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Development of novel agar media for isolating guaiacol producing *Alicyclobacillus* spp.

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ABSTRACT

The purpose of this study is to develop a selective and differential medium (SK2 agar) for isolating guaiacol producing *Alicyclobacillus*. Forty-one selected dyes and vanillic acid were incorporated in SK agar for screening selective and differential agents. Two guaiacol producing (1016, 1101) and two non-guaiacol producing (19220, C-GD 1-1) *Alicyclobacillus* isolates were streaked onto media and color differentiation of the isolates was assessed. Among 41 tested dyes, Chrome Azurol S (CAS) allowed color differentiation of the two types of *Alicyclobacillus*. Colonies of guaiacol producing *Alicyclobacillus* isolates appeared as dark purple to royal blue color with yellow background, whereas non-guaiacol producing *Alicyclobacillus* isolates produced cream colored colonies with yellow background. Vanillic acid not only served as a precursor for guaiacol formation but also inhibited non-guaiacol producing *Alicyclobacillus*. Non-guaiacol producing isolates did not grow on SK agar containing more than 70 ppm vanillic acid, whereas the recovery of guaiacol producing isolates was unaffected. When compared with other *Alicyclobacillus* isolation media, not only was SK2 agar capable of selectively recovering guaiacol-producing *Alicyclobacillus*, the degree of growth was also approximately equal if not better than orange serum agar, potato dextrose agar, and K agar. The development of SK2 agar provides the fruit juice industry with an inexpensive, simple to use alternative for the detection of guaiacol producing *Alicyclobacillus*.

1. Introduction

Alicyclobacillus is a thermoacidophilic spore forming microorganism (growth range of pH 2.5–6.0 and 25–60 °C) (Murray et al., 2007). Since the isolation of *Alicyclobacillus acidoterrestris* from pasteurized apple juice in 1982, *Alicyclobacillus* has been isolated from many juices including apple, cherry, cranberry, grapefruit, mango, orange, pear, tomato, and white grape (Cerny et al., 1984; Durak et al., 2010; Eiroa et al., 1999; Gouws et al., 2005; Groenewald et al., 2009; Splittstoesser et al., 1994, 1998; Steyn et al., 2011; Wisse and Parish, 1998). The presence of *Alicyclobacillus* in fruit juice products poses a serious problem for the juice industry, because *Alicyclobacillus* can survive under normal pasteurization procedures and results in spoilage without apparent changes to the packaging or the juice clarity (Danyluk et al., 2010; Lee et al., 2002; McIntyre et al., 1995; Spinelli et al., 2010; Walls and Chuyate, 2000).

The spoilage by *Alicyclobacillus* was characterized as having a 'medicinal', 'smoky', and 'antiseptic' off-flavor (Concina et al., 2010; Pettipher et al., 1997). The chemical compounds responsible for the distinctive off-odors are identified as guaiacol (Pettipher et al., 1997; Splittstoesser et al., 1998; Yamazaki et al., 1996), 2,6-dibromophenol, and 2,6-dichlorphenol (Baumgart et al., 1997; Borlinghaus and Engel, 1997). Though initial spoilage related interest was focused on *A. acidoterrestris*, subsequent studies reported other species of the genus that could also produce guaiacol (Goto et al., 2002, 2003; Matsubara et al., 2002). Also 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. (Chang and Kang, 2005).

Several studies have been conducted to develop various isolation methods and recovery media targeted towards *Alicyclobacillus* (Lee et al., 2002; Murray et al., 2007; Pettipher and Osmundson, 2000; Witthuhn et al., 2007). However, no selective and differential agar medium combining the recovery of *Alicyclobacillus* with instantaneous differentiation of the guaiacol producing ability of recovered colonies is available. Currently, several techniques using UV–vis spectrophotometer based on peroxidase method, HPLC, and GC–MS are available to either detect guaiacol in juice or evaluate the guaiacol

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producing ability of isolated *Alicyclobacillus* (Bahçeci and Acar, 2007; Orr et al., 2000; Pettipher et al., 1997). However, unless the goal is to solely detect the presence of guaiacol, the mentioned methods require a colony isolation procedure.

Chang and Kang (2005) previously developed *Alicyclobacillus* isolation medium (SK agar) with high recovery rates of *Alicyclobacillus* spp. The objective of this study is to develop a selective and differential agar medium (SK2 agar) with high recovery rates and instant visualization of guaiacol-producing *Alicyclobacillus* spp. SK2 agar is also compared with current widely used *A. acidoterrestris* isolation media to determine the effectiveness in isolating *Alicyclobacillus* spp.

2. Materials and methods

2.1. Bacterial strains

The bacterial cells used in this study were obtained from the School of Food Science bacterial culture collection, Washington State University (Pullman, WA, USA). *A. acidoterrestris* WAC, 1016, 1101 (ATCC 49025), and *Alicyclobacillus* spp. 97, 113 were guaiacol producing isolates. *Alicyclobacillus* spp. 849, 6348, 19220, Gala 9-2, and C-GD 1-1 were non-guaiacol producing isolates. *A. acidoterrestris* WAC was isolated from apple cranberry juice, while other strains were isolated from commercial apple juice. These isolates have been confirmed as *Alicyclobacillus* spp. through biochemical tests or the sequencing of the 16S rDNA, and some have been used in previous studies (Chang and Kang, 2005; Lin et al., 2007).

2.2. Verification of guaiacol production

The basal broth for KV broth was prepared by dissolving yeast extract 2.5 g (Difco, Sparks, MD, USA), peptone 5.0 g (Difco), glucose 1.0 g (Difco), Tween 80 1.0 g (Sigma-Aldrich, St Louis, MO, USA) in 990 ml deionized water and sterilizing at 121 °C for 15 min. A 1% (w/v) vanillic acid (Sigma-Aldrich) solution was prepared and syringe sterilized using 0.22 μm Millex®GP filter units (Millipore, Carrigtwohill, Co. Cork, Ireland) with sterile Monoject 3 ml syringes (Tyco, Mansfield, MA, USA). 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. All *Alicyclobacillus* spp. isolates were grown in 9 ml of KV broth at 43 °C for 48 h.

The identification of 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, USA). Guaiacol (Sigma-Aldrich) was used as standards. One milliliter aliquots of the 48 h old cell culture were removed and filter sterilized into amber HPLC 1.5 ml vials (Agilent Technologies) with 0.22 μm Millex®GP filter units and sterile Monoject 3 ml syringes. The filtrates were injected into the HPLC at a volume of 50 μl and analyzed for guaiacol. 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, USA). The column was operated at 25 °C with a mobile phase of acetonitrile/water/formic acid (19:80:1) at a flow rate of 0.5 ml/min. The ultraviolet wavelength used for the identification of guaiacol was 275 nm. The peaks of each isolate were compared with the standard retention time of guaiacol standards. Isolates in which a peak correlated to the standard retention time of guaiacol were designated as guaiacol producing.

2.3. Dye screening for differentiation of guaiacol producing from non-guaiacol producing Alicyclobacillus spp.

SK agar described by Chang and Kang (2005) was used as the basal medium for subsequent study. Peptone 5.0 g, yeast extract 2.5 g, glucose 1.0 g, agar (Difco) 15 g, and Tween 80 1.0 ml were dissolved in 1 l deionized water. All tested dyes listed in Table 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 medium was tempered to 48 °C. Filter sterilized 10% (w/v) $CaCl_2$ (Fisher Scientific, Fair Lawn, NJ, USA) is added to achieve Ca^{2+} concentration of 0.5 g/l. Filter sterilized 10% malic acid (Sigma-Aldrich) is used to adjust the medium pH to pH 4.0. The medium was poured into 9-cm-diameter petri dishes.

K broth was prepared as described for K agar (Walls and Chuyate, 2000) without agar. The ingredients are as follows; yeast extract 2.5 g, peptone 5.0 g, glucose 1.0 g, Tween 80 1.0 ml, and 1 l deionized water. After sterilization at 121 °C for 15 min, the medium was tempered in 48 °C. Ten percent (w/v) malic acid was prepared and filter sterilized and used to adjust the media to pH 4.0. All *Alicyclobacillus* spp. isolates were grown in K broth at 43 °C for 48 h, streaked onto each dye-containing agar with a sterile 10 μl loop (Copan Diagnostics Inc., Murrieta, CA, USA). After incubation at 43 °C for 48 h, colony morphology was compared and recorded. The experiment was repeated twice with each tested strain. The dye with the most distinctive differentiation was selected for further experimentation and optimization.

Table 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
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., New York, NY
Evan Blue	Eastman Kodak Company, Rochester, NY
Fast Green	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

2.4. Evaluation of vanillic acid and oxygen as additional selective agents

SK agar was prepared with the previously determined dye (tentatively named SKD for clarity purposes), divided into two parts: one containing 100 ppm vanillic acid (Sigma-Aldrich) (SKDV) and the other without (SKD). *Alicyclobacillus* spp. isolates (1016, 1101, 19220, C-GD 1-1) were grown in K broth at 43 °C for 48 h, streaked onto duplicate plates of SKD and SKDV agar with a sterile 10 μl loop and allowed to dry. One set of petri dishes was overlaid with 7 ml of SKD and placed in a laminar flow hood (22 ± 2 °C) for 1 h to allow solidification prior to incubation at 43 °C for 48 h. When differences in the growth or color of colonies were detected, the experiment was repeated twice 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.

2.5. The effect of Chrome Azurol S and vanillic acid on the recovery of *Alicyclobacillus spp.*

Chrome Azurol S (CAS) and vanillic acid were supplemented to basal SK agar at concentrations of 1, 5, 10, and 100 ppm (w/v), and 1, 10, and 100 ppm (w/v), respectively (pH 4.0). *Alicyclobacillus* spp. isolates (1016, 1101, 19220, C-GD 1-1) grown in K broth at 43 °C for 48 h 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 and vanillic acid. Unsupplemented basal SK agar (pH 4.0) was used as a control. After incubation at 43 °C for 48 h, colonies on each medium were enumerated and compared to a control. The experiments were repeated three times.

2.6. Determination of minimum inhibitory concentration of 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 (pH 4.0). *Alicyclobacillus* spp. isolates (1016, 1101, 19220, C-GD 1-1) grown in K broth at 43 °C for 48 h were serially diluted with 9 ml sterile deionized water to 10^2–10^3 CFU/ml. The diluted culture (100 μl) was spread plated onto the basal SK agar with different vanillic acid concentrations and plates were incubated at 43 °C for 48 h. After incubation, colonies on each medium were enumerated. The experiments were repeated three times.

2.7. Formulation of SK2 medium

The ingredients of SK2 medium are as follows: 5.0 g peptone, 2.5 g yeast extract, 1.0 g glucose, 1.0 ml Tween 80, 0.5 g $CaCl_2$, 0.01% CAS, and 70 ppm vanillic acid per liter. After sterilization, filter sterilized 10% malic acid was used to adjust the medium pH to pH 4.0, and the medium was poured into petri dishes.

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

Potato dextrose agar (PDA; Difco) and orange serum agar (OSA; Difco) were prepared according to the manufacturer's instructions. Tartaric acid (10%) was used to adjust OSA and PDA to pH 3.7. 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 ml Tween 80, 15 g agar and 1 l deionized water. Malic acid (10%) was used to adjust the media to pH 3.7. SK agar and SK2 medium were prepared as previously described. All isolates (97, 113, 849, 1016, 1101, 6348, 19220, Gala 9-2, C-GD 1-1, and WAC) were inoculated into K broth and incubated at 43 °C for 48 h. 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. Colonies were enumerated after 48 h of incubation at 43 °C. The experiments were repeated three times.

3. Results

3.1. Dye screening for differentiation of guaiacol producing from non-guaiacol producing Alicyclobacillus spp.

Among the 41 dyes listed in Table 1, 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 Fig. 1. Guaiacol producing *Alicyclobacillus* isolates (1101 and 1016) produced dark purple to royal blue colored colonies with yellow background, whereas non-guaiacol producing *Alicyclobacillus* isolates (19220 and C-GD 1-1) appeared as cream colored colonies with yellow background.

3.2. Evaluation of vanillic acid and oxygen as additional selective agents

Results for the effect of vanillic acid and exposure to oxygen on differentiating guaiacol and non-guaiacol producing *Alicyclobacillus* spp. isolates on SKD are summarized in Table 2. Addition of 100 ppm vanillic acid inhibited the recovery of non-guaiacol producing *Alicyclobacillus* (19220, C-GD 1-1). The inhibitory effect of vanillic acid was observed under both aerobic and microaerobic 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.

3.3. The effect of CAS and vanillic acid on the recovery of Alicyclobacillus spp.

Table 3 shows the effect of CAS and vanillic acid on the recovery of *Alicyclobacillus* spp. CAS did not exhibit any effect on the recovery of the tested *Alicyclobacillus* spp. isolates at the tested concentration range. Within each isolate, the recovery on SK agar supplemented with differing concentrations of CAS was not different from basal SK agar. The optimum concentration for both color intensity and color contrast for the differentiation of guaiacol and non-guaiacol producing *Alicyclobacillus* was 0.01%. Recovery of the test isolates at vanillic acid concentrations of 1 ppm and 10 ppm was 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.

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

The minimum vanillic acid concentration at which non-guaiacol producing *Alicyclobacillus* spp. were inhibited without affecting the recovery of guaiacol producing isolates was determined (Table 4). 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) was unaffected. 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. Different observations were made with isolate C-GD 1-1. 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

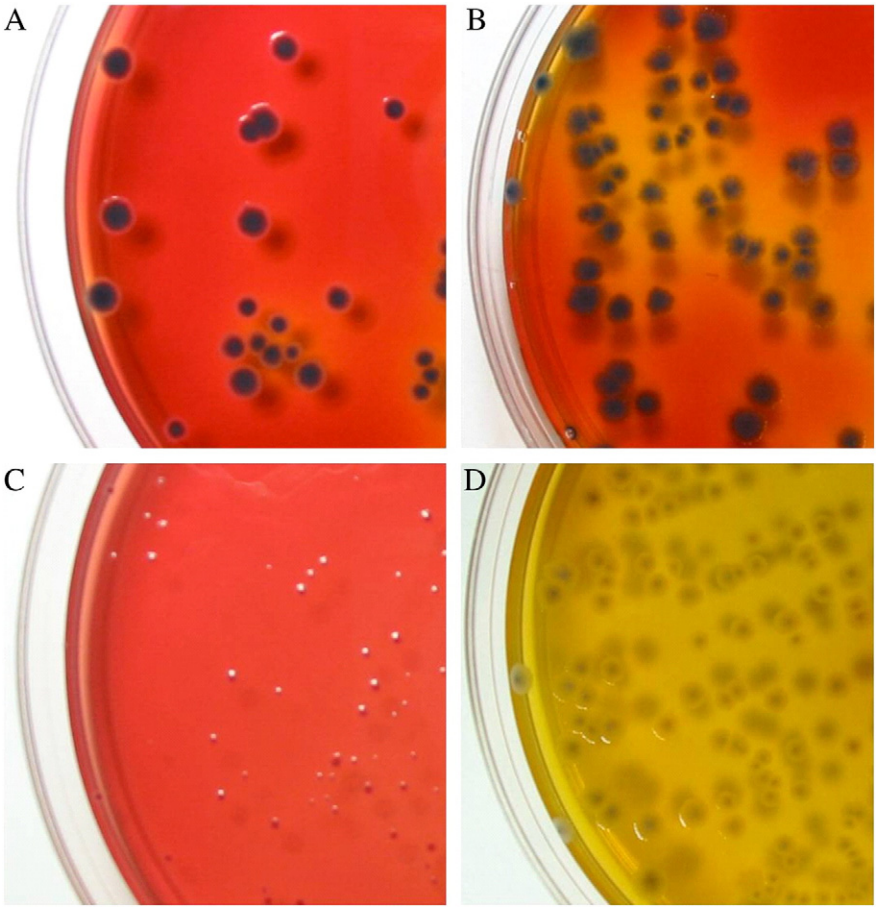

Fig. 1. Colony morphology of *Alicyclobacillus* isolates on SK agar supplemented with 0.01% Chrome Azurol S (CAS). Colonies of guaiacol producing isolates 1016 (A) and 1101 (B) appeared as dark purple to royal blue. Non-guaiacol producing isolates 19220 (C) and C-GD 1-1 (D) produced cream colored colonies.

to isolate 19220, no colony formation was detected at vanillic acid concentrations greater than 70 ppm.

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

Table 5 showed the growth of *Alicyclobacillus* spp. isolates on SK2 medium and commonly used *Alicyclobacillus* spp. isolation media. All guaiacol producing *Alicyclobacillus* isolates were detected in all tested media. The degree of recovery was approximately equal on each tested media. On PDA, K, and SK medium, all non-guaiacol producing *Alicyclobacillus* isolates were detected, whereas 3 isolates were detected on OSA. One non-guaiacol producing isolate (Gala 9-2) was detected on SK2 medium. However colonies appeared as yellow to pale white, distinctly different from the dark purple or royal blue colonies of the guaiacol producing isolates. 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*. 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.

Table 2
The effect of vanillic acid and exposure to oxygen on colony formation of *Alicyclobacillus* spp.

Recovery medium[a]	*Alicyclobacillus* spp. isolates			
	1016	1101	19220	C-GD 1-1
SKD	Purple	Dark purple to royal blue	Cream	Cream
SKDV	Purple	Blue to gray[b] Purple[d]	NG[c]	NG
OV-SKD	(m)[e] Pinpoint size colonies	(m) Purple pinpoint colonies	(m) Pinpoint size colonies	(m) Pinpoint size colonies
OV-SKDV	(m) Pinpoint colonies	(m) Purple pinpoint colonies	NG	NG

[a] SKD, SK agar supplemented with Chrome Azurol S (CAS) at 0.01% (w/w); SKDV, SKD with addition of 100 ppm vanillic acid; OV-SKD and OV-SKDV, media overlaid with SKD.
[b] Colonies in heavy growth area.
[c] NG, no growth.
[d] Single isolated colonies.
[e] Intersection between original media and overlay section.

Table 3
The effect of Chrome Azurol S (CAS) and vanillic acid concentration on the recovery of *Alicyclobacillus* spp. isolates.

Medium	*Alicyclobacillus* spp. isolates			
	1016	1101	19220	C-GD 1-1
SK agar	+++	+++	++	++
SK + 1 ppm CAS	+++	+++	++	++
SK + 5 ppm CAS	+++	+++	++	++
SK + 10 ppm CAS	+++	+++	++	++
SK + 100 ppm CAS	+++	+++	++	++
SK + 1 ppm vanillic acid	+++	+++	++	++
SK + 10 ppm vanillic acid	+++	+++	++	++
SK + 100 ppm vanillic acid	++	++	–	–

+++, $>10^2$ CFU/plate; ++, 10–10^2 CFU/plate; +, <10 CFU/plate, –, no growth.

Table 4
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	++	+++	++	++
10	++	+++	++	++
20	++	+++	++	++
30	++	+++	++	++
40	++	+++	+	++
50	++	+++	++	++
60	++	+++	+	++
70	++	+++	–	–
80	++	+++	–	–
90	++	+++	–	–
100	++	+++	–	–

+++, $>10^2$ CFU/plate; ++, 10–10^2 CFU/plate; +, <10 CFU/plate, –, no growth.

4. 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. SK agar was developed 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.

Among all tested dyes which were selected based on the availability and cost effectiveness, color differentiation was found only on the medium with Chrome Azurol S (CAS). As presented in Fig. 1, color changes and colony morphology in SK agar supplemented with CAS correlated with the guaiacol producing ability of the test isolates. CAS is widely known for use in the detection of siderophores (Milagres et al., 1999; Schwyn and Neilands, 1987; Shin et al., 2001), 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. 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*.

In previous study, no growth factors, carbohydrate utilization patterns or specific enzymes that can easily be incorporated in the differentiation of guaiacol and non-guaiacol producing *Alicyclobacillus* were determined (data not shown). 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 powerful inhibitory effect of vanillic acid on non-guaiacol producing *Alicyclobacillus* was unexpected (Table 3). 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). *Listeria monocytogenes*, *Listeria innocua*, and *Listeria grayi* did not differ in sensitivity towards vanillic acid and a concentration of less than 10 mM was sufficient to prevent the growth of these microorganisms at pH 5.0. Similarly, vanillic acid is effective against *Escherichia coli* O157:H7. In inoculated apple juice containing 5.2×10^5 CFU/ml of *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* (Delaquis et al., 2005; Nishikawa et al., 1988; Ratunanga et al., 1997). Vanillic acid is a weak carboxylic acid with two pKas of pKa1 = 4.51 and pKa2 = 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 and 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. 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. On the other hand, non-guaiacol producing *Alicyclobacillus* may lack the ability to alleviate the accumulation of vanillic acid leading to its inhibition.

To validate the efficacy of SK2 agar in recovering guaiacol producing *Alicyclobacillus*, the recovery efficiency of each isolate on selected media was compared. Comparing across recovery media, the recovery on OSA is lower than that of other media. This result is in agreement with the observations of previous findings (Chang and Kang, 2005). 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 was comparable. Non-guaiacol producing isolates were also recovered using OSA, PDA, K, and SK agar, and colony morphology was identical to the guaiacol producing isolates. Except for isolate Gala 9-2, no non-guaiacol producing isolates were recovered on SK2 agar, most likely due to the inhibitory effects of vanillic acid. Gala 9-2 grew on SK2 agar, but produced 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 guaiacol producing *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

Table 5
The growth of *Alicyclobacillus* spp. isolates on commonly used *Alicyclobacillus* isolation media.

Isolates	Guaiacol production[a]	The growth on each medium				
		OSA	PDA	K	SK	SK2
97	+	+++[b]	+++	+++	+++	+++
113	+	++	+++	+++	+++	+++
1016	+	++	+++	+++	+++	+++
1101	+	++	+++	++	+++	+++
WAC	+	++	+++	+++	+++	+++
849	–	–	+++	+++	+++	–
6348	–	–	+++	++	+++	–
19220	–	++	++	++	++	–
Gala 9-2	–	+	+++	+++	+++	+++[c]
C-GD 1-1	–	++	+++	+++	+++	–

[a] +, guaiacol producing isolate; –, non-guaiacol producing isolate.
[b] +++, $>10^2$ CFU/plate; ++, 10–10^2 CFU/plate; +, <10 CFU/plate, –, no growth.
[c] Growth was observed on the media, but colony morphology was different from typical colony formation by guaiacol producing *Alicyclobacillus*.

vanillic acid resistant non-guaiacol producing isolates, different colony colors as a result of the added CAS allow easy differentiation from guaiacol producing isolates. 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*. However, a limited number of *Alicyclobacillus* isolates (5 isolates each of guaiacol producing and non-guaiacol producing *Alicyclobacillus*) were used to evaluate the effectiveness of SK2 agar in this study. Therefore, further studies are required to evaluate the growth of more guaiacol producing and non-guaiacol producing *Alicyclobacillus* strains on SK2 agar, and it is necessary to assess the specificity of this new medium with food samples.

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