Alicyclobacillus Best Practice Guideline

A guideline for the reduction and control of thermophytic, sporeforming bacteria (Alicyclobacillus species, ACB) in the production, packing and distribution of fruit juices, juice concentrates purees and nectars

Disclaimer

Whilst every effort is made to ensure that the information contained in this Guideline is accurate, the AIJN cannot accept responsibility for errors.
Contents

1 Objectives
2 Definitions
3 Introduction
4 Summary of recommended control points
5 Water
6 Fruit processing
7 Filling factory / bottler
8 Microbiology
9 Recommendations for further research
10 Appendices
1. Objectives

1.1 To identify good manufacturing practices for the reduction and control of ACB.
1.2 To identify control measures.
1.3 To highlight control points.
1.4 To identify and suggest various testing options.
1.5 To indicate gaps in our current knowledge and recommend further research.

2. Definitions

2.1 ACB - Alicyclobacillus sp.
2.2 Product - Refers to all “relevant” liquids e.g. juices, juice concentrates, purees, drinks, nectars, sugar syrups, infusions.
2.3 EVM - Extraneous vegetable matter, e.g. Leaves.
2.4 Primary packaging - Packaging that is in contact with finished products.

3. Introduction

3.1 Alicyclobacillus (ACB) is an acid tolerant thermophyllic micro-organism which as a spore is very heat resistant and will survive the usual heat processes used in the fruit juice industry.

3.2 The presence of this organism in consumer packaged products has been widely reported to cause flavour spoilage problems described as “Smoky bacon”, “Hammy” or even “Antiseptic”.
3.3 This organism can be present and readily detectable in a wide variety of common raw materials used by packers.

3.4 Both raw material producers and packers of finished products need to be aware of this organism and understand how to control it in order to prevent unnecessary spoilage and loss of consumer confidence. This document is intended to act as a guide to help the user develop an effective ACB control programme.

3.5 ACB is not known to pose a safety hazard. Current understanding is that it is not a pathogenic organism. Not all ACB strains have the potential to spoil the final product. The best strategy for its management is to adopt the principles of HACCP, substituting Food Safety Hazards, normally associated with HACCP studies, with the risk of ACB spoilage in the final product. This document has been produced with this approach in mind.

3.6 There is a need to identify and control every point where the organism:
   • Enters the process
   • Has an opportunity to grow
   • Can be reduced or removed
   • Can be reintroduced i.e. the avoidance of cross contamination

3.7 It is necessary to adopt this approach at every stage in the production process:
   • Harvesting of the fruit
   • Storage before processing
   • Manufacture of juice/concentrate i.e. semi finished products
   • Storage and transportation of semi finished products
   • Processing and packing of finished products
   • Storage and distribution

3.8 To bring to the problem all the well known HACCP principles:
   • Identification of “Control Points”
   • Frequency of sampling
   • Define tolerances
   • Specify corrective measures in positive cases
   • Audit effectiveness

3.9 The document is as general as possible.
3.10 GMP is considered a prerequisite in line with “The AIJN guide of good hygiene practice for the fruit juice industry”

3.11 It is unrealistic to guarantee that any product will be absolutely free from ACB.

3.12 The risk of ACB contamination will vary according to product type and the process used in their production.
## 4. Summary of recommended control points

Table 1 - Summary of recommended control points at various stages of the supply chain

<table>
<thead>
<tr>
<th>#</th>
<th>Stage</th>
<th>Control Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Water</td>
<td>A testing regime for all water used in the plant should be established based on risk.</td>
</tr>
<tr>
<td>2.</td>
<td>Water</td>
<td>It should be assumed that condensate contains a high concentration of ACB. Its use, anywhere in the plant, should be examined very critically. It should be tested for the presence of ACB on a routine basis. Any water treatment, aimed at reducing / eliminating ACB, should be monitored closely to ensure that it remains effective under all operating conditions.</td>
</tr>
</tbody>
</table>
| 3. | Farm and fruit transportation| Farmers / growers / fruit suppliers should, as a minimum, be informed of the existence of ACB, of its potential to cause serious problems and be involved in discussions as to how to reduce soil contamination to a minimum. Points to consider for the involvement of the suppliers are:  
  1. Where possible – fruit should not be picked from the ground or stored in direct contact with the ground.  
  2. Fruit specifications, contracts or agreements should include a reference to contamination with soil / EVM to ensure effective information to the fruit suppliers. It is not recommended to define any limits for ACB.  
  3. The quality of fruit containers, intermediate storage and transport should be defined to ensure that it does not increase the risk of contamination with ACB. |
<p>| 4. | Fruit reception and handling | Excessively dirty fruit or fruit excessively contaminated with EVM should be rejected even if it is to be subject to a cleaning stage as part of the subsequent production process. Standards and procedures should be defined for the cleanliness (condition) of the fruit received. These standards should be communicated to the supply chain. |</p>
<table>
<thead>
<tr>
<th></th>
<th><strong>Fruit reception and handling</strong></th>
<th>The buildings, storage conditions and techniques for special fruit ripening after harvest (e.g. Mango, Papaya or Banana) should be optimised to avoid further contamination with ACB and to prevent the growth of the organism.</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.</td>
<td><strong>Fruit cleaning and sorting</strong></td>
<td>The frequency with which the flume water is changed should be linked to and influenced by the efficiency of the final fruit washing process to remove / reduce ACB contamination on the fruit.</td>
</tr>
<tr>
<td>7.</td>
<td><strong>Fruit cleaning and sorting</strong></td>
<td>The concentration and contact time of sanitisers added to wash waters must be closely controlled and monitored, especially under the most adverse production conditions. There must be no possibility of sanitiser contamination /carryover into the final product.</td>
</tr>
<tr>
<td>8.</td>
<td><strong>Filtration</strong></td>
<td>There is no guarantee that UF juice is 100% ACB free. The efficiency of the cleaning and the integrity of the membranes are control points. The continued efficiency of the filter must be monitored.</td>
</tr>
<tr>
<td>9.</td>
<td><strong>Bottler raw material QA</strong></td>
<td>Carry out supplier and raw material risk assessments to determine appropriate incoming raw material controls.</td>
</tr>
<tr>
<td>10.</td>
<td><strong>Bottler, cooling and filling temperatures</strong></td>
<td>Cool the product to less than 20 °C. as soon as possible after heat processing.</td>
</tr>
<tr>
<td>11.</td>
<td><strong>Finished product storage and distribution</strong></td>
<td>Store products at less than 20 °C.</td>
</tr>
<tr>
<td>12.</td>
<td><strong>Bottler cleaning and sanitisation</strong></td>
<td>Carry out periodic sporidical cleaning to reduce the risk of ACB spore contamination.</td>
</tr>
</tbody>
</table>
5. Water

5.01 It should be assumed that all water can contain ACB. Its status should be established by routine testing based on a risk assessment. This includes:
- Supplied Water (Incoming to the plant)
- Treated Water (Supplied water after treatments)
- Recovered water (Condensate)

5.02 A risk assessment should consider the sanitisation of water handling systems, particularly the condensate system.

5.1 Condensate

5.1.1 Introducing ACB into the concentrator dramatically increases the risk that the condensate water will become contaminated. Most evaporators are unable to prevent cross contamination from the juice stream into the condensate.

5.1.2 The exit temperature and composition of the condensate from the evaporator is ideally suited to the growth of the organism. Problems will occur if this untreated water is stored for an excessive period of time. The water should be treated prior to storage.

5.1.3 Evaporator water can contain huge numbers of spores, >1000/ml, which makes this water unsuitable for other uses within the process, unless suitably treated.

5.2 Treatments

5.2.1 Various methods are available for the control of ACB in water e.g.
- Ozone
- Hydrogen peroxide
- Chlorine dioxide
- Hypochlorite
- Peracetic acid
- UV
- Filtration
- Heat Treatment
5.2.2 Care should be taken in selecting and using an appropriate chemical to avoid contamination of it in the final product.

5.2.3 Appropriate temperatures, concentrations and contact times must be established for all chemical sterilants. Temperature, intensity, contact time and turbidity levels need to be established for UV sterilisation.

5.2.4 When testing chemically treated water the treatment chemicals must be inactivated before the water is tested for ACB.

5.2.5 If using UV, ensure the bulb is monitored for effectiveness; typically output should not fall below 75% of its initial value.

**Control Point 1**
A testing regime for all water used in the plant should be established based on risk.

**Control Point 2**
It should be assumed that condensate water contains a high concentration of ACB. Its use, anywhere in the plant, should be examined very critically. It should be tested for the presence of ACB on a routine basis. Any water treatment, aimed at reducing / eliminating ACB, should be monitored closely to ensure that it remains effective under all operating conditions.
## 6. Fruit Processing

Table 2 - Summary of factors to consider through the fruit processing stages

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Considerations</th>
</tr>
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<tbody>
<tr>
<td>Farm and fruit transportation</td>
<td>• Education</td>
</tr>
<tr>
<td></td>
<td>• Soil contamination</td>
</tr>
<tr>
<td></td>
<td>• Fallen fruit</td>
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<td></td>
<td>• Transportation cleanliness</td>
</tr>
<tr>
<td>Fruit reception and handling</td>
<td>• Inspection</td>
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<tr>
<td></td>
<td>• Unloading</td>
</tr>
<tr>
<td></td>
<td>• Ripening</td>
</tr>
<tr>
<td>Fruit cleaning / sorting</td>
<td>• Inadequate separation of damaged fruit</td>
</tr>
<tr>
<td></td>
<td>• Water as a source of contamination</td>
</tr>
<tr>
<td></td>
<td>• Inadequate final rinsing</td>
</tr>
<tr>
<td></td>
<td>• Cross contamination</td>
</tr>
<tr>
<td>Juice extraction</td>
<td>• GMP</td>
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<tr>
<td></td>
<td>• Juice peel contact</td>
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<tr>
<td>Filtration</td>
<td>• Filter integrity</td>
</tr>
<tr>
<td></td>
<td>• Filter specification</td>
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<tr>
<td></td>
<td>• Filter location</td>
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<tr>
<td></td>
<td>• Filter materials</td>
</tr>
<tr>
<td>Evaporation</td>
<td>• GMP</td>
</tr>
<tr>
<td></td>
<td>• Lethal temperatures</td>
</tr>
<tr>
<td>Storage</td>
<td>• Airborne contamination</td>
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<tr>
<td></td>
<td>• Condensation</td>
</tr>
<tr>
<td></td>
<td>• Storage temperatures</td>
</tr>
<tr>
<td>Filling</td>
<td>• Lethal temperatures</td>
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<tr>
<td></td>
<td>• Filtration</td>
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<tr>
<td></td>
<td>• Blending</td>
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<tr>
<td></td>
<td>• Container contamination</td>
</tr>
<tr>
<td></td>
<td>• Filling temperature</td>
</tr>
<tr>
<td>Shipment</td>
<td>• Temperature</td>
</tr>
<tr>
<td>Other processes</td>
<td>• Hot break vs. cold break (Tomato Juice)</td>
</tr>
<tr>
<td></td>
<td>• Resin based processes</td>
</tr>
<tr>
<td></td>
<td>• Homogenisation</td>
</tr>
</tbody>
</table>
6.1 Farm and fruit transportation

6.1.1 ACB is found in most types of soil throughout the world. Soil contamination of the fruit (and on any leaves and twigs that are mixed with the delivery) is the primary source of ACB entry into the production chain. As ACB is present in soil it will also be present in dust therefore fruit not in direct contact with the ground can also be contaminated. (Silva, FVM & Gibbs, P. (2001) Alicyclobacillus acidoterrestris spores in fruit products and design of pasteurization processes. Trends in Food Science & Technology 12 (2001) 68-74.)

Control Point 3

Farmer / growers / fruit suppliers should, as a minimum, be informed of the existence of ACB, of its potential to cause serious problems and be involved in discussions as to how to reduce soil contamination to a minimum.

Points to consider for the involvement of the suppliers are:

1. Where possible – fruit should not be picked from the ground or stored in direct contact with the ground.
2. Fruit specifications, contracts or agreements should include a reference to contamination with ACB to ensure effective information to the fruit suppliers. It is not recommended to define any limits for ACB.
3. The quality of fruit containers, intermediate storage and transport should be documented to ensure that is does not increase the risk for contamination with ACB.

6.2 Fruit Reception and handling

6.2.1 It is normal practice for fruit deliveries to be subject to a quality inspection prior to off loading. Rejection takes place if the delivery contains an unacceptable level of damage, rot, mould, EVM (extraneous vegetable matter such as leaves) and foreign material. The presence of excessively “dirty fruit” should also be a cause for rejection as it may overwhelm the subsequent cleaning stage.
**Control Point 4**

Excessively dirty fruit or fruit excessively contaminated with EVM should be rejected even if it is to be subject to a cleaning stage as part of the subsequent production process. Standards and procedures should be defined for the cleanliness (condition) of the fruit received. These standards should be communicated to the supply chain.

**Control Point 5**

The buildings, conditions and methods for fruit that requires special ripening after harvest (e.g. Mango, Papaya or Banana) should be suitable to avoid further contamination with ACB and prevent proliferation of the organism.

### 6.3 Fruit Cleaning and sorting

6.3.1 Reducing ACB as early as possible in the process is highly recommended to reduce the risk of final product contamination.

6.3.2 Washing and cleaning of fruit is performed to eliminate soil, EVM and sub-standard fruit. Traditionally the efficiency of this process has been judged visually i.e. if it looks ok it is adequate. This sort of visual assessment cannot be used to judge the level of microbiological contamination.

6.3.3 The ability of the cleaning process to reduce ACB can only be evaluated by measuring the organism at the end of this initial process step.

6.3.4 For reducing ACB at this process step, the following points should be considered:

- Removal / separation of rotten fruit.
- Removal / separation of EVM and foreign material.
- Use / effectiveness of sanitising chemicals.
- The microbiological quality of the process water.
- The source of the water.
- The effectiveness of the final rinse.
- Equipment cleanliness.
6.3.5 Water pressure, volume, contact time and adequate fruit rotation are all important parameters to ensure effective cleaning. If manual sorting is applicable; light levels, adequate inspection (this includes adequate numbers of personnel and time spent at the inspection table), table speeds and fruit levels should be considered.

6.3.6 Flume (transportation) water. Some types of fruit are often transported and even unloaded using water. This water is the first stage in the fruit washing process. How often this water is changed can influence the level of ACB contamination on the fruit.

**Control Point 6**

The frequency with which the flume water is changed should be influenced by the efficiency of the final fruit washing process to remove / reduce ACB contamination on the fruit.

6.3.7 Wash water and chemical treatments

6.3.7.1 The rinsing process is an important step as the last opportunity to reduce the concentration of the bacteria entering the process. Good design and adequate water flows are necessary to ensure effective final rinsing of the fruit. There should be procedures and standards for fruit cleaning.

6.3.7.2 Some types of fruit will tolerate the use of chemical agents in the washing and/or sanitising process to help reduce the microbiological load on the surface of the fruit. Some examples are Peracetic acid, Chlorine Dioxide and Hydrogen Peroxide. Especially for citrus, ensuring the surface of the fruit is adequately cleaned and sanitised is considered to be more important than fruit sorting in controlling the levels of ACB in the juice.

6.3.7.3 The legal application of a chemical treatment at this stage must be checked.

**Control Point 7**

The concentration and contact time of sanitisers added to wash waters must be closely controlled and monitored especially under the most adverse production conditions to ensure they are used appropriately and they have been shown to be effective. There can be no possibility of contamination /carryover into later stages of the process.
6.4 **Juice Extraction**

6.4.1 Secondary juice extraction

The water used must be subject to the same considerations as previously described.

6.5 **Pre Heating**

6.5.1 Where appropriate to the product this stage may be used to reduce ACB.

6.6 **Filtration**

6.6.1 Ultra filtration
Theoretically bacterial cells and spores should not be able to pass through the membranes of an Ultra Filter (UF) as they are far too big. After ultra filtration the juice should be sterile, however experience has shown that product after UF is not always ACB free. In other words it is possible for an UF to function as an effective producer of clear juice but could still have small lesions/surface cracks/leaks that allow the bacteria to pass. Even if it can be shown that a particular unit is functioning as an effective bacterial filter there is no guarantee that this will be the case in the future. Care needs to be taken with the cleaning of the filter particularly on the permeate side.

6.6.2 Other means of filtration
Pressure filters and vacuum filters are often used in the juice industry; however neither has an effective pore size capable of removing ACB spores.

6.6.3 Dedicated ACB filters
Plate-/Sheet-/Bag filters can be equipped with filter sheets providing an effective pore size (0, 2-0, 45 Microns). Operating details need to be obtained from the filter supplier. Factors such as flow, viscosity and temperature influence the filter’s efficiency.

6.6.4 Special care has to be taken with the continuous integrity of the filter, as the filter is often the last means of removal of ACB. Special cleaning for the plate/sheet filters is needed to prevent cross contamination when changing filters.
6.6.5 If filters are used they should be installed as early as possible in the process to prevent cross contamination and/or spreading of the spores in the plant.

6.6.6 The maximum design throughput of a filter should not exceed the manufacturer’s recommendations, unless validation studies support extended running time.

**Control Point 8**

There is no guarantee that UF juice is 100% ACB free. The efficiency of the cleaning and the integrity of the membranes are control points. The continued efficiency of dedicated ACB filters must be monitored.

### 6.7 Evaporator

6.7.1 If the fruit juice quality allows then the heating stage may go up to 125°C (for a suitable holding time), to destroy ACB spores.

6.7.2 Temperature conditions exist in evaporators that are very conducive to the growth of ACB. It is therefore important that the unit is subject to regular cleaning and sterilisation.

### 6.8 Filling and Storage

6.8.1 GMP systems will be in place to avoid cross contamination with ACB. Storage of material at less than 20°C will prevent the growth of any vegetative organisms.

6.8.2 The final UHT treatment, as part of the aseptic filling process, can be an effective heat step to destroy ACB spores and might go up to 120°C if the product tolerates it.

### 6.9 Shipping

6.9.1 Testing of the final product will be dependant upon risk assessment and any specific customer requirements. Analysis should be considered as a monitoring process, part of the QA programme and not a guarantee for the absence of ACB.
6.10 Other Processes

6.10.1 The problem of ACB should be taken into account when establishing CIP regimes.

6.10.2 Two stage heat treatments. Theoretically spores can be reduced effectively by a two step heat treatment. The first heating step activates the spores and vegetative cells are produced. The second heating step then kills these vegetative cells. This process can only be applied to certain fruit types where the final quality is not compromised.

6.10.3 A pre concentration stage can be used to reduce the water activity and therefore inhibit the subsequent growth of ACB. See Section 8.1.

6.10.4 Resin treatment systems are considered to be ACB sensitive as they are operated at optimum growth temperatures and low brix values. Resins are difficult to clean due to their large surface area. The effectiveness of the CIP is determined only by the chemicals applied, not flow rate and temperature. Optimum CIP conditions should be obtained from resin suppliers.

6.10.5 If a homogenisation step is included in the process then special care should be taken about maintenance, cleaning and effective sanitation of the equipment in order to prevent the harbourage and proliferation of ACB.

6.11 Aroma

This may be a source of ACB under certain circumstances.

6.12 Air

The extent of hazards associated with airborne contamination is not well understood.
7. **Filling Factory / Bottler**

Table 3 - Summary of issues to consider at the filler/bottler

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Considerations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation</td>
<td>• Reduction of guaicol pre cursors</td>
</tr>
<tr>
<td></td>
<td>• Antioxidants</td>
</tr>
<tr>
<td>Raw Material QA</td>
<td>• Risk Assessment of source</td>
</tr>
<tr>
<td></td>
<td>• Specification</td>
</tr>
<tr>
<td></td>
<td>• Incoming material testing</td>
</tr>
<tr>
<td></td>
<td>• Storage temperature</td>
</tr>
<tr>
<td>Packaging materials</td>
<td>• Prevention of primary packaging contamination</td>
</tr>
<tr>
<td>Sterilisation process</td>
<td>• Parameters</td>
</tr>
<tr>
<td>Oxygen availability</td>
<td>• Oxygen control</td>
</tr>
<tr>
<td>Cooling and fill</td>
<td>• Parameters</td>
</tr>
<tr>
<td>temperatures</td>
<td></td>
</tr>
<tr>
<td>Storage and distribution</td>
<td>• Temperature</td>
</tr>
<tr>
<td>CIP</td>
<td>• Hygienic design</td>
</tr>
<tr>
<td></td>
<td>• Chemical, concentration, time, temperature, frequency</td>
</tr>
</tbody>
</table>

7.1 **Formulation**

7.1.1 Consider the reduction or removal of guaicol precursors such as vanillin, vanillic acid or ferulic acid.

7.1.2 Consider the use of antioxidants.
7.2 Raw Material QA

7.2.1 Risk Assessment of source

An understanding of the supplier’s controls and processes to minimise the presence of the ACB in the raw material will help establish a specification and incoming material testing regime.

7.2.2 Specification

The specification should make a reference to this document and the agreed test method. Acceptable levels could be established in agreement with the supplier.

7.2.3. Incoming materials testing

A risk assessment on each raw material supplier and raw material should establish the frequency of incoming testing, and if the material needs to be quarantined awaiting positive release for ACB. The risk assessment will be influenced by how the raw material will be used, e.g. there is no risk of ACB spoilage in chilled (short shelf life) products.

Control point 9

Carry out supplier and raw material risk assessments to determine appropriate levels of incoming raw material controls.
7.3 Packaging Materials

7.3.1 As with other raw materials packaging suppliers should be risk assessed for ACB contamination and controls put in place to prevent cross contamination of such packaging.

7.4 Sterilisation Process

7.4.1 The typical processes used in the packing of products will not destroy ACB spores. In fact the process may heat shock the spores into germination, which could subsequently lead to finished product spoilage. This step will therefore not control the levels of ACB. A process suitable for the destruction of spores will typically not be suitable for acceptable product quality.

7.4.2 Where the product allows sterile filtration can be considered.

7.5 Oxygen Control

7.5.1 The restriction of oxygen availability may reduce the production of guaiacol and hence spoilage. Consider dissolved oxygen and headspace management.

7.6 Cooling and filling temperatures

7.6.1 For ambient shelf stable packs the fill temperature should be 20°C max. At elevated temperatures spoilage by the presence of ACB tainting spores can occur over time. This may pose a problem for hot fill processes therefore any exceptions should be carefully risk assessed. Chilled products are not subject to this type of spoilage.

Control point 10
Cool to less than 20°C as soon as possible after heat processing.
7.7 **Storage and Distribution**

7.7.1 The principle should be to maintain temperatures below 20°C. During summer months or in warm climates this may pose a challenge, therefore supply chain routes and systems should be risk assessed.

**Control point 11**
Store products at less than 20°C.

7.8 **Cleaning and sanitisisation**

7.8.1 Hygienic design
See the AIJN Guide of Good Hygiene Practice for the Fruit Juice Industry. Good standards of GMP and control should be established, particularly to prevent build up of soiling that could harbour spores and vegetative cells.

7.8.2 Chemical
Apart from the usual treatments associated with fruit juice CIP cleaning a periodic clean with a sporacide can help minimise contamination. The sporacide should be selected with expert advice to ensure it will be effective against ACB spores and suitable for the equipment to be cleaned. Concentration, contact time and temperature will be dependant upon the sporacide selected. The frequency of sporacide cleaning should be determined on a risk assessed basis. This could be weekly or monthly for example.

**Control point 12**
Periodic sporicidal cleaning should be performed to reduce the risk of ACB contamination.
8. Microbiology

8.1 Introduction

8.1.1 Alicyclobacillus species are rod-shaped, Gram-positive, aerobic, spore-forming bacteria with optimum growth at a pH of 3.5 – 4.5 (growth range pH 2.2 – 5.8) and at a temperature of 45°C (growth range 20 – 70°C).

8.1.2 The main groups of Alicyclobacillus are A. acidoterrestris (growth range 20 – 55°C) and A. acidocaldarius (growth range 45 – 70°C).

8.1.3 It is commonly believed that Alicyclobacillus acidoterrestris is the principle cause of most spoilage problems. It can grow in fruit juice and can produce tainting compounds - mainly guaiacol but also various halophenols. It should be noted, however, that A. acidophilus and A. herbarius can also produce guaiacol. Only approximately 10% of these 3 species are able to produce Guaiacol. The amount of guaiacol formed depends on a number of factors such as the Alicyclobacillus sp. concentration (In general, it is possible to detect guaiacol after the count reaches $10^4$ cfu/ml), and the presence of naturally occurring guaiacol pre cursors such as vanillin, vanillic acid and ferulic acid.

8.1.4 In general, spores need a heat shock to initiate their germination. As it is most likely that concentrate raw materials contain spores and not vegetative cells they need to be heat shocked. Finished products are usually heated during processing; therefore heating is not necessary for their investigation.

8.1.5 The pH of the detection media needs to be around 4.0. BAT medium (the name originates from Bacillus acidoterrestris) has been developed specifically for the detection of ACB and is recommended by the IFU. It contains some glucose and yeast extract and is especially characterised by the presence of many trace elements.

8.1.6 Some D and Z values have been quoted as
D-Value at 90°C = 5 – 20 min.
Z-Value = 7 – 12°C
(Silva et al., 2001, Savas Bahceci and Acar, 2007, Abecitrus)

Heat resistance increases with increasing brix values. However these values can vary according to the strain and the product.
8.1.7. It is known that growth of the organism can be affected by water activity. At brix levels of 20 – 25 and above, growth will be inhibited.

8.1.8 IFU 12 method with BAT is acceptable for determining a count in clear products, however not suitable for cloudy products.

8.2 Detection methods

8.2.1 Procedure for juice concentrates and other raw materials according to IFU Method No. 12
See the IFU method. www.ifu.org
This is the recommended method.

8.2.2 Alternative methods, alterations to the IFU No. 12 protocol

8.2.2.1 Japanese Unified Test Method
Published by the Japan Fruit Juice Association Jan 2007, “The unified test method for thermo-acidphilic Bacili”
Tel: +81 334335071

8.2.2.2 Dilution media and incubation conditions

   The product can be diluted in BAT broth or buffer instead of physiological salt solution.
   Incubation can be performed at other temperatures, e.g. 47°C.

8.2.2.3 Lowering the detection limit

   It is possible to lower the detection limit by plating bigger volumes on larger Petri-dishes

8.2.2.4 Enrichment

   In the case of low contamination levels an enrichment step is recommended. Product samples can be added to BAT broth. Incubation conditions can be varied.
   IFU No. 12 recommends 3 – 5 days 45°C.
8.2.2.5 Optimal ACB detection

Optimal ACB detection methods and recovery / cultivation media are often matrix specific and the use of multiple isolation media often yields better ACB productivity and sensitivity than the use of a single isolation medium. Examples of food/beverage matrices for which this dual medium approach applies include sweeteners, flavours, environmental swabs, rinse waters and different types of finished products.

8.2.2.6 Other media

BAT supports the growth of most Alicyclobacillus species. This medium is recommended by the IFU. Alternative media types, used in the past or for broader investigations are:

- K agar (normal pH 3.7): supports the growth of predominantly Alicyclobacillus acidoterrestris and maybe other Alicyclobacillus species, but in practice results may be variable.

- Acidified PDA (potato dextrose agar): supports the growth of Alicyclobacillus.

- OSA (orange serum agar, normal pH 5.2 – 5.5): growth of various acid tolerant micro organisms

- YSG (yeast extract, starch, glucose): pH 3, 7+/− 0.1.
  (International Federation of Fruit Juice Producers (IFU), First standard IFU-method on the detection of Alicyclobacillus in fruit juices, IFU method No. 12 January 2004 / February 2006)

The difference between this method and IFU No. 12 (at the time this document was completed) is the pre-incubation and the choice of the medium. YSG has a pH of 3.7, contains no buffering system and a little less glucose. BAT contains many trace elements, YSG none. On YSG all Alicyclobacillus species should grow, although comparative tests could not confirm this. This method is also suitable for the detection of TAB: thermo-acidophilic bacteria.
8.2.3 Alternative detection techniques

8.2.3.1 Although there are a number of alternative methods presented in the literature and on the internet, they often have a high detection limit (especially the “fast” methods) and/or are relatively expensive when compared to conventional plating techniques. If a technique claims to be able to detect <100 cells this can be the result of pre treatment such as pre-enrichment, membrane filtration or centrifugation. The principles of some of these methods are outlined below.

8.2.4 (Real time) PCR

8.2.4.1 During the PCR primers that bind specifically to the Alicyclobacillus DNA are added to the DNA isolated from the sample. If Alicyclobacillus DNA is present, this DNA is multiplied during the PCR reaction which results in a signal. During real time PCR the cycle number correlates with the input DNA concentration and thus to the number of cells in the sample.

8.2.4.2 The detection limit for PCR is estimated to be 100 cells per ml or per g. Spores can not be detected.
Results are available within a few hours.
Preincubation is necessary to detect the low levels commonly of interest.

8.2.5 Flowcytometry

Coloured reference cells are added to the sample then the total number of cells in the sample flow is counted, automatically. The number of Alicyclobacillus cells is determined by comparison to the number of reference cells.
The detection limit is considered to be relatively high, minimum ~ 103 cells per ml or per g

8.2.6 Gene probes
Specific gene probes are coupled to fluorescent markers. After incubation and preparation of approx 4 hours the samples can be tested for Alicyclobacillus using a fluorescent microscope.
The detection limit is considered to be relatively high, minimum ~ 103 cells per ml or per g.
8.2.6.1 Gene probe technology (Vermicon identification technology; VIT)

Without DNA extraction, fluorescently-labelled specific gene probes are added to the sample (enrichment or suspicious colony).

During a short incubation period the specific gene probes for Alicyclobacillus diffuse into the cells and bind to the rRNA. If viable Alicyclobacillus sp. and A. acidoterrestris are present in the sample they are detected after a subsequent washing step by different colours using a fluorescence microscope. As the target is not the DNA of the analysed bacteria, but the rRNA, solely viable cells are detected, but no irrelevant dead cells. Results are available within 3 hours. Pre-incubation is necessary to detect the low levels commonly of interest.

The detection limit is considered to be relatively high, minimum ~ 10^3 cells per ml or per g.

1-5 cells per 10 ml or 10 g are detected after enrichment of the sample in BAM/BAT broth or VCM-Select A3 broth (provided by Vermicon) for only 48 h.

The simple and robust procedure requires no special knowledge or training. No particular precautions are required as there is no risk of DNA contamination during the VIT analysis.

8.3 Characterisation and identification

8.3.1 Rough characterisation of species by selective growth

Rough discrimination between A. acidoterrestris and A. acidocaldarius:
- suspend colonies from the BAT agar in physiological salt solution and streak the obtained suspension on BAT agar
- incubate the plates at 45°C and 65°C: A. acidocaldarius is able to grow within 1 day at 65°C (A. acidoterrestris is not able to grow at 65°C)
- incubate the plates at 30°C and 45°C: some A. acidoterrestris are able to grow at 30°C within 5 days (A. acidocaldarius is not able to grow at 30°C)

8.3.2 Confirmation for guaiacol producing ACB by peroxidase test

Alicyclobacillus, which can spoil products by producing guaiacol, can be specifically identified by the following method:
- prepare YSG broth and add vanillic acid or use directly ready-to-use BAT-IB
- inoculate a fresh colony into the tubes
- incubate, shaking, at 45°C for at least 3 hours, but over night is recommended
- add buffer, H2O2 and peroxidase solution to incubated tubes and mix well
- after 5 – 10 minutes observe the change of colour by visual evaluation

The principle of the test is that guaiacol producing strains convert vanillic acid to guaiacol. Guaiacol reacts with H2O2 and peroxidase to form tetraguaiacol which is a brown coloured complex.

8.3.3 Guaiacol detection, peroxidase test.

In fruit juices tainting (not pathogenic) chemicals can be produced by Alicyclobacillus acidoterrestris. (A. Acidiphilus and A. herbarius can also produce guaiacol.)

Guaiacol (2-methoxyphenol) and the Halophenols 2, 6-dibromophenol (2, 6-DBP) and 2, 6-dichlorophenol (2, 6-DCP) can be responsible for off-flavours.

A method has been developed for the rapid determination of guaiacol. The principle of the test is that guaiacol reacts with H2O2 and peroxidase to form tetraguaiacol which is a brown coloured complex (see 8.3.2). Commercial kits are available.
8.3.4 DNA sequence analysis

A small number of cells is needed for this investigation. DNA is extracted from the cells. A great part of the 16S ribosomal RNA coding DNA is amplified and sequenced. Subsequently, these nucleotide sequences are compared with the sequences of strains available in international databases which results in identification or classification of the microbial cultures. This technique is relatively expensive. No relation between strains can be made. In most cases identification to a species is possible.

8.3.5 DNA Ribotyping

A large number of freshly grown pure cultures are needed for this investigation. Ribotyping involves the fingerprinting of genomic DNA restriction fragments that contain the genes coding for the 16S and 23S rRNA. The DNA fragments are separated by size on a gel and appear as a band. The DNA patterns (fingerprints) are matched with the patterns available in the database of the Riboprinter. In a case of more than an 85% match the isolate is identified with confidence. Also, the DNA patterns of the different isolates can be compared, to show if the isolates are identical or not. The identification to a species is not always possible, but a relation between strains can always be seen.

8.3.6 Genomotyping

The whole genome (total DNA) of a variety of Alicyclobacillus strains is randomly divided in parts and placed on a micro array as probes. A pure culture is needed for this investigation. The DNA of a strain is isolated and labelled fluorescently. Labelled DNA is then incubated with the array. During this process, only DNA fragments that match complementary parts (spots) of the micro array will bind onto the array while non-matching fragments will not. After incubation, the micro array is scanned and the fluorescence intensity is quantified. Since binding of matching DNA fragments will result in a fluorescent signal from a specific spot of the micro array, the genetic characteristics of each strain is represented by the fluorescence intensity data of all individual spots of the micro array. The power of this technique is the possibility to find markers which can predict the physiological behaviour of a strain (growth, guaiacol production). In almost every case, identification to a species and a highly specific relation between strains can be made because
the investigation is made on the total DNA and not only on a small part of the DNA as targeted in Ribotyping.

8.3.7 GC-MS

Gas chromatography / mass spectrometry (GC/MS) can be used to identify and quantify the taint producing compounds - guaiacol and 2, 6-dibromophenol (2, 6-DBP) and 2, 6-dichlorophenol (2, 6-DCP).

9. Recommendations for further research

9.1 Development of the IFU 12 method, suitable for determining a count in cloudy products.

9.2 IFU method No. 12 specifies the use of a 0.45μm filter however, for optimal retention of the bacterial spores, a 0.2μm filter may be more effective.

10. Appendices

10.1 Reference

International Federation of Fruit Juice Producers (IFU)
First standard IFU-method on the detection of Alicyclobacillus in fruit juices
IFU method No. 12 January 2004 / February 2006

Japanese unified test method.
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10.2 Working Group Members

John Collins, Director of Technical Services, Gerber Juice Co. (Chair)

Steffen Ammon, Head of Group Quality, Doehler

Morten Friis, Group Technical Manager, Agrana

Antonio Carlos Goncalves, Quality Manager, Louis Dreyfus Citrus (Also representing Citrosuco, Citrovita and Cutrale)

Erik Hoornstra, Microbiology, TNO


Susanne Koswig, Technical Manager, SGF

Jan-Erik Sommer, Process Engineer, Unipektin

Peter Spaargaren, Quality Manager Supply Chain, Cargill Flavour Systems