ELSEVIER APPLIED FOOD SCIENCE SERIES

BIOTECHNOLOGY APPLICATIONS IN BEVERAGE PRODUCTION

Edited by

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PREFACE

Beverage production is among the oldest, though quantitatively most significant, applications of biotechnology methods, based on the use of microorganisms and enzymes.

Manufacturing processes employed in beverage production, originally typically empirical, have become a sector of growing economic importance in the food industry.

Pasteur's work represented the starting point for technological evolution in this field, and over the last hundred years progress in scientifically based research has been intense. This scientific and technological evolution is the direct result of the encounter between various disciplines (chemistry, biology, engineering, etc.).

Beverage production now exploits all the various features of first and second-generation biotechnology: screening and selective improvement of microorganisms; their mutations; their use in genetic engineering methods; fermentation control; control of enzymatic processes, including industrial plants; use of soluble enzymes and immobilized enzyme reactors; development of waste treatment processes and so on.

Research developments involving the use of biotechnology for the purpose of improving yields, solving quality-related problems and stimulating innovation are of particular and growing interest as far as production is concerned. Indeed, quality is the final result of the regulation of microbiological and enzymatic processes, and innovation is a consequence of improved knowledge of useful fermentations and the availability of new ingredients.

The Council of Europe's sponsorship of the work which led to the contributions to this volume is clear evidence of the growing need for adequate information about scientific and technological progress.

The objective pursued in preparing this volume was to bring

together knowledge from various sources, thus providing an up-to-date and hopefully stimulating framework for a *unitary approach* to a number of problems common to different beverages. This kind of approach, now firmly established and widely adopted in food industry technology, should stimulate cross-fertilization between different production sectors, which suffer from an excessive compartmentalization of technologies and knowledge.

Biotechnology, which includes both basic and applied sciences, is a multidisciplinary field and can thus act as a common denominator, providing a single, integrated picture of the state of the art in beverage production.

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Chapter 1

A PROPOSAL FOR CORRECT NOMENCLATURE OF THE DOMESTICATED SPECIES OF THE GENUS *SACCHAROMYCES*

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INTRODUCTION

The ability to produce ethanol by fermentation of simple sugars is almost completely restricted to yeasts. Although many species are known to carry out this transformation, only a few are able to yield significant amounts of ethyl alcohol during the natural fermentation of the juices of various sugary fruits. Only a handful of these, are commercially exploitable as actual or potential selected starters.

Zambonelli *et al.* (see Chapter 2) list *Schizosaccharomyces pombe*, *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* with all its synonyms *sensu* Yarrow[1] as yeast species strictly related to the production of alcoholic beverages.

Even though *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* have been proposed respectively for the biological deacidification of grape musts containing high quantities of malic acid and as a possible alternative to *S. cerevisiae* for highly 'pure' fermentation processes, these applications have rarely gone beyond the laboratory or pilot plant levels.

Information on the natural flora of fermenting grape musts may be found in the review by Kunkee and Goswell,[2] while the monograph of Phaff *et al.*[3] may be consulted for a summarized review on yeast species associated with the surface of other sugary fruits or with the production of less conventional alcoholic beverages.

This review will consider only those yeasts that are traditionally considered the main protagonists of alcoholic fermentation, regardless of the name of the beverage, of the carbon source used and of the technological or natural approach to the process. In fact, it is

Saccharomyces cerevisiae, together with all its many relatives (*S. bayanus*, *S. chevalieri*, *S. oviformis*, *S. pastorianus*, etc.), that predominate in the majority of cases.[2]

For the above reasons, this discussion will be centred on the yeasts belonging to the *sensu strictu* group of the genus *Saccharomyces* (*sensu* Yarrow),[1] with emphasis on: (i) their ecology according to the latest investigations; (ii) the modern taxonomic procedures utilized for discriminating between species; (iii) the present classification based on genetic analysis as well as nDNA–nDNA reassociation data.

Before entering into the actual matter of this report, which is the taxonomy of the species of the *sensu stricto* complex of the genus *Saccharomyces*, it would seem appropriate to briefly review some recent findings on the ecology of wine yeasts in nature that denote a peculiar and unsuspected situation.

A SHORT SUMMARY ON THE ORIGIN OF YEASTS ASSOCIATED WITH GRAPE MUST AND WINE

After the first demonstration that yeasts were normal inhabitants of the epidermis of ripe grapes,[5] Hansen[6] proposed the idea that yeast cells, washed off by rain or along with ripe fruits, fall to the ground, where they somehow survive the winter. In summertime they return to the fruit surface carried by various vectors such as wind, air currents or insects. It was later found that the apiculate yeast *Kloeckera apiculata* is normally present on the surface of numerous fruits such as cherries, gooseberries, grapes, plums and strawberries.[7] More recent evidence[3] demonstrated that the elliptical yeast *S. cerevisiae* var. *ellipsoideus* (at the time considered the yeast of wine as opposed to the brewer's yeast *S. cerevisiae*) is only rarely present on fruit surfaces, while appearing on the scene only at the end of an initial occupation of the must by *K. apiculata* (double domination effect).

As a matter of fact, *S. cerevisiae* and related species were consistently isolated in the past 90 years only in those ecological surveys performed by using an enrichment culture in liquid media (sterile grape must or malt). As a result, a strong selective pressure was imposed by the high sugar concentration (up to 18–19% w/v) and by anaerobic conditions in favour of those species capable of fermenting sucrose, in particular *S. cerevisiae* and related species. On the contrary, in the few investigations carried out without enrichment,

K. apiculata always predominated (>75%), followed by *Metschinikowia pulcherrima*, a group of film-forming or pigmented species and the yeast-like organism *Aureobasidium pullulans*.

A series of ecological surveys carried out by using more vigorous preisolation treatments of samples (fast shaking, jet-streaming of surfaces, ultrasonication) confirmed the above conclusions and showed that members of the collective species *S. cerevisiae* are practically absent from natural surfaces.[8] As a result, if high ethanol tolerant yeasts are not natural residents of grape surfaces, their origin must be found elsewhere.

Following the logical supposition that wine yeasts may more easily colonize the winery environment exposed each vintage to billions and billions of cells, Peynaud and Domercq[10] demonstrated that various surfaces (floors, walls, ceilings, vats, equipment, etc.) host a yeast flora belonging in a large majority to the collective species *S. cerevisiae*. In order to verify those conclusions, Rosini[11] studied the colonization of the surfaces of a newly established winery by using a labelled yeast starter. After two consecutive years of wine-making, all surfaces of the winery were colonized by the labelled *S. cerevisiae* strain. When during the third year fermentation was allowed to proceed naturally, without the addition of a starter, the grape must was immediately taken over by the winery-resident labelled yeast.

Additional indirect evidence in favour of this peculiar ecological situation is provided by the findings of recent comparisons of electrophoretically separated yeast chromosomes. Johnston and Mortimer[12] demonstrated that *S. cerevisiae* and related strains possess a significantly higher number of medium and small-sized chromosomes than do most other yeast species. This is considered by the authors to be the result of thousands of years of continuous selection for stronger fermenting capabilities, with polymeric genes from a common progenitor continuously duplicating and rearranging in larger numbers of chromosomal units.

At this point, in order to better understand their taxonomic position, we must keep in mind the fact that the wine-associated collective yeast species *S. cerevisiae* may be considered a 'domesticated organism' living in the wineries rather than circulating in nature.

YEAST CLASSIFICATION: A BRIEF HISTORY

Since the beginning of this century, the procedures used for yeast classification have undergone profound modifications. Initially, class-

ification was mostly carried out by studying morphological characters such as the shape of the cell and/or the macroscopic appearance of the colony.[6] Other formal characters, sexual reproduction and the capability to form mycelium or pseudomycelium, were later introduced by Guilliermond.[13] In the following decades, nutritional tests based on the ability to aerobically utilize different carbohydrates as sole carbon sources (assimilation) or in the absence of oxygen (fermentation) were introduced by the taxonomic school of the Centraalbureau voor Schimmelcultures (CBS) of Delft in Holland.[14–18] Accordingly, the number of taxonomic tests kept increasing up to the 40 required today for the determination of an unknown yeast.[19]

Additional criteria proposed, such as ascospore morphology,[20] serological characteristics of the cell wall,[21] proton magnetic resonance spectra of cell wall mannans,[22] the type of coenzyme Q of the electron transport system,[23] the ability to assimilate *n*-alkanes[24] and results of numerical taxonomy[25,26] were found to be effective only for discrimination to the genus level.

In spite of the large number of tests required, the separation of two taxa was often established on a simple difference of a single character.[27,28] Classical examples of this are the results of taxonomic studies of the past 80 years in which the positive fermentation of galactose or maltose or sucrose was the sole discriminating criteria between the traditionally wine-related yeasts such as *S. cerevisiae*, *S. bayanus*, *S. chevalieri*, *S. italicus* and *S. oviformis*, while all their remaining phenotypic properties are essentially identical. Studies by Scheda and Yarrow[29,30] demonstrated that fermentation patterns can vary significantly when repeated after a period of time. More recently Rosini *et al.*,[31] in the course of a taxonomic revision of over 1000 wine-associated *Saccharomyces* strains of the Industrial Yeasts Collection of the Department of Biologia Vegetale of the University of Perugia, Italy, reported that changes in the ability to ferment various sugars appeared randomly among all the old epithets, with a relatively high frequency. Minor genetic modifications may occur so rapidly in fermenting yeast populations that the decision of separating species on the basis of differences in single phenotypic characters, often governed by a single or at the most very few genes, can no longer be accepted.[27,32,33]

Accordingly, in the latest monograph[1] the only species recognized in the *sensu strictu* group was *S. cerevisiae*. This decision was the direct consequence of the numerous observations accumulated on the

variabilty of fermentation characters but also of the pressure of the introduction of molecular taxonomy which pointed out the evident inconsistencies of the conventional classification procedure.

STUDY OF MACROMOLECULAR RELATIONSHIPS BETWEEN MICROORGANISMS

It is commonly accepted by taxonomists that relationships between organisms are based upon two postulates: common ancestral origin and differentiation due to progressive substitution in nucleotide sequences. In other words, two organisms may be considered conspecific, in spite of their phenotypic expression, only when they have conserved a major portion of their genomes directly descending from a common ancestor. This approach to the classification of microorganisms, known as 'molecular taxonomy', is based on the evaluation of affinities between two organisms at the level of their macromolecules, particularly nuclear DNA. During the past two decades several methods of genome comparison have been proposed as an aid in the classification of yeasts such as DNA base composition expressed as mole percent of guanine plus cytosine (mol %G + C) and more precisely nDNA/nDNA homology. Since it is not the scope of the present work to expand on these methods, the reader is referred to the review by Kurtzman *et al.*[28]

It must be remembered, however, that the taxonomic value of G + C percentages is mainly exclusionary. In fact, while different mol %G + C values between two strains automatically excludes conspecificity, identical values do not necessarily mean that the two taxa belong to the same species. DNA/DNA homology, on the other hand, is much more indicative on the species level since it is an in-vitro reassociation reaction of whole nuclear DNA. On the basis of extensive comparisons between species it was proposed for yeasts that strains exhibiting 80% or higher DNA/DNA relatedness be considered conspecific.[27,34] Base sequence divergence, pointing to species separation, is not yet precisely established even though it is commonly accepted that reassociation values below 20% indicate absence of complementarity.[28]

The above procedures of molecular analysis have put into dramatic evidence some of the shortcomings of conventional classification procedures and led to the unification of the numerous epithets of

Saccharomyces sensu stricto under a single species due to similar phenotypic characters as well as identical %G + C values.[1,35]

Conversely, when the same species were subjected to nuclear DNA/DNA reassociation, the situation was somewhat different. In fact, Rosini *et al.*,[31] Vaughan Martini and Kurtzman,[36] and Vaughan Martini and Martini[37] demonstrated that *Saccharomyces sensu stricto* is composed of at least three separate species: *S. cerevisiae*, *S. bayanus* and *S. pastorianus*. In addition, recent unpublished data from this laboratory introduced into the scene a fourth relative of *S. cerevisiae*, *S. paradoxus*, ecologically separated and characterized by a complete absence of relationships with the alcoholic fermentation environment. These results will be discussed later.

A PRACTICAL APPROACH TO THE CLASSIFICATION OF SPECIES OF *SACCHAROMYCES* ASSOCIATED WITH THE ALCOHOLIC FERMENTATION INDUSTRY

The old epithets to which the fermentation industry throughout the world is accustomed are essentially: *S. cerevisiae* which indicates brewer's top yeast; *S. carlsbergensis*, the agent responsible for "low" fermentation in brewing; *S. ellipsoideus*, later called *S. cerevisiae* var. *ellipsoideus*: the wine yeast '*par excellence*'; *S. oviformis*, later denominated *S. bayanus*, believed to be especially endowed for refermentation processes; and *S. pastorianus*, the agent of fermentation in cold climates.

The history of yeast classification largely coincides with that of the group of species of the genus *Saccharomyces* defined as *sensu stricto* by van der Walt,[4] which includes the most important strains mentioned above for the alcoholic beverages industry. This group, in fact, is an excellent example of the continuous changes encountered by yeast taxonomy during the last century.

Since the publication in 1912 by Guilliermond of the first taxonomic monograph, *Les Levures*,[38] throughout those of the Dutch School of Delft,[1,4,17] the genus *Saccharomyces* underwent innumerable modifications with the initial tendency of describing numerous new species followed by a period dominated by the practice of grouping more species under a single epithet. This was due to the findings of innumerable workers that the practice of separating species on the basis of the fermentation of a single or a few sugars is unsound because of the extreme variability of these properties, previously

TABLE 1

VARIATIONS IN THE FERMENTATION PROFILES OF 1014 STRAINS OF *Saccharomyces sensu stricto* CONSERVED IN THE INDUSTRIAL YEAST COLLECTION OF THE DEPARTMENT OF PLANT BIOLOGY, PERUGIA, ITALY

1. Acquisition of the ability to ferment galactose:
   S. bayanus becomes *S. cerevisiae*: (9 cases)
   S. oviformis becomes *S. cerevisiae*: (29 cases)
2. Loss of the ability to ferment galactose:
   S. cerevisiae becomes *S. bayanus*: (25 cases)
3. Acquisition of the ability to ferment maltose:
   S. chevalieri becomes *S. cerevisiae*: (50 cases)
   S. fructuum becomes *S. cerevisiae*: (21 cases)
4. Acquisition of the ability to ferment raffinose:
   S. italicus becomes *S. cerevisiae*: (10 cases)

considered cardinal criteria for speciation in *Saccharomyces sensu stricto*.[29,30] In fact, as already mentioned, the results of a study[31] on the variation of some physiological properties of 1014 *S. cerevisiae* strains conserved for up to 40 years in the Industrial Yeasts Collection of the Dipartimento di Biologia Vegetale of the University of Perugia showed that changes in fermentative characters appear randomly, though consistently, with high frequency (*c.* 16%) (Table 1). Galactose fermentation, for example, was acquired in 38 cases and lost in 25; maltose fermentation was acquired in 71 cases and raffinose fermentation in 10.

A recent genetic study[39] offers an explanation to the fact that taxonomically indistinguishable strains may exhibit rather different fermentative patterns. In the genus *Saccharomyces* the fermentation of sugars is under the control of families of genes, dispersed and repeated in the genome. Each family includes multiple copies of these genes, generally unlinked though functionally equivalent. An example of the above situation can be the SUC family that contains genes capable of coding for the synthesis of the enzyme invertase, but which can be repeated six times separately in different chromosomes of the genome. As a consequence, different copies of these SUC genes may be either present or completely lacking in different strains of *Saccharomyces cerevisiae*. In other words, genetically identical strains may appear phenotypically quite different in relation to the fermentation of sugars.

All the above fermentative versatility and variability is very useful for the yeast that can adapt to various environmental conditions, and