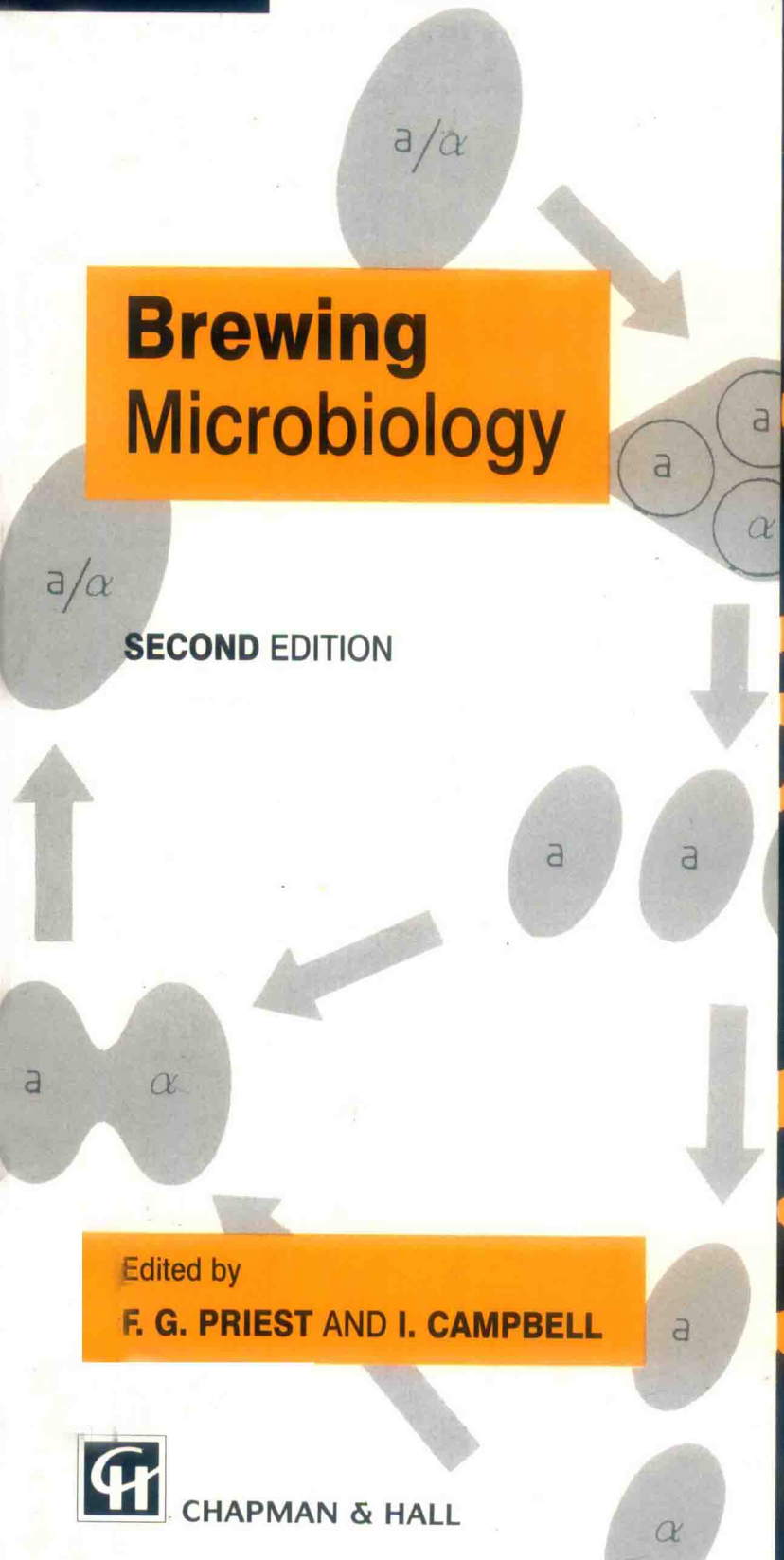
Brewing Microbiology

SECOND EDITION



Edited by

F. G. PRIEST AND **I. CAMPBELL**

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Second edition

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International Centre for Brewing and Distilling
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Preface

During the latter part of the last century and the early years of this century, the microbiology of beer and the brewing process played a central role in the development of modern microbiology. An important advance was Hansen's development of pure culture yeasts for brewery fermentations and the recognition of different species of brewing and wild yeasts. The discovery by Winge of the life cycles of yeasts and the possibilities of hybridization were among the first steps in yeast genetics with subsequent far-reaching consequences. Over the same period the contaminant bacteria of the fermentation industries were also studied, largely influenced by Shimwell's pioneering research and resulting in the improvement of beer quality.

Towards the end of the century, the influence of brewing microbiology within the discipline as a whole is far less important, but it retains an essential role in quality assurance in the brewing industry. Brewing microbiology has gained from advances in other aspects of microbiology and has adopted many of the techniques of biotechnology. Of particular relevance are the developments in yeast genetics and strain improvement by recombinant DNA techniques which are rapidly altering the way brewers view the most important microbiological components of the process: yeast and fermentation. Moreover, the changing emphasis in quality control from traditional plating techniques, which essentially provide a historical account of the process, to new rapid methodologies, which give up-to-the-minute microbiological status reports of the process upon which brewers can base decisions, is playing an important role in the way the modern brewery operates. Developments in other aspects of brewing, such as packaging under reduced oxygen levels, are affecting the range of spoilage organisms encountered in beer and have led to strictly anaerobic bacteria giving spoilage problems. In preparing this second edition of *Brewing Microbiology*, we have covered these and other developments in the microbiology of the brewing process and its products

while retaining the comprehensive yet specialist treatment of the first edition.

Once again, we thank the authors for their contributions and Nigel Balmforth of Chapman & Hall for his help and encouragement in the preparation of the book.

Fergus G. Priest, Iain Campbell

Contents

CHAPTER 1

Systematics of yeasts

I. Campbell

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

1.1 CLASSIFICATION OF YEASTS

What is a yeast? No satisfactory definition exists, and such features of commonly encountered yeasts as alcoholic fermentation or growth by budding are absent from a substantial minority of species. Although yeasts are generally accepted as fungi which are predominantly unicellular, there are various borderline 'yeast-like fungi' which are difficult to classify. Also, many yeasts are capable, under appropriate cultural conditions, of growing in mycelial form, either as true mycelium or as a pseudomycelium of branched chains of elongated yeast cells.

Most mycologists accept the series of publications of the Dutch group of yeast taxonomists as definitive. The current classification is still basically that of Kreger-van Rij (1984), although updated (Barnett, Payne and Yarrow, 1990) to recognize the discovery of new species or recent changes of attitude to the significance of taxonomic tests (Kurtzman, 1984; Kreger-van Rij, 1987; Kurtzman and Phaff, 1987).

Three of the four groups of fungi include yeasts: Ascomycetes, Basidiomycetes and Deuteromycetes. Although Zygomycetes may develop yeast morphology under unusual cultural conditions, their normal existence is in the filamentous form, as non-septate hyphae. Classification of fungi (Table 1.1) is largely on the form of vegetative growth and the nature of the spores, if formed. Physiological properties, as widely used in bacteriology, are not used in identification of filamentous fungi; so far as yeasts are

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Table 1.1 Classification of yeasts (from Kreger-van Rij, 1984)

1. 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. 1.1a); principal genera *Metschnikowia, Nematospora*.
   (b) Saccharomycetaceae (other forms of spore; Fig. 1.1b,c,d,e); four families, distinguished mainly by method of vegetative growth.
      (i) Schizosaccharomycoideae (growth by binary fission, Fig. 1.2a); one genus, *Schizosaccharomyces*.
      (ii) Nadsoniodeae (growth by polar budding, Fig. 1.2b); principal genera *Hanseniaspora, Nadsonia, Saccharomycodes*, distinguished by form of spores.
      (iii) Lipomycoideae (growth by multilateral budding, Fig. 1.2c, but the principal characteristic is the 'exozygotic ascus', a sac-like ascus with numerous oval spores, Fig. 1.1e); one genus, *Lipomyces*.
      (iv) Saccharomycoideae (growth by multilateral budding, Fig. 1.2c); numerous genera, distinguished mainly by details of the sporulation cycle and spore morphology. The most important genera in the brewing and distilling industries are *Debaryomyces, Dekkera, Kluyveromyces, Pichia, Saccharomyces, Schwanniomyces, Torulaspora* and *Zygosaccharomyces*.
2. 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.
3. 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. *Torulopsis*, formerly a separate genus including various species of spoilage yeasts of the brewing and related industries, is now incorporated into the genus *Candida* (Kreger-van Rij, 1984).

concerned, they are used to distinguish species. Formerly, the ability to utilize nitrate as a nitrogen source was used to distinguish genera but, following the demonstration by DNA analysis that species of *Hansenula* and

Pichia differing in utilization of NO_3^- were otherwise identical (Kurtzman, 1984), the 'nitrate-positive' genus *Hansenula* has now been abandoned. Former *Hansenula* spp. are now incorporated in the genus *Pichia*, or other related genera according to life cycles and sporulation characteristics (Barnett, Payne and Yarrow, 1990).

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. 1.1), 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. 1.2a) or polar budding (Fig. 1.2b) 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 related subgroup of vigorously fermentative yeasts. Indeed, by earlier classification (e.g. Lodder and 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

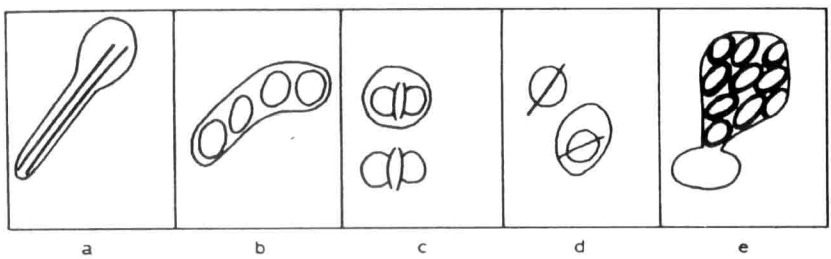

Fig. 1.1 Yeast spores. (a) Needle spores. The original vegetative cell, top right, has been distended to a club shape by development of the spores. Normally either one or two spores will be formed in *Metschnikowia* or *Nematospora* species. (b) Oval spores, as in the linear ascus of *Schizosaccharomyces 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 *Williopsis 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.

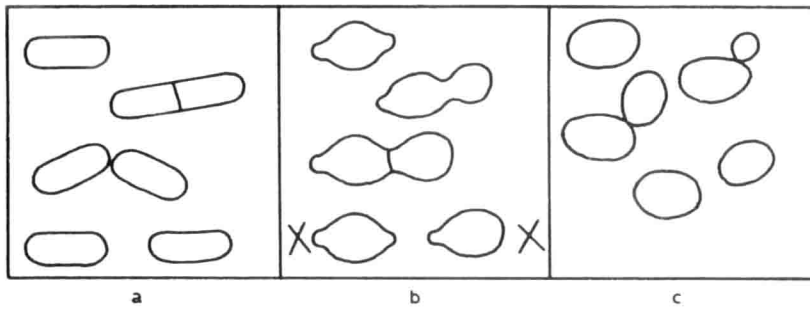

Fig. 1.2 Vegetative reproduction of yeasts. (a) Binary fission. The original cell elongates and after nuclear division a septum separates the two cells, which then complete the formation of the cell wall, and break apart. (b) Polar budding. Successive divisions are from either end (pole) of the cell, 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.

Fig. 1.3a. 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 heterothallic 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*.

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 was subsequently rectified in the more recent classification of 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

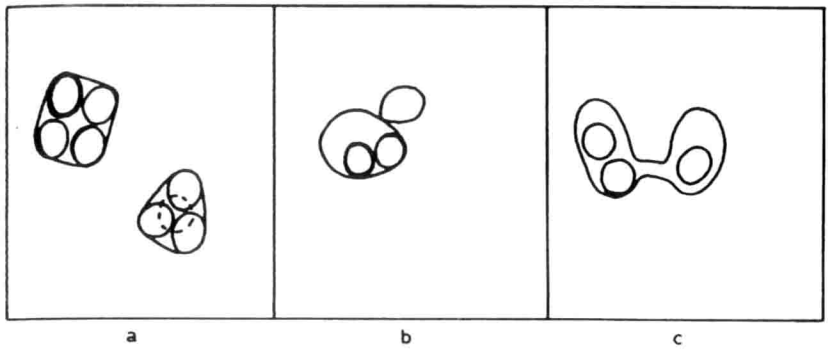

Fig. 1.3 Spores of *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces*. (a) *Saccharomyces*. Heterozygous diploid cells undergo two successive meiotic nuclear divisions to produce four haploid spores in plane (top left) or tetrahedral (bottom right) configuration. Often only two or three spores are 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.

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 1.3b and 1.3c. 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 reinfection.

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

Table 1.2 Perfect and imperfect states of common yeast contaminants of the brewing industry (Barnett, Payne and Yarrow, 1990)

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

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) a separate species 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 1.2) 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 by Barnett and colleagues, who have provided a comprehensive, but unfortunately complex, system for identification by physiological properties (see Barnett, Payne and Yarrow, 1990). A simplified version of their system is the basis of the API test kit for identification of yeasts (see Chapter 7).