11 Microbiology of soft drinks and fruit juices

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11.1 Introduction

Soft drinks and fruit juices represent an important market within the food industry. The increasing variety of products being released at a bewildering rate has altered the potential for spoilage problems. Soft drinks are generally nutrient-poor media that are spoiled by relatively few organisms – usually yeasts, and a few acid-tolerant bacteria and fungi. Carbonation shifts the spoilage flora to those organisms tolerant of carbon dioxide. Soft drinks enhanced by the addition of low levels of fruit juice tend to exhibit similar spoilage flora to fruit juices. The use of ever more exotic raw ingredients may lead to the discovery of unusual spoilage organisms in the future. Yeasts in general, and *Zygosaccharomyces bailii* in particular, remain the key spoilage organisms because of their overall physiology and resistance to organic acid preservatives (Stratford *et al.*, 2000).

Microbial problems within soft drinks and fruit juices can be divided into two groups:

(1) growth in, and deterioration of, the product by general organisms to produce spoilage;
(2) growth in, or contamination of, the product by pathogens to produce food poisoning.

There have been relatively few instances of food poisoning in fruit juices or soft drinks, but microbial spoilage is very common. The previous edition of this book contained an excellent review and practical guide to the identification of spoilage problems in the soft drink industry by the late Professor Davenport (1998). The present review will therefore keep key parts of the previous text, enhancing it with recent data, and simplifying some of the information. For example, some new information on acid-tolerant bacteria such as *Alicyclobacillus acidoterrestris*, and data on the pathogens *Escherichia coli* O157:H7 and *Salmonella* spp. within fruit juices, merit examination, as do some novel processing methods.

11.2 Composition of soft drinks and fruit juices in relation to spoilage

There is a bewildering variety of soft drink and fruit juices for sale, and many methods for their manufacture. Soft drinks can be non-carbonated, carbonated,

with or without added fruit juice, often with the addition of organic acid preservatives. They can be filled on standard or clean fill lines. Fruit juices, fruit juice concentrates and fruit nectars may be fresh, unpasteurised and clean filled, or pasteurised, then hot, aseptic, or clean filled (Stratford *et al.*, 2000; Stratford and James, 2003). Recent technology using ultra-high pressure has been used to produce 'cold pasteurised' fruit juices. These have the advantage of a fresh juice mouthfeel, but with destruction of pathogens and the majority of spoilage agents, enhancing the shelf life of an essentially fresh product (Mermelstein, 1999; Zook *et al.*, 1999).

Simple soft drinks such as orangeade and lemonade are too acidic for the growth of most organisms, so that spoilage is generally by carbonation-resistant species such as *Dekkera anomala* (Stratford and James, 2003). Yeasts usually require a carbon source such as a hexose sugar, a nitrogen source such as amino acids or ammonium salts, simple salts (phosphate, sulphate, potassium and magnesium ions), trace minerals and vitamins. Some yeasts have particular sugar requirements; for example, *Z.bailii* and *Z.rouxii* cannot utilise sucrose (Pitt & Hocking, 1997; Stratford *et al.*, 2000).

Sugars have a protective effect on the heat resistance of yeasts and bacteria; this is an important consideration at higher concentrations of sugar. Soft drinks are often nitrogen poor and thus the addition of fruit juice greatly enhances the potential for spoilage. Some yeasts, for example *Dekkera bruxellensis*, can use nitrate. Phosphate levels are often low, trace minerals satisfactory, particularly in hard water areas. The low pH value of soft drinks and fruit juices, pH 2.5–3.8 (Table 11.1), inhibits most bacteria, but leaves yeasts unaffected. In soft drinks

Table 11.1 Examples of fruit and vegetable juice pH and risk organisms

	Approximate pH ranges	Risk organisms
Fruits		
Apples	2.9–3.91	Yeasts
Grapes	3.20–4.51	Yeasts
Oranges	3.20–4.3	Yeasts
Raspberries	3.12	Yeasts
Blackcurrants	2.48–3.60	Yeasts
Pineapples	3.3–3.7	Yeasts and bacteria
Mangoes	3.95–4.50	Yeasts and bacteria
Tomatoes	3.80–4.80	Yeasts, bacteria and moulds/bacteria
Vegetables		
Carrot	4.90–6.44	Bacteria
Celery	5.7–6.1	Bacteria
Cabbage	5.4–6.0	Bacteria
Pea	6.65–6.77	Bacteria

and fruit juices, oxygen levels are usually low, and CO_2 levels either low or very high (in carbonated soft drinks). Spoilage is therefore due to facultative anaerobes, organisms that can grow with or without oxygen. In carbonated drinks, mould and bacterial growth is very unlikely as they are very sensitive to CO_2.

The recent trend towards polyethylene terephthalate (PET) packaging presents its own problems. Typically PET containers cannot be hot filled; around 50°C is the upper temperature, although some recent types can be heated to 85°C. They are also permeable to oxygen (Rodriguez *et al.*, 1992), which allows the growth of aerobic spoilage agents.

11.3 Background microbiology – spoilage

Many micro-organisms are found in soft drinks as environmental or raw material contaminants, but relatively few can grow within the acidic and low-oxygen environment. Yeasts are the most significant group of micro-organisms associated with spoilage of soft drinks and fruit juices. Spoilage will be seen as the growth and production of metabolic byproducts, for example, CO_2, acid, and tainting compounds. As noted above, most spoilage is therefore by yeasts and mould species, with yeasts most important, and some spoilage is by acid-tolerant bacteria (Hocking & Jensen, 2001; Jay & Anderson, 2001).

11.3.1 Sources

Fruit and fruit juices are commonly contaminated with yeasts and moulds, often from insect damage. Fallen fruit should thus be avoided where possible, for all of the risks outlined below. Sugars and sugar concentrates are commonly contaminated with osmophilic yeasts, for example *Z.rouxii*. Growth is slow in concentrated solutions, but one cell per container of diluted stock is enough to cause spoilage (Davenport, 1996). Flavourings, water and other chemicals are all potential sources of microbial contamination. Process machinery and filling lines are particularly problematic and strict hygiene is essential.

11.3.2 Yeasts

There are over 800 species of yeasts currently described (Barnett *et al.*, 2000), but only about 10 are commonly associated with spoilage of foods prepared in factories operating good standards of hygiene and using correctly applied chemical preservatives (Pitt & Hocking, 1997). Others are found if something goes wrong during manufacture; for example, incorrect preservative level, poor hygiene or poor-quality raw ingredients.

Davenport (1996, 1997, 1998) describes a simple classification scheme for yeasts causing spoilage in the soft drinks industry, which, for investigative purposes, is used in Sections 11.6 and 11.7 and Appendices 11.1–11.4 in this chapter. He found that the yeasts isolated could be divided into four categories: Groups 1–4. Group 1 yeasts are described as spoilage organisms that are well adapted to growth in soft drinks, able to cause spoilage from very low cell numbers (as few as one cell per container). The characteristics of Group 1 yeasts are osmotolerance, aggressive fermentation, resistance to preservatives (particularly weak organic acids) and a requirement for vitamins. *Z.bailii* is a typical example of this group, and this group corresponds closely with Pitt and Hocking's (1997) 10 key spoilage yeasts. Davenport describes Group 2 organisms as spoilage/hygiene types, able to cause spoilage of soft drinks, but only if something goes wrong during manufacturing, for example, too low a level (or absence) of preservative, ingress of oxygen, failure of pasteurisation or poor standards of hygiene. Group 2 organisms are common contaminants in factories, but can be severely restricted if good manufacturing practices (GMP) are adhered to strictly. Group 3 organisms are indicators of poor hygiene standards. These yeasts will not grow in soft drinks, even if present in high numbers, and are typical of the yeasts found in many factories; the higher the count, the worse the hygienic state of the factory. Group 4 yeasts, called 'aliens' by Davenport, are those out of their normal environment. An example would be *Kluyveromyces lactis*, a dairy spoilage yeast. Table 11.2 details the most common organisms in each group.

Table 11.2 Examples of yeast species found in soft drink factory environments

Group 1 – Fermentation and preservative resistance	Group 2 – Spoilage and hygiene	Group 3 – Hygiene	Group 4 – Aliens
Dekkera anomala	*Candida davenportii*	*Aureobasidium pullulans*	*Kluyveromyces marxianus* (dairy yeast)
D. bruxellensis	*C. parapsilopsis*	*Candida sake*	*K. lactis* (dairy yeast)
D. naardenensis	*Debaryomyces hansenii*	*C. solani*	
Saccharomyces cerevisiae (atypical strains)	*Hanseniaspora uvarum*	*C. tropicalis*	
S. exiguus	*Issatchenkia orientalis*	*Clavispora lusitania*	
Schizosaccharomyces pombe	*Lodderomyces elongisporus*	*Cryptococcus albidus*	
Zygosaccharomyces bailii	*Pichia anomala*	*C. laurentii*	
Z. bisporus	*P. membranifaciens*	*Debaryomyces etchellsii*	
Z. lentus	*Saccharomyces bayanus*	*Rhodotorula glutinis*	
Z. rouxii	*S. cerevisiae*	*R. mucilaginosa*	

Sources: Based on Davenport (1996); Stratford & James (2003).

Table 11.3 Names of the most important yeasts within the soft drinks industry, with synonyms

Current Name	Synonym
D. bruxellensis	*Brettanomyces intermedius/B.bruxellensis*
D. naardenensis	*B. naardenensis*
Saccharomyces cerevisiae	*Saccharomyces carlsbergensis*
S. bayanus	*S. uvarum/S.heterogenicus*
S. exiguus	*Candida/Torulopsis holmii*
Zygosacchaaromyces bailii	*Saccharomyces bailii*
Z. bisporus	*Saccharomyces bisporus*
Z. lentus	*S. lentus*
Z. rouxii	*S. rouxii*
Torulaspora delbreuckii	*S. delbreuckii/S. rosei /Candida colliculosa*
Pichia anomala	*Hansenula anomala*
P. membranifaciens	*Candida valida*
Issatchenkia orientalis	*C. krusei*
Kluyveromyces marxianus	*C. kefyr*
K. lactis	*C. sphaerica*
Debaryomyces hansenii	*C. famata*
Pichia guilliermondii	*C. guilliermondii*
Hanseniaspora uvarum	*Kloeckera apiculata*
Metchnikowia pulcherrima	*Candida pulcherrima*

One of the most confusing aspects of mould and yeast taxonomy is the frequency with which names are changed. Many yeasts and moulds have sexual and asexual stages that may have different names. Sometimes, the association of a yeast with a particular genus is recognised as wrong, and a name change then occurs. The current trend is to name the organism by the sexual name, with the asexual stage described as a synonym. Thus, *Candida famata* (asexual) becomes *Debaryomyces hansenii* (sexual). It is important to be aware of this variation when conducting literature searches. Names do not always reflect the spoilage potential – *Saccharomyces cerevisiae* is an important yeast for the brewing and baking industries, but a disaster for the soft drinks and fruit juice industry. Table 11.3 details alternative names for important yeasts.

11.3.3 Bacteria

Bacteria that have been associated with spoilage in the soft drinks industry include Acetobacter, Alicyclobacillus, Bacillus, Clostridium, Gluconobacter, Lactobacillus, Leuconostoc, Saccharobacter, Zymobacter and Zymomonas (Vasavada, 2003). Gluconobacter is a common spoilage agent of fruit juices; it is a strict aerobe, requiring free oxygen (Stratford *et al.*, 2000).

Alicyclobacillus spoilage is becoming an important issue in heat-treated fruit juices, and the bacterium is commonly found contaminating fruit in the field. Growth of the organism is associated with the production of antiseptic and 'smoky' taints within juice. The former is due to 2,6-dibromophenol (2,6-DBP), giving a 'TCP' flavour (Jensen & Whitfield, 2003); the latter is due to guaiacol (2-methoxyphenol) (Jensen, 1999). Heat shock, taint precursors, incubation temperature and oxygen are all important factors in the production of taints (Jensen, 1999).

Alicyclobacillus acidoterrestris is a thermotolerant, spore-forming aerobic organism that can survive fruit juice heat treatments to grow in the juice. Spoilage can occur from inoculum levels as low as 1 spore per 10 ml (Pettipher & Osmundon, 2000; Walls & Chuyate, 2000a). Growth minima and maxima at temperatures of 26–50°C, minimum pH 2.0, D_{95} of 2.4 min. Isolation methods have been compared (Pacheco, 2002; Pettipher & Osmundson, 2000; Silva & Gibbs, 2001, Walls & Chuyate 2000). Effective control can be achieved by rapid chilling of juice to below 20°C after pasteurization, use of sorbate or benzoate, removal of oxygen or addition of ascorbic acid (Cerny *et al*., 2000) or by using an appropriate pasteurisation regime (Silva & Gibbs, 2001). Surface disinfection of fruit using chlorine or 4% peroxide has also proved effective (Orr & Beuchat, 2000).

11.3.4 Moulds

Mould problems can be divided into two types: growth of a variety of moulds due to poor hygiene within the factory or field environment, and growth of heat-resistant moulds within heat-processed juices. The former type can cause tainting, discolouration and other general problems associated with gross mould growth. The latter type can result in slow growth of the mould within the processed product. There is some overlap between the two groups. Xerophilic (highly sugar-tolerant) fungi are likely contaminants if hygiene is poor.

Heat-resistant moulds able to cause spoilage of fruit juices and soft drinks include *Aspergillus ochraceus*, *Aspergillus tamarii*, *Aspergillus flavus*, *Byssochlamys nivea*, *Byssochlamys fulva*, *Paecilomyces variotii*, *Neosartorya fischeri*, *Eupenicillium brefeldianum*, *Phialophora mustea*, *Talaromyces flavus*, *Talaromyces trachyspermus* and *Thermoascus aurantiacum*. Others include *Penicillium notatum*, *Penicillium roquefortii* and *Cladosporum* spp. (de Nijs *et al*., 2000; Pitt & Hocking, 1997; Stratford *et al*., 2000). Mould spoilage within the factory is associated with poor hygiene. Various types of heat-resistant spores can be produced: ascospores, chlamydospores and sclerotia.

Many of the above moulds are found on fruits pre- and post-harvest. Most moulds require oxygen to grow. Growth is exhibited as surface mats, sometimes

producing copious spores. Some moulds produce extracellular degradative enzymes, such as pectinases. Detection of heat-tolerant moulds is usually carried out by plating out a sample of heat-shocked juice. Mould identification is carried out by reference to standard morphological texts, and is difficult unless carried out by experienced personnel. An excellent scheme that combines basic physiology with simple morphological attributes was developed by Pitt and Hocking (1997). It uses growth on three media at three temperatures to differentiate moulds: 5, 25 and 37°C, a high sugar content (xerophilic), a closely defined medium with nitrate as a nitrogen source and sucrose as hexose sugar, and a general medium. This technique is particularly useful for the differentiation of *Penicillium* species.

11.3.4.1 Mycotoxins

Mycotoxins are toxic secondary metabolites produced by fungi growing within or on foods. They can be a serious threat to human and animal health (Nagler *et al*., 2001). Table 11.4 details mycotoxins associated with soft drinks and fruit juice manufacture and raw materials. Patulin is the most common mycotoxin associated with fruit juice, particularly apple juice (Pitt & Hocking, 1997). It commonly occurs if juice is produced from stored apples. Mould growth in infected apples increases with time, raising levels of patulin. The use of windfall apples for juice is also a factor. Avoidance of windfall apples, filtration of juice and pressing quickly after harvest are all methods to reduce the incidence of patulin in juice. Patulin can be destroyed by fermentation to cider or by the addition of ascorbic acid (Marth, 1992). Within Europe, the European Union has set a limit of 50 μg/kg for patulin in both apple juice and cider. A recent survey of apple products in Chile found that 28% of samples of juice and concentrate exceeded this limit (Canas & Aranda, 1996).

Table 11.4 Examples of toxin-producing moulds associated fruit with fruit raw materials and soft drinks products

Fruit(s)	Mould	Toxin(s)
Apple	*Penicillium expansum*	Patulin, citrinin, roquefortine C
Citrus	*P. digitatum*	Trptoquivalins
Carbonated beverages	*P. glabrum*	Citromycetin
Bottled water	*P. roqueforti*	Roquefortine C
Diluted fruit/water beverages	*P. roqueforti*	Isofumigaclavine A and B
Treated orange juice	*Fusarium oxysporum*	Oxysporone
Fruit juices	*Aspergillus versicolor*	Geosmin sterigmatocystin

11.4 Microbiological safety problems

E. coli and enterococci have been isolated from citrus juices, and apple juice has been associated with Cryptosporidiosis. Contamination by hepatitis A and Norwalk-like viruses has been reported in fruit juices (Vasavada, 2003).

11.4.1 Escherichia coli

There have been a number of cases of food poisoning associated with pathogenic *E. coli* O157 contamination of fresh apple juice (Battey & Schaffner, 2001; Czajka & Batt, 1996; Miller & Kasper, 1994; Parish, 1997, 2000; Splittsoesser *et al*., 1996; Zhao *et al*., 1993), which was found to be able to grow in apple juice with low pH. The organism potentially has a low infective dose of $<$100 cells, and this, coupled with the occasional severe side-effect of haemolytic uraemic syndrome, makes *E. coli* O157 a severe hazard within unpasteurised juices.

11.4.2 Salmonella

Salmonella is generally only a problem in fresh, unpasteurised fruit juices, due to its low thermal tolerance. There have been three major outbreaks of Salmonellosis, all from unpasteurised orange juice, in the United States, Canada and Australia, involving 62, 298 and 400 reported cases in 1995 and 1999 (Bell & Kyriakides, 2001). *Salmonella* Hartford, *Salmonella* Muenchen and *Salmonella* Typhimurium were involved. The main risk factors were associated with the fertilization of agricultural crops: crops were grown in orchards where sheep grazed, manuring the soil. Fallen fruit were often used, allowing soil and faecal contamination. In addition, poor decontamination of fruit occurred in the factory; poor-quality wash water was used; pest control within the factory was poor; and the cleanup of fruit boxes and conveyors was poor. Data indicated that *Salmonella* could survive for up to 300–968 days in soil treated with animal slurry. Other studies showed that fresh juice had to be stored for 15–24 days at 4°C to achieve a 10^6 reduction from 10^7 to 10^1 (Bell & Kyriakides, 2001).

11.5 Control Measures

Control measures include maintaining the cleanliness of equipment, the control of storage temperature, hot water immersion, chemical sanitizers, surfactants, surface waxes (particularly on oranges) and UV irradiation. The chemical preservative dimethydicarbonate (Velcorin) has been used to ‘cold pasteurise’ fresh juice products to reduce microbial loading, minimising the use of sulphur dioxide (Threlfall & Morris, 2002; Vasavada & Heperkan, 2002). High-pressure

processing, a pulsed electric field and ultraviolet light have also been investigated as control measures. Modelling of yeast and bacterial growth has been used as a technique for determining effective preservative and other control regimes (Battey & Schaffner, 2001; Shearer, *et al*., 2002).

Good hygienic practices and adherence to GMPs are the most effective control measures for microbial contamination in the soft drinks industry, particularly for yeasts. Sticky sugar and fruit residues are ideal food sources for yeasts and moulds. The Hazard Analysis and Critical Control Point (HACCP) approach has been adopted by food processors around the world. In the United States, HACCP is mandaory for fruit juice processors, with good agricultural practices (GAP) as the foundation of a successful HACCP system. In Europe, growers, distributors and packaging houses must meet the EUREGAP protocols if they wish to be certified to sell their products to certain markets or established buyers (Stier & Nagle, 2003).

11.6 Forensic grouping and applications

11.6.1 Forensic grouping

Forensic grouping involves consideration of simple behaviour patterns of micro-organisms as evidence for decision-making. Three answers are obtained from each test, namely, activity (growth/no growth), potential significance/role (related to product and/or product environment) and tentative recognition/identification (i.e. most probable genus; sometimes the name of five possible species). Emphasis is placed on simple economic methods where selected raw materials and products can be used as liquid or solid media. Some commercial bottles and containers can be used in place of expensive laboratory glassware. This latter method, combined with some conventional testing, gives the best evidence for decision-making, rather than providing data for historical records. The forensic system is also a practical approach that marries well with HACCP procedures.

11.6.2 Group formats

Each micro-organism group has a basic behaviour pattern common to all members. Members will have other characteristics that separate them as individuals. However, the group system is best operated in a series of simple steps where behaviour patterns are determined by a combination of some conventional identification methods and technology parameters (e.g. preservative resistance/sensitivity). Each step provides evidence for current use but can also indicate the best direction for further testing steps in both technology and taxonomy. Table 11.5 gives the essential patterns of the micro-organism groups that are suitable for soft drink and fruit juice products (see appendices for further details).

Table 11.5 Baseline behaviour patterns of the most common micro-organisms associated with soft drinks and fruit juices (Forensic Groups for Practical Applications and Assessments)

Group	Status	Behaviour pattern						Micro-organisms
		Fe	Fi	Pr	Ac	Os	Vi	
1	Spoilage	+	−	+	−	+	+	*Zygosaccharomyces bailii* *Saccharomyces cerevisiae* *Torulaspora delbrueckii* *Schizosaccharomyces pombe*
2	Spoilage and hygiene	−	+	−	+	±	−	*Pichia anomala* *P. membranaefaciens* *Debaryomyces hansenii* *Acetic acid bacteria*
3	Hygiene	−	±	−	+	−	−	*Geotricium candidum* *Trichosporon* spp. *Rhodotorula glutinis* (red/pink yeast) *Bacillus* spp. (bacteria with spores)
4	Special Indicators	+	+	±	+	−	+	*Kluveromyces marxianus* (dairy yeast) *Brettanomyces/Dekkera anomalus* (brewery origin)

Table contents modified from Davenport (1995a,b, 1996, 1997).
Fe = fermentation (strong, i.e. in presence of/production of <5% v/v alcohol).
Fi = surface film formation.
Pr = preservative resistance, principally common chemical agents, e.g. benzoic acid.
Ac = acitidione (antifungal compound) >10 ppm.
Os = growth at high osmotic pressure sugar >60°Brix.
Vi = one or more B group vitamins required.
Note: All B group vitamins are essential for yeast growth. Some yeasts can synthesize all their requirements (e.g. *Pichia anomala*); the remainder can synthesize only part of the vitamin B group, hence the remainder of the set must be supplied externally.

11.7 Suggested test programme

11.7.1 Packaged products

11.7.1.1 Spoilage symptoms: dominant feature fermentation

Action points:

(i) Check formulation data and. other product history.
(ii) Assess spoilage incidence, that is, spasmodic, increasing, major, etc.
(iii) Plan an appropriate programme, that is, sample:
- direct microscopy
- record cell shape
- follow outline as in Table 11.6

Table 11.6 Scheme for soft drink forensic microbiological investigations

1	All possible information on history, analyses and other documentation should be assessed, i.e. environmental characteristics and micro-organism characteristics together determine the outcome of the organism.
2	An overall objective should be stated based on (1). In practice, this objective will have to be divided into a series of sub-objectives. The most efficient practical test regime(s) can then be designed to meet the objective(s).
3	The investigation should be designed on the following criteria:
3.1	Both academic and technical aspects must be covered (academic = foundation of good recognised knowledge for some routine testing plus 'extra' input to allow data/responses to be assessed for future needs; technical = testing designed specifically for the objective in hand).
3.2	Testing programmes should be significant, i.e. designed to suit the problem, not to copy an accepted publication. Too often the preoccupation with standard methods to get counts within range or correct names can be expensive and time-consuming.
3.3	Testing should be kept as simple as possible.
3.4	Staff should be suitably trained in bench skills and should have a clear simple under standing of beverage microbiology.

(iv) For spasmodic spoilage outbreaks, the capping system is likely to be the most frequent source of contamination.

(v) Method for (iv)
Place a new autoclave bag over the end of the closure jaws. Take a sterile swab and knock about 30–50 caps into the bag. Pour on sterile mixture of 80% product plus 20% water. Tie up the bag and leave in a warm place at 22–26°C. Observe for up to one week. If no fermentation occurs, then caps are an unlikely contamination source. If fermentation does occur, then action is required. If results indicate activity, this is evidence that technology needs attention. The most probable identity of the micro-organisms is restricted to a very few in Group 1 (see Table 11.5).

(vi) Major spoilage outbreaks mean either an inefficient cleaning programme or a possible formulation fault.

(vii) With (vi) a survey is required to determine whether this spoilage is restricted to one or two places or is more widespread.

11.7.1.2 No obvious symptoms

11.7.1.2.1 Clear products – suitable for membrane filtration. Action points:

(i) Proceed with routine membrane filtration test.

(ii) Count colonies on the membrane or place the membrane in a product/water mixture and observe for fermentation (Section 11.7.1.1(v)) or

(iii) Pour plates: count colonies, recount colonies that look like 'stars' and calculate the percentage of stars. This percentage gives an index of strong fermentative organisms.
(iv) Pour plate contents may be put into product/water mixture and observed for fermentation (Section 11.7.1.1(v)).
(v) For further identification the test in Table 11.6 can be used.

11.7.1.2.2 Fruit juices and unfilterable drinks. Split bottle method. Action points:

(i) Proceed with routine test.
(ii) Take a very clean container (plastic or glass bottle). Carefully decant half the product contents into the container. Add about quarter sterile water to one bottle – both bottles will have heat spaces. Leave for one week at 22–26°C. Observe for fermentation.
(iii) Further identification can be carried out based on the behaviour of the organism in different media (see Table 11.7).

11.7.2 Key raw materials

Raw materials are the main means of carrying spoilage yeasts into the factory. Materials include liquid sugars, juice concentrates, comminutes and natural colors and flavours. Sample size is 250 ml (minimum).

Table 11.7 Group pattern-making: simultaneous assessment of behaviour and tentative identification of yeasts (part of a model for soft drinks)

Groups	Fermentation YM broth or product	YM	YMS	YM1	YM60	ACT	Sexual
1	+	+	+	+	+	−	+
2	±	+	±	−	±	±	+
3	−	+	−	−	−	+	±
4	+	+	+	−	−	+	+

AGARSYM
YM (oxoid difco).
YM5YM + 0.5% acetic acid (add just before pouring plate).
YM1YM + 1% acetic acid (add just before pouring plate).
YM60YM + 60% (w/w) glucose.
ACT: Actidione agar
Sexual states: ascospores for Zygosaccharomyces, Saccharomyces, Torulaspora, Schizosaccharomyces, Pichia, Debaromyces, Geotrichum (very rare), Kluveromyces and Dekkera (rare).

Action points:

(i) Collect data.
(ii) Make up a solution of the raw material at 20°Brix using sterile water or glucose solution
(iii) Add 0.5 ml if 10% yeast extract solution (10 g yeast extract in 100 ml water) and autoclave 115ºC for 10 min) to ensure sufficient vitamins and nitrogen sources.
(iv) Set up a test series as shown below to register the presence or absence of Group 1 organisms

	Additions			
	None	5% Ethanol	1% acetic acid	2% acetic acid
Zygosaccharomyces bailii group	+	+	+	+
Saccharomyces cerevisiae	+	+	−	−
Atypical *Saccharomyces cerevisiae*	+	+	−	−
Schizosaccharomyces pombe (fission cells)	+	+	+	+

11.7.3 Environmental samples

The main sites to be inspected for dominant microflora include

- drains
- raw material store
- near filler
- soap lubrication from jets
- electric motor fins (all areas)
- bottle tracks
- ultraviolet insectocutors
- soak baths
- capping systems
- swabs from brushes and squeegees.

Action points:

(i) Swabs may be smeared across a series of agar plates to give evidence for activity, technology and identity simultaneously (see example given below).
(ii) Swabs must be returned to their cases and an appropriate liquid medium (e.g. YM ± modification) or product water mixture added. The samples are then observed for fermentation.
(iii) Liquid and debris samples can be streaked out on agar plates (use swab) and an enrichment culture of YM + 20% (w/w) used to select yeasts.

Example: suggested solid (agar) medium pattern for soft drink and related beverages.

YM (YM1) YM + 1% acetic acid (add acid just before pouring)
(YM20) YM + 20% (w/w) glucose
(YM40) YM + 40% (w/w) glucose
(YM60) YM + 60% (w/w) glucose
(ACT) actidione agar (Oxiod)

Characteristics of strains of spoilage yeasts

	YM	YM1	YM20	YM40	YM60	ACT
Zygosaccharomyces bailii	+	+	+	+	+	−
Saccharomyces cerevisiae	+	±	+	±	±	−
Pichia anomala	+	−	+	+	−	−
Torulaspora delbrueckii	+	−	+	±	−	−
Hygiene yeasts						
Aureobasidium pullulans	+	−	±	−	−	+
Rhodotorula clutinis	+	−	+	−	−	+
Geotrichum candidum	+	+	+	−	−	+

+ = growth; − = no growth.

11.8 Conclusions

This chapter has set out one approach to conventional microbiological testing and identification of organisms within the soft drinks industry. A choice can be made, therefore, between classical testing regimes, which are time-consuming and expensive, and a simple programme that gives evidence in the form of an assessment in the areas of activity/no activity, technology significance and tentative identity.

The time-scale for this approach ranges from a few minutes (sample data plus direct microscopy) up to a maximum of 5–7 days, without the need for obtaining pure cultures. Sample sizes can be large or small. With conventional testing, which includes most high-technology microbiological methods, a pure culture (which takes about 3 weeks) has to be used to ensure that only the characteristics of the individual organism are determined. The conventional approach presumes that once an organism is identified other conclusions are readily obtained. It has, therefore, been seen as an essential part of the problem-solving process.

In identifying dominant important organisms for the soft drinks industry, there are many pitfalls for the inexperienced yeast microbiologist. Recently, there have been three major investigations of beverage and food problems that have cost the industry millions of pounds in lost product and raw materials. These losses were due to inexperience in the identification of micro-organisms and making appropriate assessment and recommendations. Other limitations of

classical or conventional methods are time (e.g. up to 3 months required for yeast identifications) and the expense of a programme involving some 80–90 individual tests and observations per organism.

For routine monitoring of raw materials, packaged product and processing and packaging environments, a simple forensic microbiological programme is, with appropriate training, straightforward to set up. The system works towards prevention and prediction at an economic cost. At any point the system is flexible enough to add or subtract any other simple method and, if necessary, to introduce more conventional programme test(s).

Appendix 11.1 Conclusions from environmental audit inspections

Table 11A.1 Examples of management indicators for conclusions of environmental audits

Geotrichum candidium *Trichosporon* spp. Acetic acid bacteria Common moulds	Unsatisfactory
Pichia anomala *Pichia membranaefaciens* Acetic acid bacteria	Cleaning programme not efficient
Aureobasidium pullulans *Rhodotorula glutinis* *Debaryomyces hansenii* *Cryptococcus* spp.	Excessive dust-like particles

The factory hygiene status in the right hand column of Table 11A.1 predicts, in the left hand column, the most likely microflora to be found. Conversely, a predication of factory status can be made if the corresponding microflora are present.

Appendix 11.2 Examples of environmental microbial indicators

During environmental audit inspections residues may be observed. The predicted dominant micro-organisms are those in the left-hand column of Table 11A.2.

Table 11A.2 Examples of environmental indicators

Zygosaccharomyces spp. *Saccharomyces cerevisiae* strains *Schizosaccharomyces pombe*	Residues Fruit concentrates Sugars syrups
Kloechera apiculata *Metchnikowia pulcherrima*	Fresh fruit and juices
Saccharomycopsis lipolytica *Kluyveromyces* spp.	Oil and/or dairy products

Appendix 11.3 Simple recognition and identification of Group 1–4 micro-organisms

This brief outline model demonstrates how simple systems can be designed to give a practical application for micro-organism recognition and identification.

Observations

Fermenting soft drink
preserved with benzoic acid

Assessment of microorganisms

1. Predicted spoilage yeasts
   Zygosaccharomyces bailii (Zb)
   Z. bisporus (Zbi)
   Z. rouxii (Zr)
   Atypical *Saccharomyces cerevisiae* (ASc)
   S. *cerevisiae* (Sc)
   Torulaspora delbrueckii (Td)
   Schizosaccharomyces pombe (Sz)

Direct microscopy

Fission cells = *Schizosaccharomyces pombe*
Budding cells

2. Set up split bottles test with 80% sterilised product and 20% distilled water (v/v) as follows:

Split bottle test separation of yeasts

		Zb	Zbi	Zr	ASc	Sc	Td
2.1	Product + water only	+	+	+	+	+	+
2.2	Product + water + 5% (v/v) ethanol	+	+	+	+	+	+
2.3	Product + water + 1% (v/v) acetic acid	+	+	−	+	−	−
2.4	Product + water + 2% (v/v) acetic acid	+	−	−	−	−	−

NB: Zb, Zbi and ASc are very similar in behavioural traits, and therefore collectively can be called the 'bailii group' since assessments and subsequent action would be the same for one or more members present.

3. Confirmation test

Growth on solid medium/microscopy

		Zb*	Zbi*	ASc**
3.1	YM agar	+	+	+
3.2	YM agar + 1% (v/v) acetic acid	+	−	−
3.3	YM agar + 2% (v/v) acetic acid			

Microscopy

*Spores with conjugation tubes
**Spores without conjugation tubes

Observations
Swabs from factory environment can be immersed in the product/water mixture as 2.1–2.4

Assessment of micro-organisms
Assess as for products, i.e. absence/presence of one or more Group 1 organisms

Note any fermentation

Surface growth (colonies on plates)	**Microscopy (cell-shapes)**	**Yeasts (yeast-like fungi)**
Red/pink	Oval-round budding cells	*Rhodotorula glutinis*
Very rough, almost mould-like. Wrinkled, often tough cream-brown	Threads with cross-walls. Large splitting cells. Budding cells	*Trichosporon* spp.
White/grey, dusty	Box shapes with cross-walls. Some threads with cross-walls	*Geotrichum candidum*
Rough brain/cauliflower-like, white/dirty cream sometimes colony top chocolate brown *or* smooth creamy pearl shape	Mixture of cell shapes and sizes (budding). Hat-shaped ascospores	*Pichia anomala*
	Short fat cylindrical cells. Short chains of budding cells	*P.memberanaefaciens*
Smooth white/cream/ brownish conical shape	Circular, often with ring of buds on mother cell surface	*Debaryomyces hansenii*
White/cream/brownish wrinkled/striated to rough	Oval and short cylindrical	*Kluyveromyces marxianus*
Rough, small, often slow growing colonies. Distinctive smell – acidic/ wine/mouse	Mixture of cell shapes and sizes. Some pointed at one end (ogive)	*Brettanomyces anomalus*

Recognition/identification separation tests for yeasts of Group 2–4 micro-organisms

	Observations/Tests					
Yeasts	Colony	Fe	Fi	Cells	44°C	Acid Pr
Pichia anomala	S/R	+	+	M	−	−
P. membranaefaciens	R	−	+	M	−	−
Debaryomyces hansenii	S	−	−	C	−	−
Kluyveromyces marxianus	S	+	−	M	+	−
Brettanomyces anomalus	S/R	+	+	M	−	++

Code
S = Smooth colonies
R = Rough colonies
Fe = Fermentation in presence of 5% (v/v) ethanol:product or medium
Fi = Film formation

Cells
M = Mixture of shapes
C = Circular/short oval shape
Acid Pr = Acid production: confirm with YM or WA or ME agar +0.5% calcium carbonate

Recognition of indicator organisms not included in the foregoing table

	Recognition/identification				
Yeasts	Act	Col	Fe	Cells	Fat splitting
Aureobasidium pullulans	+	F−	L−	M*	+weak
Kloechera apiculata	+	S	+	L	−
Metchnikowia pulcherrima	+	S(Pi)	+	C/O	−
Saccharycopsis lipoltica	+	R	−	M	+++ strong
Cryptococcus spp. (**not** pink forms)	+	Mu	−	C	± weak

Code
Act = Growth on acitidione agar
Col = Colony type
F = Flat
Fe = Fermentation
S = Smooth colonies
S(Pi) = Red/purple pigment, if enough iron and biotin are present
L = Lemon-shaped
C = Circular
O = Oval
R = Rough
M* = Mixture of large budding cells

Moulds – various
Woolly, hair, dusty mat-like – colours and textures vary according to mould type. Common examples on

Apple	(Blue)	*Penicillium expansum*
Oranges	(Blue/green)	*P. italicum*
Onions	(Black)	*Aspergillus niger*

Common bacteria

Colonies	**Microscopy (cells)**	**Bacteria**
Transparent/watery buff/fawn/smooth	Mixture of rod shapes	*Acetomonas* spp.
Small/cream/brown Smooth to wrinkled	Predominantly small fat rods	*Acetomonas* sp.

Appendix 11.4 Media for cultivation of industrial micro-organisms[a]

Bacteria	**Media** (Oxoid or Difco or other commercial brands)
Acetic acid bacteria }	Actidione agar (PM1 118); also certain yeasts can be detected
Lactic acid bacteria }	(Difco–WLD–B425)
	Wort agar (CM247)
	Malt extract agar (CM59)
	Tomato juice agar (CM133)
	Rogosa agar (PM221)
	MRS agar (CM361)
Other bacteria[b]	Plate count agar (CM 183)
	Nutrient agar (CM3)

Yeasts and fungi	Actidione agar (PM118) (e.g. *Kloeckera and Brettanomyces* spp.) contains 10 ppm actidione (cycloheximide)	
	WLD (Difco–B425) contains 4 ppm Actidione (cycloheximide)	
	Wort agar (CM 247)[c]	
	Malt extract agar (CM 59)[c]	
	Corn meal agar (CM 103)	for mycelium formation
	Potato dextrose agar (CM 139)	
	WL agar (Difco–B424) (CM309)	
	Buffered yeast agar (CM 153)	

Storage medium for yeast cultures (MYGP/YM medium)

(See Wickerham, 1951)

Malt extract (powdered)	3 g
Yeast extract (powdered)	3 g
Glucose	10 g
Peptone (mycological)	5 g
Water	1000 ml

for solid medium (20 g) is added

YM agar (Difco–0712)
YM broth (Difco–0711)
Antimicrobial supplement to restrict bacteria, some moulds thus giving maximum chance for soft drink yeast – use 20% (w/w) glucose in solid and liquid YM.

[a] Based on data in Davenport (1980).
[b] For assessment of wide range of microbes (many yeasts and moulds grown on these).
[c] pH 4.5 or 3.5 (use of concentrated HCL two drops per 100 ml before pouring plates can help with selection of yeasts of particular industrial significance in some products).

References

Battey, A.S. and Schaffner, D.W. (2001) 'Modelling bacterial spoilage in cold-filled ready to drink beverages by *Acinetobacter calcoaceticus* and *Gluconobacter oxydans*.' *Journal of Applied Microbiology* **91**(2), 237–247.

Bell, C. and Kyriakides, A. (2001) *Salmonella: A Practical Approach to the Organism and its Control in Foods*, Blackwell Science, Oxford, 336pp.

Canas, P. and Aranda, M. (1996) 'Decontamination and inhibition of patulin-induced cytotoxicity.' *Environmental Toxicology and Water Quality* **11,** 249–253.

Cerny, G., Duong, H.-A., Hennlich, W. and Miller, S. (2000) '*Alicyclobacillus acidoterrestris*: influence of oxygen content on growth in fruit juices.' *Food Australia* **52**(7), 289–91.

Czajka, J. and Batt, C.A. (1996) 'A solid phase fluorescent capillary immunoassay for the detection of *Escherichia coli* O157:H7 in ground beef and apple cider.' *Journal of Applied Bacteriology* **81**(6), 601–7.

Davenport, R.R. (1980) An outline guide to the use of media for yeasts and yeast-like organisms, in *Biology and Activities of Yeasts* (ed. F.A. Skinner, S.M. Passmore and R.R. Davenport), Academic Press, London.

Davenport, R.R. (1995a) Forensic investigation for yeasts. European Commission Science Research Development, *Proceedings of Meeting on Fungal Identification Techniques*, Barcelona, Spain.