Chapter 5
Organisms Associated with Acetic Acid Bacteria in Vinegar Production

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5.1 Introduction

Vinegars are the product of scalar fermentations carried out by several groups of microorganisms acting at different moments in time. The initial phase is generally represented by an alcoholic fermentation commonly carried out by yeasts. Lactic acid bacteria (LAB) can also play a role in releasing ethanol and acetic acid from heterofermentative lactic acid fermentations. Depending on the nature of the substrate, the production of ethanol can be preceded by a transformation that induces the release of fermentable sugars from complex substrates. This is the case of rice vinegars, which require the action of some moulds of the genus *Aspergillus* to break the starch into fermentable sugars. The ethanol originating from the alcoholic fermentation is finally oxidized by acetic acid bacteria (AAB) and the alcoholic beverage is turned into vinegar (see Table 5.1). Even though acetic acid bacteria play the leading role in vinegar production, the metabolic activity of yeasts, moulds and lactic acid bacteria is also crucial for guaranteeing the manufacture of the product. These microorganisms, in fact, modify the fermentative substrates in order to allow the final stage of ethanol oxidation. This chapter provides an overview of their taxonomy, their nutritional requirements, their metabolic activity and their relevance in the vinegar manufacturing process (see Table 5.2). Brief descriptions of vinegar eels and *Drosophila* are also given to complete the variety of organisms that are involved in vinegar production.

Table 5.1 Summary of the technological steps in vinegar production, the microorganisms involved, and their metabolic activity

Step 1[a]	Step 2	Step 3
Saccharification	Ethanol production	Acetic oxidation
Aspergillus spp.	Yeasts (alcoholic fermentation)	Acetic acid bacteria (AAB)
	Lactic acid bacteria (LAB) (heterofermentative lactic fermentation)	

[a] Necessary only for substrates containing complex sugars.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

Table 5.2 Microorganisms involved in the production of vinegars obtained from different substrates

Substrate	Step 1	Step 2	Step 3
Apple	NA	Yeast	AAB
Cassava	NA	Yeast/LAB	AAB
Grapes	NA	Yeast	AAB
Honey	NA	Yeast	AAB
Malted barley	NA	Yeast	AAB
Palm	NA	Yeast	AAB
Rice	*Aspergillus* spp.	Yeast	AAB
Tea	NA	Yeast	AAB
Whey	NA	Yeast/LAB	AAB

NA, not applicable. AAB, acetic acid bacteria. LAB, lactic acid bacteria.

5.2 Yeasts

Yeasts are fungi with vegetative states that predominantly reproduce by budding or fission, which results in growth that comprises mainly single cells. The variety of yeasts is rather large: the current classification embraces over 700 species grouped in approximately 70 genera (Kurtzman and Fell, 1998).

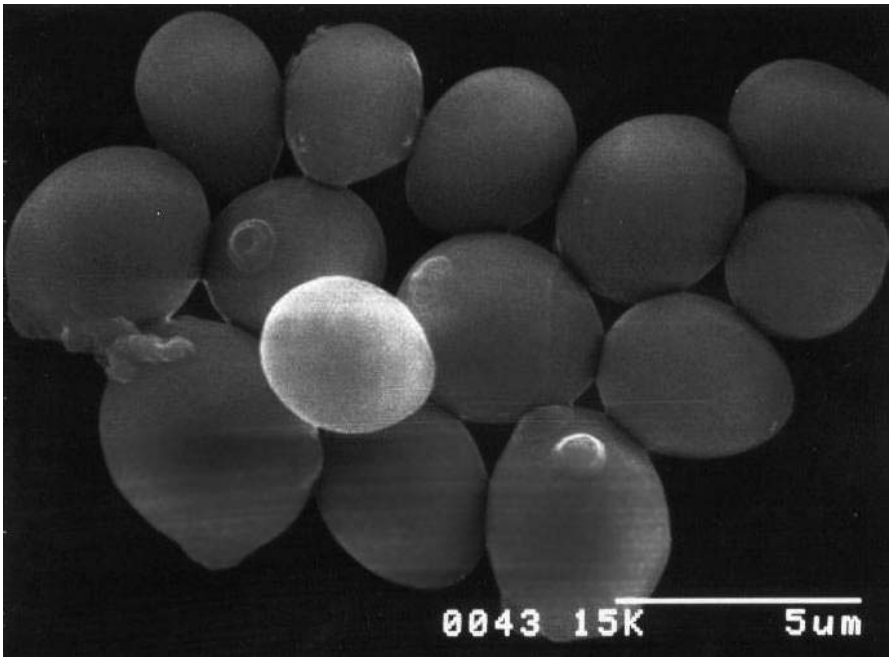

Figure 5.1 *Saccharomyces cerevisiae* wine yeast cells photographed under a scanning electron microscope (SEM) at 6000× magnification

Most yeasts, as well as most fungi, can reproduce both sexually and asexually. Often only one method of reproduction is observable at a specific moment in time or under specific environmental conditions. A fungus that reproduces mainly sexually is referred to as teleomorphic; a fungus that reproduces solely asexually as anamorphic; and a fungal form combining both states is referred to as holomorphic. Figure 5.1 shows the cells of the typical yeast *Saccharomyces cerevisiae*.

5.2.1 Yeast Classification

Yeasts belong to the Kingdom of Fungi, or *Eumycotes*, which has traditionally been divided into the following five divisions, depending on the mode of sexual reproduction:

- *Ascomycota*
- *Basidiomycota*
- *Chytridiomycota*
- *Glomeromycota*
- *Zygomycota*

Recently some rearrangements of such a grouping has been proposed: the divisions *Ascomycota* and *Basidiomycota* have been grouped under a subkingdom named *Dikarya*, reflecting the putative synapomorphy of dikaryotic hyphae. Moreover, groups that had commonly been included in the phyla *Chytridiomycota* and *Zygomycota* have been shifted to new groups; in particular, taxa traditionally placed in *Zygomycota* are now distributed among *Glomeromycota* and in several subphyla *incertae sedis* (Hibbett et al., 2007).

The fungi that are of interest in vinegar production are placed within the phylum *Ascomycota*. In particular, yeasts are mostly part of the subphylum *Saccharomycotina*, which includes the class *Saccharomycetes*. The organization of this class is shown in Table 5.3.

Table 5.3 Current classification of the class *Saccharomycetes*

Subphylum	Order	Family	Genera
Saccharomycotina	*Saccharomycetales*	*Ascoidaceae*	1
		Cephaloascaceae	2
		Dipodascaceae	4
		Endomycetaceae	4
		Eremotheciaceae	5
		Lipomycetaceae	9
		Metschnikowiaceae	6
		Phaffomycetaceae	3
		Saccharomycetaceae	53
		Saccharomycodaceae	9
		Saccharomycopsidaceae	7

From: Eriksson and Winka, 1997; Hibbett et al., 2007

5.2.1.1 Genus

Yeast identification at the genus level is based on some simple determinations:

- mode of multiplication
- number of spores per ascus
- spore shape
- mode of sporulation (direct transformation of the cell into an ascus or by sexual conjugation followed by the conversion of the cells into asci)
- ability to ferment some sugars.

5.2.1.2 Species

Until the 1980s, yeast species were defined exclusively on the basis of yeast phenotypic characteristics, the most important of which being the ability to ferment and assimilate a variety of carbon sources (e.g. glucose, galactose, maltose, sucrose, lactose, melibiose and raffinose). Yeast taxonomists have always been aware that the merely phenotypic characterization of cultures was not exhaustive for establishing the taxonomic status of yeasts; however, up to the classification of Kreger van Rij (1984), the determination of phenotypic characteristics was the only approach used in yeast taxonomic studies. Starting from the early 1990s novel approaches based on yeast genotypic characterization have been developed and successfully employed in yeast taxonomy. In particular, the development of molecular techniques, such as nucleotide sequencing and polymerase chain reaction (PCR), has allowed us to study the yeast genome in depth and to detect DNA regions with a large amount of interspecific polymorphism. The polymorphism of these regions, located in specific portions of the ribosomal DNA (rDNA), namely within the subunits 26S and 18S (the internal transcribed spacer – ITS; the non-transcribed spacer – NTS) and in a small portion of the subunit 26S (D1/D2 region), detected by sequencing or restriction fragment length polymorphism (RFLP) have recently been given a major taxonomic value. In addition, the determination of the chromosome banding pattern by contour-clamped homogeneous electric field (CHEF), the DNA composition (expressed as percentage of G+C), and the DNA reassociation value are considered useful tools in yeast taxonomy. The employment of a polyphasic approach in yeast taxonomy, which includes the determination of several genotypic characteristics, as well as a classical phenotypic characterization, is currently considered the most rational and accurate way to proceed in yeast species determination. For an overview of the techniques currently used for yeast species determination see Giudici and Pulvirenti (2002).

5.2.2 Growth and Nutritional Requirements

5.2.2.1 Temperature

Yeasts are typical mesophilic organisms with an optimum temperature for growth ranging between 20 °C and 40 °C. Yeasts do not generally demonstrate optimal

temperatures for growth higher than 40 °C, and therefore cannot be considered as thermophilic microorganisms; or below 20 °C (with the only exception being a few strains isolated from Polar environments), and therefore cannot be considered as psychrophilic microorganisms.

However, some yeast species can still grow and be metabolically active at extreme temperatures. Some species of the genus *Kluyveromyces*, for example, maintain the ability to reproduce and to ferment far above 40 °C. Some species of the genus *Saccharomyces* have a very low minimum temperature for growth and can still ferment vigorously at approximately 0 °C (cold-fermenting yeasts). Some species of the genus *Zygosaccharomyces* are resistant to high temperatures and can survive at temperatures that are lethal for the cells and spores of all the other genera.

5.2.2.2 pH

Yeasts prefer acidic substrates and grow well at pH values between 3 and 5. At neutral pH, they sometimes grow with difficulty.

5.2.2.3 Growth Media Composition

The following compounds are essential for yeast growth; (i) carbon compounds; (ii) nitrogen compounds; (iii) growth factors (vitamins and minerals); (iv) minor elements.

Carbon Compounds Like all fungi, yeasts have a respiratory metabolism and grow vigorously in the presence of oxygen, drawing energy from numerous carbon compounds: carbohydrates (monosaccharides, some disaccharides and trisaccharides); several organic acids (e.g. acetic acid, lactic acid and ketoglutaric acid); a number of alcohols (such as methanol and ethanol); dihydroxyacetone (DHA) and other substances. Some yeasts can also use carbohydrates with an anaerobic metabolism, thus carrying out alcoholic fermentation.

Nitrogen Compounds Yeasts can use a great variety of nitrogen compounds, with the exception of elementary nitrogen and proteins. Ammonia can be metabolized by all yeasts, whereas only a few yeast species can metabolize nitrates. The ability of yeasts to grow in the presence of ammonia as the sole nitrogen source indicates that they are capable of synthesizing all the amino acids and DNA components. Generally, ammonia compounds are good sources of nitrogen, especially ammonium phosphate, which is perhaps the most important. This compound is frequently employed by fermentation industries to stimulate yeast growth. Ammonia represents the best source of nitrogen for yeasts; however, their growth is faster in media containing a variety of amino acids. Many yeasts can grow well even in media containing only one amino acid; particularly glutamic acid, aspartic acid or asparagine. Amino acid metabolism occurs by direct absorption or alternatively by a preceding transamination. Amino acids represent the starting point for the formation of a number of minor fermentation by-products.

Growth Factors Yeasts have the ability to synthesize most vitamins except for biotin, pantothenic acid, thiamine, pyridoxine, nicotinic acid and inositol. Occasionally, some strains require parabenzoic acid, whereas folic acid and riboflavin are not generally required. Different yeast species have different nutritional requirements and in some cases such diversity can be detected even at strain level.

Minor Elements Phosphorus, sulphur, potassium and magnesium are necessary for yeast growth under all conditions. In the absence of these elements, yeast growth is minimal and terminates when all the cell supplies have been exhausted.

5.2.2.4 Oxygen

Yeasts are organisms with a respiratory metabolism that can grow in the presence of oxygen using a great variety of carbon compounds. Many types of yeasts in the absence of oxygen also show a fermentative metabolism especially when metabolizing monosaccharides with six atoms of carbon. This double activity was discovered by Pasteur, who established that, if provided with a sufficient amount of oxygen, yeast behaves like all other fungi growing actively; whereas, if deprived of air, yeasts grow very little but ferment instead. This means that in an abundance of oxygen (i.e. in forced aeration), from 4 g of sugar, 1 g of yeast cells (dry weight) are produced. In this case the sugar is metabolized following the classic respiration reaction:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2$$

totally exploiting the potential energy of 688 kcal · mol^{-1}.

In the absence of oxygen, yeasts grow very little, and 176 g of sugar are required to produce 1 g of yeast cells:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

Through fermentation, the potential energy of sugar is only partially exploited. In fact, the major product released, i.e. ethanol, is still rich in energy and develops 56 kcal · mol^{-1}.

A phenomenon known as the ‘Pasteur effect’ dictates that, with a sugar concentration above 5%, the fermentation activity of fermenting yeasts prevails over the respiratory activity. However, when the glucose concentration is higher, some *Saccharomyces* yeasts produce ethanol aerobically rather than producing biomass. This phenomenon is known as the ‘Crabtree effect’.

5.2.3 Alcoholic Fermentation

5.2.3.1 Fermentation and Assimilation of Sugars

For almost a century, the ability to ferment sugars has been the major key to yeast species identification and differentiation. However, sugar metabolism has now lost its taxonomic value. Nevertheless, the knowledge of sugar fermentation metabolism is still valuable from a technological point of view; in fact, the sugars tested

for species identification are exactly those present in fermenting substrates used for the production of alcoholic beverages.

Yeasts ferment only carbohydrates with six atoms of carbon (hexoses). All fermenting yeasts can ferment glucose, fructose and mannose. In general, glucose and fructose are fermented with the same degree of vigour, whereas mannose is fermented more slowly. Galactose can be fermented by some yeasts. The speed and intensity of galactose fermentation depends on the species and on the strain. Disaccharides, particularly sucrose, maltose, lactose and melibiose, are fermented only by those yeasts possessing the specific hydrolytic enzyme. None of the fermenting yeasts possess the ability to ferment both maltose and lactose.

Raffinose is a trisaccharide composed of one molecule of fructose and one of melibiose; this sugar can be: (i) not fermented; (ii) completely fermented, by yeasts possessing both raffinase and melibiase; or (iii) partially fermented, by yeasts possessing raffinase but not melibiase. Starch is generally not fermented by yeasts, except for some species such as, for example, *Schwanniomyces occidentalis* (McCann and Barnett, 1986). Inulin (a polymer of fructose) can also be fermented by some yeast species, particularly those of the genus *Kluyveromyces* (Rouwenhorst et al., 1990).

Yeasts generally ferment sugars vigorously at concentrations up to 20%; at higher concentrations the fermentative metabolism slows down. If sugar concentration is above 50%, the osmotic pressure becomes excessively high and inhibits most yeasts. Only a few species, referred to as osmophilic, can grow in such conditions.

Several yeasts can assimilate certain sugars aerobically, but not anaerobically. This respiration-dependent sugar utilization is known as the 'Kluyver effect' and respiration-dependent species are referred to as Kluyver effect positive. A yeast can be Kluyver effect positive for some sugars and not for others. The cause of the Kluyver effect seems to be the low level of sugar transporters which, although it cannot sustain the high substrate flow required for fermentative growth, is sufficient to guarantee the energy-efficient respiratory growth that does not require a high rate of sugar uptake. *Saccharomyces cerevisiae*, which is predominantly fermentative, is Kluyver effect negative on most sugars (Fukuhara, 2003).

5.2.3.2 Fermentation By-Products

The alcoholic fermentation produces two molecules of ethanol (PM $46 \times 2 = 92$) and two molecules of carbon dioxide (PM $44 \times 2 = 88$) starting from one molecule of glucose (PM 180). The ethanol yield is slightly above 50% (w/w) and above 65% (v/w). These yields are theoretical, as, in reality: (i) part of the glucose is used by the yeast to grow; (ii) in addition to ethanol and carbon dioxide, yeasts produces numerous minor compounds. Pasteur formulated the following balance for products formed by 100 g glucose:

- ethanol 48.4 g
- CO_2 46.6 g
- glycerol 3.3 g
- succinic acid 0.5 g
- dried yeast 1.2 g

In current practice we assume that the ethanol yield is approximately 60% (60 mL per 100 g of fermented glucose). Ethanol that accumulates during the process of alcoholic fermentation exerts an inhibitory action on yeast growth and metabolic activity. Yeasts species show a different degree of ethanol tolerance; apiculate yeasts, such as those of the genus *Kloeckera*, cannot grow at an ethanol concentration higer than 4% (v/v), whereas more tolerant species, such as those of the genus *Saccharomyces*, can grow at up to 14% (v/v) ethanol.

During alcoholic fermentation, numerous other compounds are also released, generally at very low concentrations. These compounds can have three origins:

- they can originate from the alcoholic fermentation of sugars through anabolism: their formation occurs independently of the composition of the medium
- they can originate from the catabolism of some compound present in the medium
- they can originate via both anabolism and catabolism.

Table 5.4 shows the most important minor products of fermentation produced by *Saccharomyces cerevisiae*, the most common fermenting yeast.

S. cerevisiae is the yeast providing the purest alcoholic fermentation, achieving the maximum ethanol yield and the lowest concentration of minor fermentation compounds (Giudici et al., 1993; Antonelli et al., 1999; for a complete review, see Zambonelli, 2003).

Glycerol is quantitatively the third fermentation by-product. The amount produced is very variable among yeast strains, even within the same species. In alcoholic beverages, glycerol provides body and is generally regarded as a desirable compound. In addition to the compounds listed in Table 5.2, numerous other compounds are formed during alcoholic fermentation, such as lactic acid, fatty acids, acetic acid, succinic acid and esters. In many cases, nearly a hundred compounds can be found at the end of alcoholic fermentation. There is considerable variability between the different yeast species in the production of such minor fermentation compounds. For example, apiculate yeasts of the genus *Hanseniaspora* (*Kloeckera*) produce acetic acid at much higher concentrations than *S. cerevisiae* does. There is also a great difference between yeast species with regard to ethanol production.

Table 5.4 Minor products of alcoholic fermentation produced by *S. cerevisiae*, with reference to 100 vol of ethanol produced

Compound	Amount	Origin
Glycerol	4-7 g	Sugar
Succinic acid	0.30-0.6 g	Sugar
Acetic acid	0.1-1 g	Sugar
Acetic aldehyde	10-80 mg	Sugar
Propanol	20-50 mg	Sugar and amino acids
Isobutanol	30-90 mg	Sugar and amino acids
Amylic alcohol	30-60 mg	Sugar and amino acids
Isoamylic alcohol	100-300 mg	Sugar and amino acids
Phenylethyl alcohol	10-100 mg	Sugar and amino acids

Many species are inhibited when the ethanol concentration reaches levels that are far lower than those that can be tolerated by *S. cerevisiae*.

5.2.4 Important Genera and Species for Vinegar Production

Fermenting yeasts are of significant importance for vinegar production as they are responsible for the production of the alcoholic substrate from which vinegar is obtained. The most important groups of yeasts are the following:

- yeasts belonging to the genus *Saccharomyces*
- apiculate yeasts of the genera *Hanseniaspora* and *Kloeckera*
- lactose-fermenting yeasts of the genus *Kluyveromyces*
- osmophilic yeasts of the genus *Zygosaccharomyces*

5.2.4.1 The Genus *Saccharomyces*

The genus belongs to the family *Saccharomycetaceae* (see Table 5.1) and has the following general characteristics: multiplication by multilateral budding; elliptical or cylindrical cells that are generally diploid. Asci are formed without previous conjugation and contain from one to four elliptical or round smooth spores. Asci are persistent (do not release the spores into the medium). The genus is characterized by vigorous fermentative activity. Although the genus *Saccharomyces* is rather homogeneous; it has always been difficult to organize, especially at species level, and over the years it has undergone several changes. Since the 1970s, such species have been divided into three groups (Van der Walt, 1970):

- *Saccharomyces sensu stricto*, including *S. cerevisiae* and other species characterized by carrying out vigorous fermentations, showing good ethanol tolerance.
- *Saccharomyces sensu lato*, including less vigorous species with restricted habitat.
- The third group consist of just one species, *S. kluyveri*, which is phylogenetically distant from both *sensu stricto* and *sensu lato* groups.

Saccharomyces sensu stricto Currently this group includes the following species: *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, *S. paradoxus*, *S. cariocanus*, *S. mikatae* and *S. kudriavzevii* (Kurtzman, 2003).

S. cerevisiae is the most important yeast for the fermentation industry, being able to ferment efficiently almost all fermentative substrates, especially those originating from plants (fruit, vegetables, grains), but to a lesser extent also those of animal origin. *S. cerevisiae* is, in fact, the most vigorous and ethanol-tolerant yeast. Even though it is not naturally abundant in the wild, it is able to outcompete other microorganisms, including other yeasts that cannot tolerate high ethanol concentrations, to carry out the latest stages of alcoholic fermentation.

S. bayanus yeasts of interest for the fermentation industry are referred to as *S. uvarum*. These yeasts are frequently responsible for spontaneous fermentations that occur at low temperatures, as they can grow and ferment well even at 1-2 °C (Giudici et al., 1999). Generally they are isolated from the oenological environment

(Torriani et al., 1999; Naumov et al., 2002; Demuyter et al., 2004). They possess fermentation characteristics that differ from *S. cerevisiae* and generally produce a high concentration of glycerol, a low concentration of acetic acid and, interestingly, unlike *S. cerevisiae* strains they can synthesize malic acid, thus increasing the concentration of this compound in wine (Kishimoto et al., 1993; Castellari et al., 1994). They have been proposed as starter cultures for certain types of wine production (Giudici et al., 1995).

S. pastorianus is the typical yeast species used for lager brewing fermentations; it includes all lager brewing strains and is therefore important for the production of malt vinegar, which originates from the oxidation of fermented malt or beer. The species is thought to have originated through the natural hybridization between a *S. cerevisiae* and non-*S. cerevisiae* yeast similar to *S. bayanus* (see Kodama et al., 2006, and references therein). The genome of this species is indeed very complex and this reflects the metabolic properties of this organism. It grows and ferments well at low temperatures and produces a unique fermentation profile.

S. paradoxus is generally considered a yeast that is not of interest in the production of fermented foods; however, recent studies have highlighted its presence as the dominant agent of fermentation in Croatian wines, and it seems that one of the peculiarities of this species is its ability to metabolize malic acid efficiently, diminishing its concentration in the final product (Redzepovic et al., 2002, 2003).

Saccharomyces sensu lato Currently this group includes the following species: *S. barnettii*, *S. castellii*, *S. dairenensis*, *S. exiguus*, *S. rosinii*, *S. servazzii*, *S. spencerorum*, *S. transvaalensis* and *S. unisporus*. More recently, three new species have been added: *S. naganishii*, *S. humaticus* and *S. yakushimaensis* (Mikata et al., 2001). Among this group only *S. unisporus* can be considered relevant for the production of fermented beverages. This yeast takes its name from the fact that it forms asci containing only one single spore. Yeasts belonging to this species do not ferment lactose; however, they ferment glucose and galactose, the latter more vigorously. Originally *S. unisporus* was named *S. mongolicus* and was detected in a number of foods of dairy origin such as whey, cheese, kefir and other fermented milks (Engel et al., 1986; Montanari et al., 1996). The reason for the presence of a yeast not fermenting lactose in substrates where the main source of carbon is actually lactose can be explained by the concomitant presence of some lactic acid bacteria (such as *Streptococcus thermophilus* and *Lactobacillus kefir*) that grow by metabolizing lactose, but using only the glucose fraction of the disaccharide, thus releasing the galactose fraction into the medium. This encourages the growth of the microorganisms that can ferment this monosaccharide more vigorously (Montanari et al., 1996). *S. unisporus* is important for the production of whey vinegars, obtained by the oxidation of fermented whey.

5.2.4.2 Apiculate Yeasts (Genera *Hanseniaspora* and *Kloeckera*)

Apiculate yeasts are named after the shape of their cells, which have extended extremities. This peculiar morphology is the consequence of the mode of multipli-

cation, which occurs by budding alternately at the two opposite extremes of the cell. They belong to the family *Saccharomycoidaceae* and have a corresponding anamorphic species in the family *Candidaceae*. The apiculate yeasts that more frequently occur in the production of alcoholic beverages are those of the genus *Hanseniaspora*, which corresponds with the asporigenous genus *Kloeckera* (*Candidaceae*). Currently the six species shown in Table 5.5 are recognized.

Table 5.5 Species of *Hanseniaspora* and corresponding anamorphic species of *Kloeckera* currently recognized

Sporigenous apiculate species (*Hanseniaspora*)	**Asporigenous anamorphic species (*Kloeckera*)**
H. vinae	*K. africana*
H. uvarum	*K. apiculata*
H. guillermondii	*K. apis*
H. ospmophila	*K. corticis*
H. valbyensis	*K. japonica*
H. occidentalis	*K. javanica*

Hanseniaspora and *Kloeckera* yeasts are widespread in nature. The most frequent species, which are also the most active in natural alcoholic fermentations, are *H. uvarum* (*K. apiculata*) and *H. guillermondii* (*K. apis*). These yeasts have a vigorous fermentation activity limited to monosaccharides (maltose, sucrose and lactose are not fermented). They have a very low ethanol tolerance; the growth of *H. uvarum* stops when the ethanol concentration reaches 4% by volume. Alcoholic fermentations carried out by apiculate yeasts are rich in minor fermentation compounds, especially acetic acid, and for this reason they are generally considered negatively within the fermentation industries producing alcoholic beverages. In fact, they are responsible for one of the most frequent causes of wine spoilage due to their high level of volatile acidity they produce, often above the legal limits. The growth of apiculates, and of *H. uvarum* in particular, can be prevented by adding SO_2, to which these yeasts are very sensitive, or by using selected starter cultures of *S. cerevisiae*.

5.2.4.3 Lactose-Fermenting Yeast (Genus *Kluyveromyces*)

The genus *Kluyveromyces* belongs to the family *Saccharomycetaceae* (see Table 5.3) and shows the following general characteristics: cells can be ovoid, ellipsoidal, cylindrical or elongated and they can produce a pseudomycelium. Reproduction occurs by multilateral budding. Conjugation may or may not precede ascus formation. One to four smooth, spherical, reniform, ellipsoidal spores are present in the ascus; one species is known to produce up to 100 spores per ascus. Glucose is fermented vigorously and nitrate is not assimilated. Currently this genus contains 15 species, some of which (*K. marxianus*, *K. lactis* and *K. thermotolerans*) show important technological characteristics such as the ability to ferment lactose, the ability to directly ferment inulin, and the ability to grow at high temperatures (over 40 °C).

K. marxianus is probably the most important species of the genus for the fermentation industry. This species comprises strains with different characteristics, and these are generally considered as varieties. The most important variety is *K. marxianus* var. *marxianus*, which includes strains that in the past were ascribed to two different species: *K. fragilis* and *K. marxianus*. Both these former species included strains growing well at high temperatures and possessing the ability to ferment inulin. However, only *K. fragilis* strains were able to ferment lactose. According to the currently accepted classification, both *K. fragilis* and *K. marxianus* are now grouped under the species *K. marxianus*. *Kluyveromyces marxianus* var. *lactis* can ferment lactose but not inulin.

All the strains of *K. marxianus* var. *marxianus* grow well at relatively high temperatures and can directly ferment inulin because they possess the specific enzyme that hydrolyses this polysaccharide (Rouwenhorst et al., 1990). Inulin is a polymer of fructose and has a wide distribution in nature; it accumulates in tubers, such as yam and cassava, as well as onions, which can therefore undergo fermentation. They can therefore intervene in the fermentation of such products, preparing the substrate for oxidation to yam, cassava and onion vinegars.

Some of the strains of the variety *marxianus* (those formerly referred to as *K. fragilis*) as well as all the strains belonging to the variety *lactis*, can ferment lactose. They are therefore able to grow in milk and to carry out an alcoholic fermentation from this substrate and are therefore of interest for whey vinegar production (Parrondo et al., 2003).

5.2.4.4 Osmophilic Yeasts of the Genus *Zygosaccharomyces*

The genus *Zygosaccharomyces* belongs to the family *Saccharomycetaceae* and shows the following general characteristics: multiplication by multilateral budding; spheroidal, ellipsoidal or elongate cells that can form pseudohyphae. Cells are prevalently haploid, sporification is generally preceded by cell conjugation, more rarely between cells and buds. Asci are persistent and contain from one to four spherical or ellipsoidal spores. Glucose is fermented and nitrate is not assimilated. They provide a vigorous fermentation. Currently, nine species are acknowledged within this genus: *Z. bailii*, *Z. bisporus*, *Z. cidri*, *Z. fermentati*, *Z. florentinus*, *Z. mellis*, *Z. microellipsoideus*, *Z. mrakii* and *Z. rouxii*. More recently, based on the nucleotide sequence of the 18S subunit and of the ITS2 region of rDNA, a number of new *Zygosaccharomyces* type of yeasts have been proposed (Steel et al., 1999; Solieri et al., 2007), as well as a species of particular interest for vinegar production named *Z. kombuchaensis* (Kurtzman et al., 2001) which is typically isolated from 'tea fungus' – a complex of several yeast and *Acetobacter* strains that ferment sweetened tea, producing a vinegar beverage known as kombucha (Greenwalt et al., 2000).

Zygosaccharomyces yeasts are ubiquitous, always present in sugar-based substrates. They can predominate in some specific circumstances due to their distinctive characteristics. Their cells and spores are highly thermoresistant and some species of the genus can survive the thermal treatments to which some food products are subjected. *Zygosaccharomyces bailii*, in particular, is considered to be one

of the most frequent agents of spoilage of soft drinks, even after the products have been pasteurized (see Stratford, 2006). *Zygosaccharomyces* yeasts are osmophilic and can grow in media with a very high sugar concentration. *Zygosaccharomyces rouxii* and *Z. mellis* were originally isolated from fermented honeys with a sugar concentration close to 80%. Besides honey, they can grow in media such as syrups or concentrated grape must, which represent the fermentative basis for balsamic vinegar production (Solieri et al., 2006). Only a few other yeasts can grow in such environments, among which are yeasts of the genus *Hanseniaspora*.

5.3 Moulds

Mould is a generic name indicating a large group of multicellular organisms distributed in different divisions of the Kingdom of Fungi. Typically, they grow as filaments named hyphae of 5-10μm in diameter. Hyphae are surrounded by a wall and extend at their extremities, while drawing the protoplasm forwards as they grow. The hyphae branch repeatedly to form a mycelium and together they constitute the thallus or 'body' of the mould. Moulds are generally disseminated by spores that can be of many different varieties and produced by either an asexual or a sexual process. Moulds that are primarily of interest in vinegar production are mainly in the genus *Aspergillus*.

5.3.1 Classification

Moulds have traditionally been classified according to the morphology of the mycelium and of the spores. The moulds of industrial and biotechnological interest are basically grouped in the class *Ascomycetes* and are part of the subclass *Eurotiomycediade*, order *Eurotiales*, family *Trichocomaceae*, which includes the genera *Aspergillus* and *Penicillium*. Within the *Ascomycetes*, the genus *Aspergillus* is the most important for vinegar production. The classification of *Aspergillus* is primarily based on the morphology of some macroscopic (i.e. colony shape and colour) and microscopic features (i.e. shape and type of spores). The most relevant features of *Aspergillus* colonies are the following:

- colour of the vegetative mycelium and of the aerial portion
- pigmentation of the basal mycelium and of the substrate underneath
- texture of the basal mycelium
- development of concentric rings.

The numerous species that are part of this genus have been divided into subgroups or sections; the sections *Fumigati*, *Circumdati*, *Flavi* and *Nigri* contain the most important human pathogens, as well as the fungi of biotechnological interest. In particular the section *Flavi* includes both species used for rice vinegar production, namely *A. oryzae* and *A. sojae*, currently considered the domesticated forms of *A. flavus* and *A. parasiticus*, respectively.

The taxonomy of the genus *Aspergillus*, as well as most other fungi, has undergone several changes according to the taxonomic criteria adopted over time. As for most microorganism classification, the most recent trend is to apply a polyphasic approach that links the evaluation of the micro- and macromorphology of the fungus with an evaluation of its physiology and production of metabolites, as well as information on its genome (Samson et al., 2006).

5.3.2 Nutritional Requirements

All types of mould share a strictly aerobic metabolism and depend on pre-formed organic nutrients for energy as well as for the synthesis of cellular materials. They obtain these nutrients by absorbing simple, soluble nutrients (e.g. sugars, amino acids) through their walls, and by releasing extracellular enzymes to degrade polymers that they cannot absorb directly.

Moulds grow well in presence of a source of organic carbon for energy, a source of nitrogen for protein and vitamin synthesis, and several minerals; basically, they are ubiquitous.

5.3.3 Metabolic Activity

Moulds produce a wide range of secondary metabolites, including compounds with antibiotic properties, pigments, flavour and odour components. The release of a large variety of enzymes is a trait common to most moulds and this has been largely exploited in an industrial context. Moreover, the relatively high efficiency of growth of some moulds has made them the ideal organism to be used biotechnologically to extract a number of compounds such as organic acids.

Among the most important metabolites produced by moulds are potent toxins termed mycotoxins, which are highly toxic to some animals, causing liver and kidney damage and also showing carcinogenic potential (see Cary et al., 2000). The most notorious mycotoxins are aflatoxin and ochratoxin, which are primarily produced by *A. flavus* and *A. ochraceus*. However, with a different degree of toxicity, they can potentially be produced by other members of the *Aspergillus* genus as well. The topic is still the subject of numerous studies and some mycotoxins have not been identified yet; however, the spontaneous growth of moulds, especially on foodstuffs, is always considered undesirable.

5.3.4 Genera and Species Relevant to Vinegar Production

Aspergillus oryzae and *A. sojae* are the most common moulds associated with vinegar production. They constitute part of the so-called *koji*, a mouldy rice substance that has the function to break down the starch molecules of rice into simple fermentable sugars in the production of the alcoholic base for rice vinegars. *A. oryzae*

is considered the domesticated form of *A. flavus*; however, unlike *A. flavus*, it does not release mycotoxin and is therefore considered a safe organism to be used in foodstuff production (Matsushima et al., 2001). Due to its high biotechnological value, it is a very well-studied species used by the enzyme industry to produce numerous enzymes that are applied in a variety of fields. Its genome has been fully sequenced (Machida et al., 2005) and numerous cultures are being genetically manipulated in order to optimize the production of enzymes and other metabolites of industrial interest (e.g. Christensen, 1994).

5.4 Lactic Acid Bacteria

Lactic acid bacteria (LAB) represent a homogeneous group of microorganisms showing the following general characteristics: cells are regular, in the shape of cocci or rods, they are not mobile, Gram-positive and do not form spores. Figure 5.2 shows an example of lactic acid bacteria.

5.4.1 Classification

The earliest LAB classification was established by Orla-Jensen in 1919, and over the years it has undergone several modifications that, however, have maintained the

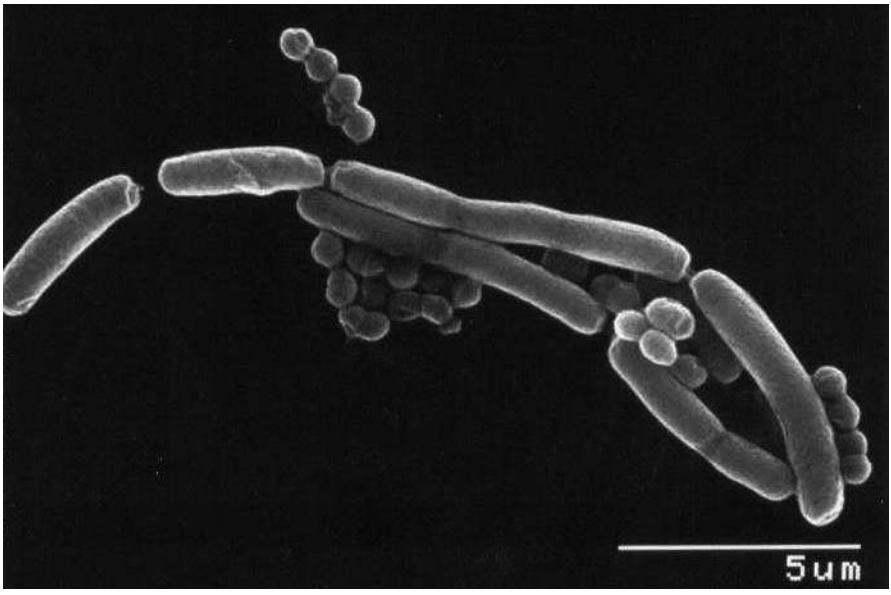

Figure 5.2 Cells of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, typical LAB of fermented milk and whey, photographed under a scanning electron microscope (SEM) at 6000× magnification

Table 5.6 Lactic acid bacteria classification according to Orla-Jensen (1919)

Cell shape	Type of fermentation	Genus	Notes
Cocci	Homofermentative	*Streptococcus*	–
	Heterofermentative	*Betacoccus*	–
Rods	Homofermentative	*Thermobacterium*	Thermophilic
		Streptobacterium	Mesophilic
	Heterofermentative	*Betabacterium*	–

Table 5.7A Current status of cocci-shaped lactic acid bacteria classification

Cell organization	Fermentation	Lactic acid	Genus of 1986[a]	Current genus
Chains	Homofermentative	L(+) lactic acid	*Streptococcus*	*Streptococcus* *Lactococcus* *Enterococcus*
Tetrads	Homofermentative	DL lactic acid	*Pediococcus*	*Pediococcus*
Chains	Heterofermentative	D(–) lactic acid	*Leuconostoc*	*Leuconostoc* *Oenococcus* *Weisella*

[a] Sneath et al. (1986).

Table 5.7B Current status of rod-shaped lactic acid bacteria classification

Genus	Group	Fermentation
Lactobacillus	Group I	Homofermentative
	Group II	Homofermentative, facultatively heterofermentative
	Group III	Heterofermentative

original basic structure. From a physiological point of view, LAB are rather homogeneous and thus they have long been gathered into one single family. Since 1986 they have been grouped on the basis of their cell morphology (Sneath et al., 1986).

Tables 5.6 and 5.7 show the earliest and the currently acknowledged classifications (Stiles and Holzapfel, 1997), respectively.

5.4.2 Nutritional Requirements

Lactic acid bacteria are facultative anaerobes and have complex nutritional requirements. To grow they require a medium containing all the amino acids and many vitamins that they are not able to synthesize. They are catalase-negative, with the exception of a number of species of the genus *Pediococcus*.

Some LAB species are mesophilic and some are thermophilic. The latter have an optimal temperature of growth above 40 °C and are all homofermentative, with cells shaped as rods (genus *Lactobacillus*). Among the homofermentative cocci, the species *Streptococcus thermophilus*, in spite of having an optimal temperature of growth below 40 °C, can also grow at an extremely high temperature; it therefore grows well in the widest range of temperatures.

Lactic acid bacteria grow well at pH 7; however, they can also grow in habitats with very low pH values. Some species, either homo- or heterofermentative, can grow at pH values between 3 and 4. Moreover, LAB produce acids that lower the pH values of their growth substrate.

5.4.3 Metabolic Activity

Lactic acid bacteria can ferment sugars following two different metabolic pathways; (i) via the glycolytic pathway (Embden-Meyerhof-Parnas pathway) that, under standard conditions, causes the conversion of sugars almost exclusively to lactic acid; and (ii) via the 6-phosphogluconate/phosphoketolase pathway, through which sugars are converted into lactic acid, acetic acid, ethanol and CO_2. Lactic acid bacteria that metabolize sugars according to the first pathway are referred to as homofermentative; whereas lactic acid bacteria following the second pathway are defined as heterofermentative.

In homolactic fermentation, 1 mol of glucose yields 2 mol of lactic acid:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Lactic acid}}{2\,CH_3CHOHCOOH}$$

In heterolactic fermentation, 1 mol of glucose yields 1 mol each of lactic acid, ethanol and carbon dioxide:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Ethanol}}{CH_2H_5OH} + \underset{\text{Carbon dioxide}}{CO_2}$$

When glucose is used as carbon source, two fermentation patterns can be described for heterofermentative lactic acid bacteria (Nelson and Werkman, 1935):

The production of equimolar quantities of lactate, ethanol, and carbon dioxide, with occasional traces of acetate.

The formation of glycerol along with lactate, acetate and carbon dioxide.

Most LAB can ferment lactose and can therefore grow and reproduce in milk and dairy products. The physiology, metabolism and classification of LAB have recently been reviewed by Axelsson (2004).

5.4.4 Importance for Vinegar Production

Lactic acid bacteria are ubiquitous, widespread in soil and water, common contaminants of foodstuffs. Because of their characteristics they are widely employed in the fermentation and food industries. In particular, they play a major role in the production of several fermented foods, such as all cheeses, cured sausages (Italian salami), bakery products and preserved forages.

As a consequence of producing acid and lowering the pH of the environment, LAB can counteract putrid reactions; in fact, putrifying bacteria are very sensitive to low pH values and usually cannot live in the same habitat as LAB. Some LAB

inhabit the digestive tracts of mammals; 'probiotics' is a science based on the properties of this type of lactic acid bacteria. They are used industrially for the production of lactic acid obtained by fermentation.

The growth of some LAB is at times undesirable; this is the case, for example, for some *Enterococci* that, being thermotolerant, can survive after some fermented sausages are cooked and can be the cause of the souring of the final product.

5.4.4.1 Lactic Acid Bacteria in Alcoholic Fermentations

Lactic acid bacteria can be of interest in the production of fermented beverages, as they can grow both before and after the fermentation process. On some occasions they are desired and are used as starter cultures; however, in some other cases their growth is considered detrimental.

In the case of beer, for example, specific LAB can be used to lower the pH of wort, guaranteeing the biological stability of the final product (Lowe and Arendt, 2004). In the case of wine, lactic acid bacteria can be helpful in reducing the total acidity by fermenting malic acid and encouraging the wine maturation process. Malolactic fermentation is generally determined by the heterofermentative *Oenococcus oeni*, often together with homo- or heterofermentative lactobacilli (Liu, 2002).

The growth of LAB in beer after wort fermentation is considered negatively, as it causes hazing of the product and increases the level of acidity excessively. In wines, their growth at the expense of residual sugars can cause spoilage, and the spoiled product can then only be employed in distillery.

For vinegar production, the growth of LAB in the above-mentioned alcoholic beverages is not necessary and does not affect the acetic acid bacteria (AAB) activity.

Milk and whey, a by-product of cheese making, are not ideal substrates for yeast growth, due to their high pH value and their sugar composition. Yeasts can grow in milk only if its pH is lowered to a value of 5, and this occurs as a consequence of a previous lactic acid fermentation carried out by LAB. Lactic acid bacteria therefore play a major role in the production of alcoholic beverages obtained from milk and whey. Lactic acid bacteria that use lactose as a sole carbon source are numerous and widespread in all genera and species. Some conditions, primarily temperature, can favour one or another species. Just after milking, at 37 °C, homofermentative bacteria with a high optimum or maximum growth temperature will grow: these belong to the species *Streptococcus thermophilus* and to some thermophilic *Lactobacillus* species, such as *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. lactis* and *Lb. helveticus*. At lower temperatures, up to 30 °C, *St. thermophilus*, a highly adaptable species, can still grow; however, mesophilic homo- and heterofermentative species generally prevail.

Several LAB, among which *St. thermophilus* and the heterofermentative *Lb. kefir*, are very frequent in fermented milks. They hydrolyse lactose but ferment only its glucose component and not galactose, which is therefore released into the medium and remains available as a nutrient together with some lactose. As a con-

sequence, both lactose-fermenting yeasts, such as those belonging to the genus *Kluyveromyces*, as well as galactose-fermenting yeasts such as *Saccharomyces* yeasts, can grow in such fermented milks (Giudici et al., 1996; Montanari et al., 1996).

5.5 Nematodes

The nematodes or roundworms are one of the most common phyla of animals, with over 20,000 different species described (including over 15,000 parasitic species). They are ubiquitous in freshwater, marine and terrestrial environments.

Nematodes are one of the simplest animal groups to have a complete digestive system, with a separate orifice for food intake and waste excretion; a pattern followed by all subsequent, more complex animals. Their nutrition generally depends upon the genus and species and includes bacteria, fungus, algae, protozoa, and animal and vegetal protoplasm. Reproduction is usually sexual; however, hermaphroditic and parthenogenetic species are also known.

Ecologically, they can be divided into free-living and parasitic forms. Free-living nematodes generally measure 1 mm in length, whereas parasitic species can be bigger (on average 8 mm of length). Taxonomically, nematodes can be divided into two classes (Klingler, 1986):

- Secernentea (or Phasmidiae), including most terrestrial free-living and parasitic species.
- Adenophorea (or Aphasmidia), generally represented by marine or free-living animals.

5.5.1 Nematodes in Vinegar Production

Nematodes are ubiquitous, inhabiting damaged fruit such as grapes and apples, and as a consequence they can often be seen swimming on the surface of vinegars. Not much is known about the role and the effect of vinegar eels on vinegar production and not many studies have been carried out to clarify this issue.

Shann (1987) reported for the first time the presence of a nematode (*Panagrellus zymosiphilus*) on damaged grapes of an Italian vineyard. He carried out a pioneer study in which demonstrated that such nematodes, besides carrying a yeast-like fungus (*Botryzyoma nematodophila*), also harbour a large amount of AAB on their surface. This saprophytic system seems to have an influence on the development of sour rot of grapes.

Recently, Buchholz et al. (2005) identified a large number of vinegar nematodes in traditional balsamic vinegar. The authors classified the nematode as *Turbatrix aceti*, formerly known as *Anguillula aceti* and colloquially referred to as the 'vinegar eel' or 'vinegar worm'. *Turbatrix aceti* is often found in vinegars obtained from apples or other fruits as well as in other fermented foods. Figure 5.3 shows a sample of *Turbatrix aceti* isolated from traditional balsamic vinegar.

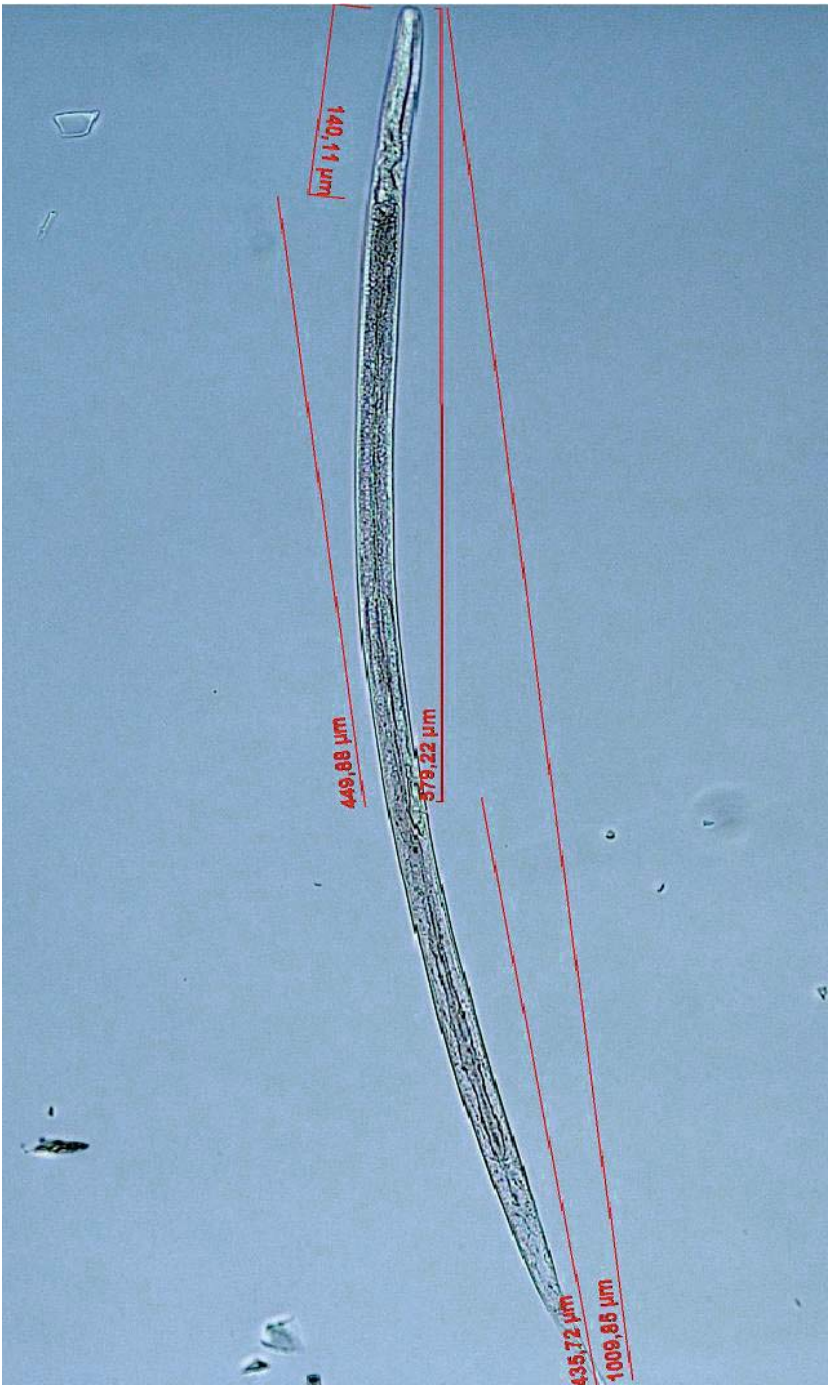

Figure 5.3 The nematode *Turbatrix aceti*: body of the nematode with key measurements

5.6 Insects

Small flies belonging to the family Drosophilidae play a major role in vinegar production, especially in the case of wine and cider vinegars. The genus *Drosophila* hosts the most common flies that can be found in vinegar, and it also contains about 1500 species that are very diverse in appearance, behaviour and breeding habitat. One species of *Drosophila* in particular, *D. melanoganster*, has been extensively used as a model organism in genetic and developmental biology studies. *Drosophila* flies are commonly known as 'fruit flies' or 'vinegar flies'; in fact their existence is very often associated with the presence of such substrates.

5.6.1 The Role of Fruit Flies in Vinegar Production

Fruit flies feed on fresh as well as rotting and fermenting fruit and vegetables, and are therefore always found in such habitats. Generally they are considered as a negative, undesired element in food fermentations as well as in ethanol oxidation processes, as they compromise the sanitary quality of the final product. However, fruit flies play a relevant biological role both in fermentation and ethanol oxidation, as they are basically the vector to most microorganisms involved in such processes. Several studies have highlighted, for example, the finding that fermenting yeasts are often transported by insects such as fruit flies, which feed on them and carry yeast spores into their digestive tract (Mortimer and Polsinelli, 1999; Pulvirenti et al., 2002). This system allows the spreading of fermenting yeasts as well as other organisms. Shann (1987), in fact, reported that fruit flies were responsible not only for the transport of yeasts but also of nematodes that in turn were found to carry a large amount of AAB on their surface. Unfortunately, besides good fermenting yeasts and oxidising bacteria, other more undesirable microorganisms, responsible for fermentation defects or grape diseases and further oxidation processes, are also transported.

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