Brewing Microbiology

Edited by

F. G. PRIEST and I. CAMPBELL

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BREWING MICROBIOLOGY

Preface

The art of brewing is as old as human civilization. The scientific knowledge of the microbiology of brewing, and other alcoholic fermentations, developed largely from the researches of Pasteur little over a century ago. An important advance was Hansen's development of pure cultures of yeasts for brewery fermentations, and the recognition of different species of brewing and wild yeasts. During the present century the discovery by Winge of the life cycles of yeasts and the possibilities of hybridization were the first steps in the development of yeast genetics as a research topic with implications far wider than the alcoholic beverage industries. Over the same period the contaminant bacteria of the fermentation industries were also studied, largely influenced by Shimwell's pioneering research, with resultant improvement in beer quality.

The breadth of microbiological influence on the brewing process is, we hope, reflected in the range of reviews included in this book. We have attempted to review not only the nature and effects of the yeasts, moulds and bacteria which are culture organisms or contaminants, but also the application of modern biotechnological knowledge to the traditions of 'the oldest biotechnology'. In particular, the recent spectacular advances in the application of yeast genetics, and the further advances which can be predicted in the near future, will provide yeasts which could synthesize a variety of products in addition to ethanol, or possess novel desirable physiological traits. New approaches to the rapid detection of spoilage micro-organisms are revolutionizing the quality control laboratory and the application of computers and commercial kits for the identification of micro-organisms simplifies the task of monitoring the microbiological quality of beer. This book reviews these and other aspects of modern brewing microbiology.

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Contents

Chapter 1

Systematics of Yeasts

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Systematics includes classification, nomenclature and identification. Routine identification of both culture and contaminant yeasts will be discussed later (Chapter 7); this present chapter is concerned primarily with the classification and nomenclature aspects of systematics, and only the general principles of yeast identification are considered here.

1. CLASSIFICATION OF YEASTS

Most mycologists accept the series of taxonomic studies produced by the Dutch group of taxonomists as definitive: the current classification is that of Kreger-van Rij (1984). Although no satisfactory definition of yeasts exists, they are generally accepted as fungi which are predominantly unicellular (Kreger-van Rij, 1984). However, many yeasts are capable under appropriate cultural conditions of growing in mycelial form, either true mycelium or a pseudomycelium of branched chains of elongated yeast cells.

Three of the four groups of fungi include yeasts: Ascomycetes, Basidiomycetes and Deuteromycetes. Although Zygomycetes may grow in yeast morphology under certain cultural conditions, their normal existence is in the filamentous form. Classification of fungi (Table 1) is

TABLE 1
Classification of Yeasts (from Kreger-van Rij, 1984)

Group 1: Ascomycetes
The sexual spores are formed endogenously, i.e. within the cell. In filamentous fungi a specialized spore-bearing ascus is formed; in yeasts, the spores develop within the former vegetative cell which is then correctly termed the ascus (but note the exception, *Lipomyces*).

(a) Spermphthoraceae (needle-shaped spores; Fig. 1a); principal genera *Metschnikowia*, *Nematospora*.
(b) Saccharomycetaceae (other forms of spore; Fig. 1b,c,d,e)
Four families, distinguished mainly by method of vegetative growth.
  (i) Schizosaccharomycoideae (growth by binary fission, Fig. 2a); one genus, *Schizosaccharomyces*.
  (ii) Nadsoniodeae (growth by polar budding, Fig. 2b); principal genera *Hanseniaspora*, *Nadsonia*, *Saccharomycodes*, distinguished by form of spores.
  (iii) Lipomycoideae (growth by multilateral budding, Fig. 2c, but the principal characteristic is the 'exozygotic ascus', a sac-like ascus with numerous oval spores, Fig. 1e); one genus, *Lipomyces*.
  (iv) Saccharomycoideae (growth by multilateral budding, Fig. 2c); numerous genera, distinguished mainly by details of the sporulation cycle and spore morphology. The most important genera in the fermentation industries are *Debaryomyces*, *Dekkera*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schwanniomyces*, *Torulaspora* and *Zygosaccharomyces*.

Group 2: Basidiomycetes
The sexual spores are formed exogenously. Yeasts of this group are unimportant in the fermentation industries, but their non-sporing, 'imperfect' forms, especially *Sporobolomyces*, *Rhodotorula* and *Cryptococcus*, are common surface organisms of plant materials, including barley and malt.

Group 3: Blastomycetes, Deuteromycetes or Fungi Imperfecti
No sexual spores are formed.

(a) Sporobolomycetaceae (forming exogenous asexual spores, i.e. ballistospores); two genera, *Bullera* (relatively rare) and *Sporobolomyces* (very common).
(b) Cryptococcaceae (no exogenous asexual spores). Genera of this family, which represent those yeasts of groups 1 and 2 which have lost the ability to form sexual spores, are classified by different characteristics from the sporing yeasts and therefore do not necessarily coincide with the equivalent 'perfect' genera. Genera are distinguished by the form of vegetative growth, fermentative ability and a somewhat haphazard selection of other tests. Principal genera of importance to the fermentation industries are *Kloeckera* (which grows by polar budding), *Brettanomyces*, *Candida*, *Cryptococcus* and *Rhodotorula* (growth by multilateral budding). The genus *Trichosporon*, often a contaminant of cereal grains, grows both by multilateral budding and a form of fission. Note that *Torulopsis*, formerly a separate genus including various species of spoilage yeasts of the brewing and related industries, has recently (Kreger-van Rij, 1984) been incorporated into the genus *Candida*.

largely on the basis of the mode of vegetative growth and the nature of the spores, if formed. Physiological properties, as widely used in bacteriology, are of only minor importance in classification of yeast families or genera. One of the few physiological tests of importance in generic differentiation at present is for the ability to utilize nitrate as sole source of nitrogen for growth: *Hansenula* species grow on nitrate, but the otherwise similar genus *Pichia* does not. If the recent recommendation by Kurtzman (1984) is generally accepted that these genera be combined, physiological tests will no longer be required for generic differentiation, and will be limited to distinguishing species within genera.

The definitive property of Ascomycetes is the production of endogenous sexual spores (ascospores). Further subdivision as families and genera is based on the type of spores (Fig. 1), and the nature of the life cycle by which they are formed. The mode of vegetative multiplication is also relevant, since growth by binary fission (Fig. 2a) or polar budding (Fig. 2b) is sufficiently different from the more common multilateral budding to be of taxonomic importance. Within the group of yeasts growing by multilateral budding, the genera *Saccharomyces*, *Kluyveromyces*, *Torulaspora* and *Zygosaccharomyces* form a closely-

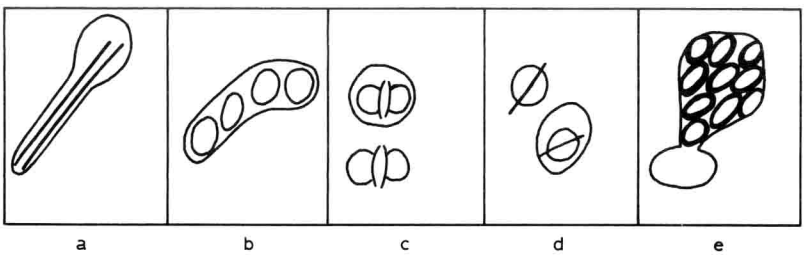

Fig. 1. Yeast spores. (a) Needle spores. The original vegetative cell, top right, has been distended to a club shape by development of the spores. Normally either one or two spores will be formed in *Metschnikowia* or *Nematospora* species. (b) Oval spores, as in the linear ascus of *Schizosaccharomyces pombe*. (c) 'Hat' spores in the ascus, and free. The hat appearance is created by a tangential plate or ring forming the 'brim'. *Pichia membranaefaciens* is a brewery contaminant forming this type of spore. (d) 'Saturn' spores, as in *Hansenula saturnus*. The ring is located equatorially on the spore, giving the appearance of the planet Saturn. (e) Ascus of *Lipomyces*. Although the ascospores are endogenous, i.e. within the structure of the ascus, the ascus itself is a separate structure (above) from the original vegetative cell (below). In all other ascosporogenous yeasts the spores develop within the original vegetative cell which is then, by definition, called the ascus.

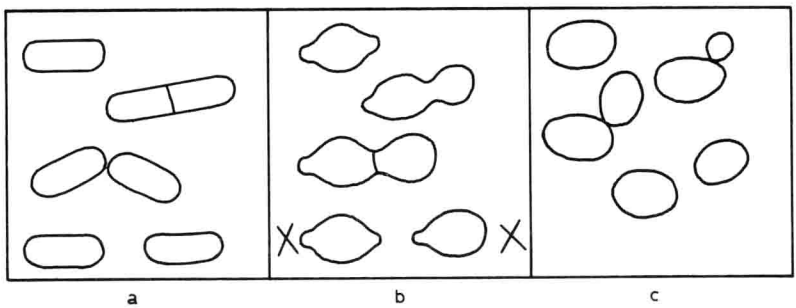

Fig. 2. Vegetative reproduction of yeasts. (a) Binary fission. The original cell elongates and after nuclear division a septum separates the two cells, which then complete the formation of the cell wall, and break apart. (b) Polar budding. Successive divisions are from either end (pole) of the cell, alternately. The scar tissue from budding gives the cell a 'lemon' shape. Note also the broad base to the developing bud, characteristic of this method of growth. In the next generation the two cells will bud from end X. (c) Multilateral budding, i.e. budding from many different points on the surface in successive generations. Note the typical narrow base to the developing bud.

related subgroup of vigorously fermentative yeasts. Indeed, by earlier classification (e.g. Lodder & Kreger-van Rij, 1952), species at present in these four genera were all allocated to one genus, *Saccharomyces*. Subsequently, the differences in vegetative growth cycles and sporulation were considered sufficiently different to justify four separate genera. *S. cerevisiae* is of such industrial importance that its vegetative and sporulation cycles have been carefully studied, in recent years largely from the viewpoint of applied genetics. Wild sporulating strains of *S. cerevisiae* are normally diploid, and meiosis on sporulation produces asci containing four haploid spores (Chapter 3). The typical appearance of asci of *S. cerevisiae* is illustrated in Fig. 3a. Often tetrahedral asci are formed, as the most compact arrangement of the four spores. However, occasional failure of meiotic nuclear divisions can result in two- or three-spored asci. Spores of *Saccharomyces* species are not liberated immediately on maturation; spores of *Kluyveromyces* species, although superficially similar in appearance in some species, are rapidly released.

Kluyveromyces is composed of both homo- and hetero-thallic species with spherical or ellipsoidal spores, and homothallic species producing reniform (kidney-shaped) spores. The number of spores produced by *Kluyveromyces* species is often large: up to 60 in *K. polysporus*.

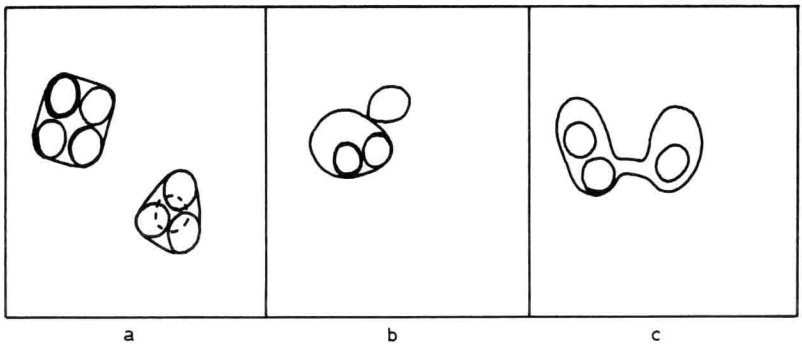

Fig. 3. Spores of *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces*. (a) *Saccharomyces*. Heterozygous diploid cells undergo two successive meiotic nuclear divisions to produce four haploid spores in plane (top left) or tetrahedral (bottom right) configuration. Often only two or three spores are formed, by failure of nuclear division. (b) *Torulaspora*. Under conditions which stimulate sporulation, the haploid cell undergoes nuclear fusion with its bud, which although not yet fully developed is already, in nuclear terms, a distinct individual. In this way a transient diploid cell is formed, and then two haploid spores. (Note that in some species of *Debaryomyces*, which have a similar method of sporulation, the spores are formed in the buds. Development in the mother cell, as shown, is more normal, and invariable practice in *Torulaspora*.) (c) *Zygosaccharomyces*. Conjugation of independent haploid cells precedes ascus formation. The ascus, composed of the two former vegetative cells and the conjugation tube, contains one to four haploid spores; three, as shown, is a common occurrence.

The sporulation process of *Kluyveromyces* species, and in particular the early liberation of mature spores, was judged to be sufficiently distinctive to justify a separate genus in the previous classification of yeasts (Lodder, 1970). The remaining species of actively fermenting yeasts were, somewhat illogically, retained in a single genus *Saccharomyces*, even though there were substantial differences in methods of sporulation within the genus. This has subsequently been rectified in the most recent classification (Kreger-van Rij, 1984), with the transfer of species to the genera *Torulaspora* and *Zygosaccharomyces*.

Torulaspora is haploid in the vegetative growth cycle, but sporulation results from homothallic fusion between the nuclei of parent cell and bud, under the cultural conditions which promote sporulation. *Zygosaccharomyces* is also haploid in the vegetative state, but fusion leading to sporulation is between independent cells, forming a diploid

zygote which produces two spores, one in each parent, or subsequent meiotic division may result in two spores in each former cell. Asci of *Torulaspora* and *Zygosaccharomyces* are illustrated in Figs 3b and 3c. The haploid vegetative cycle and sporulation in the manner of *Torulaspora* and *Zygosaccharomyces* spp. are also properties of the common wild yeasts of the genera *Debaryomyces*, *Hansenula* and *Pichia*. However, these genera ferment sugars only weakly, or not at all (Kreger-van Rij, 1984).

Yeasts of the genera included in the Basidiomycetes are unlikely to occur as contaminants of brewery or related fermentations, but are common surface contaminants of barley and malt (Chapter 4). These yeasts of malt are killed during mashing and hop boiling, and their poor growth at pH 5 or below prevents later re-infection.

It is reasonable to believe that the imperfect, non-sporing yeasts are derived from heterothallic sporing yeasts, but in the absence of the opposite mating type are unable to conjugate and form spores. Single spores germinated in the absence of the opposite mating type grow indefinitely as haploid cells, provided nutrients are available. Previously the principles of classification were applied rigidly and yeasts were allocated to perfect or imperfect genera according to sporulation. In the most recent classification this principle has been less rigidly applied. *Candida robusta* (Lodder, 1970) had the same properties as *S. cerevisiae*, except that no spores were formed. Sporing yeasts were obviously *S. cerevisiae*, but the logic of the classification broke down over the nomenclature of the majority of yeasts of the brewing and related industries, which, in the course of their long history of artificial cultivation, had lost the ability to form spores. To apply the name *S. cerevisiae* was technically wrong, but was nevertheless accepted practice. In the classification of Kreger-van Rij (1984) it is interesting to note that a separate species *C. robusta* is not listed; it is now a synonym of *S. cerevisiae*. Similarly the various other examples of identical pairs of sporing and non-sporing yeasts (Table 2) are now recognized by the name of the sporing species.

Largely to avoid the complications arising from the use of sporulation as a fundamental property for 'classical' yeast taxonomy, and the requirement to observe spores as a first step in classical identification, various authors have provided alternative identification schemes which ignore sporulation. A simple, effective scheme introduced by Beech *et al.* (1968) has been superseded by a succession of diagnostic keys published by Barnett and colleagues, who have provided a compre-

TABLE 2
Perfect and Imperfect States of Common Yeast Contaminants of the Brewing Industry (Barnett *et al.*, 1983; Kreger-van Rij, 1984)

Spore-forming yeast	*Non-spore-forming synonym*
Dekkera bruxellensis	*Brettanomyces bruxellensis*
Dek. intermedia	*B. intermedius*
Debaryomyces hansenii	*Candida famata*
Hanseniaspora uvarum	*Kloeckera apiculata*
Hsp. valbyensis	*Kl. japonica*
Hsp. vineae	*Kl. africana*
Hansenula anomala	*C. pelliculosa*
H. fabianii	*C. fabianii*
Kluyveromyces marxianus	*C. kefyr*
Pichia fermentans	*C. lambica*
P. guilliermondii / *P. ohmeri*	*C. guilliermondii*
P. orientalis (*Issatchenkia orientalis*)	*C. krusei*
P. membranaefaciens	*C. valida*
Saccharomyces cerevisiae	*C. robusta*
S. exiguus	*C. holmii*
Torulaspora delbrueckii	*C. colliculosa*

hensive, but unfortunately complex, system for identification by physiological properties (Barnett & Pankhurst, 1974; Barnett *et al.*, 1979, 1983). A simplified version of this system is the basis of the API test kit for yeast identification (Chapter 7).

2. NOMENCLATURE OF YEASTS

Hansen, the pioneer yeast taxonomist, applied the specific name *S. cerevisiae* to the traditional top-cropping ale yeast of the British and German brewing industries. The different properties of lager yeast were acknowledged by allocation to a different species, *S. carlsbergensis* (Gilliland, 1971; Maule, 1977). Other specific names were applied to yeasts of different properties with respect to fermentation of sugars, industrial properties or cell morphology. Ale yeast *S. cerevisiae* (spherical, or almost so) and wine yeast *S. ellipsoideus* (ellipsoidal) were distinguished by Hansen partly on the basis of shape, but also by their different behaviour in fermentation. In the reclassification of yeasts by

Lodder & Kreger-van Rij (1952) the sole difference detectable in the laboratory, morphology, was judged insufficient for separation of two species and the wine yeast became *S. cerevisiae* var. *ellipsoideus*. However, another wine yeast, *S. uvarum*, and lager yeast *S. carlsbergensis*, despite their biochemical similarities, remained distinct species. In the next reclassification of yeasts (Lodder, 1970) *S. cerevisiae*, its *ellipsoideus* variety and *S. willianus*, identical in fermentation properties but of distinctively elongated cell morphology, were merged as a single species *S. cerevisiae*. An equivalent series of yeasts, comprising *S. carlsbergensis*, *S. uvarum* and *S. logos*, were united as *S. uvarum*. *S. cerepvisiae* and *S. uvarum* were distinguished by the ability of only the latter to ferment melibiose. Subsequently, extensive research on yeast hybridization demonstrated interbreeding, with the production of fertile progeny, between *S. cerevisiae* and *S. carlsbergensis*, which suggested that only one species was justified. Also, the availability of these same hybrids as industrial yeasts completely blurred any distinction in physiological terms between the species. Therefore the allocation of both groups to a single species *S. cerevisiae* by Kreger-van Rij (1984) was understandable. Indeed, many other former species were also merged in the new definition of *S. cerevisiae*, as shown in Table 3.

It was unfortunate that 'classical' identification methods failed to distinguish the very different industrial properties of these yeasts. Gilliland's (1971) objection that 'industrial' properties were sufficient to justify separate species was confirmed by numerical analyses of data including not only the standard morphological and physiological properties, but also such industrially useful properties as tolerance to ethanol, flocculation and fining ability and optimum growth temperature (Campbell, 1972). More detailed analysis of the effect of temperature on growth of lager and non-brewing strains of *S. uvarum* (i.e. *S. carlsbergensis* and *S. uvarum* respectively as defined by Lodder & Kreger-van Rij in 1952) by Walsh & Martin (1977) confirmed the distinctive nature of lager yeast, however alike they may have been in the test of 'classical' taxonomy. Unfortunately, the ability to ferment various sugars, some (galactose, melibiose, raffinose) unlikely to occur in brewery fermentations, has arbitrarily been given more importance than industrially useful properties. However, the fundamental difference between ale, lager and non-brewing strains of *S. cerevisiae* was demonstrated again by Pedersen (1983, 1985) in 'fingerprint' analyses of the genetic material of these yeasts.

A selective history of the re-naming of *Saccharomyces* species has

TABLE 3
Nomenclature of Important Yeast Species of the Brewing and Related Industries, 1952–1984

1952 classification (*Lodder & Kreger-van Rij, 1952*)	*1970 classification* (*Lodder, 1970*)	*1984 classification* (*Kreger-van Rij, 1984*)
Saccharomyces bayanus *S. oviformis* *S. pastorianus*	*S. bayanus*	*S. cerevisiae*
S. cerevisiae *S. cerevisiae* var. *ellipsoideus* *S. willianus*	*S. cerevisiae*	
S. carlsbergensis *S. logos* *S. uvarum*	*S. uvarum*	
S. chevalieri	*S. chevalieri*	
S. italicus	*S. italicus*	
	S. aceti[a]	
	S. diastaticus[a]	
S. delbrueckii	*S. delbrueckii*	*Torulaspora delbrueckii*
S. rosei	*S. rosei* *S. vafer*[a]	
S. acidifaciens *S. bailii* *S. elegans*	*S. bailii*	*Zygosaccharomyces bailii*
S. fragilis *S. marxianus*	*Kluyveromyces marxianus*	*K. marxianus*
S. lactis	*K. lactis*	

[a]New species.

been presented as Table 3, showing species which are now (Kreger-van Rij, 1984) merged as *S. cerevisiae.* Other changes in nomenclature also of importance in the fermentation industries are included in Table 3, comparing the classifications of Lodder & Kreger-van Rij (1952), Lodder (1970) and Kreger-van Rij (1984) with respect to these species.

An unfortunate result of this series of taxonomic revisions is that many formerly distinct species of yeast contaminants of the brewing and related industries are all grouped together as *S. cerevisiae*, because the standard, 'classical', tests fail to recognize differences sufficient to justify the rank of species. Yet the industrial microbiologist must continue to distinguish these organisms, but now, unfortunately, without the assistance of distinctive specific names. Therefore the benefits of a standardized nomenclature are lost, as each microbiologist develops individualistic classification schemes for the different contaminant strains of *S. cerevisiae.*

3. PROPERTIES FOR IDENTIFICATION OF YEASTS

The properties used for yeast taxonomy according to the Dutch mycologists include both morphological and physiological properties (Table 4). The large number of growth tests listed by Lodder (1970), including over 30 carbon or nitrogen sources, has now been substantially reduced (Kreger-van Rij, 1984) to 18. Barnett *et al.* (1983) listed 60 characters based on sugar fermentation, or aerobic growth on C or N compounds, but suggested substantially fewer tests for routine identification. There is a fundamental difference between the identification procedures of the Dutch school and that of Barnett *et al.* (1979, 1983): the former identify strictly in descending hierarchical order, i.e. family, genus, species, whereas the latter subdivide first into largely unrelated groups. The subdivision suggested by Barnett *et al.* was based on a small number of key tests, designed to subdivide all known yeasts into groups of approximately equal numbers of species. Then these groups were in turn subjected to a small number of tests chosen to identify species in that group; different tests were required for different groups. The complications largely negated the biochemical good sense of the system, and a simpler system introduced by API, although based on the same principles, effectively identifies in a single set of tests. The three systems are compared in Table 4.

Numerous biochemical properties other than fermentation or aerobic