

Control of hazardous bacteria in acidic beverages by using a guaiacol detection kit (peroxidase method)

Motohiro Niwa
R&D Laboratory, Kirin Beverage Corporation

Introduction

After the incident of turbidity of transparent apple juice in the west Germany market in 1984, Cerny et al. isolated a species of thermo-acidophilic bacilli (TAB) [1]. This was named *B. acidoterrestris* (sp. nov.) by Deinhard et al. and was classified into the new genus *Alicyclobacillus* proposed by Wisotzkey et al. in 1992.

The *Alicyclobacillus* species, which are spore-forming acidophil bacilli, remain in fruit juices, survive through usual sterilizing conditions for acidic beverages, and may cause deterioration. *A. acidoterrestris* has a $D_{95^{\circ}\text{C}}$ value of 5.3 min, a growth pH range of 2.5 to 5.5, and a growth temperature range of 20°C to 55°C. A major consequence of deterioration in fruit juice is production of guaiacol, which causes off-flavor (Figure 1). This bacillus, with its low level of required nutrition, may cause deterioration even when the concentration of fruit juice is low, like in near-water beverages.

Because TAB, which are thermophilic aerobic

bacteria, cannot be detected with usual, commercially-available culture media, a suitable medium (in particular on pH) must be selected. Although YSG broth is generally used for culturing TAB, additional procedures to differentiate and identify harmful *A. acidoterrestris* is required; TAB are almost constantly found in fruit juices. Differentiation and identification after detection have been required because there has been no practical method to detect *A. acidoterrestris* directly on a selective basis [2].

The erythritol-BAM culture medium is a simple method to identify the detected bacteria on the basis of differences in sugar utilization, but this

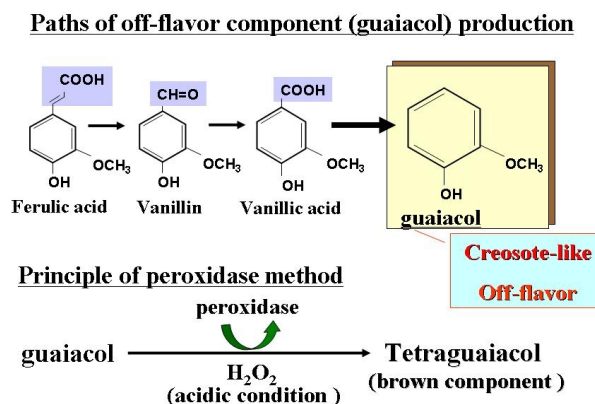


Figure 1 Principle of guaiacol generation and detection

method requires a few days for judgement [3]. Quick methods of judgement include the RT-PCR method, using a primer for *A. acidoterrestris*, and the enzymatic method, in which vanillic acid is added to a culture medium to produce a large amount of guaiacol [4]. The latter method is now available in the form of a commercial test kit, the practicality of which is detailed in this paper. This kit also allows direct detection from specimens on a selective basis.

1. Present status of control in Japan

A unified test method for thermo-acidophilic bacilli was published in the Report of Japan Fruit Juice Association in March 2003. This method detects total TAB by using YSG broth for culturing at 45°C for 3 to 5 days; *A. acidoterrestris* (AAT) is detected by using the peroxidase and temperature difference methods. In the temperature difference method, colonies that grew at 45°C for 18 to 20 hours are judged to be AAT. As a control test, colony growth at 65°C for the same time period is investigated; *A. acidocaldarius* (AAC) can but AAT cannot grow at this temperature.

On the other hand, the peroxidase method, which allows judgement within a short period (about 3 hours), looks into guaiacol productivity. This method is detailed in this paper.

2. Trends outside Japan

After the unified test method proposed by Japan Fruit Juice Association was presented at the 21st

IFU-WG Microbiology Meeting held at Koeln in April 2003, unification of test methods is being pursued also outside Japan. While the IFU member states have been evaluating the BAT culture medium, decisions were made to compare the precision of detection by both the BAT and YSG media and to evaluate the precision of AAT detection by the peroxidase method once the test kit is made available. Conclusions are to be drawn regarding the above two issues at the 22nd IFU-WG Microbiology Meeting to be held in April 2004.

3. Principle of the peroxidase method

Use of Va-YSG broth allows production of high concentrations of guaiacol (a few tens of ppm) within a short period, and the produced guaiacol can easily be detected by using the peroxidase method [6].

The principle of guaiacol production is that the expression of Vdc (vanillic acid decarboxylase) genes is induced by adding vanillic acid of high concentration to AAT during the logarithmic breeding stage. The details of the principle are given in a published paper [4].

The principle of the peroxidase method is as follows. Presence of hydrogen peroxide under an acidic condition leads to oxidation of peroxidase, production of guaiacol radicals from guaiacol, and then production of brown-colored tetraguaiacol by polymerization of four guaiacol radicals (Figure 1).

4. Test kit development and evaluation

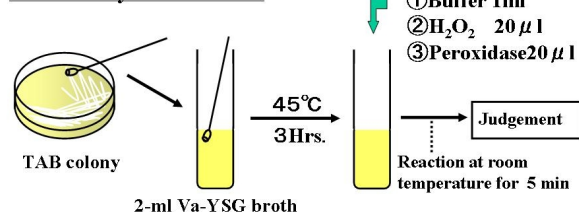
4-1 AAT differentiation by using the test kit

A combined set of Va-YSG broth for guaiacol production and a guaiacol test kit was manufactured (Photo 1). The guaiacol production broth can be used for two purposes: colony judgement and direct detection of AAT from bacteria-bred specimens. One set contains 100 disposable 7-ml test tubes, each containing 2 ml of Va-YSG broth. Colony judgement is a promising quick method to judge, within a few hours, whether guaiacol is produced. The procedure of colony judgement is shown in Figure 2.



Photo 1 Kit for quick detection of guaiacol-producing TAB

Case1 colony differentiation



Visual judgement:

If the specimen is markedly brown in reference to the blank, the specimen is judged to be positive for AAT or guaiacol-producing bacteria.

Figure 2 Colony judgement procedure

Eight species of TAB (53 strains) were evaluated and the 14 strains of *A. acidoterrestris* (AAT) were all positive (guaiacol production). *A. acidiphilus* (weakly positive) and *A. herbarius* were also positive but these are very rare species. The AAT detection kit, which provides a simple and very sensitive method, is considered to be useful on a practical basis (Table 1). A precaution for colony judgement is to use fresh colonies without spore and inoculate at least a loopful of colony into the broth with a 10- μ l loop (Photo 2).

4-2 Direct detection of AAT from specimen with the test kit

In the proposed test scheme, a specimen is cultured at 45°C for 3 to 5 days (bacteria-bred specimen) according to the preliminary culture of the unified test method of Japan Fruit Juice Association, and a 100- μ l sample is directly added to 2 ml of Va-YSG broth and incubated at 45°C for 5 to 24 hours. Then, a buffer, hydrogen peroxide, and peroxidase are sequentially added, and judgement is made after 5 min based on observation of brown coloring (Figure 3) [5].



Photo 2 Colony judgement using the test kit

Table 1 Guaiacol production for 53 TAB strains using Va-YSG broth

a stench	Bacterial species	Strains	Judgement
non-production species	<i>Alicyclobacillus genomic sp.1</i>	6DSM strains and 6 isolated bacterial strains	- (all 12 strains)
	<i>A.acidocaldarius</i>	6DSM strains and 6 isolated bacterial strains	- (all 12 strains)
	<i>A. hesperidum</i>	3DSMstrains and 4isolated bacteria strains	- (3strains) +(4strains) 1)
	<i>A.cycloheptanicus</i>	3DSM strains including a type strain	- (all 3 strains)
	Other species	3 strains	- (all 3 strains)
production species	<i>A.acidoterrestris</i>	ATCC49025 ^T	+++
		DSM2498	+++
		DSM3923	+++
		DSM3924	+++
		OR3	+++
		RB1	+++
		RB221	+++
		RB253	+++
		RB346	+++
		RB359	+++
	TAB-H1	+++	
B2065	+++		
B2066	+++		
B2067	+++		
<i>A.acidiphilus</i>	TA67 ^T	+	
<i>A.herbarius</i>	CPI ^T	+++	

Judgement on guaiacol production: +++stands for 40 ppm or more, ++20 to 40 ppm, and 20 ppm or less.

Judgement on guaiacol non-production: - (the specimen is similar to the blank in color).

1) see Table2

Case 2: direct detection of AAT from fruit juice

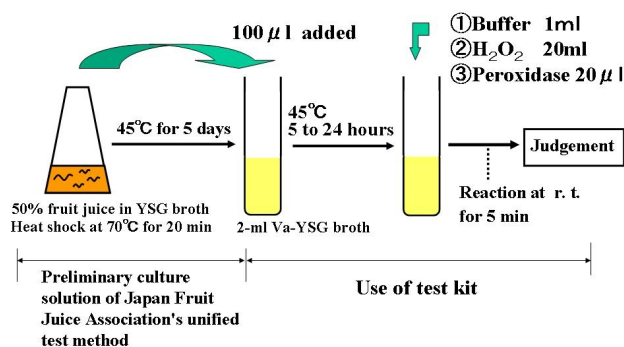


Figure 3 Direct detection of AAT from a bacteria-bred specimen of fruit juice

As a model experiment, 10^1 cfu/ml of AAT spores (TA-27) was inoculated into orange juice, 100-µl samples were subjected to the preliminary culture of the unified test method for 2 and 5 days, and the samples were tested by using the kit. In the case of the 2-day culturing (bacterial

Case 2: test example

Orange juice is subjected to preliminary culture at 45°C for 5 days according to the unified test method of Japan Fruit Juice Association

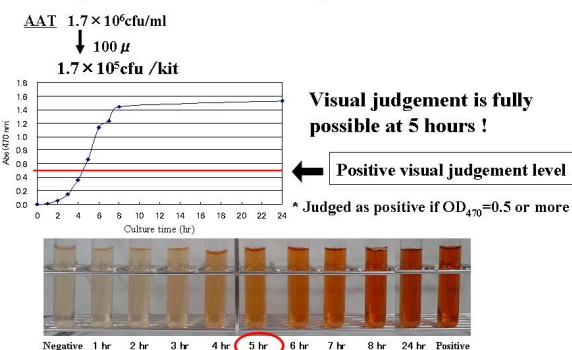


Figure 4 Example of direct detection of AAT

tube in the kit), a clear positive judgement was achieved 24 hours after the start of kit use (Va-YSG broth). In the case of the 5-day culturing, a clear positive judgement was achieved 5 hours after the start of kit use.

The results of actual evaluation of samples are shown in Figure 4.

5. Actual control by using the test kit

5-1 Colony judgement

Only AAT turned brown markedly as shown in Photo 3. The results of evaluation for different species of *Alicyclobacillus* are given in Table 1. Other than AAT, positive strains were found in *A. acidiphilus*, *A. hesperidum* (Table 2; some strains were positive), and *A. herbarius*.

The strains of *A. acidiphilus* and *A. hesperidum* were weakly positive (Photo 4). These two species are not frequently found but may affect products adversely. Therefore, all the guaiacol-producing strains should be subjected to testing.

5-2 Direct detection of AAT

The number of bacteria sampled in each test tube of the kit and the time required for visual judgement were investigated for AAT, which strongly produces guaiacol, and *A. hesperidum* (AHes), which mildly produces guaiacol.

In AAT, visual judgement could be achieved after 5 and 15 hours of incubation when bacterial concentration was 10^5 and 10^1 cfu or more per test tube, respectively (Figure 5). In AHes, visual judgement could be achieved after 24 hours of incubation when bacterial concentration was 10^2 cfu or more per test tube (Figure 6).

When dealing with guaiacol-producing bacteria

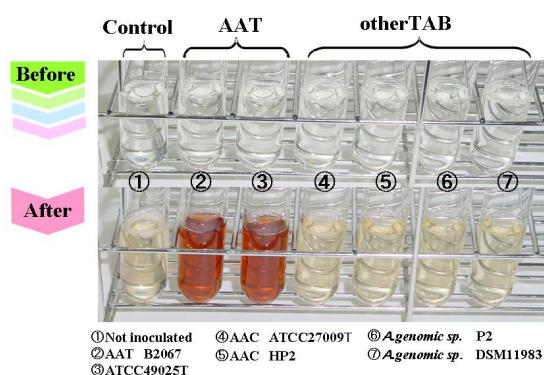


Photo 3 Example of colony judgement

Table 2 Guaiacol productivity of *A. hesperidum*

Bacterial species	Group	No.	Strains	Productivity
<i>A. hesperidum</i>	A	1	DSM12489 ^T	-
		2	DSM12766	-
<i>A. hesperidum</i>	B	3	DSM11985	+
		4	Isolated strain 1	+
		5	Isolated strain 2	+
		6	Isolated strain 3	+
<i>A. hesperidum</i>	C	7	Isolated strain	-

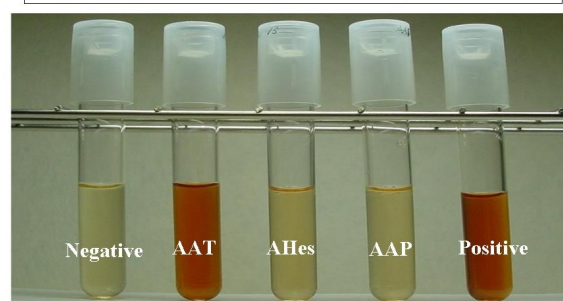
conditions: static culture at 45°C for 3 hours

* All bacterial species were identified based on the base sequence of 16SrDNA.

* Strains having the same base sequence were classified into different groups (two differences in the HV region between groups A and B).

- Some strains of *A. hesperidum* having a different genotype were found to be guaiacol-productive (group B).
- Guaiacol productivity of this species is lower than that of *A. acidoterrestris*.
- Because this species is quite similar in nature to AAT, the two species may be controlled under the same conditions.

A. hesperidum (AHes) and *A. acidiphilus* (AAP) show weak reaction when cultured at 45°C for 3 hours.



Positive control: standard guaiacol solution (50 ppm)

Photo 4 Weakly reacted strains (AHes and AAP)

other than AAT, visual judgement could be achieved after 24 hours of incubation by using 100- µl of bacteria-bred culture solution.

Relationship between the initial number of bacteria and time for judgement (1)

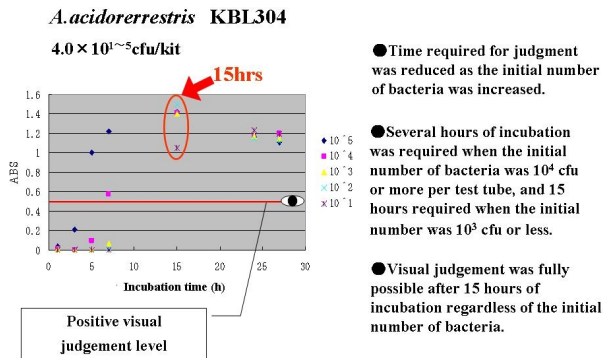


Figure 5 Initial number of bacteria (AAT) and incubation time required for judgement using the kit

Relationship between the initial number of bacteria and time for judgement (2)

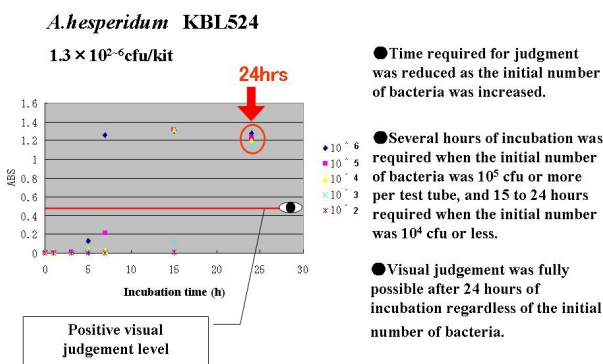


Figure 6 Initial number of bacteria (AHes) and incubation time required for judgement using the kit

6. Method of detecting guaiacol generating bacteria (*Bacillus*) in food

Regular testing for *A. acidoterrestris* in acidic beverages is now a standard practice. Occurrence of off-flavor has been found also in neutral food, and investigation into such cases revealed that *B. subtilis* was responsible. Nine species of the former genus *Bacillus* (22 strains) were investigated for guaiacol productivity (Table 3),

and *B. subtilis*, *B. megaterium*, *P. polymyxa*, and *B. licheniformis* were found to be guaiacol-productive. Because contamination of *Bacillus* into food is unavoidable, off-flavor may occur if there is a matrix required for guaiacol production. Guaiacol-producing *Bacillus* can be detected by using YSG broth added by 100 ppm of vanillic acid (pH 7), YPG broth, or TSB broth and incubation at 35°C for a few hours to three days.

Table 3 Guaiacol productivity of *Bacillus*

Bacterial species	StrainsNo.	4hrs	72hrs incubation
<i>B. subtilis</i>	15	+++	N.T.
	47 IFO3134	+	N.T.
	48 IFO13719	+++	N.T.
	159	—	++
	181 ATCC6633	++	N.T.
	347	+++	N.T.
	379	—	++
	382	—	++
	397	++	N.T.
414	—	—	
<i>B. megaterium</i>	155	+++	N.T.
	156	+++	N.T.
	157	++	N.T.
	158	++	N.T.
	381	++	N.T.
	383	++	N.T.
<i>B. licheniformis</i>	50 IFO12195	++	N.T.
<i>B. polymyxa</i>	274	++	N.T.
<i>B. sphaericus</i>	303	—	—
	54	—	—
<i>B. coagulans</i>	350 JCM2257	—	—
<i>B. circulans</i>	166	—	—

"+++" stands for 40 ppm or more, "++" 20 to 40 ppm, "+" 20 ppm or less, "—" undetected, and "NT" not tested.

Final remarks

The unified test method of Japan Fruit Juice Association is given in Report of Japan Fruit Juice Association, No. 535 issued in March 2003. The test kit used in this study is based on the peroxidase method adopted in the unified test method.

References

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- 4) **Niwa, M.** et al.: DEVELOPMENT OF A RAPID DETECTION METHOD OF *A.acidoterrestris*,HAZARDOUS BACTERIA TO ACIDIC BEVERAGE",*Fruit Processing* **13**, 103-107 (2003)
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