-guaiacol producing and (b) guaiacol producing *Alicyclobacillus* using KV test after incubation in KV broth at 43°C for 48 h and the addition of 25 μl peroxidase and 75 μl 3% hydrogen peroxide.

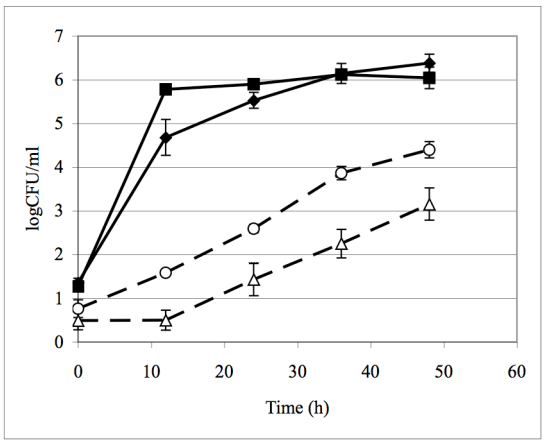
(a) 25°C



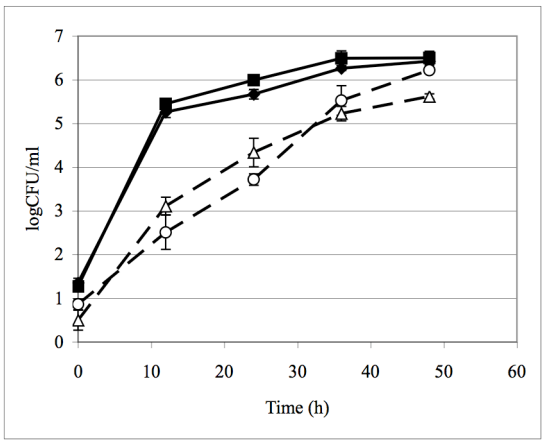
(b) 32°C



(c) 37°C



(d) 43°C



(e) 55°C

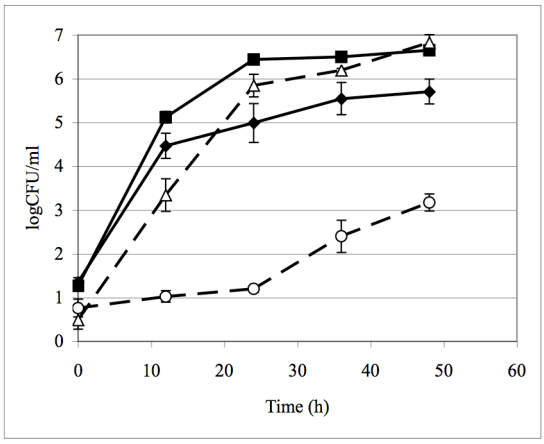


Figure 2-3. Growth of *Alicyclobacillus* spp. isolates 1016 (—◆—), 1101 (—■—), 19220 (--△--), and C-GD 1-1 (--○--) in K broth (pH 4.0) at (a) 25°C, (b) 32°C, (c) 37°C, (d) 43°C, and (e) 55°C. Error bars indicate a 5% error rate.

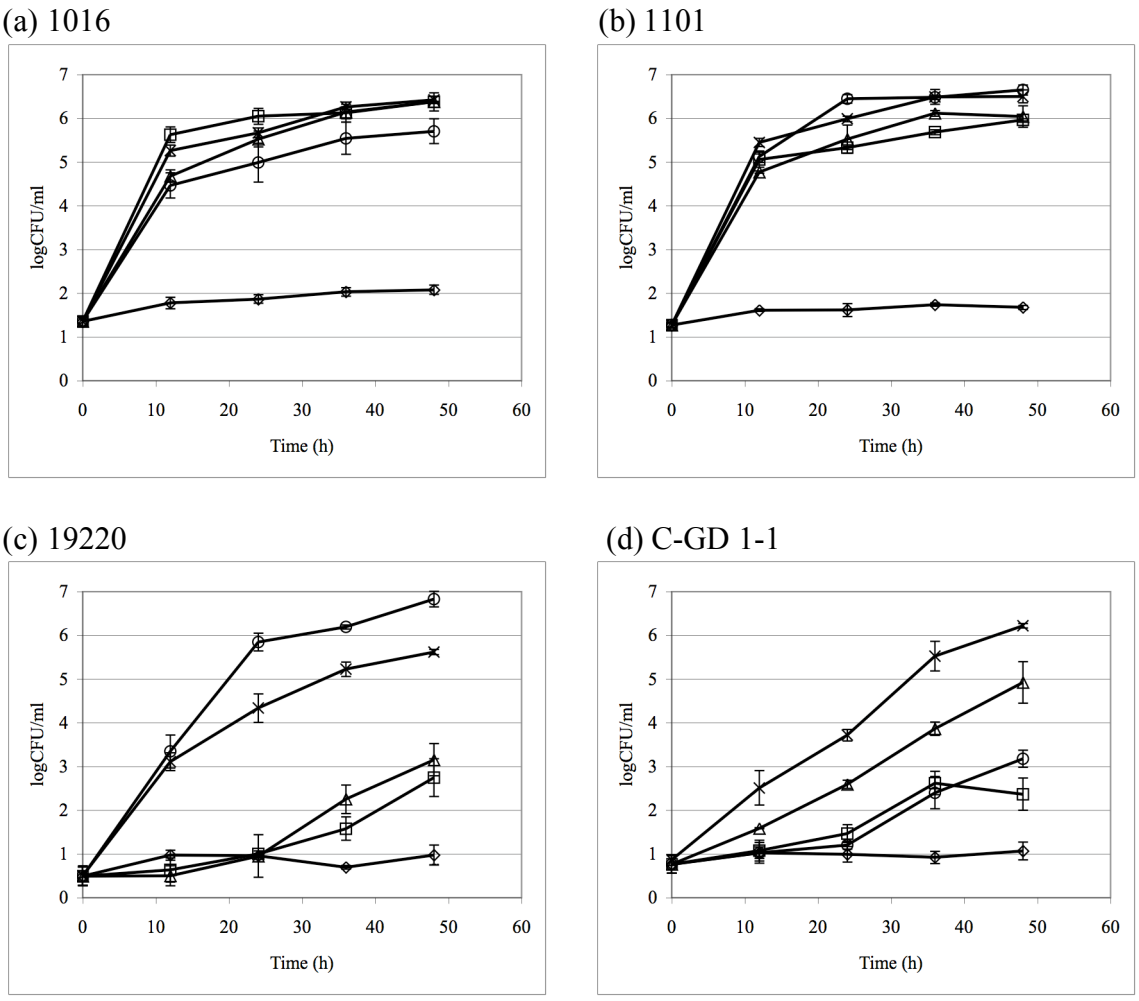
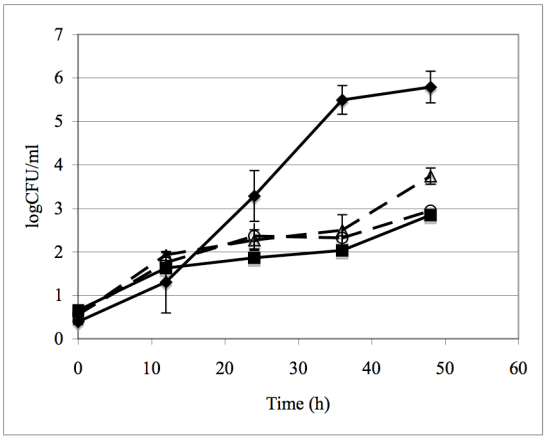
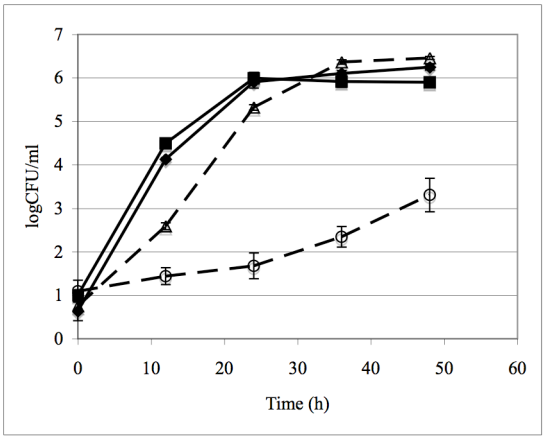


Figure 2-4. Growth of *Alicyclobacillus* spp. isolates (a) 1016, (b) 1101, (c) 19220, and (d) C-GD 1-1 in K broth (pH 4.0) at 25°C (—◇—), 32°C (—□—), 37°C (—△—), 43°C (—✕—), and 55°C (—○—). Error bars indicate a 5% error rate.

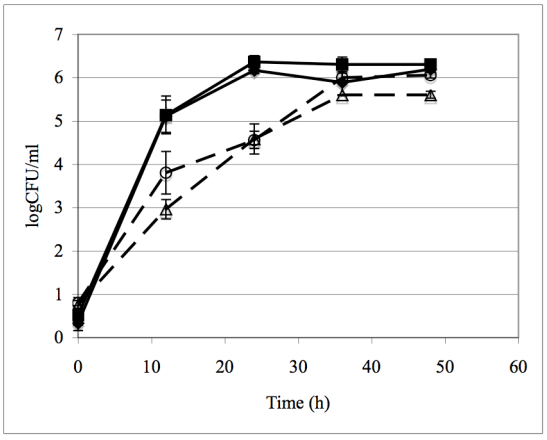
(a) pH 3.0



(b) pH 3.5



(c) pH 4.0



(d) pH 4.5

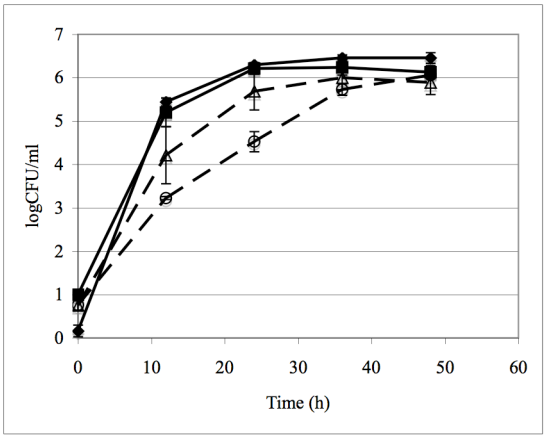
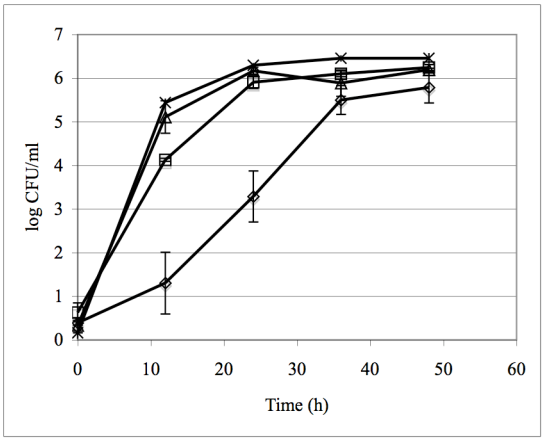
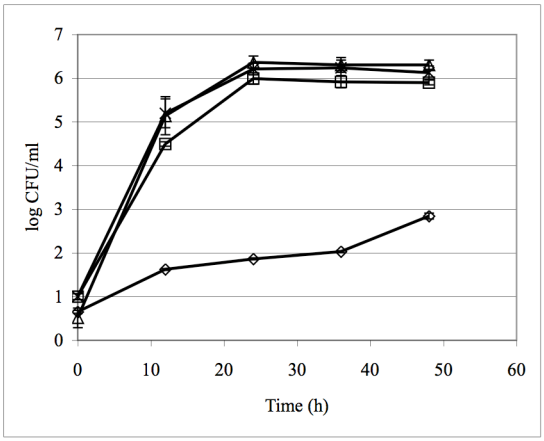


Figure 2-5. Growth of *Alicyclobacillus* spp. isolates 1016 (—◆—), 1101 (—■—), 19220 (--△--), and C-GD 1-1 (--○--) in K broth at (a) pH 3.0, (b) pH 3.5, (c) pH 4.0, and (d) pH 4.5. Error bars indicate a 5% error rate.

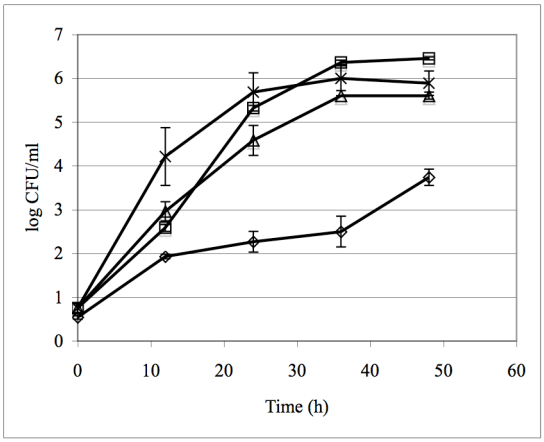
(a) 1016



(b) 1101



(c) 19220



(d) C-GD 1-1

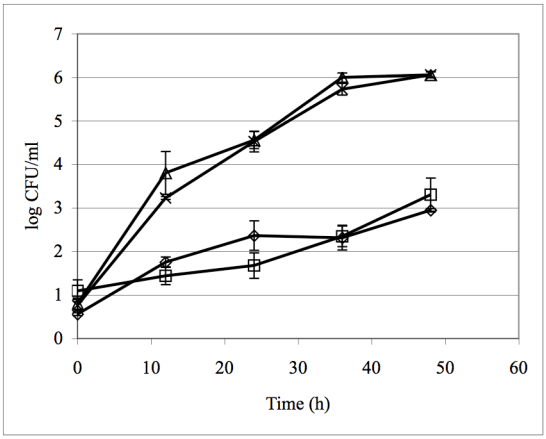
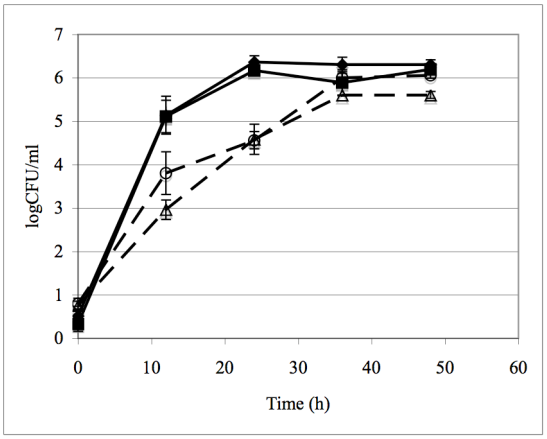
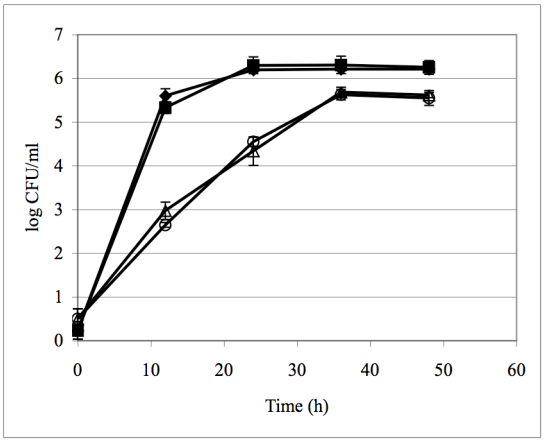


Figure 2-6. Growth of *Alicyclobacillus* spp. isolates (a) 1016, (b) 1101, (c) 19220, and (d) C-GD 1-1 in K broth at 43°C at pH 3.0 (—◇—), pH 3.5 (—□—), pH 4.0 (—△—), and pH 4.5 (—×—). Error bars indicate a 5% error rate.

(a) aerobic



(b) microaerophilic



(c) anaerobic

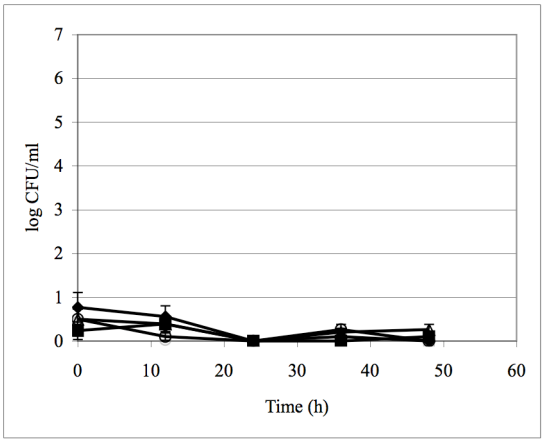
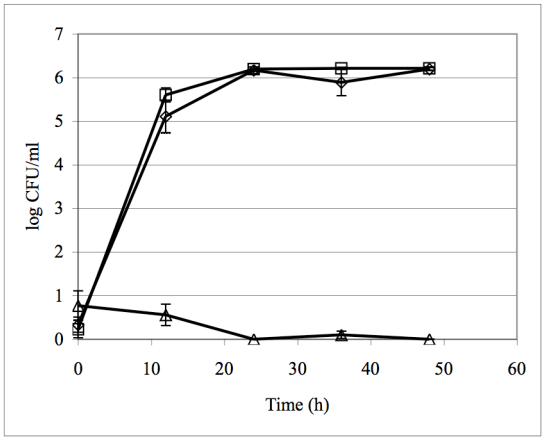
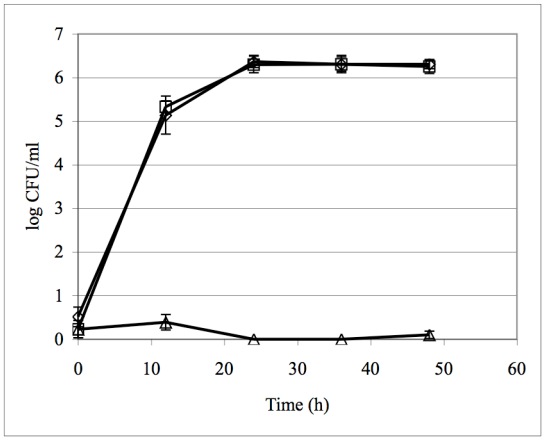


Figure 2-7. Growth of *Alicyclobacillus* spp. isolates 1016 (—◆—), 1101 (—■—), 19220 (--△--), and C-GD 1-1 (--○--) in K broth (pH 4.0) at 43°C under (a) aerobic, (b) microaerophilic, and (c) anaerobic conditions. Error bars indicate a 5% error rate.

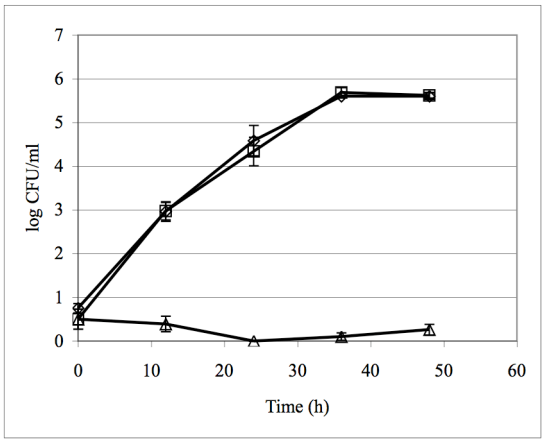
(a) 1016



(b) 1101



(c) 19220



(d) C-GD 1-1

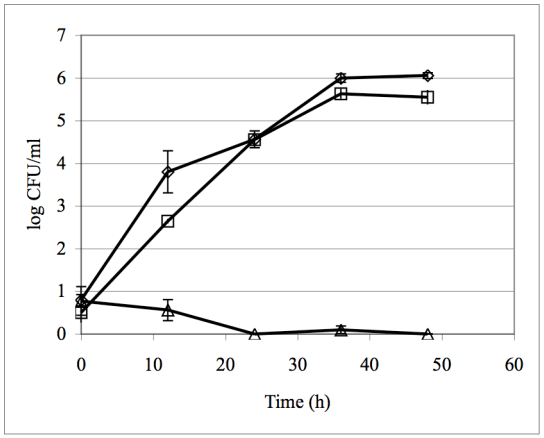
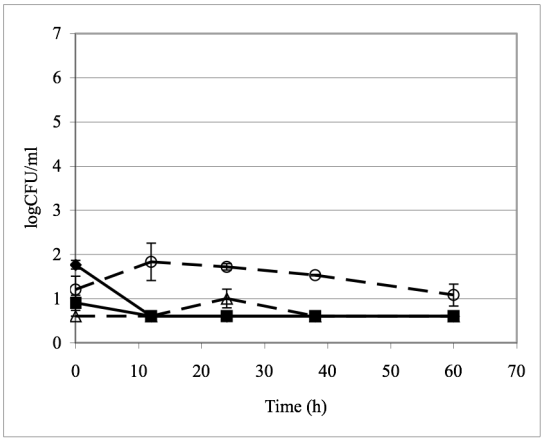
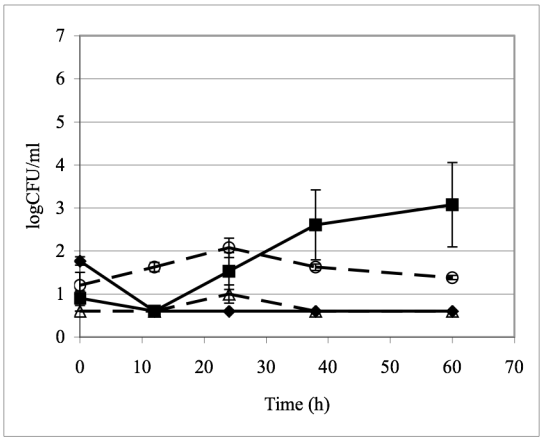


Figure 2-8. Growth of *Alicyclobacillus* spp. isolates (a) 1016, (b) 1101, (c) 19220, and (d) C-GD 1-1 in K broth (pH 4.0) at 43°C under aerobic (—◇—), microaerophilic (—□—), and anaerobic (—△—) conditions. Error bars indicate a 5% error rate.

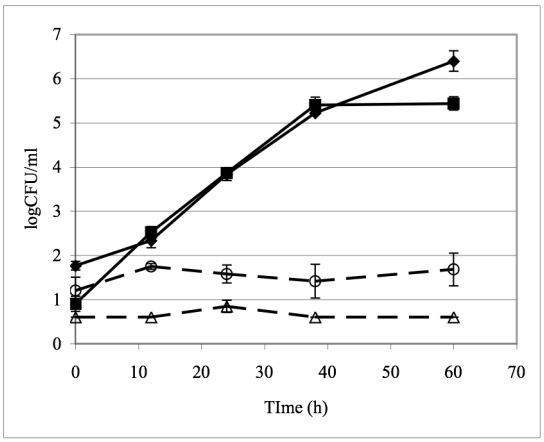
(a) 19.2°Brix



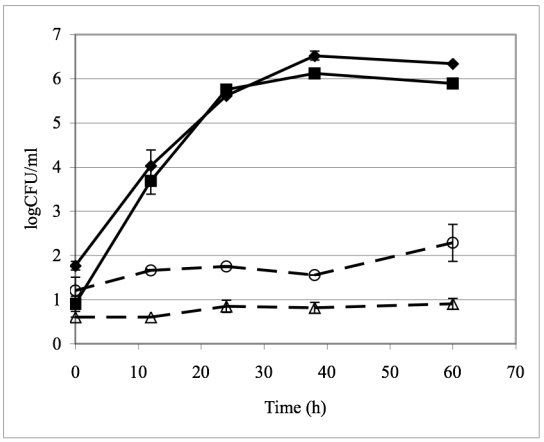
(b) 14.4°Brix



(c) 9.7°Brix



(d) 4.9°Brix



(e) 0.2°Brix

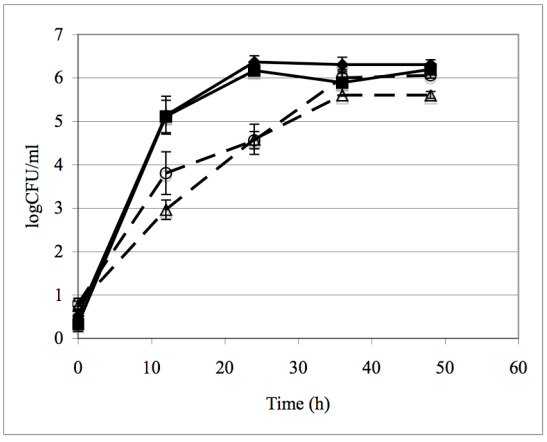
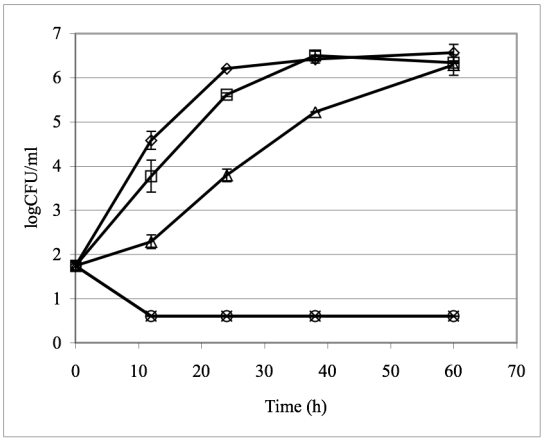
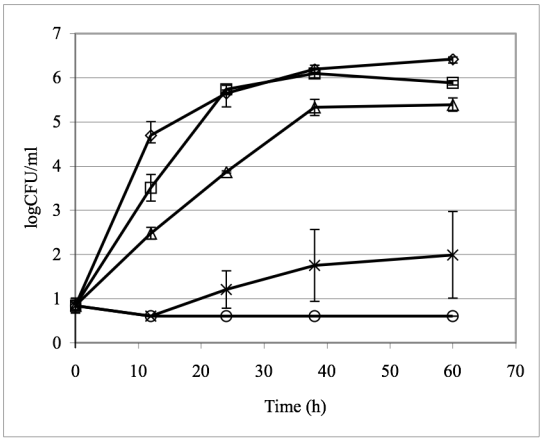


Figure 2-9. Growth of *Alicyclobacillus* spp. isolates 1016 (—◆—), 1101 (—■—), 19220 (--△--) and C-GD 1-1 (--○--) in K broth (pH 4.0) with soluble solid concentrations of (a) 19.2, (b) 14.4, (c) 9.7, (d) 4.9, and (e) 0.2 °Brix at 43°C. Error bars indicate a 5% error rate.

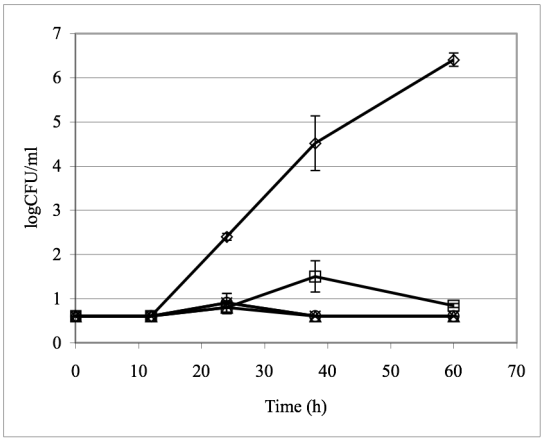
(a) 1016



(b) 1101



(c) 19220



(d) C-GD 1-1

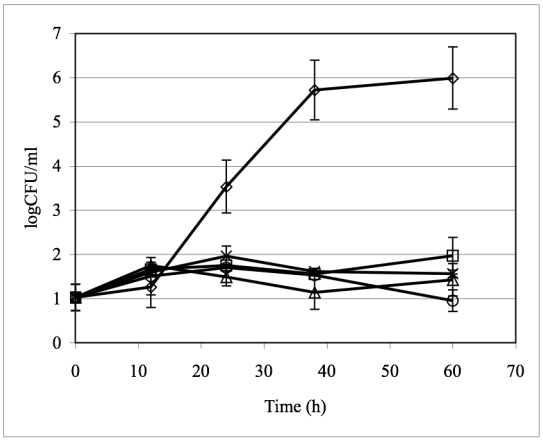


Figure 2-10. Growth of *Alicyclobacillus* spp. isolates (a) 1016, (b) 1101, (c) 19220, and (d) C-GD 1-1 in K broth (pH 4.0) at 43°C with soluble solid concentrations of 0.2 (—◇—), 4.9 (—□—), 9.7 (—△—), 14.4 (—×—), and 19.2 (—○—) °Brix. Error bars indicate a 5% error rate.

CHAPTER THREE

EXTRINSIC FACTORS AFFECTING THE PRODUCTION OF GUAIACOL IN *ALICYCLOBACILLUS* SPP.

ABSTRACT

Alicyclobacillus spp. is of significance to the fruit juice industry due to the production of guaiacol. Studies on *Alicyclobacillus* in regard to guaiacol focused mainly on novel ways to detect guaiacol (HPLC, GC) or evaluate guaiacol producing potential of isolated *Alicyclobacillus* (KV method). Basic studies on factors that induce or affect the production of guaiacol and the conversion pathway of vanillic acid to guaiacol are not available. The goal of this study is to evaluate how extrinsic factors can affect the production of guaiacol. Guaiacol producing *Alicyclobacillus* isolates 1016 and 1101 were used in this study and the effects of temperature (25-55°C), pH (3.0-5.5), and oxygen concentration on guaiacol production in laboratory media was investigated. Maximum production of guaiacol by isolate 1016 was detected within 9 h when incubated at 43°C, pH 4.0 under microaerophilic conditions. Isolate 1101 produced detectable amounts of guaiacol within 8 h at pH 5.0. However, maximum guaiacol production was achieved within 14 h by isolate 1101 when incubated at 50°C. Our results indicate that the production of guaiacol, contrary to common belief, is a rapid reaction under desirable conditions specific to each isolate. The acceleration of guaiacol production by altering growth factors can be useful for developing rapid guaiacol monitoring methods for *Alicyclobacillus* related spoilage or be applied to more detailed enzyme related studies.

INTRODUCTION

Alicyclobacillus spp. are continuously implicated in juice spoilage since the isolation of *A. acidoterrestris* in apple juice in 1984 (Cerny et al., 1984; McIntyre et al., 1995; Eiroa et al., 1999; Duong and Jensen, 2000; Jensen, 2000). Spoilage is often characterized as "medicinal", "phenolic", or "smoky", and the chemical compound responsible for this off-odor has been identified as guaiacol (Yamazaki et al., 1996; Orr et al., 2000; Walls and Chuyate, 2000; Jensen and Whitfield, 2003). Originally, the primary means for juice companies to be aware of an *Alicyclobacillus* spp. spoilage incidence was through consumer complaints. Currently, identification of guaiacol using HPLC, GC, or colorimetric tests such as KV method is possible (Pettipher et al, 1997; Orr et al., 2000; Chang, 2003). However, due to the slow production of guaiacol, tests usually require at least 48 h before results can be obtained. Research to monitor guaiacol production or investigate growth factors that may induce and accelerate the production of guaiacol by *Alicyclobacillus* spp. is not available. The goal of this study is to investigate factors that may accelerate the conversion of vanillic acid to guaiacol.

MATERIALS AND METHODS

1. Bacterial strains and spore suspension preparation.

Frozen spore stocks of *Alicyclobacillus* spp. isolates 1016 and 1101 were obtained from the Washington State University Department of Food Science and Human Nutrition Culture Collection (Pullman, WA). Ten microliters of each frozen stock spore suspension was inoculated into individual 9 ml deionized water using sterile 10 μl disposable loops (Copan Diagnostics Inc., Murrieta, CA), heat shocked at 80°C for 10

min, chilled in an ice bath to terminate the heat shock effect. Heat shocked spore suspensions were freshly prepared prior to each experiment.

2. Preparation of laboratory media and chemical solutions.

Preparation of K agar.

K agar was prepared from individual components as described by Walls and Chuyate (2000): 2.5 g yeast extract, 5.0 g peptone, 1.0 g glucose, 1.0 g Tween 80®, 15 g agar and 1.0 L deionized water. The mixture was combined and brought to a slight boil using a stirrer-hot plate to facilitate the solubilization of agar and dispersion of Tween® 80 prior to sterilization at 121°C for 15 min. Following sterilization and termpering in a 48°C water bath, 10% (w/v) filter sterilized malic acid was used to adjust the pH to pH 4.0. The agar medium was poured into disposable sterile petri dishes and air dried at room temperature for 48 h prior to use.

Preparation of KV broth.

The basal broth for KV broth was prepared by dissolving 2.5 g yeast extract, 5.0 peptone, 1.0 g glucose, 1.0 g Tween® 80 in 990 ml deionized water and sterilizing at 121ºC for 15 min. A 1% (w/v) vanillic acid (Sigma®, St. Louis, MO) solution was prepared and syringe sterilized using 0.22 µm Millex®GP filter units (Millipore, Carrigtwohill, CO) with sterile Monoject 3 ml syringes (Tyco, Mansfield, MA). KV broth was prepared by adding 10 ml of the 1% sterilized vanillic acid solution to the basal broth and adjusting the pH to 4.0 with 10% malic acid.

Reagents and solvents.

Acetonitrile and formic acid used for the mobile phase were obtained from J.T. Baker (Phiilipsburg, NJ), and water was treated with the MilliQ water purification system

(Millipore, Billerica, MA) and sterilized using a using a 0.22 µm 500 ml filter sterilization unit (VWR International, West Chester, PA) prior to use. Vanillic acid and guaiacol were purchased from Sigma-Aldrich (St. Louis, MO) and used to prepare 100 ppm standards for HPLC analysis.

3. Identification of vanillic acid utilization/guaiacol production using HPLC.

HPLC instrumentation and chromatographic conditions.

The quantification of vanillic acid and guaiacol was performed on an Agilent 1100 system equipped with CHEMSTATION software, a degasser, a gradient pump, a thermoautosampler, and a column oven (Agilent Technologies, Santa Clara, CA). The column system consisted of a Nova-Pak® 4 µm C18 guard column (3.9×20 mm) followed by a Nova-Pak® 4 µm C18 cartridge (3.9×150 mm), both obtained from Waters (Milford, MA). The column was operated at 25°C using a mobile phase of acetonitrile:water:formic acid (20:80:1) at a flow rate of 0.5 ml/min. The ultraviolet wavelength used to monitor the utilization of vanillic acid and the production of guaiacol was 275 nm.

HPLC sample analysis.

Cell suspension aliquots of one ml were removed from each medium at the designated time interval. Aliquots were filter sterilized into amber HPLC 1.5 ml vials (Agilent Technologies, Santa Clara, CA) using 0.22 µm Millex®GP filter units (Millipore, Carrigtwohill, CO) and sterile Monoject 3 ml syringes (Tyco, Mansfield, MA). The sterilized cell suspensions (50 µl) were injected and monitored for vanillic acid utilization and guaiacol production using the previously described chromatographic conditions.

Conversion calculations for the utilization of vanillic acid and the production of guaicaol.

Peak areas calculated by the CHEMSTATION software for the 100 ppm vanillic acid and guaiacol standards were designated as 100%. Vanillic acid catabolism/guaiacol production percentages at each sampling interval were calculated by dividing the detected sample peak areas for vanillic acid and guaiacol against the peak areas of their relative standards. Vanillic acid catabolism/guaiacol production graphs were generated by plotting time (h) against the calculated vanillic acid utilization and guaiacol production percentages.

4. Correlation of cell growth and guaiacol production.

The heat shocked spore suspension was 10-fold serially diluted with deionized water and inoculated into KV broth (pH 4.0) to achieve an initial starting concentration of 10^{1-2} CFU/ml. The inoculated KV broth was incubated at 43°C and sampled every 3 h for a total of 48 h. At each sampling time, 2.5 ml of the cell suspension were removed. One milliliter was prepared for HPLC analysis using the previously described method and monitored for the utilization of vanillic acid and production of guaiacol. Another one ml aliquot was used for conventional K agar spread plating to monitor the growth of *Alicyclobacillus* in the media.

5. Growth factors and the production of guaiacol.

Incubation temperature and guaiacol formation.

Heat shock spore suspensions were inoculated into KV broth (pH 4.0) as previously described and incubated at 25, 32, 37, 50, and 55°C. Aliquots of one ml were removed at appropriate time intervals and subjected to HPLC analysis to monitor the

effect of incubation temperature on the catabolism of vanillic acid and formation of guaiacol.

Media pH and guaiacol formation.

KV broth was prepared as described previously but adjusted to pH 3.0, 3.5, 4.0, 4.5, and 5.0 with 10% (w/v) malic acid. The diluted heat shock spore suspensions were inoculated into KV broth with selected pH followed by incubation at 43°C. Each treatment was sampled at appropriate time intervals and subjected to HPLC analysis to assess the effect of media pH on the catabolism of vanillic acid and formation of guaiacol.

Incubation oxygen concentration and guaiacol formation.

The heat shocked spore suspension of each *Alicyclobacillus* isolate was inoculated into duplicate tubes of KV broth (pH 4.0) to achieve the starting concentration of 10^{1-2} CFU/ml. One inoculated KV broth from each isolate was placed into an anaerobic jar containing an activated GasPak™ EZ CampyPouch™ System (BBL, Franklin Lakes, NJ) and tightly sealed. The remaining inoculated KV broth tubes were placed in the incubator without additional adjustments. Both sets were incubated at 43°C. At suitable time intervals, samples were removed and analyzed for guaiacol formation using HPLC to determine the effect of oxygen concentration on guaiacol formation.

RESULTS

Correlation of cell growth and guaiacol formation.

The relationship between cell growth and the production of guaiacol of *Alicyclobacillus* isolates 1016 and 1101 are illustrated in Figure 3-1. Initial production of guaiacol occurred within 12-15 h of incubation at 43°C and maximum guaiacol production was achieved after 21-24 h for both isolates. With regard to cell growth, the

initial production of guaiacol occurred at the mid-log phase of cell growth while maximum concentrations of guaiacol production was achieved at the late log phase/early stationary phase.

Incubation temperature and guaiacol formation.

The catabolism of vanillic acid and the formation of guaiacol as a function of time at selected incubation temperatures by isolates 1016 and 1101 are presented in Figure 3-2 and Figure 3-3, respectively. Time required for vanillic acid catabolism and guaiacol production was inversely correlated with temperature. Vanillic acid catabolism and guaiacol formation accelerated as incubation temperature increased in the range of 32°C-50°C. No vanillic acid catabolism or guaiacol formation was detected at 25°C. The time required for initial detection and maximum formation of guaiacol at 55 °C was slightly longer than that at 50°C for both isolate 1016 and 1101. Table 3-1 summarizes the detection time for initial and maximum guaiacol production at each incubation temperature for each isolate.

Media pH and guaiacol formation.

As presented in Figure 3-4, vanillic acid catabolism and guaiacol formation by isolate 1016 was only observed in KV broth with a pH range of pH 3.5-4.5. Isolate 1101 utilized vanillic acid to produce guaiacol from pH 3.5 to pH 5.0 (Figure 3-5). No guaiacol production was observed for both isolates at pH 3.0. Similar to the effect of temperature on guaiacol production and within the pH range where guaiacol was produced, the time required for guaiacol production was inversely related to growth medium pH (Table 3-1).

Incubation oxygen concentrations and guaiacol formation.

Guaiacol formation by isolates 1016 and 1101 was observed under both aerobic and microaerophilic conditions. When isolates 1016 and 1101 were incubated under microaerophilic conditions, catabolism of vanillic acid and formation of guaiacol was observed earlier than when incubated under aerobic conditions (Figure 3-6). Initial detection of guaiacol formation was detected after 6-9 h under microaerophilic conditions compared to 12-15 h under aerobic conditions for isolate 1016. Isolate 1101 produced detectable amounts of guaiacol after 8-10 h under microaerophilic conditions, 2-7 h less than the time needed under aerobic incubation. Whereas both isolates required 21-24 h to reach maximum guaiacol concentrations under aerobic incubation, isolates 1016 and 1101 produced maximum concentrations of guaiacol under microaerophilic conditions after 9-12 h and 14-16 h, respectively (Table 3-1).

DISCUSSION

The production of guaiacol by *Alicyclobacillus* spp. is established and Alicyclobacillus spp. is recognized as a major spoilage microorganism in the pasteurized fruit juice industry (Yamazaki et al., 1996a; Silva et al., 1999). Since the initial isolation of *A. acidoterrestris* by Cerny et al. (1984) in a spoiled apple juice, many studies were conducted, providing better understanding of *Alicyclobacillus* spp. characteristics, methods for improved isolation and detection, and a wide variety of control methods to minimize spoilage incidents (Chang and Kang, 2004). The majority of studies accumulated in the three decades following its discovery focused mainly on the microorganism and little is known about factors that contribute to the formation of guaiacol and the mechanism of guaiacol formation by *Alicyclobacillus*. This study

provides an alternative look at the spoilage of *Alicyclobacillus* by the production of guaiacol through investigating factors that facilitate/accelerate the production of guaiacol.

Previous studies on the detection of guaiacol by *Alicyclobacillus* recommended a 48 h incubation for the production and stabilization of guaiacol (Chang and Kang, 2005; Orr and Beuchat, 2000). Our results indicate that when incubated under standard conditions (KV broth adjusted to pH 4.0 and incubated at 43°C), guaiacol is produced within 12-15 h and achieves maximum concentrations within 24 h (Table 3-1). When correlated to the growth of *Alicyclobacillus*, initial detection of guaiacol occurred when cell growth reached 10^5 log CFU/ml and 10^4 CFU/ml for isolate 1016 and 1101, respectively (Figure 3-1). These observations confirmed results reported by Pettipher et al. (1997) and Komitopoulou et al. (1999). Pettipher et al. (1997) reported that spore germination and outgrowth to a level of 10^5-10^6 cells/ml in apple and orange juices formed adaquate concentrations of guaiacol to produce sensory taint (Pettipher et al., 1997). Taint was also subjectively noticed after four days storage at 30°C when *Alicyclobacillus* concentrations increased from 10^2 to 10^5 CFU/ml in apple, orange, and grapefruit juices (Komitopoulou et al., 1999).

Based on the results summarized in Table 3-1, the production of guaiacol by *Alicyclobacillus* is affected by incubation temperature, growth medium pH, and oxygen concentrations. The rate of guaiacol formation generally correlates with the optimal conditions of growth for guaiacol producing *Alicyclobacillus*. The absence of guaiacol formation at 25°C most likely is due to the limited ability of *Alicyclobacillus* to grow at this temperature (Cerny et al., 2000). As the incubation temperature increases to the optimum growth temperature of guaiacol producing *Alicyclobacillus*, production of

guaiacol is accelerated. The most rapid production of guaiacol was observed at 50°C for both isolates. With regard to pH, production of guaiacol was observed between pH 3.5-4.5 for isolate 1016 and between pH 3.5-5.0 for isolate 1101. Isolates 1016 and 1101 did not produce guaiacol at pH 3.0 and pH 5.5, most likely due to limited growth at extreme pH concentrations. When incubated at 43°C, the fastest guaiacol production was observed at pH 4.5 for isolate 1016 and at pH 5.0 for isolate 1101. However, the most pronounced acceleration of guaiacol production was observed when the isolates were incubated under microaerophilic conditions. Detectable concentrations of guaiacol were produced within 10 h by both 1016 and 1101. The rapid production of guaiacol is likely linked to the rapid outgrowth of the inoculated *Alicyclobacillus* spores. Cerny et al. (2000) reported that *Alicyclobacillus* grew more rapidly in apple juices with limited oxygen concentrations than in aerobic conditions.

The accelerated production of guaiacol under microaerophilic conditions may be useful in furthering research of guaiacol production by *Alicyclobacillus*. Details about the conversion of vanillic acid to guaiacol by guaiacol producing *Alicyclobacillus* isolates are currently unavailable. Much of what is known about guaiacol formation by *Alicyclobacillus* is speculated from scientific studies conducted with *Bacillus magaterium* (Crawford and Olson, 1978), *Streptomyces setonii* (Pometto et al., 1981), and *Rhodotorula rubra* (Huang et al., 1993). Our results demonstrate the utilization of vanillic acid in conjunction with the formation of guaiacol, but the mechanism of guaiacol production by guaiacol producing *Alicyclobacillus* spp. remains to be elucidated. Questions such as (1) is an intracellular/extracellular enzyme involved in the formation of guaiacol, (2) metabolic details of guaiacol formation, and (3) why guaiacol formation is

limited to some *Alicyclobacillus* should be investigated to better understand guaiacol producing *Alicyclobacillus* spp.

In addition to being a time saving procedure for metabolic studies of guaiacol producing *Alicyclobacillus*, accelerated production of guaiacol under microaerophilic conditions is also noteworthy to the fruit juice industry. Most pasteurized fruit juices are bottled in plastic containers that are economical and can withstand pasteurization temperatures. Since all polymers used in packaging are to some extent permeable to oxygen (Axelson-Larsson, 1995), the exchange of small amounts of oxygen will create a microaerophilic environment which inevitably will lead to spoilage should guaiacol-producing *Alicyclobacillus* be present in the juice.

In summary, guaiacol production is not a slow process and can be completed at 43°C within 24 h. Among the three growth factors tested, incubation under microaerophilic conditions accelerates guaiacol formation to within 10 h. The results reported here provide preliminary but important information on factors that enhance guaiacol production, and can be further applied in metabolic studies regarding guaiacol producing *Alicyclobacillus*.

Table 3-1. Selected factors and the production of guaiacol by *Alicyclobacillus* isolates 1016 and 1101.

Treatment Factor	*Alicyclobacillus* spp. Isolate			
	1016		1101	
	Initial Guaiacol Formation[1] (h)	Maximum Guaiacol Formation[2] (h)	Initial Guaiacol Formation (h)	Maximum Guaiacol Formation (h)
Temperature				
25°C	None	None	None	None
32°C	36-48	>48	30-36	36-48
37°C	21-24	27-30	15-18	21-24
43°C	12-15	21-24	12-15	21-24
50°C	11-12	15-18	8-10	14-16
55°C	12-15	18-21	12-15	15-18
pH				
3.0	None	None	None	None
3.5	18-21	24-27	15-18	21-24
4.0	12-15	21-24	12-15	21-24
4.5	12-15	18-21	8-10	12-14
5.0	None	None	6-8	12-14
5.5	None	None	None	None
O_2 Concentration				
Aerobic	12-15	21-24	12-15	21-24
Microaerophilic	6-9	9-12	8-10	14-16

[1]: time required for guaiacol formation to be detected by HPLC.
[2]: time required for guaiacol formation to reach maximum concentration and stabilize.

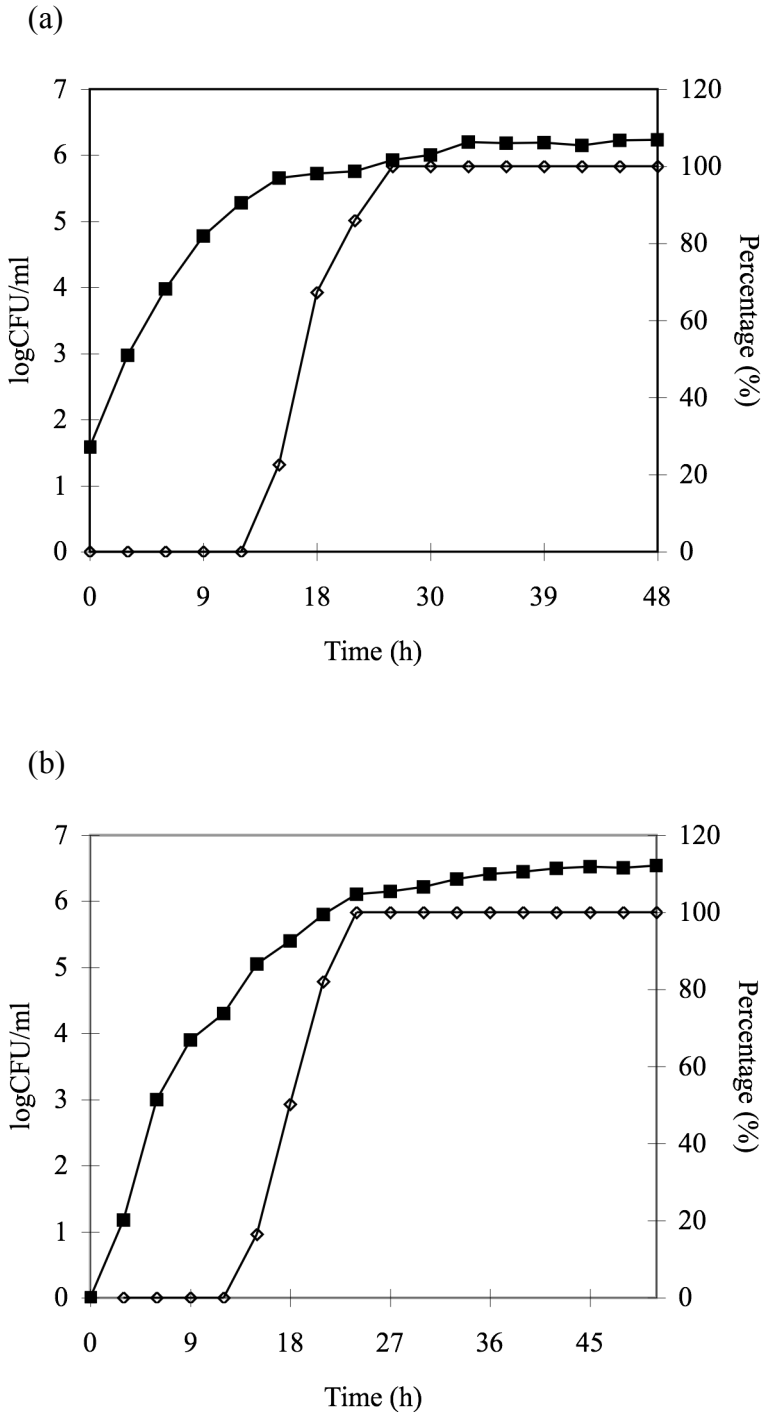


Figure 3-1. Relationship between cell growth (—■—) and guaiacol formation (—◇—) in guaiacol producing *Alicyclobacillus* (a) 1016 and (b) 1101 at 43°C under aerobic conditions. The left and right y-axis represent growth in log CFU/ml and the production of guaiacol relative to the 100 ppm standard, respectively.

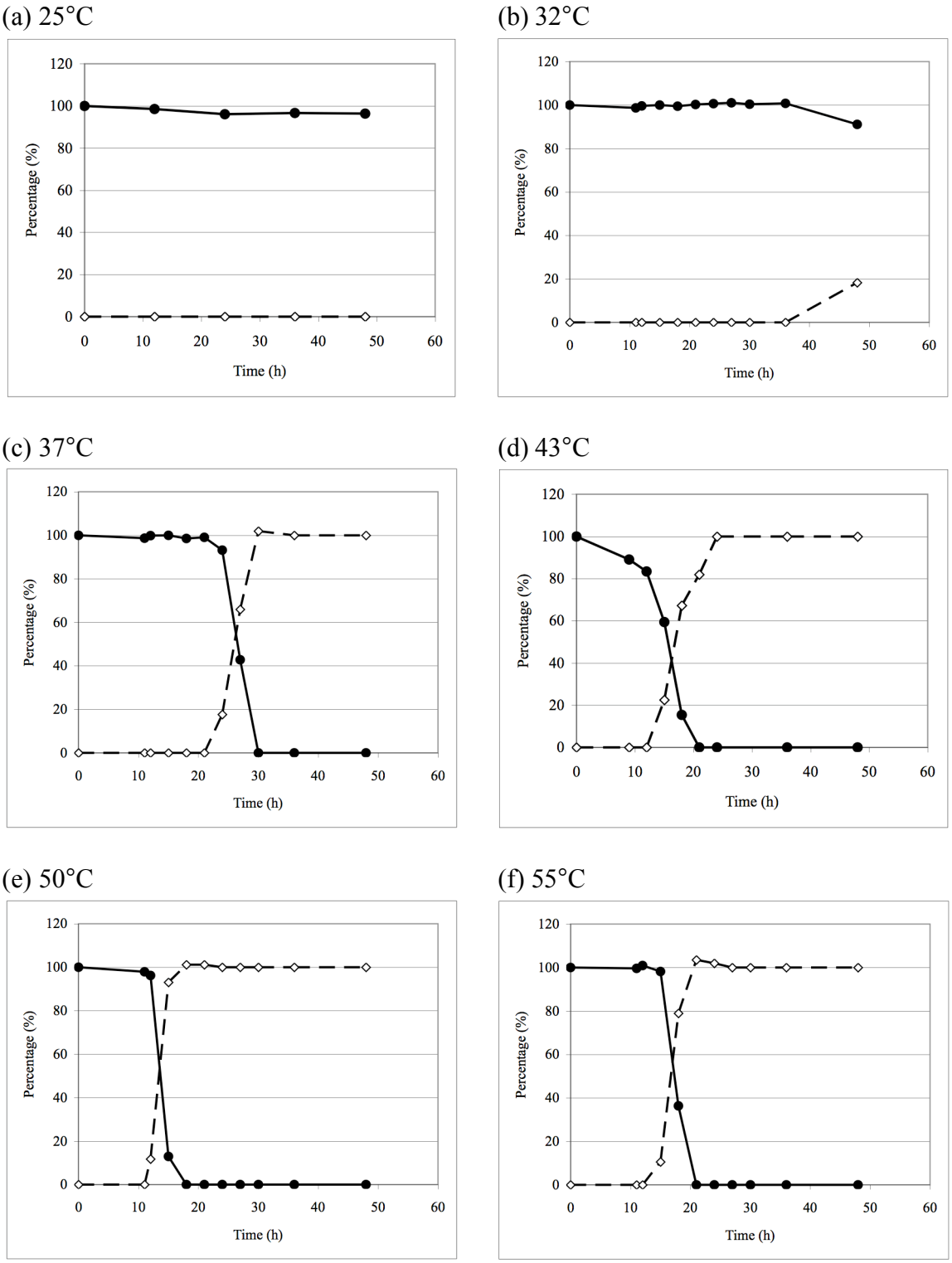


Figure 3-2. Temperature and the utilization of vanillic acid (—●—) and production of guaiacol (--◇--) by *Alicyclobacillus* isolate 1016.

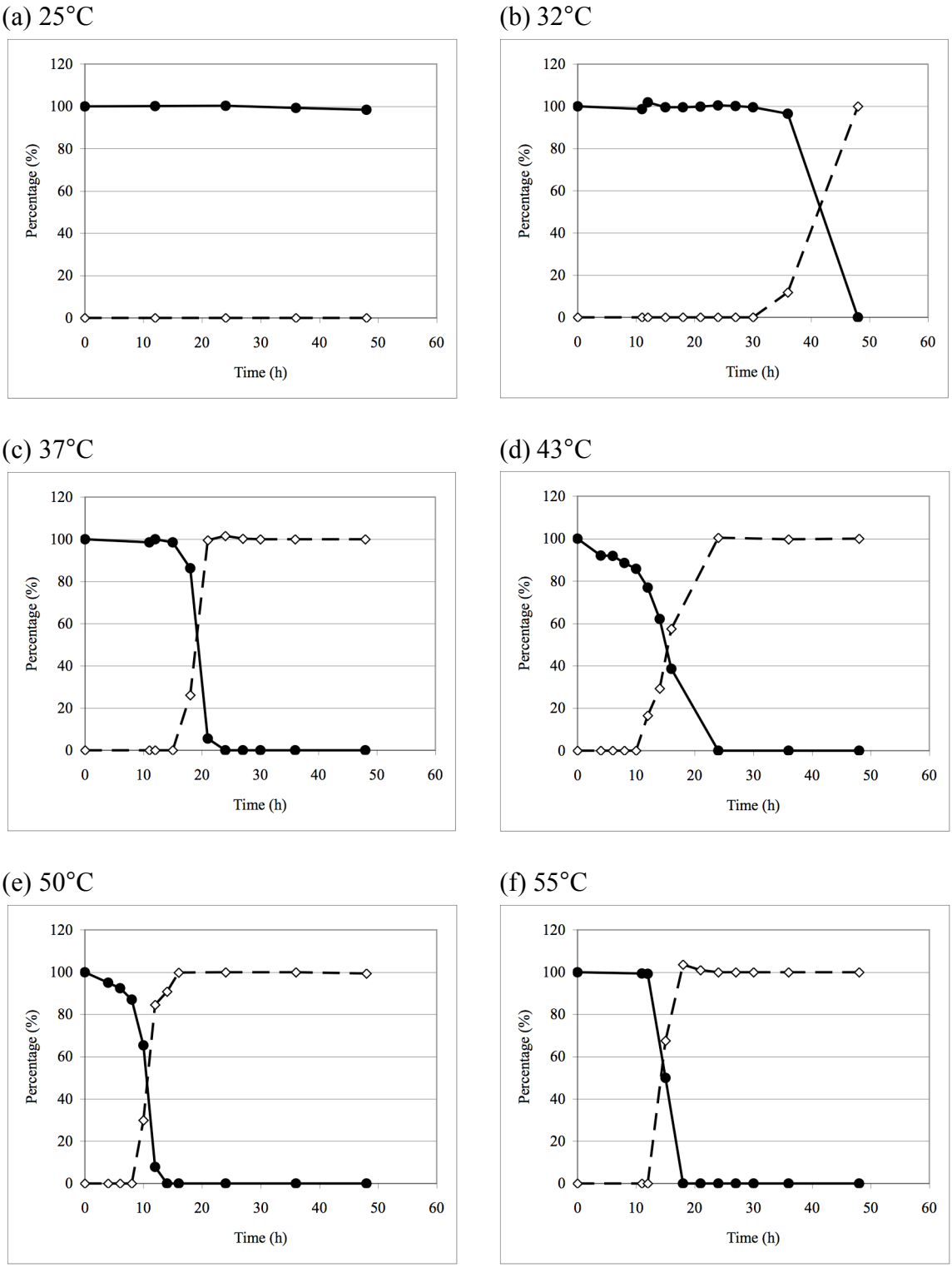


Figure 3-3. Temperature and the utilization of vanillic acid (—●—) and production of guaiacol (--◇--) by *Alicyclobacillus* isolate 1101.

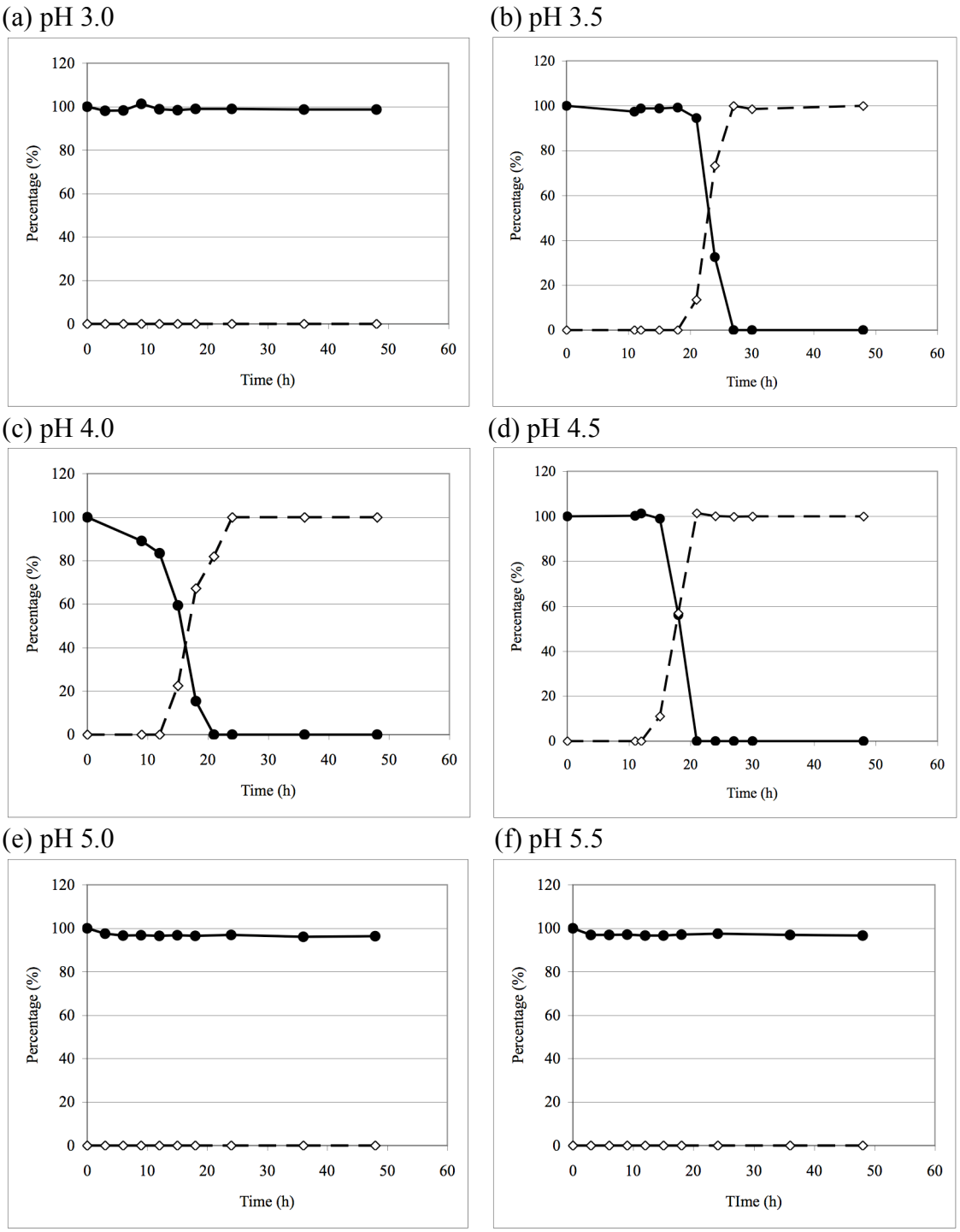


Figure 3-4. pH and the catabolism of vanillic acid (—●—) and production of guaiacol (--◇--) by *Alicyclobacillus* isolate 1016.

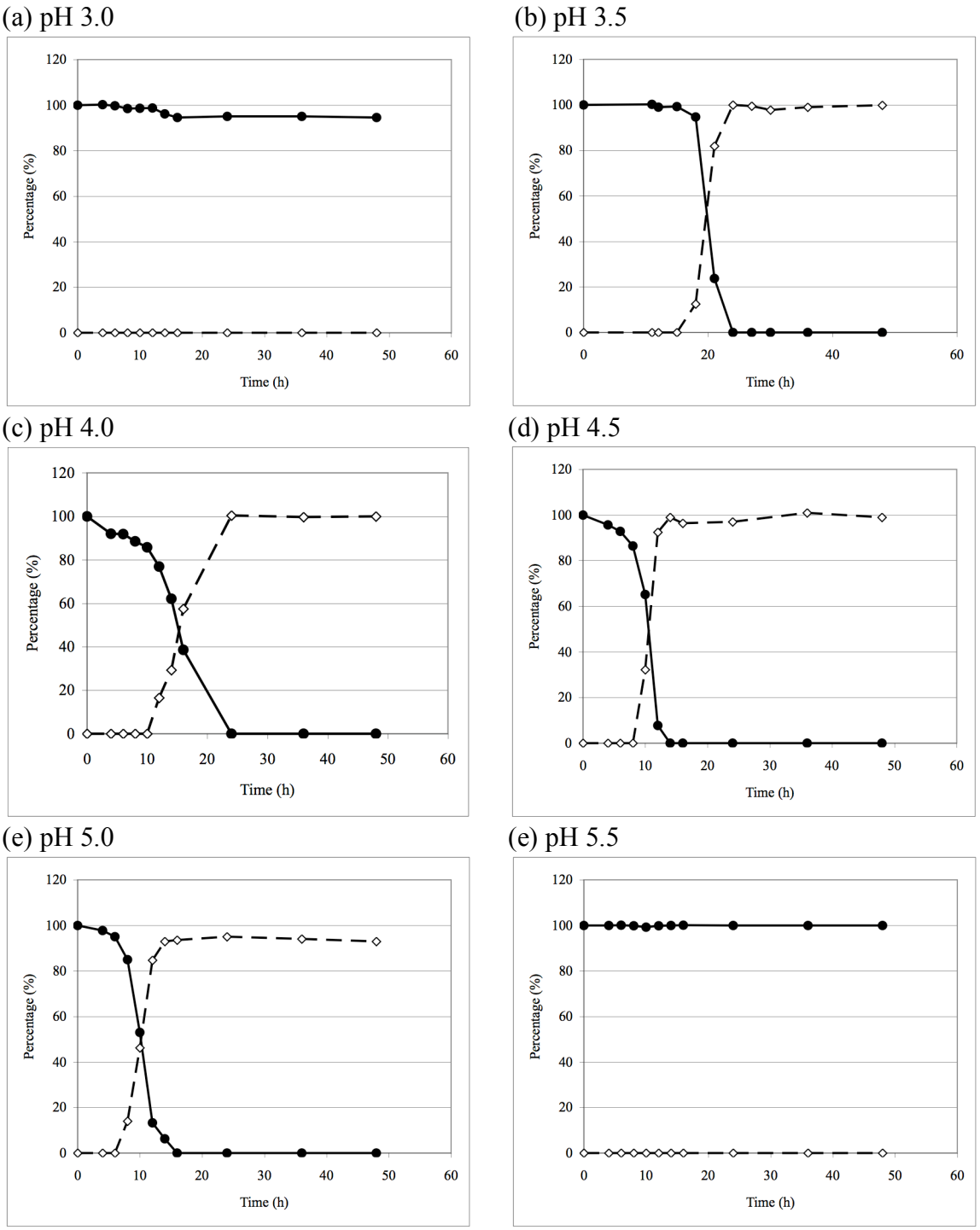


Figure 3-5. pH and the catabolism of vanillic acid (—●—) and production of guaiacol (--◇--) by *Alicyclobacillus* isolate 1101.

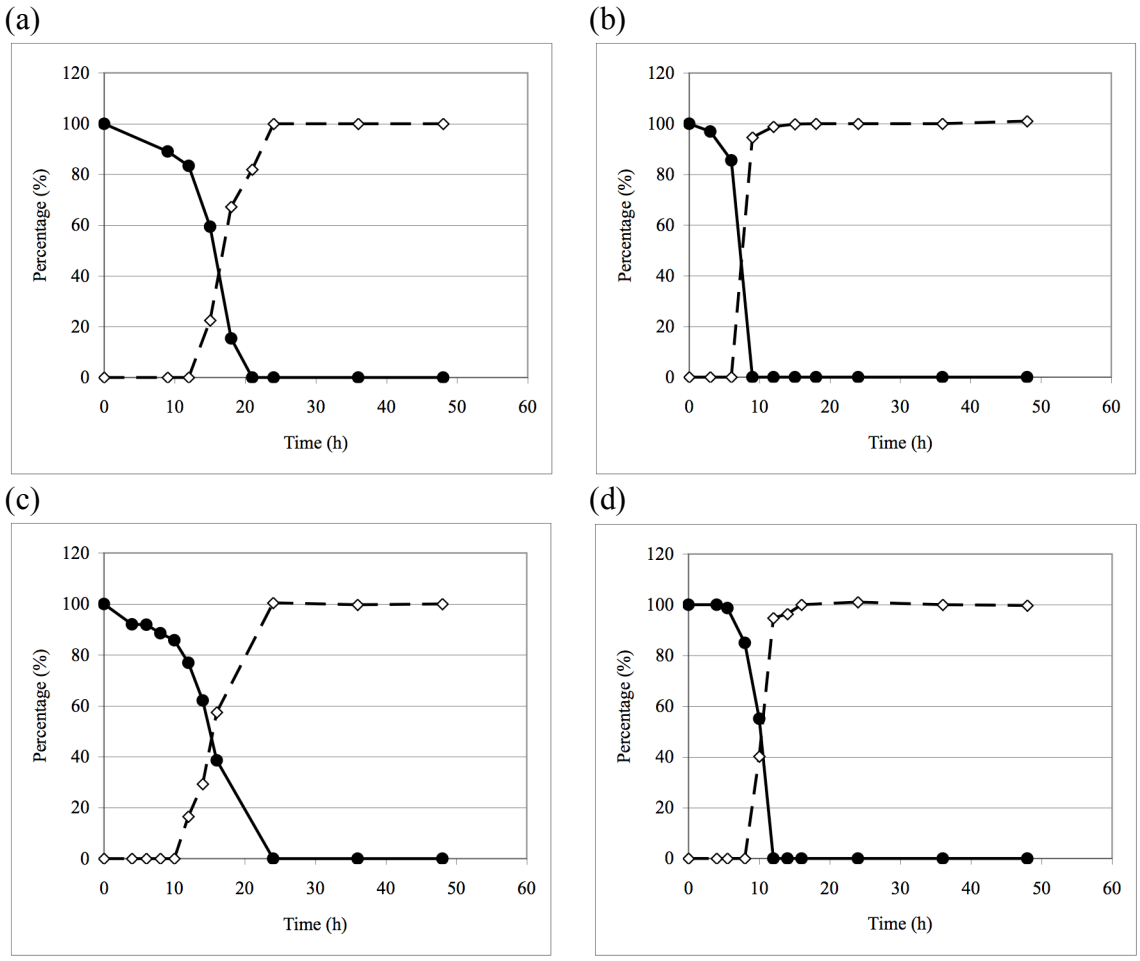


Figure 3-6. Oxygen concentration and the catabolism of vanillic acid (—●—) and production of guaiacol (--◇--) by *Alicyclobacillus* isolates: (a) isolate 1016, aerobic; (b) isolate 1016, microaerophilic; (c) isolate 1101, aerobic; and (d) isolate 1101, microaerophilic.

CHAPTER FOUR

DEVELOPMENT OF NOVEL AGAR MEDIA FOR ISOLATING GUAIACOL PRODUCING *ALICYCLOBACILLUS* SPP.

ABSTRACT

The role of *Alicyclobacillus* spp. on the spoilage of pasteurized fruit juices is widely reported since 1982. Previous research reported that the ability to produce guaiacol and cause spoilage is not present in all *Alicyclobacillus* spp. and recommended the revision of fruit juice/beverage control procedures to focus solely on guaiacol-producing *Alicyclobacillus* spp. Though peroxidase tests, gas chromatography, and high pressure liquid chromatography methods were developed to detect the presence of guaiacol and/or guaiacol-producing *Alicyclobacillus*, no procedure currently exists that can isolate and identify guaiacol producing *Alicyclobacillus* in one single step. Thus, the purpose of this study is to develop a selective or differential media for isolating guaiacol-producing *Alicyclobacillus*. Forty-two selected dyes were added at a concentration of 0.01% into SK agar (pH 4.0). As a documented antimicrobial compound and the precursor of guaiacol, the potential of vanillic acid as a selective agent was also investigated. Two guaiacol producing (1016, 1101) and two non-guaiacol producing (19220, C-GD 1-1) *Alicyclobacillus* isolates were streaked onto the agar media and color differentiation of the isolates was assessed. Chrome Azurol S allowed color differentiation of the two types of *Alicyclobacillus* with guaiacol-producing isolates forming purple irregular colonies with yellow background pigmentation while non-guaiacol producing isolates were yellow. Vanillic acid not only served as a precursor for

guaiacol formation but also inhibited non-guaiacol producing *Alicyclobacillus*. When compared with other *Alicyclobacillus* isolation media, not only was the novel medium capable of selectively recovering guaiacol-producing *Alicyclobacillus*, the recovery rate was also equal if not better than PDA and K agar. The new selective medium was named SK2 agar and consisted of 0.5 g peptone, 0.25 g yeast extract, 1.0 g glucose, 1.0 g Tween® 80, 70 ppm vanillic acid, and 0.01% Chrome Azurol S per liter. The development of SK2 agar provides the fruit juice industry with an inexpensive, simple to use alternative for monitoring guaiacol-producing *Alicyclobacillus*.

INTRODUCTION

Since the isolation of *A. acidoterrestris* from pasteurized apple juice in 1982 (Cerny et al., 1984), the role of *Alicyclobacillus* in the fruit juice and beverage industry is increasingly important and recognized worldwide (Yamazaki et al., 1996a; Pettipher et al., 1997; Walls and Chuyate, 1998). The acidophilic and thermophilic nature of *Alicyclobacillus* enables its survival under normal pasteurization procedures and results in spoilage without apparent changes to the packaging or the juice clarity (McIntyre et al., 1995; Walls and Chuyate 1998; Lee et al., 2002). Though initial spoilage related interest was focused on *A. acidoterrestris*, subsequent studies reported other species of the genus that are could also produce guaiacol and thus broadened the target for control procedures (Goto et al., 2002a; Matsubara et al., 2002; Goto et al., 2003). *Alicyclobacillus* spp. are soilborne microorganisms, can easily enter into production via water, fruit, or air, and contaminant fruit juices and concentrates. However, Chang and Kang (2005) demonstrated that the presence of *Alicyclobacillus* spp. does not necessarily indicate a spoilage concern as the ability to produce guaiacol is not consistent across all *Alicyclobacillus* spp. Currently, several techniques such as HPLC, GC, KV (peroxidase) method are available to either detect guaiacol in juice or evaluate the guaiacol producing ability of isolated *Alicyclobacillus* (Pettipher et al., 1997; Orr et al., 2000; Chang, 2003). Unless the goal is to solely detect the presence of guaiacol, the mentioned methods require a colony isolation procedure. No differential/selective agar medium combining the recovery of *Alicyclobacillus* with instantaneous differentiation of the guaiacol producing ability of recovered colonies is available. Thus, the objective of this study is to

develop a differential or selective agar medium with high recovery rates and instant visualization of guaiacol-producing *Alicyclobacillus* spp.

MATERIALS AND METHODS

Preparation of *Alicyclobacillus* isolates.

Alicyclobacillus spp. isolates 1016, 1101, 19220, and C-GD 1-1 were selected for use in this experiment. All isolates were grown in K broth from their respective frozen stock culture by transferring 10 µl into 9 ml of K broth and incubating at 43°C for 48h prior to use.

Dye screening for differentiation/selection of guaiacol producing and non-guaiacol producing *Alicyclobacillus* spp.

SK agar was prepared as described by Chang and Kang (2005) and used as the basal medium for subsequent studies. All tested dyes listed in Table 4-1 were added to the basal SK agar at a concentration of 0.01% prior to sterilization. After sterilization at 121°C for 15 min, the media were tempered to 48°C adjusted to pH 4.0 with 10% malic acid, poured into sterile agar plates and air dried at 25°C for 48 h prior to use. Actively growing *Alicyclobacillus* vegetative cells prepared as mentioned previously were streaked onto each dye-containing agar with a sterile 10 µl loop (Copan Diagnostics Inc., Murrieta, CA). The potential use of each dye for differentiating guaiacol and non-guaiacol producing *Alicyclobacillus* was evaluated after incubation at 43°C for 48h. The dye(s) with the most distinctive differentiation was selected for further experimentation and optimization.

Evaluation of vanillic acid and oxygen concentration as additional differentiation/selective agents for guaiacol producing and non-guaiacol producing *Alicyclobacillus* spp.

To amplify differentiation/selection of *Alicyclobacillus* based on guaiacol producing ability, the effects of vanillic acid and oxygen concentration were investigated. SK agar with the addition of the suitable dye determined from the prior experiment (tentatively named "SKD" for clarity purposes) was prepared, divided into two parts: one containing 100 ppm vanillic acid ("SKDV") and the other without ("SKD"), poured and air dried as previously mentioned. Once the petri dishes were ready for use, actively growing vegetative cells of 1016, 1101, 19220, and C-GD 1-1 were streaked onto duplicate plates of SKD and SKDV agar with a sterile 10 μl loop and allowed to dry. One set of petri dishes were overlaid with tempered SKD and placed at room temperature for 4 h to allow solidification prior to incubation with the other set at 43°C. The potential differentiation/selection effects of vanillic acid and oxygen concentration was evaluated and recorded after 48 h. When differences were detected, the experiment was repeated by serially diluting the actively growing *Alicyclobacillus* spp. cultures to approximately 10^2-10^3 CFU/ml and plating 100 μl on each respective media rather than streaking. Observations of individual colonies were recorded after incubation at 43°C for 48 h.

Chrome Azurol S (CAS) and the recovery of *Alicyclobacillus* spp.

To analyze the effect of Chrome Azurol S (CAS) on the recovery of *Alicyclobacillus*, basal SK agar was prepared and the pH was adjusted as previously described. Chrome Azurol S was supplemented to the medium at concentrations of 0.0001%, 0.0005%, 0.001%, and 0.01% (w/v). Actively growing cell cultures of the test

isolates were serially diluted with 9 ml sterile deionized water to 10^2-10^3 CFU/ml and 100 µl of the diluted culture was spread plated onto the basal SK agar containing different concentrations of CAS. Unsupplemented basal SK agar (pH 4.0) was used as the control. After incubation at 43°C for 48 h, cell growth on each medium were enumerated and compared to the control to assess possible inhibitory effects of Chrome Azurol S to the recovery of *Alicyclobacillus* spp.

Vanillic acid and the recovery of *Alicyclobacillus* spp.

Basal SK agar was prepared as previously described and supplemented with vanillic acid at concentrations of 1 ppm, 10 ppm, and 100 ppm prior to adjusting the pH to 4.0 with 10% malic acid. Actively growing cell cultures of the test isolates were serially diluted with 9 ml sterile deionized water to 10^2-10^3 CFU/ml and 100 µl of the diluted culture was spread plated onto the basal SK agar containing different concentrations of vanillic acid. Unsupplemented basal SK agar (pH 4.0) was used as the control. After incubation at 43°C for 48 h, cell growth on the respective medium were enumerated and compared to the control to assess possible inhibitory effects of vanillic acid to the recovery of *Alicyclobacillus* spp.

Minimum inhibitory concentrationof vanillic acid on non-guaiacol producing *Alicyclobacillus* spp.

Vanillic acid was supplemented to prepared basal SK agar at concentrations from 0 to 100 ppm at 10 ppm increments. The pH was adjusted to pH 4.0 with 10% malic acid. Actively growing cell cultures of the test isolates were serially diluted with 9 ml sterile deionized water to 10^2-10^3 CFU/ml and 100 µl of the diluted culture was spread plated onto the basal SK agar with different vanillic acid concentrations. Unsupplemented basal

SK agar (pH 4.0) was used as the control. After incubation at 43°C for 48 h, cell growth on each medium was enumerated and the minimum inhibition concentration of non-guaiacol producing *Alicyclobacillus* by vanillic acid was determined.

Evaluation of guaiacol production ability by non-guaiacol producing *Alicyclobacillus* at vanillic acid concentrations under the minimum inhibitory level.

K broth (9 ml) tubes were prepared, supplemented with 10, 20, 30, 40, 50 ppm vanillic acid and adjusted to pH 4.0 with malic acid. Frozen stocks of non-guaiacol producing isolates 19220 and C-GD 1-1 were inoculated (10 μl) into each respective K broth tube containing different concentrations of vanillic acid. Prior to incubation, 0.5 ml aliquots were removed from each tube for HPLC analysis and 1 ml aliquots were removed for spread plating onto K agar. After incubation at 43°C for 48 h, each tube was assessed for growth, vanillic acid degradation and guaiacol production.

Comparison of recovery efficiency of novel SK2 medium with other commonly used *Alicyclobacillus* spp. isolation media.

Potato dextrose agar (PDA), orange serum agar (OSA) were obtained from Difco (Franklin Lakes, NJ) and prepared according to the manufacturer's instructions. K and SK agar were prepared as previously described. In addition to the previously used test isolates, isolates 97, 113, 849, 6348, A-Gala 9-2, and WAC were also used to validate the ability of SK2 to selectively isolate guaiacol producing *Alicyclobacillus* spp. All isolates were inoculated into K broth (pH 4.0) and incubated at 43°C for 48 h. The actively growing cell cultures were serially diluted to allow approximately 10^2-10^3 CFU/ml and 100 μl of each diluted cell culture was plated onto each type of media. All plates were enumerated after 48 h of incubation at 43°C.

RESULTS

Dye screening for differentiation/selection of guaiacol producing and non-guaiacol producing *Alicyclobacillus* spp.

Among the 42 dyes listed in Table 4-1, distinction among the test isolates was identified in basal SK agar containing the following dyes: acid fuchin, acid green B, bromcresol purple, chrome azurol S, and resazurin. Detailed colony morphology and color formation of individual *Alicyclobacillus* spp. isolates on basal SK agar supplemented with the dyes are listed in Table 4-2. Color differentiation corresponding with the guaiacol producing ability of the test isolates was found only on basal SK agar supplemented with chrome azurol S (CAS) as presented in Figure 4-1. Thus CAS was selected as the differential dye for subsequent studies.

Evaluation of vanillic acid and oxygen concentration as additional differentiation/selective agents for guaiacol producing and non-guaiacol producing *Alicyclobacillus* spp.

Results for the effect of vanillic acid and oxygen concentration on differentiating guaiacol and non-guaiacol producing *Alicyclobacillus* spp. isolates on SKD is summarized in Table 4-3. Addition of 100 ppm vanillic acid resulted in a selective effect by inhibiting the recovery of non-guaiacol *Alicyclobacillus* (Figure 4-2). The inhibitory effect of vanillic acid was observed under both aerobic and microaerophilic conditions. Limiting oxygen by overlaying did not enhance color formation or selectiveness of the media. Growth of colonies on media with overlays formed pinpoint colonies, with no color differences among the guaiacol and non-guaiacol producing isolates, thus counteracting the differential effect of CAS useless.

Chrome Azurol S (CAS) and the recovery of *Alicyclobacillus* spp.

Chrome Azurol S did not exhibit any effect on the recovery of the tested *Alicyclobacillus* spp. isolates at the tested concentration range of 0.0001% to 0.01% (Table 4-4). Within each isolate, recovery by SK agar supplemented with differing concentrations of CAS were not different from basal SK agar. No differences were observed between the guaiacol producing isolates and the non-guaiacol producing isolates at any given CAS supplemented concentration. The optimum concentration for both color intensity and color contrast for the differentiation of guaiacol and non-guaiacol producing *Alicyclobacillus* was 0.01%.

Vanillic acid and the recovery of *Alicyclobacillus* spp.

Table 4-5 summarizes the effect of vanillic acid on the recovery of selected *Alicyclobacillus* isolates. Recovery of the test isolates at vanillic acid concentrations of 1 ppm and 10 ppm were similar to recovery in basal SK agar. At vanillic acid concentrations of 100 ppm, guaiacol producing isolates were recoverable but slightly inhibited while no growth was observed for non-guaiacol producing isolates (Figure 4-3).

Determination of the minimum inhibitory concentration of vanillic acid on non-guaiacol producing *Alicyclobacillus*.

Since vanillic acid was inhibitory towards guaiacol producing *Alicyclobacillus* isolates at 100 ppm, further research was conducted to determine the minimum vanillic acid concentration at which non-guaiacol producing *Alicyclobacillus* spp. were inhibited without affecting the recovery of guaiacol producing isolates. The recovery of *Alicyclobacillus* spp. isolates on SK supplemented with vanillic acid concentrations ranging from 10-100 ppm are presented in Table 4-6. In general, recovery of the test

isolates decreased as vanillic acid concentrations increased. At 70 ppm vanillic acid, non-guaiacol producing isolates 19220 and C-GD 1-1 did not grow whereas the recovery of guaiacol producing isolates 1016 and 1101 were unaffected. Thus, the minimum inhibitory concentration of non-guaiacol producing isolates was 70 ppm.

The effect of vanillic acid on isolates 19220 and C-GD 1-1 differed slightly based on colony formation. Isolate 19220 grew within vanillic acid supplement concentrations of 10-60 ppm and colony morphology was identical to colonies on the basal SK agar. In SK agar supplemented with ≥ 70 ppm vanillic acid, colonies were not formed (Figure 4-4). Different observations were made with isolate C-GD 1-1 as illustrated in Figure 4-5. In SK agar containing 10-30 ppm vanillic acid, colony morphology of isolate C-GD 1-1 was identical to unsupplemented SK agar. However, C-GD 1-1 formed pinpoint colonies (approximately 1 mm) on SK agar supplemented with 40-60 ppm vanillic acid. Similar to isolate 19220, no colony formation was detected at vanillic acid concentrations greater than 70 ppm.

Evaluation of guaiacol production ability by non-guaiacol producing *Alicyclobacillus* at vanillic acid concentrations under the minimum inhibitory level.

The previous method used to determine the guaiacol producing ability of *Alicyclobacillus* utilized 100 ppm of vanillic acid. Since growth of isolates 19220 and C-GD 1-1 were inhibited when grown in media containing 100 ppm vanillic acid (Table 4-5), it was critical to clarify if lack of guaiacol production by non-guaiacol producing isolates was due to a "true" inability to produce guaiacol or the result of no growth at 100 ppm vanillic acid. Isolates 19220 and C-GD 1-1 were monitored for growth, vanillic acid

catabolism, and guaiacol formation under small vanillic acid concentrations uninhibitory to growth as presented in Table 4-6.

Table 4-7 and Table 4-8 summarize the results for isolates 19220 and C-GD 1-1, respectively. Reduced recovery of isolate 19220 was observed at vanillic acid concentrations of 40 and 50 ppm, whereas growth in K broth containing 10-30 ppm vanillic acid was no different from the control. Isolate C-GD 1-1 was more sensitive to vanillic acid compared to isolate 19220. Inhibited growth of isolate C-GD 1-1 was observed in K broth containing more than 30 ppm vanillic acid. At the vanillic acid concentrations which inhibited growth for both 19220 and C-GD 1-1, C-GD 1-1 was inhibited to a greater extent than 19220. For both isolates at vanillic acid concentrations that do not adversely affect growth, no utilization of vanillic acid or formation of guaiacol was detected.

Composition of the novel SK2 medium.

The composition of the novel selective/differential medium for guaiacol producing *Alicyclobacillus* is as follows (per liter): 5.0 g peptone, 2.5 g yeast extract, 1.0 g glucose, 1.0 g Tween® 80, 0.5 g $CaCl_2$, 0.01% Chrome Azurol S, and 70 ppm vanillic acid, adjusted to pH 4.0 with 10% malic acid after sterilization. As the new developed media was modified and optimized for differentiation and selectivity using SK agar as a basal media, we propose that the novel media be named SK2.

Comparison of recovery efficiency of novel SK2 medium with other commonly used *Alicyclobacillus* spp. isolation media.

Table 4-9 summarizes the ability of different media to detect the presence or absence of *Alicyclobacillus* test isolates. The presence of all test isolates were detected in

potato dextrose agar (PDA), K agar, and SK agar, whereas only some isolates were recovered with orange serum agar (OSA) and SK2 agar. OSA detected the presence of guaiacol producing *Alicyclobacillus* and could only recover 2/5 non-guaiacol producing *Alicyclobacillus* isolates. SK2 recovered all guaiacol producing isolates and one non-guaiacol producing isolate, A-Gala 9-2. The color formation of isolate A-Gala 9-2 on SK2 was yellow to pale white, distinctly different from the dark purple/royal blue colonies of the guaiacol producing isolates. The recovery rates of *Alicyclobacillus* isolates by different media are presented in Table 4-10. The recovery efficiency of the five media for guaiacol producing isolates and isolate A-Gala 9-2 is SK≥SK2≥PDA≥K>OSA. For the recovery of other non-guaiacol producing isolates where SK2 is not applicable, the recovery efficiency was SK≥PDA>K>OSA. In general, OSA could detect the presence of *Alicyclobacillus* (Table 4-9) but the recovery rate was low compared to other media (Table 4-10). Considering the ability of the recovery medium to differentiate between guaiacol producing and non-guaiacol producing *Alicyclobacillus*, SK2 was the only medium that provided selectivity/differentiation between the two types of *Alicyclobacillus* (Figure 4-6). Colonies formed on OSA, PDA, K agar, and SK agar were off-white and no differences were detected between guaiacol producing and non-guaiacol producing isolates. Growth of guaiacol producing isolates on SK agar were dark purple to royal blue compared to non-guaiacol producing isolates that were completely inhibited or formed white to cream colonies.

DISCUSSION

Since the recognition of *Alicyclobacillus* as an important spoilage microorganism to the juice and beverage industry, relatively small improvements are made regarding the

recovery media. A review on currently available recovery media are detailed elsewhere (Chang and Kang, 2004). The most recently developed media is SK agar with enhanced recovery of *Alicyclobacillus* compared to OSA, PDA, and K agar (Chang and Kang, 2005). The development of SK2 agar provides an alternative for simultaneously recovering and detecting the guaiacol producing ability of the isolated *Alicyclobacillus* colonies.

The major differential/selective agents for SK2 media are Chrome Azurol S and vanillic acid, respectively. As presented in Figure 4-1, color changes/colony morphology in SK agar supplemented with Chrome Azurol S (CAS) correlated with the guaiacol producing ability of the test isolates. Chrome Azurol S is widely known for use in the detection of siderophores (Schwyn and Neilands , 1987; Shin et al., 2001; Milagres et al., 1999), low-molecular mass (<1000 Da) compounds with high iron affinity to assist microorganisms in sequestering and solubilizing ferric iron (Hider, 1984; Neilands, 1984, 1995). The general idea behind CAS-siderophore detection tests is based on a competition for iron between the ferric complex of CAS and a siderophore (Machuca and Milagres, 2003). A color change from blue to orange/yellow is observed as the iron is removed from CAS-iron complex to form a chelate with the siderophore (Schwyn and Neilands,1987). In this study, the prominent differential effect of CAS lies not in the color change of the solid media but in the color of the colonies growing on the media (Figure 4-1). The underlying mechanism on the formation of different colony colors on CAS supplemented medium consistent with guaiacol producing ability remains to be explored and may provide valuable information on internal differences that exist between guaiacol producing and non-guaiacol producing *Alicyclobacillus*.

As previously reported, no growth factors, carbohydrate utilization patterns or specific enzymes that can easily be incorporated in the differentiation of guaiacol and non-guaiacol producing *Alicyclobacillus* was determined. The major distinction remains in the ability or inability to produce guaiacol. As vanillic acid is the direct precursor of the off-flavor compound guaiacol (Crawford and Olson, 1978; Huang et al., 1993), the addition of vanillic acid to basal SKD media was used to enhance any differences between the two types of *Alicyclobacillus* through the production of guaiacol; thus the powerful inhibitory effect of vanillic acid on non-guaiacol producing *Alicyclobacillus* was unexpected (Table 4-5). Nonetheless, the inhibitory effects of vanillic acid have been studied and well documented in other microorganisms. The effect of vanillic acid on *Listeria* species was investigated by Delaquis et al. (2005). *L. monocytogenes*, *L. innocua*, and *L. grayi* did not differ in sensitivity towards vanillic acid and a concentration of less than 10 mM was sufficient to prevent the growth of *L. monocytogenes*, *L. innocua*, and *L. grayi* at pH 5.0. Similarly, vanillic acid is effective against *Escherichia coli*. In inoculated apple juice containing 5.2×10^5 CFU/ml *E. coli* O157:H7, no colonies could be recovered after seven days when the apple juice was supplemented with 10 mM vanillic acid (Moon et al., 2006). A 50% inhibition was observed for a non-pathogenic *E. coli* strain that ferments hemicelluloses at vanillic acid supplementation of 1.15 g/L (Zaldivar and Ingram 1999). Other microorganisms inhibited by vanillic acid include *Saccharomyces cerevisiae, Zymomonas mobilis*, and *Klebsiella pneumoniae* (Nishikawa et al., 1988; Ratunanga et al., 1997; Delaquis et al., 2005). Weak organic acids are commonly used as preservatives, and the antimicrobial activities are highly dependent on pH. Optimum inhibitory activities are observed at low pH where the

undissociated weak acid can enter into the cell through permeation across the plasma membrane (Wen et al., 2003). Once within the cell, the higher intracellular pH will result in dissociation of the weak acid and the release of charged anions and protons which are unable to cross the plasma membrane (Brul and Coote, 1999). The weak acid will diffuse into the cell until equilibrium is reached in accordance with the pH gradient across the membrane resulting in the accumulation of anions and protons inside the cell (Booth and Kroll, 1989). Cellular damage from the permeation action of weak acids include membrane disruption (Freese et al., 1973; Stratford and Anslow, 1998; Bracey et al., 1998; Zaldivar and Ingram, 1999), the inhibition of essential metabolic reactions (Krebs et al, 1983), stress on intracellular pH homeostasis (Salmond et al., 1984; Cole and Keenan, 1987; Bracey et al., 1998), and the accumulation of toxic anions (Eklund, 1985). Vanillic acid is a weak carboxylic acid with two pK_as of pK_{a1}=4.51 and pK_{a2}=9.39. As the pH of the growth media used within this study was adjusted to pH 4.0 for optimal growth of *Alicyclobacillus* species, vanillic acid within the media would be in its undissociated and most lethal form. Since both types of *Alicyclobacillus* were exposed to identical concentrations of vanillic acid, the permeation of vanillic acid into the cells and the extent of membrane disruption/leakage would be the same. The different growth responses of guaiacol producing and non-guaiacol producing *Alicyclobacillus* should be due to different intracellular mechanisms to minimize the adverse effects of vanillic acid. Guaiacol producing *Alicyclobacillus* may possess a detoxifying mechanism, most likely the ability to convert vanillic acid to guaiacol (Figure 4-7). This postulation is supported by observations in Chapter 3 that guaiacol formation by *Alicyclobacillus* isolates 1016 and 1101 are approximately simultaneous to vanillic acid utilization and without the

formation of other phenolic products. Slight inhibition of guaiacol producing *Alicyclobacillus* in the presence of 100 ppm vanillic acid may be due to the detrimental effects of vanillic acid that are not converted to guaiacol (Table 4-5). On the other hand, non-guaiacol producing *Alicyclobacillus* may lack the ability to alleviate the accumulation of vanillic acid leading to its inhibition. The absence of vanillic acid utilization by non-guaiacol producing *Alicyclobacillus* at sublethal vanillic acid concentrations presented in Tables 4-7 and 4-8 support this assumption.

To validate the efficacy of SK2 agar in recovering guaiacol producing *Alicyclobacillus*, the relative recovery percentage of each isolate by selected media was calculated (Table 4-11). Recovery by K agar, the most commonly used recovery media in the United States, was designated as 100% to facilitate comparison. Comparing across recovery media, recovery by OSA is lower than that of other media. This result is in agreement with the observations of previous findings (Chang and Kang, 2005). PDA (with the exception of isolate 97), SK agar, and SK2 agar exhibited greater recovery percentages than K agar. All guaiacol producing isolates were recovered on SK2 agar and at higher percentages than on K agar. The addition of vanillic acid as the selective agent did not adversely affect the recovery on SK2 agar as the recovery on SK and SK2 agar were comparable. Non-guaiacol producing isolates were also recovered using OSA, PDA, K, and SK agar, and colony morphology were identical to the guaiacol producing isolates (Figure 4-6). Except for isolate A-Gala 9-2, no non-guaiacol producing isolates were recovered on SK2 agar, most likely due to the inhibitory effects of vanillic acid. A-Gala 9-2, a non-guaiacol producing *Alicyclobacillus*, grew on SK2 agar. However, due to the differential agent CAS, the formed colonies were cream colored rather than the dark

purple/royal blue of the guaiacol-producing isolates, making them easily distinguishable as non-spoilage related *Alicyclobacillus*.

As demonstrated in this study, the novel SK2 agar is a new alternative for the detection of *Alicyclobacillus*. Previously, confirmation of the presence of guaiacol producing *Alicyclobacillus* would require an isolation procedure and a guaiacol formation analysis, requiring at least 96 h before results can be obtained. The unique composition of SK2 agar allows simultaneous recovery and detection of guaiacol producing *Alicyclobacillus* in one 48 h test. In the case of vanillic acid resistant non-guaiacol producing isolates, different colony colors as a result of the added CAS allows easy differentiation from guaiacol producing isolates. In summary, the time-saving dual function SK2 agar can be easily incorporated by juice manufacturing facilities and may be a valuable tool in the quality control and detection of spoilage related *Alicyclobacillus*.

Table 4-1. Dyes used for selective agent in development of SK2 agar.

Dye	Company
Acid Fuschin	Spectrum Chemicals & Laboratory Products Inc., Gardena, CA
Acid Green B	Hartman-Leddon Co., Philadelphia, PA
Alizarin	Allied Chemical Corporation, New York, NY
Amido Black 10B	E. Merck AG, Darmstadt, Germany
Aniline Blue	Allied Chemical Corporation, New York, NY
Bacto Basic Fuchsin	Difco Laboratories, Detroit, MI
Benzyl Violet	The Coleman & Bell Co., Norwood, OH
Brilliant Green	Sigma-Aldrich Inc., St. Louis, MO
Brom Chlor Phenol Blue	Allied Chemical Corporation, New York, NY
Bromcresol Purple	Sigma Chemical Company, St. Louis, MO
Brom Thymol Blue	National Aniline & Chemical Co, Inc., New York, NY
Bromcresol Green	Sigma Chemical Company, St. Louis, MO
Bromcresol Purple	Sigma Chemical Company, St. Louis, MO
Bromophenol Blue	LaMotte Chemical Products Co., Baltimore, MD
Carmine	Mereck & Co., New York, NY
Chrome Azurol S	Sigma-Aldrich Inc., St. Louis, MO
Eosin Y	Difco Laboratories, Detroit, MI
Erie Garnet B	Hartman-Leddon Co., Philadelphia, PA
Erythrosine, bluish	National Aniline & Chemical Co, Inc., New York, NY
Evan Blue	Eastman Kodak Company, Rochester, NY
Fast Green FCF	Allied Chemical Corporation, New York, NY
Giemsa Stain	National Aniline & Chemical Co, Inc., New York, NY
Indigo Carmine	J. T. Baker Chemical Co., Phillipsburg, NJ
Lacmoid	The Coleman & Bell Co., Norwood, OH
Light Green SF Yellow	National Aniline & Chemical Co, Inc., New York, NY
“Luxol” Fast Red B	Du Pont de Nemours & Company, Inc., Wilmington, DE
“Luxol” Fast Scarlet 2R	Du Pont de Nemours & Company, Inc., Wilmington, DE
Malachite Green-Oxalate	J. T. Baker Chemical Co., Phillipsburg, NJ
May-Greenwald’s Stain	Allied Chemical Corporation, New York, NY
Meta Cresol Purple	LaMotte Chemical Products Co., Baltimore, MD
Methylene Blue	Hartman-Leddon Co., Philadelphia, PA
Nile Blue A	Allied Chemical Corporation, New York, NY
Oil Red O	Sigma-Aldrich Inc., St. Louis, MO
Orange I	National Aniline & Chemical Co, Inc., New York, NY
Orange G	Allied Chemical Corporation, New York, NY
Resazurin	Eastman Kodak Co., Rochester, NY
Rhodamine 6G	Eastman Kodak Co., Rochester, NY
Safranin O	Allied Chemical Corporation, New York, NY
Tartrazine	National Aniline & Chemical Co, Inc., New York, NY
Thiazol Yellow GGM	Eastman Kodak Co., Rochester, NY
Thionin	Dr. G. Griibler & Co., Leipzig
Thymol Blue	Eastman Kodak Co., Rochester, NY

Table 4-2. Dye screening results[1] for possible differentiation between guaiacol producing and non-guaiacol producing *Alicyclobacillus* spp. isolates.

Dye	Colony Color/Morphology	Background color	
		Under colony	Without growth
Acid Fuchsin			
1101	pink, irregular	light pink	hot pink
1016	pink, irregular	(S)[2] hot pink (A)[3] light pink	hot pink
19220	pink, circular	(S) hot pink (A) light pink	hot pink
C-GD 1-1	pink, circular	(S) hot pink (A) light pink	hot pink
Acid Green B			
1101	teal, circular	(S) teal (A) lighter teal	teal
1016	teal, circular	lighter teal halo	teal
19220	teal, circular	(S) teal (A) lighter teal	teal
C-GD 1-1	teal, circular	(S) teal (A) lighter teal	teal
Bromcresol Purple			
1101	white irregular purple center	(S) yellow to purple (A) purple	yellow
1016	greenish to white	(S) yellow (A) purple	yellow
19220	white, circular purple center	(S) yellow to purple (A) purple	yellow
C-GD 1-1	white, circular purple center	(S) yellow to purple (A) purple	yellow
Chrome Azurol S			
1101	dark purple to royal blue, irregular	yellow	red
1016	purple, irregular	yellow	red
19220	cream, circular	yellow	red
C GD 1-1	cream, circular	yellow	red
Resazurin			
1101	cream with pink center, irregular	(S) cream (A) pink	cream
1016	cream, irregular	(S) cream (A) pink	cream
19220	cream, circular	(S) cream (A) pink	cream
C-GD 1-1	cream, circular	(S) cream (A) pink	cream

[1]: results that showed no difference among the tested isolates are omitted
[2]: color under single isolated colony
[3]: color under streak of abundant colony growth

Table 4-3. Colony formation of *Alicyclobacillus* spp. isolates on recovery medium (pH 4.0) at 43°C for 48h.

Recovery Medium	*Alicyclobacillus* spp. Isolate			
	1016[3]	1101[3]	19220[4]	C-GD 1-1[4]
SKD[1]	purple with yellow background	dark purple to royal blue with yellow background	cream with yellow background	cream with yellow background
SKDV[2]	purple with yellow background	(h)[5] blue to gray with yellow backgrounds (s)[6] purple with yellow background	NG	NG
ov[9]SKD	(m)[7] pinpoint size colonies, no background color (t)[8] no growth	(m) purple pinpoint colonies (t) large purple colonies with yellow background at heavy growth areas	(m) pinpoint size colonies, no background color (t) NG	(m) pinpoint size colonies, no background color (t) NG
ov[9]SKDV	(m) pinpoint colonies, no background color (t) NG	(m) purple pinpoint colonies (t) large purple colonies with yellow background at heavy growth areas	NG	NG

[1]: Chrome Azurol S (CAS) was supplemented to SK agar at 0.01% (w/w)
[2]: SKD with addition of 100 ppm vanillic acid
[3]: guaiacol producing *Alicyclobacillus* spp. isolates
[4]: non-guaiacol producing *Alicyclobacillus* spp. isolates
[5]: heavy growth
[6]: single isolated colony
[7]: intersection between original media and overlay section
[8]: surface of overlay section
[9]: media overlaid with SKD (pH 4.0)
NG: no growth

Table 4-4. Chrome Azurol S (CAS) concentration and the recovery of *Alicyclobacillus* spp. isolates.

Medium (pH 4.0)	Recovery (CFU/plate)			
	Alicyclobacillus spp. Isolate			
	1016	1101	19220	C-GD 1-1
SK Agar (control)	183	127	55	87
SK+0.0001% CAS	194	123	54	88
SK+0.0005% CAS	208	113	60	73
SK+0.001% CAS	190	114	46	86
SK+0.01% CAS	210	122	47	85

Table 4-5. Vanillic acid concentration and the recovery of *Alicyclobacillus* spp. isolates.

Medium (pH 4.0)	Recovery (CFU/plate)			
	Alicyclobacillus spp. Isolate			
	1016	1101	19220	C-GD 1-1
SK Agar (control)	183	127	55	87
SK+1 ppm	180	147	53	85
SK+10 ppm	207	145	42	80
SK+100 ppm	84	81	0	0

Table 4-6. Minimum inhibitory concentration of vanillic acid on non-guaiacol producing *Alicyclobacillus* spp. isolates.

Vanillic Acid Concentration (ppm)	Growth of *Alicyclobacillus* spp. Isolates (CFU/plate)			
	1016	1101	19220	C-GD 1-1
0	91	207	43	73
10	90	206	39	65
20	85	203	40	69
30	89	192	35	58
40	83	199	8	59
50	86	196	10	55
60	82	192	7	21
70	85	189	0	0
80	69	164	0	0
90	54	128	0	0
100	50	120	0	0

Table 4-7. *Alicyclobacillus* sp. 19220 growth and vanillic acid catabolism under low concentrations of vanillic acid.

Vanillic Acid Concentration in K Broth (ppm)	Growth (log CFU/ml)		Vanillic Acid Concentration[1] (ppm)		Guaiacol Concentration[1] (ppm)	
	0h	48h	0h	48h	0h	48h
0	0.48	6.03	0	0	0	0
10	0.48	5.95	10.00	9.80	0	0
20	0.48	6.12	20.00	19.16	0	0
30	0.48	6.02	30.00	30.26	0	0
40	0.48	3.90	40.00	41.50	0	0
50	0.48	2.99	50.00	50.96	0	0

[1]: As determined by HPLC.

Table 4-8. *Alicyclobacillus* sp. C-GD 1-1 growth and vanillic acid catabolism under low concentrations of vanillic acid.

Vanillic Acid Concentration in K Broth (ppm)	Growth (log CFU/ml)		Vanillic Acid Concentration[1] (ppm)		Guaiacol Concentration[1] (ppm)	
	0h	48h	0h	48h	0h	48h
0	0.48	6.22	0	0	0	0
10	0.48	6.24	10.00	8.69	0	0
20	0.48	6.17	20.00	19.90	0	0
30	0.48	3.40	30.00	30.26	0	0
40	0.48	1.94	40.00	40.60	0	0
50	0.48	1.08	50.00	50.32	0	0

[1]: As determined by HPLC

Table 4-9. Presence/absence results of various *Alicyclobacillus* spp. isolates in commonly used *Alicyclobacillus* isolation media.

Isolate	Guaiacol Production	Recovery Media				
		OSA	PDA	K	SK	SK2
97	+	+	+	+	+	+
113	+	+	+	+	+	+
1016	+	+	+	+	+	+
1101	+	+	+	+	+	+
WAC	+	+	+	+	+	+
849	-	-	+	+	+	-
6348	-	-	+	+	+	-
19220	-	+	+	+	+	-
A-Gala 9-2	-	+	+	+	+	+*
C-GD 1-1	-	+	+	+	+	-

*: Growth was observed on the media, but colony morphology was different from typical colony formation by guaiacol producing *Alicyclobacillus*.

Table 4-10. Recovery of various *Alicyclobacillus* spp. isolates by different media.

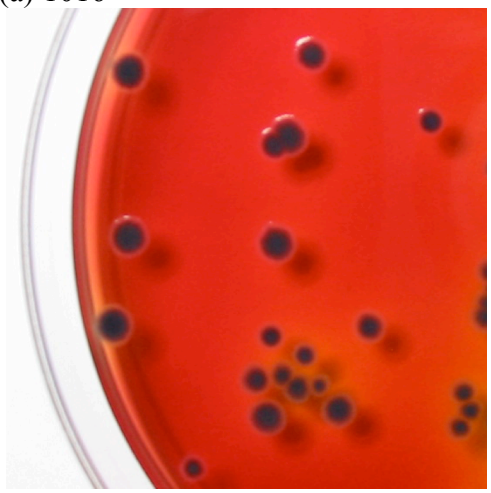
Isolate	Guaiacol Production	Recovery (CFU/plate)				
		OSA	PDA	K	SK	SK2
97	+	168	267	328	363	360
113	+	71	338	316	414	399
1016	+	56	145	143	157	158
1101	+	22	108	97	125	119
WAC	+	14	378	190	296	317
849	-	0	415	397	448	0
6348	-	0	271	68	275	0
19220	-	36	67	55	88	0
A-Gala 9-2	-	2	235	148	544	324*
C-GD 1-1	-	79	234	211	264	0

*: Growth was observed on the media, but colony morphology was different from typical colony formation by guaiacol producing *Alicyclobacillus*.

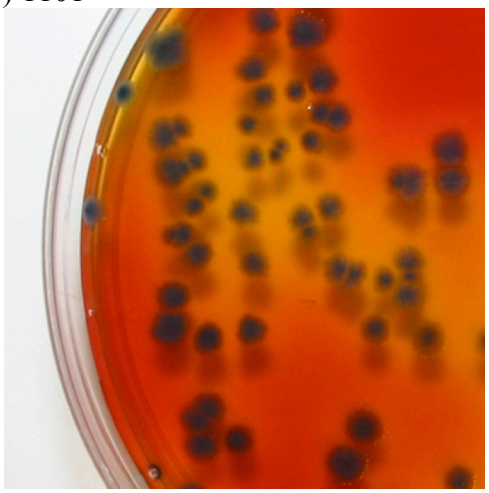
Table 4-11. Relative recovery percentages of selected *Alicyclobacillus* spp. isolates by different media.

Isolate	Guaiacol Production	Recovery percentage (%)				
		OSA	PDA	K	SK	SK2
97	+	51.22	81.40	100	110.67	109.76
113	+	49.65	106.96	100	131.01	126.27
1016	+	39.16	101.39	100	109.79	110.49
1101	+	22.68	111.34	100	128.86	122.68
WAC	+	7.37	198.95	100	155.79	166.84
849	-	0	104.53	100	112.85	0
6348	-	0	158.78	100	367.57	0
19220	-	65.45	398.53	100	404.41	0
A-Gala 9-2	-	1.35	121.82	100	160.00	218.92
C-GD 1-1	-	37.44	110.92	100	125.12	0

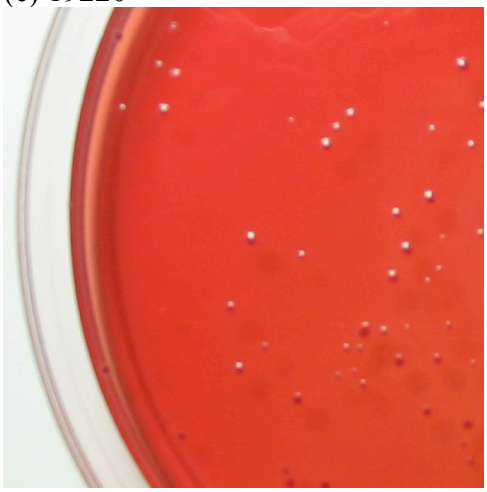
(a) 1016



(b) 1101



(c) 19220



(d) C-GD 1-1

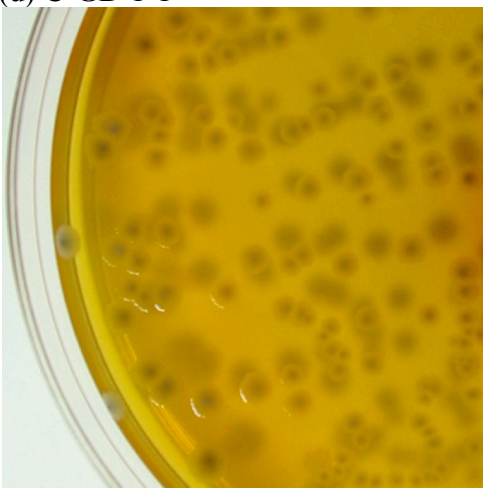
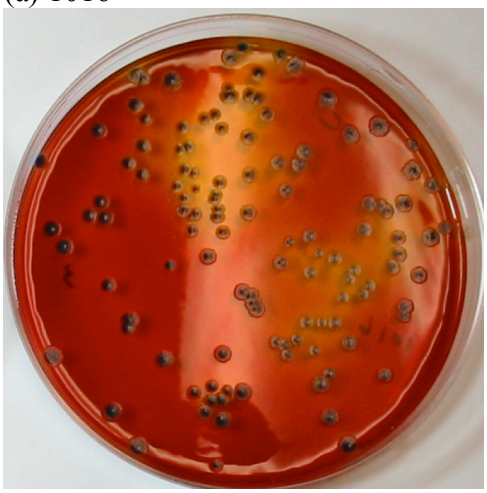
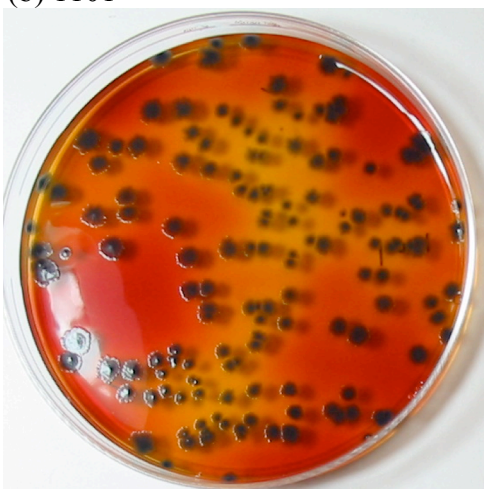


Figure 4-1. Color formation of *Alicyclobacillus* isolates on SK agar supplemented with 0.01% Chrome Azurol S (CAS).

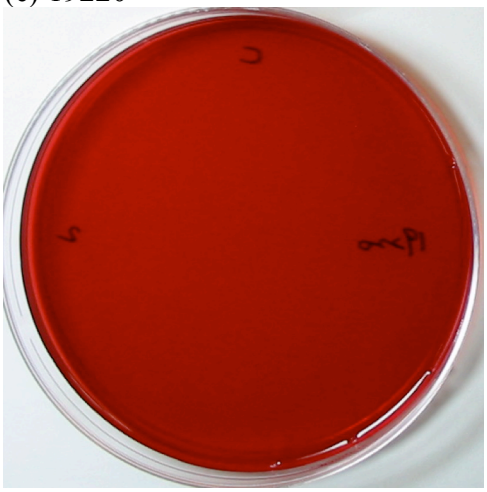
(a) 1016



(b) 1101



(c) 19220



(d) C-GD 1-1

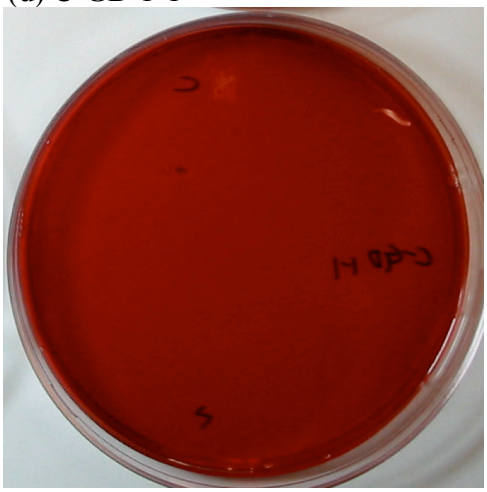
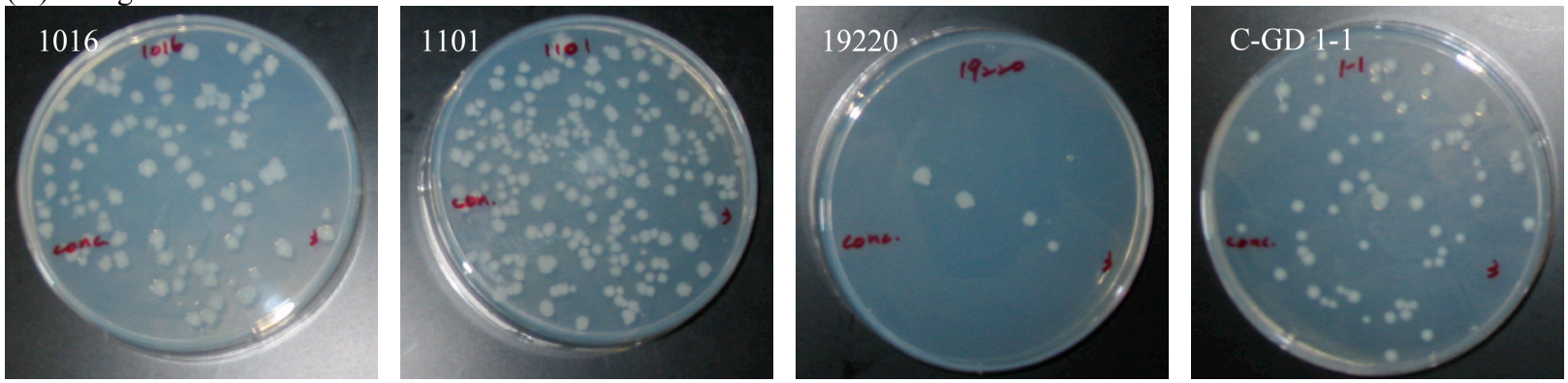


Figure 4-2. Effect of vanillic acid (100 ppm) on the recovery of *Alicyclobacillus* isolates on SK agar supplemented with 0.01% Chrome Azurol S (CAS).

(A) SK agar



(B) SK agar supplemented with 100 ppm vanillic acid

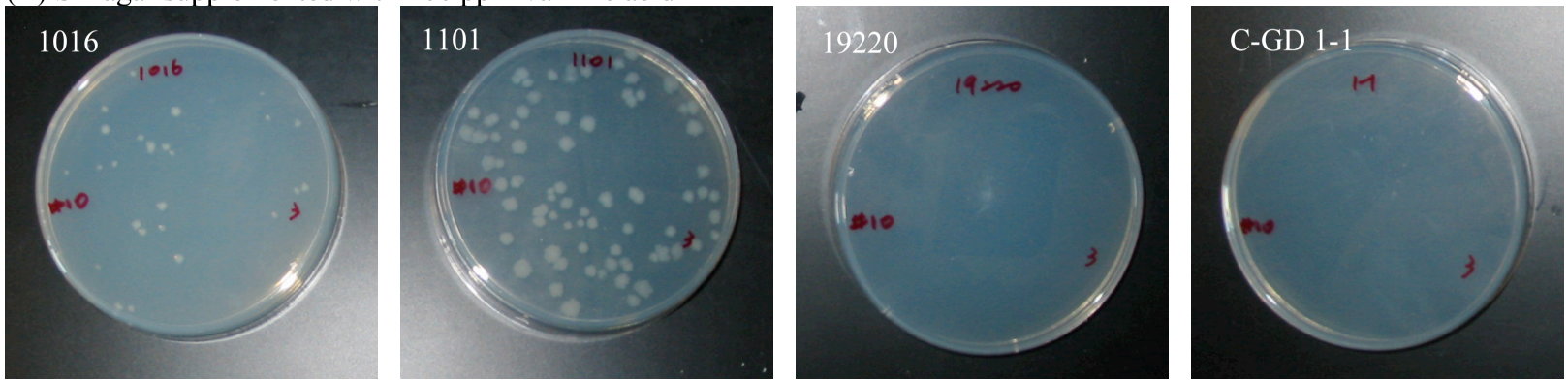


Figure 4-3. Recovery of guaiacol producing (isolates 1016 and 1101) and non-guaiacol producing (isolates 19220 and C-GD 1-1) *Alicyclobacillus* on (A) SK agar and (B) SK agar supplemented with 100 ppm vanillic acid. The pH of both agar media were adjusted to pH 4.0 with 10% malic acid.

(a) (b)

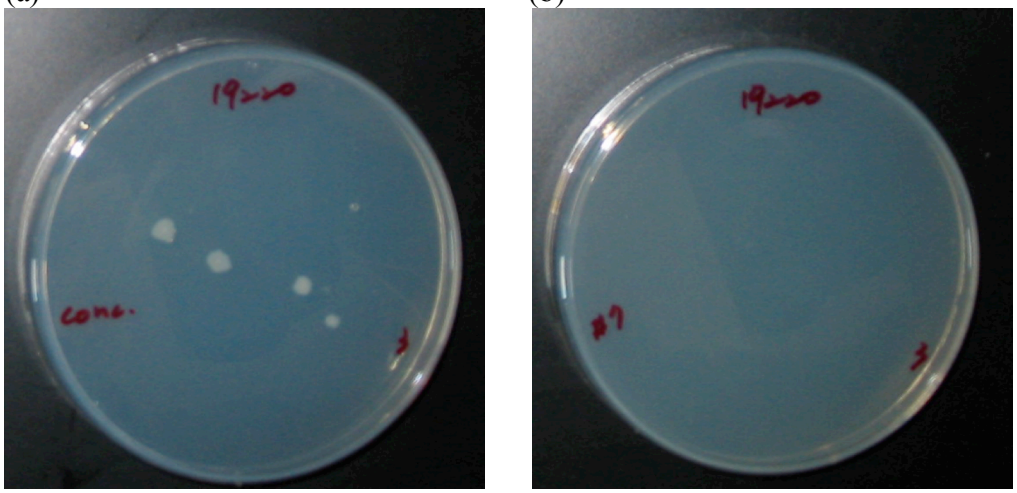
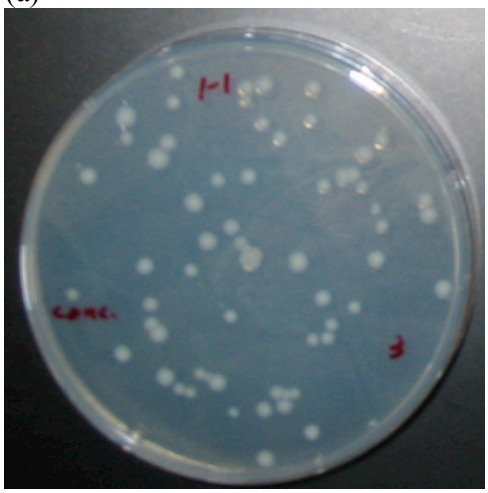
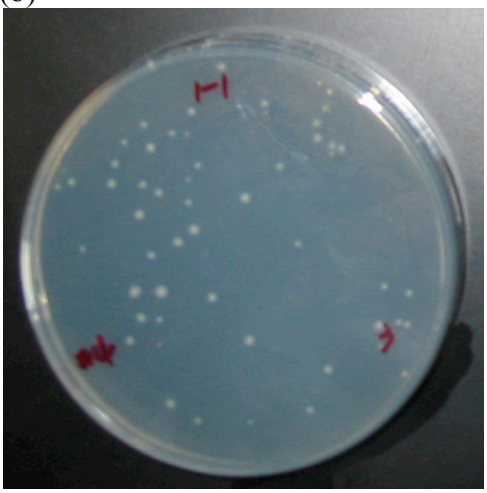


Figure 4-4. Vanillic acid concentration and colony formation of *Alicyclobacillus* sp. isolate 19220. (a) 0-30 ppm vnaillic acid; (b) 70-100 ppm vanillic acid.

(a)



(b)



(c)

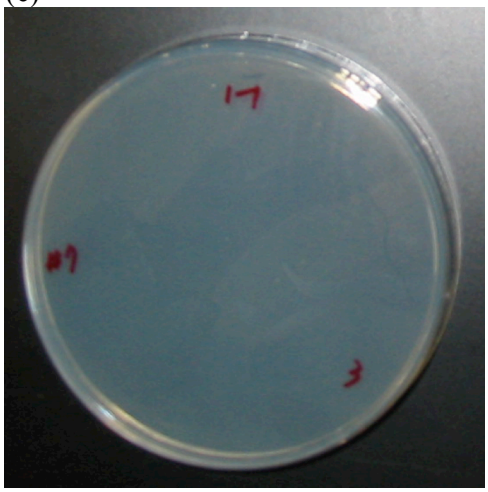
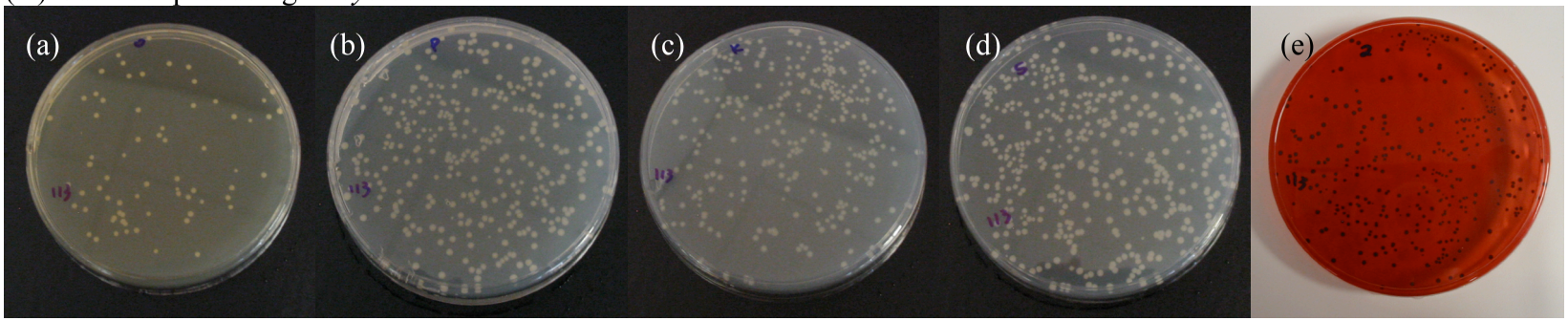
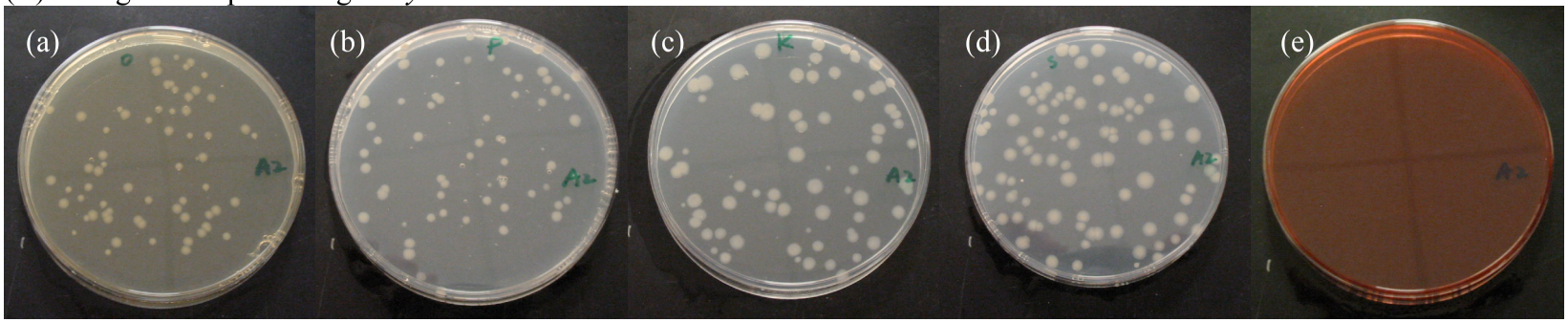


Figure 4-5. Vanillic acid concentration and colony formation of *Alicyclobacillus* sp. isolate C-GD 1-1. (a) 0-30 ppm; (b) 40-60 ppm; (c) 70-100 ppm.

(A) Guaiacol producing *Alicyclobacillus*



(B) Non-guaiacol producing *Alicyclobacillus*



(C) Atypical reaction for non-guaiacol producing *Alicyclobacillus* (Isolate A-Gala 9-2)

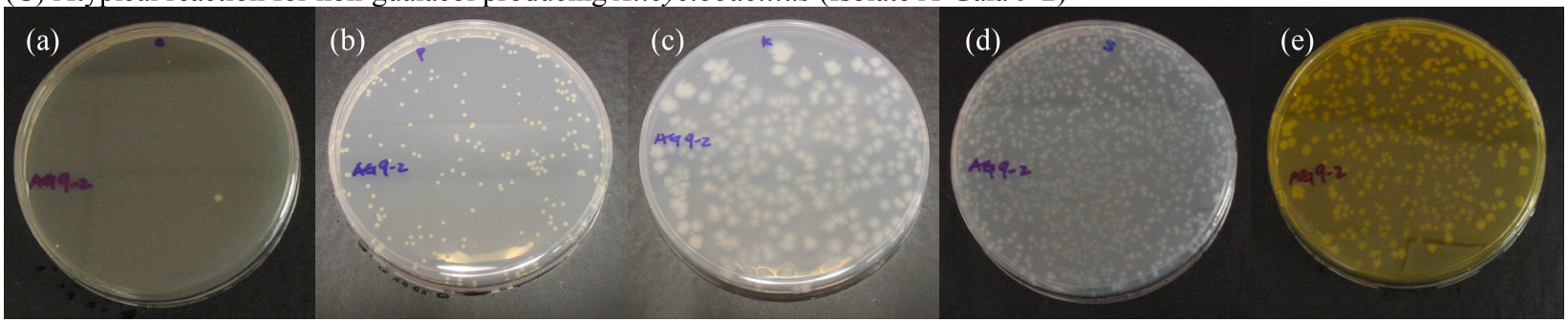


Figure 4-6. Validation of the selectivity/differential ability of guaiacol producing Alicyclobacillus by SK2 Agar against other commonly used recovery medium; (a) Orange Serum Agar (OSA), (b) Potato Dextrose Agar (PDA), (c) K Agar, (d) SK Agar, and (e) SK2 Agar.

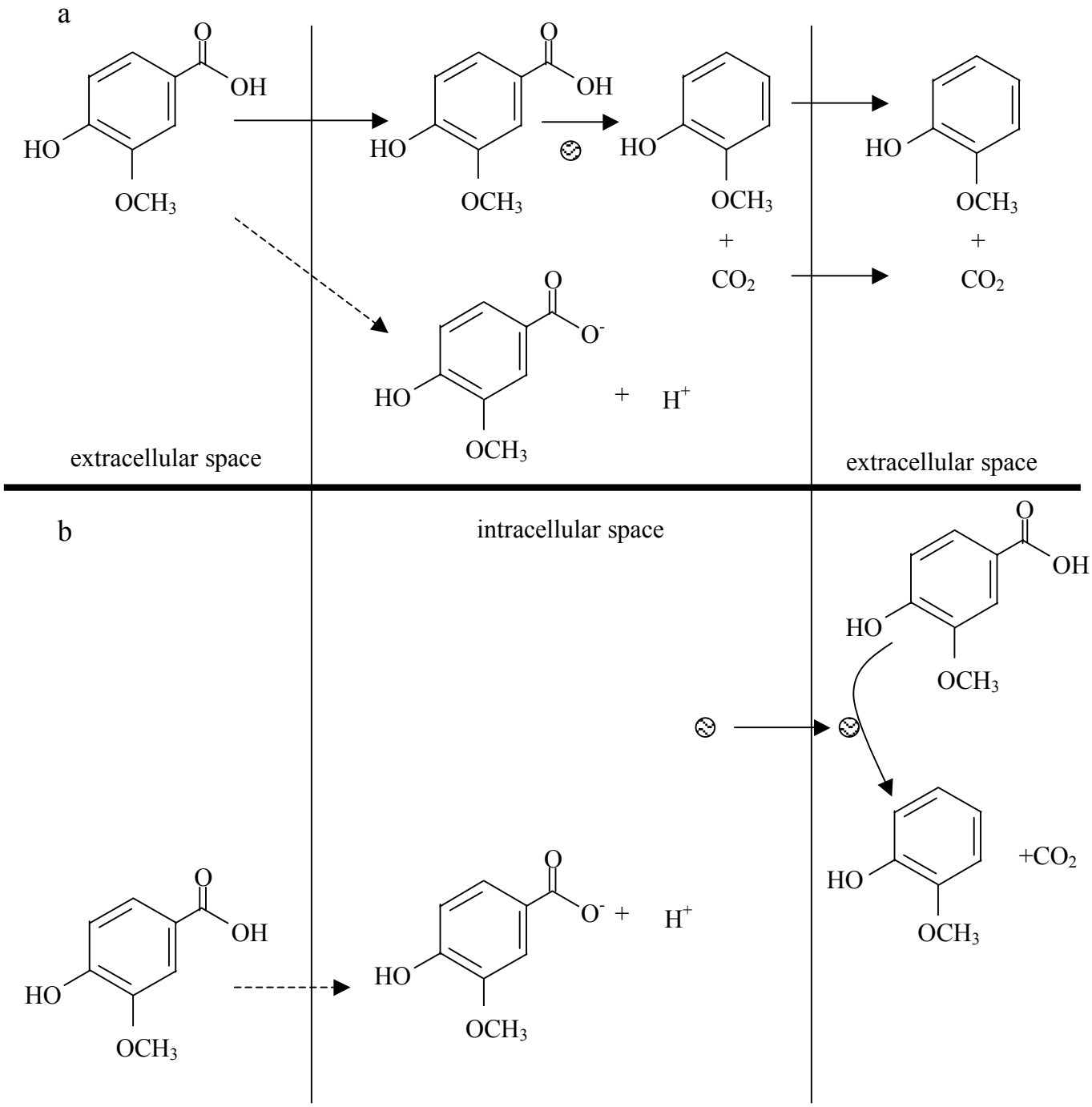


Figure 4-7. Possible mechanisms for vanillic acid detoxification in guaiacol producing *Alicyclobacillus*: (a) conversion of vanillic acid occurs within the cell; (b) conversion of vanillic acid occurs extracellularly. Major and minor reactions are depicted by solid and dotted arrows, respectively. ⊜ indicates a chemical compound or enzyme responsible for the decarboxylation of vanillic acid to guaiacol.

CHAPTER FIVE

CONTROL OF *ALICYCLOBACILLUS* SPORES ON APPLE SURFACES BY AQUEOUS AND GASEOUS CHLORINE DIOXIDE

ABSTRACT

Alicyclobacillus spp. are soilborne spoilage microorganisms contaminating pasteurized fruit juices. In the orchard, apples may be contaminated with spores which can potentially grow in juice and result in spoilage. This study was undertaken to evaluate the efficacy of aqueous and gaseous chlorine dioxide controlling *Alicyclobacillus* spores. *Alicyclobacillus* spores in aqueous suspension treated for 5 min were reduced by more than 4 log with 40 ppm $ClO_{2(aq)}$, and were <0.7 log CFU/ml at 80 ppm and 120 ppm $ClO_{2(aq)}$. On apple surfaces, 40 ppm $ClO_{2(aq)}$ reduced *Alicyclobacillus* spore concentrations by 1.5, 3.2, 4.5, > 4.8 log after 1, 2, 3, and 4 min treatments, respectively. Spore concentrations were reduced by > 4.8 log with 120 ppm $ClO_{2(aq)}$ after one min. With $ClO_{2(g)}$, exposure to both rapid and intermediate release sachets for one h resulted in a 5 log CFU/ apple reduction; however, visual quality was compromised. Spore reductions after exposure to slow release sachets for 1, 2, and 3 h were 2.7, 3.7, and 4.5 log, respectively. Exposure to slow release sachets for 30 min did not significantly reduce spore concentrations compared to the control. No significant visual quality differences between the apples exposed to low release sachet for all treatment times when compared to the control after seven days storage. These results demonstrate the potential applications of ClO_2 in controlling *Alicyclobacillus* spores. Aqueous

chlorine dioxide may be used as a dipping/washing s olution while chlorine dioxide gas sachets may be useful to maintain apple quality during storage and shipping.

INTRODUCTION

Alicyclobacillus spp. are thermoacidophilic sporeformers that are implicated in fruit juice spoilage incidences in the United Kingdom, Germany, Australia, Japan, and the United States (Chang and Kang, 2004). *Alicyclobacillus* spores are highly resistant to conventional juice pasteurization and can germinate and result in spoilage in finished fruit juice products (Yamazaki et al., 1996a). Spoilage by *Alicyclobacillus* is characterized by the production of off-odors without apparent appearance changes to the juice. The major compound responsible for the medicinal-like off-odor of *Alicyclobacillus* related spoilage has been identified as guaiacol (Yamazaki et al., 1996; Jensen and Whitfield, 2003). To a lesser extent 2,6-dibromophenol and 2,6-dichlorophenol are reported as additional off-odor compounds produced by *Alicyclobacillus* contributing to the medicinal-like odor(Baumgart et al., 1997; Jensen, 1999). As a producer of off-flavors, *Alicyclobacillus* is currently an important quality assurance target for acidic beverages. Since *Alicyclobacillus* spores are not affected by pasteurization and find suitable growth conditions in the high acidic conditions of fruit juice products (Yamazaki et al., 1996a; Baumgart et al., 1997; Murakami et al., 1998; Pontius et al., 1998), there is an urgent need to develop a suitable method for inhibiting or controlling the germination and outgrowth of *Alicyclobacillus* to ensure the quality of pasteurized fruit juice products.

Several investigators reported that apples can become contaminated with *Alicyclobacillus* spores in the orchard (Splittstoesser et al., 1994; Orr and Beuchat 2000; Walls and Chuyate 2000). Because spores of *Alicyclobacillus* on apple surfaces survive pasteurization, contamination of the final products is inevitable. Therefore, reducing

Alicyclobacillus spores on apple surfaces is a necessary step for reducing microbial spoilage in apple juice. To reduce the possibility of contamination of juice, apples must be washed prior to processing. Traditionally, aqueous chlorine at concentrations between 50-200 ppm are used to wash fruits and vegetables, which results in a microbial reduction of less than two logs on fresh fruits and vegetables (Beuchat, 1992; Brackett, 1992). A variety of commercial cleansers are available for washing apples, but one study (Kenney and Beuchat, 2002) reported that when used according to the manufacturer's instructions, the maximum reduction achieved by commercial apple washing solutions was three logs. Sanitizers are even less effective against *Alicyclobacillus* spores, as the resistance of spores to chemical sanitizers is well documented (Gorman et al., 1984; Orr and Beuchat, 2000).

Chlorine dioxide (ClO_2) is a strong oxidizing and sanitizing agent that may have a practical application for sanitizing surfaces within the food industry. Chlorine dioxide can be used to sanitize food contact surfaces and food surfaces as a gas or in an aqueous form. Benarde et al. (1965) reported that ClO_2 exhibits about 3.5 times the oxidation capacity of chlorine and Lillard (1979) reported that aqueous ClO_2 produces a bactericidal effect equivalent to that of a seven times stronger concentration of chlorine when applied to poultry processing chilled water. Winniczuk and Parish (1997) reported the minimum inhibitory concentrations of aqueous chlorine dioxide against *Lactobacillus plantarum, Leuconostoc mesenteroides*, and *Saccharomyces cerevisiae* were 0.0031, 0.0048 and 0.0014% (w/v), respectively. *S. cerevisiae* was the most sensitive to $ClO_{2(aq)}$ and *L. mesenteriodes* was the most resistant. Moreover, the bactericidal activity of $ClO_{2(aq)}$ was not affected by high pH or the presence of ammonia or nitrogenous

compounds, and was less reactive with organic compounds compared to chlorine (White 1972). The FDA approved the use of $ClO_{2(aq)}$ for washing fruits and vegetables in 1998 (FDA et al., 1998).

Aqueous chlorine dioxide is a relatively effective sanitizer for fruits and vegetables (Costilow et al., 1984; Brown and Wardowski, 1986). However, microorganisms bound to fruit surfaces may be protected (Han et al., 2001). *Escherichia coli* O157:H7 cells may become trapped in the floral tube wall, lenticels, and damaged cuticle surrounding puncture wounds on apple surfaces, which protects against aqueous disinfection (Kenney et al., 2001; Burnett and Beuchat, 2002). Therefore, it will be advantageous to develop a sanitizing method with greater penetration power.

Gaseous chlorine dioxide is a potent sanitizer/disinfectant for over 30 years (Bernarde et al., 1965). Recently, use of $ClO_{2(g)}$ in food processing environments and as a food sanitizer is widely investigated. Han et al. (1999) reported that 10 ppm $ClO_{2(g)}$ reduces spoilage organisms by six logs on juice tank surfaces. The use of $ClO_{2(g)}$ against foodborne pathogens and spoilage yeasts and molds is also tested and effective on foods including apples, blueberries, green peppers, lettuce, onions, peaches, raspberries, strawberries, and tomatoes (Han et al., 2001; Lee et al., 2004; Sy et al., 2005a,b; Popa et al., 2007). Effective sporicidal activity against spores are also observed using $ClO_{2(g)}$. Foegeding et al. (1986) demonstrated the sporicial activity of $ClO_{2(g)}$ against *Bacillus* spp. and *Clostridium* spp. spores in K_3PO_4 buffer at pH 4.5, 6.5 and 8.0. Chlorine dioxide gas was also reported effective against the spores of *B. thuringiensis* on a wide variety of surfaces, including wood, paper, epoxy, and plastic (Han et al., 2003).

Aqueous and gaseous chlorine dioxide were found effective against several sporeforming bacteria, foodborne pathogens, yeasts and molds. However, to date, the efficacy of chlorine dioxide on *Alicyclobacillus* spores has not been reported. Different types of ClO_2 may exhibit the potential for selected applications during apple juice production and may serve to further ensure the quality of apples before juicing. Therefore, the objective of this study was to evaluate the effect of aqueous and gaseous chlorine dioxide on the reduction of *Alicyclobacillus* spores on the surface of apples.

MATERIALS AND METHODS

1. Bacterial strains.

Two strains of guaiacol producing *Alicyclobacillus* spp. ATCC # 49025 and ATCC # 1013 were obtained from the National Food Processors Association (NFPA, Seattle, WA, USA). Both strains were isolated from spoiled apple juice. The cultures were grown on Orange Serum Agar (OSA; Becton Dickinson, Cockeysville, MD) adjusted to pH 3.7 with sterile filtered 10% tartaric acid for 48 h at 43°C, then stored at 4°C as stock cultures.

2. Preparation of spore cocktail inoculum.

Stock cultures of *Alicyclobacillus* spp. ATCC#49025 and ATCC 1013 were spread plated onto Potato Dextrose Agar (PDA, pH 5.6; Becton Dickinson) in petri plates and incubated at 43°C for 7 days until at least 80% of cells sporulated as determined by microscopic examination. Spores of each strain were individually harvested by depositing 1 to 2 ml of sterile water onto the surface of PDA culture plates. Spores were dislodged by gently rubbing with a sterile swab. Pooled suspensions of 15 plates of each strain were centrifuged at 4,000×g for 20 min (4°C), followed by resuspension in sterile water and

centrifugation at 4,000×g for 10 min (4°C). The washing procedure was repeated three times. The final pellet was resuspended in sterile phosphate buffer (pH 7.0), corresponding to approximately 10^{7-8} spores/ml as enumerated on K agar. The spore suspensions of the isolates 49025 and 1013 were combined to construct the spore cocktail. The mixed spore cocktail was stored in 1.8 ml cryogenic tubes (Fisher Scientific, Pittsburgh, PA) at -20°C until use.

3. Chemical reagents and growth media.

Preparation and measurement of aqueous chlorine dioxide.

Aqueous chlorine dioxide ($ClO_{2(aq)}$) was prepared by adding 6.1 ml Oxine sanitizer (Bio-Cide International, Inc., Norman, OK) to 0.63 g food-grade citric acid crystals in a 600 ml beaker. After 5 min, the beaker was gently swirled to dissolve remaining crystals and deionized water was added. For 20 ppm $ClO_{2(aq)}$, 600 ml of water was added. For higher concentrations of $ClO_{2(aq)}$ proportionately less water was added. The $ClO_{2(aq)}$ concentrations were ascertained using a chlorine dioxide test kit supplied by Bio-Cide International Inc. (Norman, OK).

Generation and measurement of gaseous chlorine dioxide.

Chlorine dioxide gas sachets with selected release rates (slow, intermediate, and rapid) were obtained from ICA TriNova, LLC (Forest Park, GA) and used in this study. Sachets were activated with 10 ml sterile water, suspended 1 to 2 cm above 50 ml of 10% (w/w) KI solution in a 200 ml glass jar, sealed with a rubber gasket and a screw-on lid. After 30 min, the sachet was transferred to another jar containing fresh KI solution. The transfer was repeated at 1 h, 2 h and 3 h. Chlorine dioxide gas ($ClO_{2(g)}$) produced in each

jar was quantified by iodometric titration. The total chlorine dioxide generation is represented as a sum of the titration results at 1, 2, and 3 h.

Preparation of K agar.

K agar was prepared from individual components as described by Walls and Chuyate (2000): 2.5 g yeast extract, 5.0 g peptone, 1.0 g glucose, 1.0 g Tween® 80, 15 g agar and 1.0 L deionized water. The mixture was mixed and brought to a slight boil using a stirrer-hot plate to facilitate the solubilization of agar and dispersion of Tween® 80 prior to sterilization at 121°C for 15 min. Following tempering in a 48°C water bath, the pH of K agar was adjusted to pH 4.0 with 10% (w/v) filter sterilized malic acid (J.T Baker, Phillipsburg, NJ). The agar medium was poured into disposable sterile petri dishes and air dried at room temperature for 48 h prior to use.

4. Inoculation of apples.

Unwashed and unwaxed apples were obtained from Ronald B. Tukey Orchard, Washington State University, Pullman, WA. Apples were placed stem side up in a biosafety laminar flow hood with the fan off at room temperature (25°C). One hundred microliters of spore suspension were applied to the skin on top of each apple by depositing droplets at ten locations with a micropipettor. Inoculated apples were held in the hood overnight with the fan on.

5. Effectiveness of aqueous chlorine dioxide on *Alicyclobacillus* spores.

Aqueous chlorine dioxide and planktonic *Alicyclobacillus* spores.

Two hundred microliters of planktonic *Alicyclobacillus* spp. spores were added to 10 ml of 0, 20, 40, 80 and 120 ppm ClO_2 solution. After 0.5, 1.0, and 5.0 min, 2 ml aliquots were withdrawn and added to 8 ml Dey/Engley (DE) neutralizing broth (Difco, Detroit,

MI) composed of pancreatic digest of casein (5.0 g), yeast extract (2.5 g), dextrose (10.0 g), sodium thioglycollate (1.0 g), sodium thiosulfate (6.0 g), sodium bisulfite (2.5 g), polysorbate 80 (5.0 g), lecithin (7.0 g), and bromcresol purple (0.02 g) adjusted to pH 7.0 with HCl and tempered to 80°C, heat-shocked for 10 min at 80°C, and cooled in an ice-water bath. Ten-fold serial dilutions were performed in sterile water and 100 µl was duplicate spread-plated onto K agar. Where low spore survival was anticipated, 250 µl was spread-plated onto 4 plates. Plates were incubated at 43°C for 48 h.

Aqueous chlorine dioxide and *Alicyclobacillus* spp. spores on apple surfaces.

Unwashed and unwaxed 'Red Delicious', 'Golden Delicious', 'Gala' and 'Fuji' apples inoculated with *Alicyclobacillus* spore cocktail and placed into pint-size ziplock bags sterilized with ultraviolet light. Fifty ml of $ClO_{2(aq)}$ solution with 40 or 120 ppm $ClO_{2(aq)}$ were poured into the bag, and the apple vigorously hand-massaged for one minute. Aliquots of 2.5 ml were collected at one min intervals for five min, mixed with 2.5 ml double-strength pH 7.0 DE broth (tempered to 80°C) by vortex mixer, heat-shocked at 80° C for 10 min, cooled in ice-water, then tenfold serially diluted, plated, and enumerated as described previously.

6. Effectiveness of gaseous chlorine dioxide on *Alicyclobacillus* spp. spores.

Gaseous chlorine dioxide and *Alicyclobacillus* spp. spores on apple surfaces.

A 20 L polypropylene bucket was used as a model gas treatment chamber (Figure 5-1). A small electric fan (Hankscraft Motors, Inc., Reedsburg, WI) was installed onto the lid to facilitate circulation of gas in the chamber. Unwashed, unwaxed Fuji apples from Ronald B. Tukey Orchard, Washington State University (Pullman, WA) were used in this study and inoculated as described. Chlorine dioxide gas sachets were activated with 10

ml sterile water, added to the bucket with an inoculated apple, bucket sealed, and the fan started. Controls were added to the bucket and sealed without a sachet. Control and gas treated apples were aseptically removed from buckets at 30 min, 1 h, 2 h, and 3 h. Apples were placed in sterile stomacher bags containing 50 ml D/E broth and massaged vigorously by hand for one minute to remove spores from surface. One milliliter aliquots of the D/E broth was removed and mixed with 1ml D/E broth (pH 7.0) tempered at 80°C. The D/E broth mixture was heat shocked at 80°C for 10 min, chilled in a water bath, diluted with sterile deionized water, and spread plated (100 µl) onto K agar in duplicate. Where low spore survival was anticipated, 250 µl was spread-plated onto four plates. Plates were incubated at 43°C for 48 h.

Gaseous chlorine dioxide and the visual quality of apples.

Following hand massage for 1 min, treated and control apples were transferred to ultraviolet sterilized plastic zip lock bags (G.T. Bag Company, Novato, CA) and stored at 4°C for 7 days. Evaluation of apple visual quality was performed daily to assess the effect of gaseous chlorine dioxide on apple appearance during storage.

7. Statistical analysis.

Triplicate data from each treatment were collected and analyzed statistically using Minitab Student Version 9.0 (Minitab Inc., State College, PA). Type I error rate was designated at 5%. Analysis of variance was first performed to determine the occurence of significant differences ($P<0.05$). When significant differences between the treatments were detected by ANOVA, Tukey's multiple range test was performed to identify the the variable for significant differences ($P<0.05$).

RESULTS

Aqueous chlorine dioxide and planktonic *Alicyclobacillus* spp. spores.

The survival of planktonic *Alicyclobacillus* spores treated with aqueous chlorine dioxide is presented in Table 5-1. Spore reduction with 20 ppm $ClO_{2(aq)}$ was not significantly different from the control (P>0.05). Spores treated with 40 ppm $ClO_{2(aq)}$ for 5 min were reduced by more than four logs. Significantly smaller spore concentrations were observed when *Alicyclobacilus* spores were treated with 80 ppm and 120 ppm $ClO_{2(aq)}$ for 1 min and 30 sec, respectively. Aqueous chlorine dioxide concentrations of 80 ppm and 120 ppm reduced spore concentrations to undetectable levels (<0.7 log CFU/ml) after 5 min (Table 5-1). Within the $ClO_{2(aq)}$ treatment range of 40-120 ppm, significant decrease in the number of viable *Alicyclobacillus* spores with each increase in $ClO_{2(aq)}$ concentration was observed. Significant differences (P<0.05) in spore concentrations were detected among the different $ClO_{2(aq)}$ concentration treatments after 1 min exposure.

Aqueous chlorine dioxide and *Alicyclobacillus* spores on apple surfaces.

Based on favorable results of $ClO_{2(aq)}$ in reducing planktonic *Alicyclobacillus* spores, the effectiveness of $ClO_{2(aq)}$ was further tested on spore inoculated on apple surfaces. Table 5-2 presents the concentration of *Alicyclobacillus* spores (CFU/apple) recovered from apple surfaces following $ClO_{2(aq)}$ treatment. As 20 ppm $ClO_{2(aq)}$ was not effective in reducing planktonic spore concentrations, it was not tested against spores inoculated onto apples. Four apple cultivars (‘Red Delicious’, ‘Golden Delicious’, ‘Gala’, and ‘Fuji’) were tested for survival differences due to cultivars. No significant differences were observed among different apple cultivars (P>0.05). When treated with 40 ppm

$ClO_{2(aq)}$, a significant decrease in the concentration of viable *Alicyclobacillus* spores was observed with increasing treatment time; spore viability was reduced more than five logs CFU/apple after 4 min (Table 5-2). Spores were reduced to undetectable concentrations (< 2 log CFU/apple) after 1 min in 120 ppm $ClO_{2(aq)}$ for the four apple cultivars. Spore reductions on apples were greater than on planktonic spore suspensions treated with equivalent $ClO_{2(aq)}$ concentrations at equivalent time intervals.

Gaseous chlorine dioxide and *Alicyclobacillus* spores on apple surfaces.

Based on the results from aqueous chlorine dioxide, the difference between apple cultivars on the $ClO_{2(aq)}$ effectiveness on spores was negligible, thus the effect of $ClO_{2(g)}$ on *Alicyclobacillus* spp. spores was conducted using only Fuji apples. The peak concentration of $ClO_{2(g)}$ generated each hour for 3 h by each type of sachet is summarized in Table 5-3.

Treatment of inoculated Fuji apples with intermediateand rapid release sachets for 1 h achieved a reduction greater than 5 logs, reducing the spores to undetectable levels (Figure 5-2). A 2.7 log reduction in *Alicyclobacillus* spores was observed for slow release sachets after 1 h. Further exposure to slow release $ClO_{2(g)}$ for 2 and 3 h resulted in spore reductions of 3.7 logs and 4.5 logs.

Gaseous chlorine dioxide and the visual quality of apples.

Chlorine dioxide gas treated apples were stored at 4°C for 7 days for visual quality assessments. Although intermediate and rapid release sachets were effective against *Alicyclobacillus* spp. spores, the skin of the apples receiving the treatment developed small black spots within 3 days of storage at 4°C (Figure 5-3). Therefore, the concentration of $ClO_{2(g)}$ generated by these sachets was too great for apple treatment

from a visual quality standpoint. No significant differences in visual quality between control (untreated) and apples treated with slow release sachets were determined for the 7 d storage duration. Thus, treatment with slow release chlorine dioxide gas sachets did not affect the visual quality of apples over the 7 d period (Figure 5-3a).

DISCUSSION

Table 5-1 and 5-2 illustrate the effectiveness of $ClO_{2(aq)}$ in reducing the number of *Alicyclobacillus* spores in suspension and apple surfaces, respectively. Despite the significant (P<0.05) pH difference between 20 ppm and 40 ppm $ClO_{2(aq)}$ solutions, survival spore concentrations were not significantly different when treated for 0.5 min and 1 min. Aqueous ClO_2 of 40 ppm, 80 ppm, and 120 ppm were similar in pH values, but resulted in significantly different (P<0.05) *Alicyclobacillus* spore survival numbers when exposed for 1 min. In addition, *Alicyclobacillus* spores are reported to grow in a pH range of 2.5-6.0 (Yamazaki et al., 1996a), thus the reduction of *Alicyclobacillus* spore concentrations in this study can be attributed solely to the effect of various concentrations of ClO_2.

Orr and Beuchat (2000) also evaluated the effectiveness of disinfectants against planktonic *Alicyclobacillus* spp. spores. The effectiveness of NaOCl, $NaClO_2$, Na_3PO_4, H_2O_2, and Tsunami™ against planktonic *Alicyclobacillus* spores were examined, with H_2O_2 and NaOCl being the most effective in reducing *Alicyclobacillus* spores. When spores were treated at room temperature (23±2°C) for 10 min, 4% H_2O_2 and NaOCl concentrations exceeding 1,000 ppm reduced spores to less than the detection limit of 5 CFU/ml. Under equivalent treatment time and temperature, 2% H_2O_2 also significantly reduced planktonic *Alicyclobacillus* spore concentrations from 6.28 log CFU/ml to 0.75

log CFU/ml. Despite a significantly (P<0.05) larger number of spores inactivated with each increasing concentration of $NaClO_2$, spore concentrations were reduced less than 2 logs at the largest tested concentration of 1,200 ppm. Spores inactivated by Na_3PO_4 and Tsunami™ were substantially lower in concentration compared to the other disinfectants (Orr and Beuchat, 2000). Based on our results, exposure of *Alicyclobacillus* spp. spores to $ClO_{2(aq)}$ of 80 ppm for 5 min or 120 ppm for 1 min resulted in comparable spore reductions by 4% H_2O_2 and NaOCl exceeding 1,000 ppm (Orr and Beuchat, 2000). The effect of NaOCl, $NaClO_2$, and H_2O_2 in inactivating *Alicyclobacillus* spores on apple surfaces were further investigated (Orr and Beuchat, 2000). Treatment with 500 ppm NaOCl or 1,200 ppm $NaClO_2$ for 1 min significantly (P≤0.05) reduced the number of viable spores compared to the control water wash, but reductions were less than 1 log CFU/apple. Hydrogen peroxide (2%) was ineffective in reducing *Alicyclobacillus* spores on apple surfaces. Orr and Beuchat (2000) contributed the lower disinfectant efficacy to the inaccessibility of spores located in cracks, crevices or lenticels on apple skin and to the reduced concentration of the active form of the treatment chemicals, particularly chlorine and $NaClO_2$, through contact with the apple skin. On the contrary, the results from this study indicate $ClO_{2(aq)}$ was equally effective towards planktonic and apple-surface attached *Alicyclobacillus* spores.

Gaseous ClO_2 was also effective in reducing *Alicyclobacillus* spores on apple surfaces. After 1 h, intermediate and rapid release gas sachets produced $ClO_{2(g)}$ concentrations of 1.78 mg/L and 4.32 mg/L, respectively; both of which resulted in 5 log reductions of *Alicyclobacillus* spores. Smaller concentrations of $ClO_{2(g)}$ were produced with slow release $ClO_{2(g)}$ sachets and reduction of *Alicyclobacillus* spores was not as

rapid as with the intermediate and rapid release gas sachets. Nonetheless, slow release $ClO_{2(g)}$ sachets were still effective in reducing *Alicyclobacillus* spores, and a 4.5 log reduction per apple was observed in systems with peak $ClO_{2(g)}$ concentrations of 0.6 mg/L. While the concentrations of $ClO_{2(g)}$ generated by intermediate and rapid release sachets were effective at reducing *Alicyclobacillus* spores on apple surfaces, visual damage to the treated apples in the form of black spot formation was apparent after 3 d storage. From a fresh produce point of view, black spots are undesirable to consumers, however, they may not be of critical importance in juice production, particularly if weighed against the potential impact of *Alicyclobacillus* spoilage. According to USDA standards for apples used for processing, the most stringent grade of apples used for processing (U.S. No. 1) adheres to the following standard: "not overripe, free from decay, worm holes, freezing injury and internal breakdown, and free from any other defect, or combination of defects, the removal of which in the usual commercial preparation for use will cause a loss of more than 5 percent, by weight, of the apple" (AMS-USDA, 2008). With regard to the USDA standards, apples treated with $ClO_{2(g)}$ at intermediate or rapid release concentrations can still be used for juicing, and may be beneficial for reducing *Alicyclobacillus* related spoilage incidences. In contrast, smaller concentrations of $ClO_{2(g)}$ generated by slow release sachets reduced *Alicyclobacillus* spores without visible damage to apple skin, which may be a more appropriate treatment for apples that endure a longer holding time prior to juicing.

The efficacy of $ClO_{2(g)}$ against *Alicyclobacillus* spores are in contrast to previous studies conducted on *Bacillus thuringiensis* (Han et al., 2003). When held in a system with a peak $ClO_{2(g)}$ concentration of 5 mg/L over a period of 12 h, reductions of *B*.

thuringiensis spores on paper and wood were 2.5 log and 3.6 log, respectively. One major factor that may contribute to the differences in spore reduction may be the sustainability of $ClO_{2(g)}$ for the duration of the test. As presented in Table 5-3, all three $ClO_{2(g)}$ sachets were capable of generating and maintaining $ClO_{2(g)}$ levels for the duration of the 3 h treatment time. For the *B*. *thuringiensis* study, the peak $ClO_{2(g)}$ concentration of 5 mg/L was reached immediately after activation, and fell to 0.8 mg/L after 6 h. No $ClO_{2(g)}$ was detected after 12 h (Han et al., 2003). Continuous exposure to $ClO_{2(g)}$ may result in more spore damage than sudden exposure to massive $ClO_{2(g)}$ concentrations that subsequently taper off. Another reason for the differences in reduction may be attributed to adherence of *Alicyclobacillus* and *B*. *thuringiensis* spores. The more porous surfaces of wood and paper may provide additional protection to *B*. *thuringiensis* while *Alicyclobacillus* spores may be more exposed to $ClO_{2(g)}$ on the smoother apple surface.

Previous studies investigating the lethal activity of ClO_2 against other bacterial spores are summarized in Table 5-4. In addition to the effectiveness of ClO_2 against bacterial spores, several researchers reported that spores treated with ClO_2 exhibit decreased tolerance toward heat (Beuchat et al., 2004; Ryu and Beuchat, 2005; Kreske et al., 2006a). The amount of injured spores caused by sublethal heat treatment following a 5 min exposure to $ClO_{2(aq)}$ was investigated by Kreske et al. (2006) by calculating the difference between spore recovery without heat treatment and spore recovery after heat treatment of 80°C for 11 min. At $ClO_{2(aq)}$ of 50 µg/ml, injured spore concentrations of >2.53 log CFU/ml and 2.11 log CFU/ml was observed for *B*. *cereus* and *B*. *thuringiensis*, respectively. When the $ClO_{2(aq)}$ concentration was increased to 75 µg/ml, *B*. *cereus* injured spores were >0.37 log CFU/ml, and *B*. *thuringiensis* injured spores were >3.46

log CFU/ml. The production of injured spores by sublethal heat treatment following $ClO_{2(aq)}$ exposure was also observed for *B. cereus* spores embedded within biofilms (Ryu and Beuchat, 2005). A major mechanism for the observed sensitivity to subsequent sublethal heat treatment is likely oxidative damage to inner membrane of spores (Young and Setlow, 2003) The mechanism of *Bacillus* spore sensitization by oxidizing agents to subsequent sublethal heat was investigated by Cortezzo et al. (2004). Normally, the inner membrane of a dormant spore is relatively compressed and most lipids in this membrane are immobile, making it difficult for dodecylamine to initiate spore germination by the formation of channels or pores through which DPA can exit (Setlow et al., 2003; Cortezzo et al., 2004; Cowan et al., 2004). The observation that all spores treated with oxidative agents including $ClO_{2(aq)}$ were sensitized to subsequent germination with dodecylamine is in agreement with other studies indicating that oxidatively damaged membranes become leaky (Hurst, 1977; Gutteridge and Halliwell, 1990; Imlay, 2002; Cortezzo et al., 2004). The large increase in permeation rate of methylamine into the spore core of spores treated with oxidizing agents (Cortezzo et al., 2004), and the great amount of dipicolinic acid (DPA) released by spores incubated at 80-85°C following exposure to oxidizing agents (Young and Setlow, 2003) also support the proposition that inner membranes are ruptured by oxidizing agents, leading to a significant alteration in the ability of the damaged spore to maintain its permeability barriers upon exposure to high temperature (Cortezzo et al., 2004). The increased sensitivity of oxidatively damaged spores to heat is most likely due to the absence of DPA in spores (Wise et al., 1967; Balassa et al. 1979; Paidhungat et al., 2000).

Chlorine dioxide has several advantages over chlorine, the primary sanitizer for fruit washing (Beuchat et al., 2004). Chlorine dioxide can break down phenolic compounds and remove phenolic tastes and odors in water, does not form trihalomethanes, is capable of eliminating cyanides, sulfides, and mercaptans from wastewater, and is not reactive with ammonia (Cords and Dychadala, 1993; Beuchat, 1998). The oxidizing power of ClO_2 is approximately 2.5 times of chlorine (Bernarde et al., 1967), and antimicrobial effectiveness is less affected by pH. However, like chlorine, the oxidizing power of ClO_2 diminishes on contact with organic matter resulting in reduced lethality (Zhang and Farber, 1996; Lindsay et al., 2002; Stampi et al., 2002; Rye and Beuchat, 2005; Kreske et al., 2006b).

As *Alicyclobacillus* spores can survive pasteurization, eliminating spores prior to milling is the most critical step in securing the quality of apple juice against *Alicyclobacillus*-related storage. The efficacy of aqueous and gaseous ClO_2 against *Alicyclobacillus* spores are demonstrated in this study and may be applicable to the juice industry. The standard procedures for apple handling and storage prior to milling are presented in Figure 5-4. $ClO_{2(g)}$ can be applied to harvested fruits during closed transportation or storage prior to entering the juicing facility. Aqueous ClO_2 application in transport/flume water is not recommended as the high organic concentration in transport/flume water would reduce $ClO_{2(aq)}$ efficacy. Alternatively, a $ClO_{2(aq)}$ soak following the transport/flume water and prior to the final water rinse is recommended to maximize the effectiveness of $ClO_{2(aq)}$ treatment. As previously mentioned, exposure to ClO_2 results in oxidative damage to spore inner membranes and sensitizes spores to sublethal heat treatments. Spores surviving ClO_2 treatment may be more susceptible to

the normally sublethal pasteurization treatment, thus providing a possible measure for controlling *Alicyclobacillus* spoilage.

In summary, ClO_2 in both aqueous and gaseous form can be used to reduce *Alicyclobacillus* spores on apple surfaces. The effectiveness of ClO_2 against fruit postharvest decay fungi and filamentous fungi occurring on fruit packinghouse surfaces (Roberts and Reymond, 1994) makes it a dual function food quality/safety alternative to the more conventional chlorine wash.

ACKNOWLEDGEMENTS

The author wishs to thank ICA TriNova, LLC (Forest Park, GA) for supplying the chlorine dioxide gas sachets, and especially Joel Tenney and Bill Ernst for their technical assistance and support.

Table 5-1. Aqueous chlorine dioxide and the survival of planktonic *Alicyclobacillus* spores.

Concentration of free ClO_2 (ppm)	pH	Treatment time (min)			
		0	0.5	1	5
10	-------	6.10±0.11[1]A[2]a	6.08±0.08Aa	6.07±0.23Aa	6.05±0.14Aa
20	4.62	6.06±0.30Aa[3]	5.97±0.43Aa	5.94±0.21Aa	4.37±1.41Aa
40	2.75	6.12±0.12Aa	5.95±0.22Aa	5.38±0.84Aa	1.62±0.94Bb
80	2.62	6.02±0.23Aa	5.08±0.95Aab	3.25±1.68Bb	<0.70[4]Bc
120	2.58	6.03±0.48Aa	3.92±0.41Bb	1.26±0.07Cc	<0.70Bd

[1]Data represent means±standard deviations of three measurements.

[2]Means with the same upper case letter within a row (following the values) are not significantly different (P>0.05).

[3] Means with the same lower case letter within a column (following the values) are not significantly different (P>0.05).

[4]Detection limit was 0.7 log CFU/ml.

Table 5-2. Recovery of *Alicyclobacillus* spp. spores (log CFU/apple) from various apple surfaces treated with aqueous chlorine dioxide.

Free $ClO_{2(aq)}$ concentration (ppm)	Treatment time (min)	Apple cultivar			
		Red Delicious	Golden Delicious	Gala	Fuji
0	1	6.76±0.05[1]A[2]a[3]	6.81±0.09Aa	6.78±0.13Aa	6.81±0.07Aa
40	1	5.00±0.71Ab[3]	4.89±0.75Ab	5.58±0.75Ab	5.57±0.50Ab
	2	3.64±1.64Ac	3.37±0.73Ac	3.74±0.16Ac	3.46±0.22Ac
	3	2.40±0.69Acd	<2.00[4]Ac	2.40±0.35Ad	2.55±0.96Acd
	4	<2.00Ad	<2.00Ac	<2.00Ad	<2.00Ad
	5	<2.00d	<2.00c	<2.00d	<2.00d
120	1	<2.00d	<2.00c	<2.00d	<2.00d
	2	<2.00d	<2.00c	<2.00d	<2.00d
	3	<2.00d	<2.00c	<2.00d	<2.00d
	4	<2.00d	<2.00c	<2.00d	<2.00d

[1] Data represent means±standard deviations of three measurements.
[2] Means with the same upper case letter within a row are not significantly different (P>0.05).
[3] Means with the same lower case letter within a column are not significantly different (P>0.05).
[4]Detection limit was 2.0 logCFU/apple.

Table 5-3. Peak concentrations of chorine dioxide gas generated by slow, intermediate, and rapid release sachets after each reaction time.

Reaction time (h)	Peak $ClO_{2(g)}$ Concentration (mg/L)		
	Slow	Intermediate	Rapid
0	0.00	0.00	0.00
1	0.39	1.78	4.32
2	0.50	2.42	5.95
3	0.60	2.69	6.55

Table 5-4. Efficacy of chlorine dioxide on various sporeformers on different types of surfaces.

Suspension/ Adherence Surface	ClO_2 Treatment			Subsequent Treatment	Reduction	Reference
	Phase	Concentration	Time			
B. cereus						
planktonic	aq	50 µg/ml	15-38 min	None	3 log	Foegeding et al., 1986
	aq	50 µg/ml	5 min	None	1.19-1.33 log CFU/ml	Kreske et al., 2006
	aq	50 µg/ml	5 min	80°C, 11 min	3.09-4.74 log CFU/ml	Kreske et al., 2006
	aq	75 µg/ml	5 min	None	3.65-4.97 log CFU/ml	Kreske et al., 2006
	aq	75 µg/ml	5 min	80°C, 11 min	>4.49 to >5.49 log CFU/ml	Kreske et al., 2006
	aq	85 µg/ml	5 min	None	5.7 log CFU/ml	Beuchat et al., 2004
coupon	aq	50 µg/ml	5 min	None	1.85 log CFU/coupon	Kreske et al., 2006
	aq	100 µg/ml	5 min	None	2.59 log CFU/coupon	Kreske et al., 2006
	aq	100 µg/ml	5 min	None	3.74 log CFU/coupon	Kreske et al., 2006b
	aq	200 µg/ml	5 min	None	>2.55 log CFU/coupon	Kreske et al., 2006
	aq	200 µg/ml	5 min	None	4.48 log CFU/coupon	Kreske et al., 2006b
biofilm	aq	50 µg/ml	5 min	None	1.8 log CFU/coupon	Ryu and Beuchat, 2005
	aq	50 µg/ml	5 min	80°C, 10 min	2.3 log CFU/coupon	Ryu and Beuchat, 2005
	aq	100 µg/ml	5 min	None	2.7 log CFU/coupon	Ryu and Beuchat, 2005
	aq	100 µg/ml	5 min	None	3.37 log CFU/coupon	Kreske et al., 2006b
	aq	100 µg/ml	5 min	80°C, 10 min	4.2 log CFU/coupon	Ryu and Beuchat, 2005
	aq	200 µg/ml	5 min	None	3.85 log CFU/coupon	Kreske et al., 2006b
apple surface	aq	50 µg/ml	5 min	None	3.2 log CFU/apple	Kreske et al., 2006
	aq	100 µg/ml	5 min	None	3.79 log CFU/apple	Kreske et al., 2006
B. subtilis						
planktonic	aq	10-14 µg/ml	9 min	None	3 log	Young and Setlow, 2003

Table 5-4. Efficacy of chlorine dioxide on various sporeformers on different types of surfaces (cont'd).

Suspension/ Adherence Surface	ClO_2 Treatment			Subsequent Treatment	Reduction	Reference
	Phase	Concentration	Time			
B. thuringiensis						
planktonic	aq	50 µg/ml	5 min	None	0.84 log CFU/ml	Kreske et al., 2006
	aq	50 µg/ml	5 min	80, 11	2.66 log CFU/ml	Kreske et al., 2006
	aq	75 µg/ml	5 min	None	2.73 log CFU/ml	Kreske et al., 2006
	aq	75 µg/ml	5 min	80°C, 11 min	>5.90 log CFU/ml	Kreske et al., 2006
coupon	aq	50 µg/ml	5 min	None	1.6 log CFU/coupon	Kreske et al., 2006
	aq	100 µg/ml	5 min	None	3.65 log CFU/coupon	Kreske et al., 2006
	aq	200 µg/ml	5 min	None	>2.71 log CFU/coupon	Kreske et al., 2006
apple surface	aq	50 µg/ml	5 min	None	3.83 log CFU/apple	Kreske et al., 2006
	aq	100 µg/ml	5 min	None	4.25 log CFU/apple	Kreske et al., 2006
paper	gas	5 mg/l	12 h	None	2.5 log CFU/ml	Han et al., 2003
	gas	10 mg/l	12 h	None	3.6 log CFU/ml	Han et al., 2003
	gas	15 mg/l	12 h	None	>5 log CFU/ml	Han et al., 2003
wood	gas	5 mg/l	12 h	None	3.6 log CFU/ml	Han et al., 2003
	gas	10 mg/l	12 h	None	5.0 log CFU/ml	Han et al., 2003
	gas	15 mg/l	12 h	None	>5 log CFU/ml	Han et al., 2003
epoxy	gas	5 mg/l	12 h	None	4.0 log CFU/ml	Han et al., 2003
	gas	10 mg/l	12 h	None	5.7 log CFU/ml	Han et al., 2003
	gas	15 mg/l	12 h	None	>5 log CFU/ml	Han et al., 2003
plastic	gas	5 mg/l	12 h	None	4.9 log CFU/ml	Han et al., 2003
	gas	10 mg/l	12 h	None	5.8 log CFU/ml	Han et al., 2003
	gas	15 mg/l	12 h	None	>5 log CFU/ml	Han et al., 2003

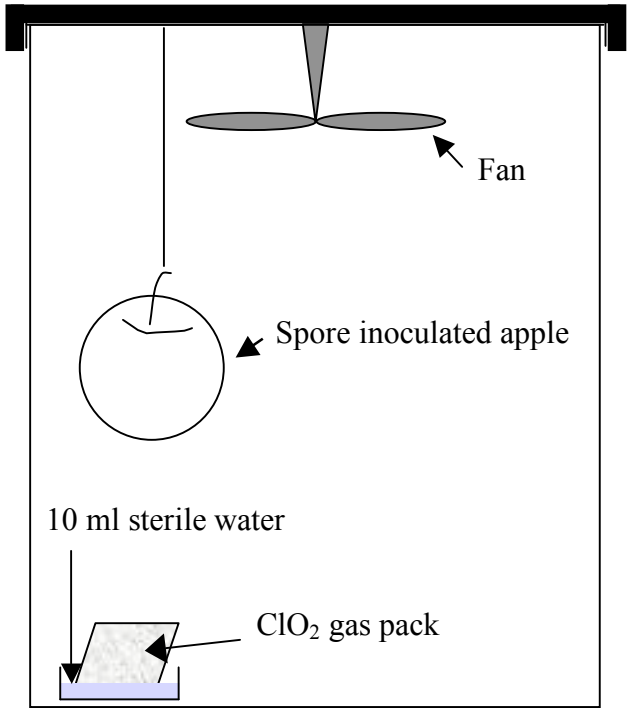


Figure 5-1. Diagram of experimental gas cabinet.

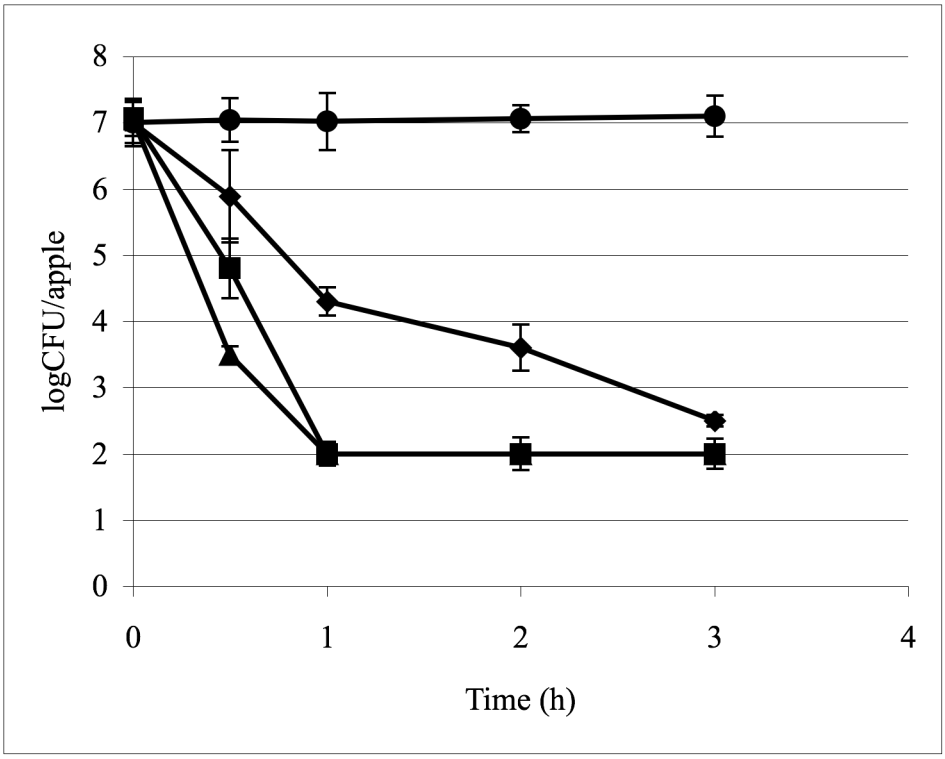


Figure 5-2. Survival curves for *Alicyclobacillus* spp. spores on Fugi apple surfaces exposed to slow release (—◆—), intermediate release (—■—), and rapid release (—▲—) chlorine dioxide gas at 25°C. Control treatments (—●—) were sealed in a 20 L polypropylene bucket without any $ClO_{2(g)}$. The error bars indicate a 5% error rate.

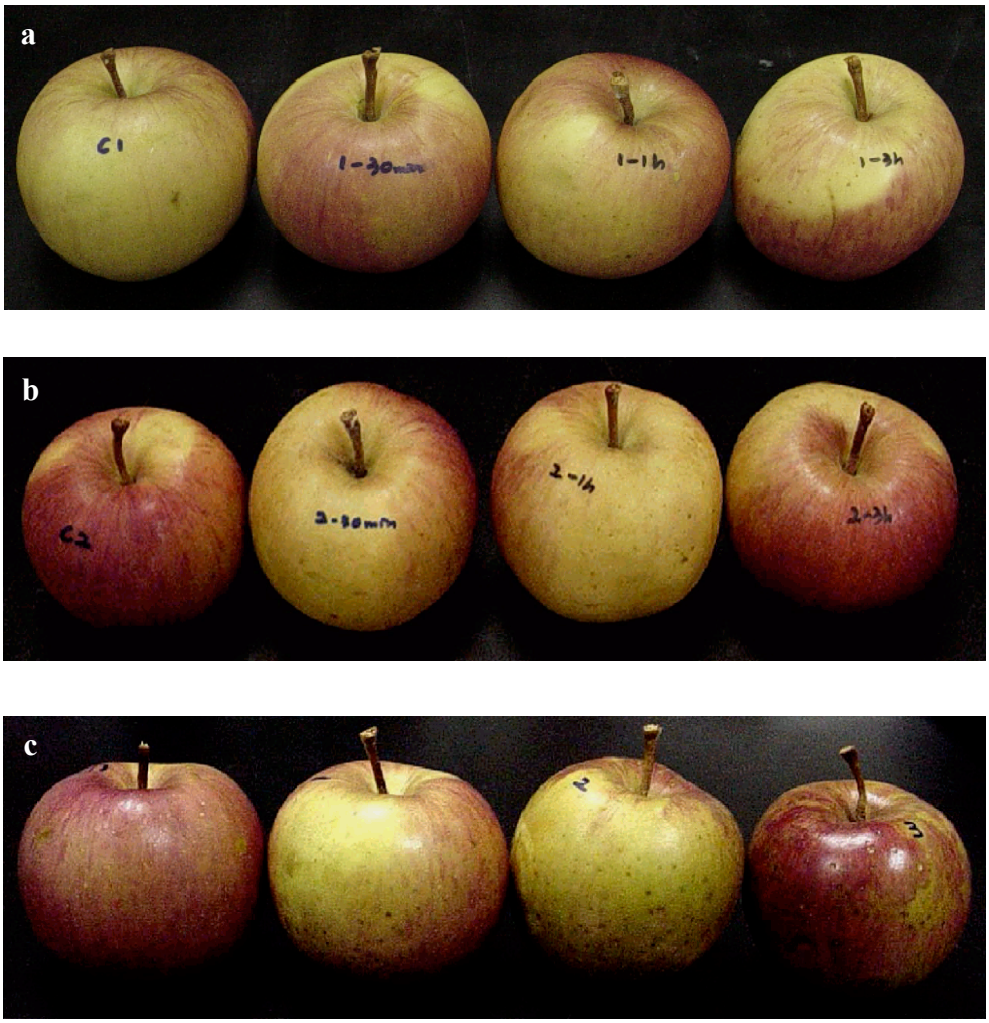


Figure 5-3. Digital photographs of apples stored for (a) 7 days or (b and c) 3 days at 4 °C after treatment with chlorine dioxide gas. Apples treated with (a) ClO_2 gas using slow release sachets; (b) intermediate release sachets; and (c) rapid release sachets before storage. All pictures from left to right indicate untreated apple (control), and apples treated for 30 min, 1 h, and 3 h.

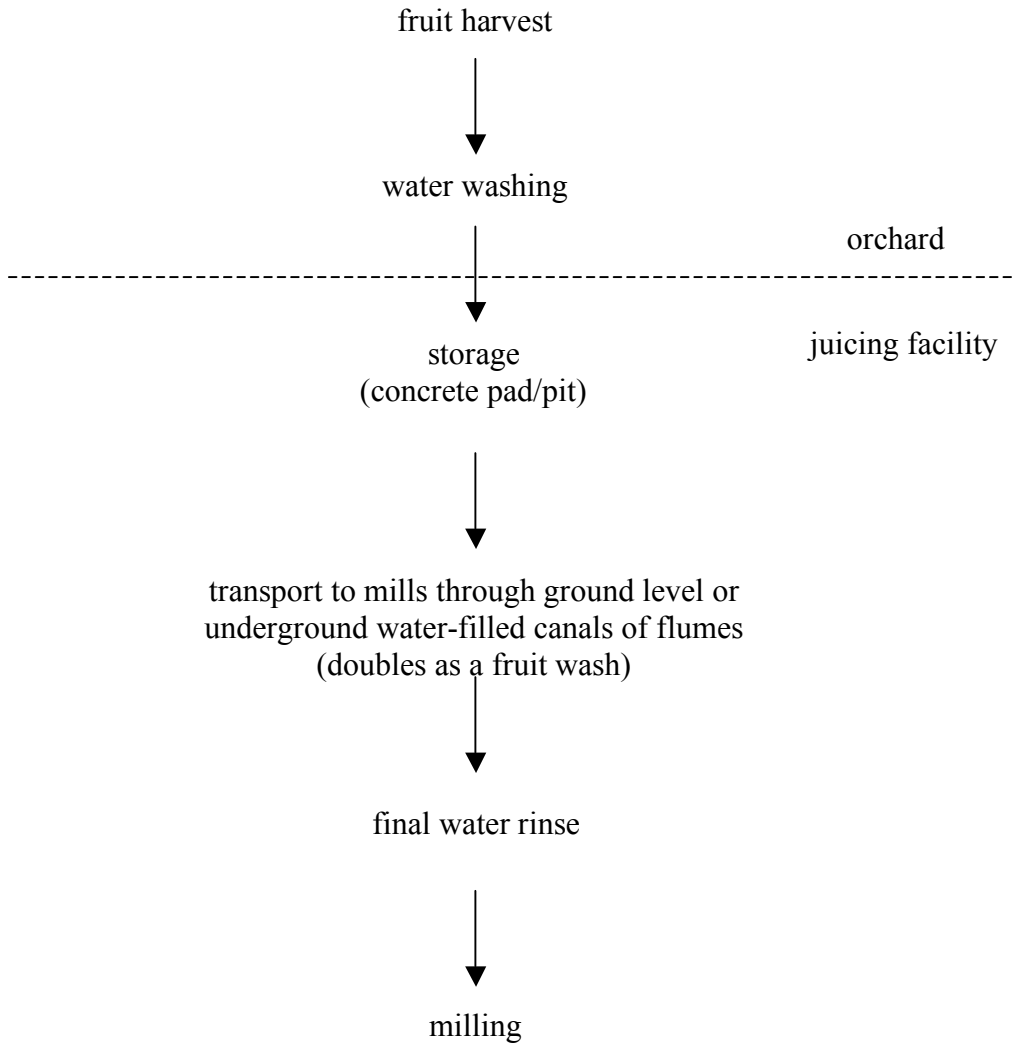


Figure 5-4. Current standard procedures for apple storage and handling prior to milling (*Source*: Downes, 1995).

CHAPTER SIX

CONCLUSIONS AND FUTURE RESEARCH

Based on this research, differences exist between guaiacol producing and non-guaiacol producing *Alicyclobacillus* spp. Guaiacol producing *Alicyclobacillus* spp. have a wider range of optimum growth and utilize less carbohydrates compared to non-guaiacol producing *Alicyclobacillus* spp. The most prominent difference between guaiacol producing and non-guaiacol producing *Alicyclobacillus* is the ability to produce guaiacol.

Guaiacol production by guaiacol producing *Alicyclobacillus* spp, occurs during the log phase of cell outgrowth and is affected by incubation temperature, medium pH, and incubation oxygen concentration. Guaiacol formation was detectable within 12 h under standard incubation conditions of 43°C and pH 4.0 and further accelerated under microaerophilic incubation.

The developed SK2 agar provides a convenient single procedure that selectively detects for guaiacol producing *Alicyclobacillus* spp. without the need of additional equipment or specialized personnel training. SK2 agar has comparable recovery rates to PDA and K Agar, the most commonly used *Alicyclobacillus* recovery media used in Japan and the United States. SK2 combines the microbial isolation and guaiacol identification procedures and reduces the typical sampling-to-results time of 96 h to 48 h. SK2 is the first selective/differential media reported for guaiacol producing *Alicyclobacillus* spp.

Both aqueous and gaseous chlorine dioxide were effective against *Alicyclobacillus* spores. Aqueous chlorine dioxide can be applied as a fruit dip following transport water and prior to the final water rinse. Gaseous chlorine dioxide can be applied

during apple transportation or hold time prior to juicing. Chlorine dioxide may be used as an effective alternative to the commonly used chlorine to minimize the entrance of *Alicyclobacillus* spores into pasteurized juice beverages.

Spoilage related *Alicyclobacillus* research is focused on practical applications such as the control and detection of *Alicyclobacillus*. To date, no investigations are reported on the phylogenetic relationship between guaiacol producing and non-guaiacol producing *Alicyclobacillus*, or the significance of guaiacol production to guaiacol producing *Alicyclobacillus*. Metabolic pathways on the formation of guaiacol by guaiacol producing *Alicyclobacillus* still remain unknown. Fundamental research in *Alicyclobacillus* metabolism can provide insights into the mechanism of guaiacol production and may serve as critical information for reducing spoilage caused by guaiacol producing *Alicyclobacillus*.

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