Yeasts:
From Nature to Bioprocesses

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Yeasts: From Nature to Bioprocesses

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Mycology: Current and Future Developments

Volume # 2

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Yeasts: From Nature to Bioprocesses

ISSN (Online): 2452-0780

ISSN (Print): 2452-0772

ISBN (Online): 978-981-5051-06-3

ISBN (Print): 978-981-5051-07-0

ISBN (Paperback): 978-981-5051-08-7

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PREFACE

Yeasts: from nature to bioprocesses travels back in time through the history of yeasts from the early days up to now, with an evolutionary, taxonomic, and biotechnological approach. Along this journey, its chapters present numerous bioprocesses which use these microorganisms, from the Neolithic revolution to the present.

While the budding yeasts subphylum has been estimated to appear on earth about 400 million years ago, some yeast species known today are certainly more recent, such as the workhorse *Saccharomyces cerevisiae*, which probably diverged from its sister species approximately 5 million years ago. Indeed, yeasts play a fundamental ecological role in nutrient recycling and angiosperm reproduction. Thus, directly and indirectly, they have guaranteed the maintenance of the biodiversity of plants and, consequently, animals that establish an ecological relationship with them. Yeast ecology has a chapter of its own in this book, although other chapters have also punctuated this theme in different contexts.

The main yeast genera are discussed in specific chapters of the book. Likewise, important biotechnological applications of these microorganisms are also addressed in different chapters. It should be noted that industrial sectors dependent on yeasts comprise a trillion-dollar annual market value. Therefore, yeasts stand out as the most profitable microorganisms in industrial microbiology.

Although humans appeared on earth much more recently, several yeast species have been widely domesticated by them, aiming for yeast-based bioprocesses. Given the benefits that yeasts provide to humanity, either as the leading figures in various bioprocesses or indirectly through their ecological role, the book ends up bringing up a question that has already been asked other times before: would yeasts be the best friends of humans? Although the question does not need to be categorically answered, the reading of *Yeasts: from nature to bioprocesses* will undoubtedly convince the reader of the importance of these microorganisms for the development of civilization, economy, and science.

We wish everybody an excellent reading.

Beyond grateful,

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CHAPTER 1

Origin and Evolution of Yeasts

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Abstract: Yeasts are generally unicellular fungi that evolved from multicellular ancestors in distinct lineages. They have existed in this form for millennia in various habitats on the planet, where they are exposed to numerous stressful conditions. Some species have become an essential component of human civilization either in the food industry as drivers of fermentative processes or health sector as pathogenic organisms. These various conditions triggered adaptive differentiation between lineages of the same species, resulting in genetically and phenotypically distinct strains. Recently genomic studies have expanded our knowledge of the biodiversity, population structure, phylogeography and evolutionary history of some yeast species, especially in the context of domesticated yeasts. Studies have shown that a variety of mechanisms, including whole-genome duplication, heterozygosity, nucleotide, and structural variations, introgressions, horizontal gene transfer, and hybridization, contribute to this genetic and phenotypic diversity. This chapter discusses the origins of yeasts and the drivers of the evolutionary changes that took place as organisms developed niche specializations in nature and man-made environments. The key phenotypic traits that are pivotal to the dominance of several yeast species in anthropic environments are highlighted.

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Keywords: Adaptation, Abiotic stressors, Aneuploidy, *Brettanomyces*, Crabtree effect, Copy number variations, Domestication, Dimorphism, Fermentation, Fructophilic, Glucophilic, Horizontal gene transfer, Pathogenicity, Saccharomy cotina, *Starmerella*, *Saccharomyces cerevisiae*, Whole-genome duplication, *Wickerhamiella*, 4-vinylguiaiacol.

INTRODUCTION

The term "yeast" generally refers to a polyphyletic group of unicellular or dimorphic fungi that maintain a unicellular cell structure through most of their life cycle, divide asexually through budding or fission, and have a sexual structure not enclosed in fruiting bodies [1]. As members of the Kingdom Fungi, yeasts share a common ancestor with other opisthokonts, all of which are believed to have transitioned from unicellular to multicellular organisms. However, the yeasts seem to have subsequently "de-evolved" back to unicellularity from multicellular filamentous ancestors in distinct lineages of Ascomycota, Basidiomycota and certain Mucoromycota, containing more complex forms of fungi [2] and have lost most of the genes associated with multicellularity. This "de-evolution" was accompanied by convergent changes in regulatory networks, reduction and compaction of the genome marked by extensive gene losses [1 - 3]. Evidently, 3000 – 5000 genes, including those encoding plant cell wall degrading enzymes, fungal cell wall synthesis and modification, hydrophobins and fungal lysozymes, were dispensed, while genes required for essential cellular processes such as DNA replication, sequence recognition, chromatin binding and chromosome segregation were retained [4]. Moreover, it is hypothesized that the transcription factors regulating the Zn-cluster gene family, which contributes to the suppression of filamentous forms throughout the life cycle and under different conditions, were expanded [4]. Yeasts have evolved at least five times independently within the Kingdom Fungi. Today, yeasts are mainly distributed in two phyla, the Ascomycota and Basidiomycota. Within the Ascomycota, they are distributed between two subphyla, the Saccharomycotina (representing almost two-thirds of all known yeast), the Taphrinomycotina (representing ~ 3% of the total of members of the Ascomycota) [5].

Interestingly, even in their unicellular life forms, some yeasts can display multicellular growth under specific environmental conditions. For instance, dimorphic yeasts can switch from yeast to multicellular hyphae or pseudohyphae. These include pathogenic fungi of mammals, such as *Candida* spp. (*e.g.*, *C. albicans, C. parapsilosis, C. dubliensis, C. guilliermondii* and *C. lusitaniae*), *Exophiala dermatidis* and *Trichosporon cutaneum*, as well as phytopathogens such as *Taphrina deformans*, *Ustilago maydis*, *Ophiostoma ulmi* and saprophytic biotechnologically important yeasts such as *Saccharomyces cerevisiae*, *Yarrowia*

lipolytica and *Debaryomyces hansenii*. In pathogenic fungi, the yeast-mycelial switch is involved in virulence; however, in other yeasts, this switch is induced in response to environmental stimuli, *e.g*., nutrient limitation, pH, oxygen availability, ethanol concentrations, *etc* [6, 7]. Pseudohyphal or hyphal growth leads to clonal multicellularity, where daughter cells "stay together" after mitotic divisions. Alternatively, individual single cells can form multicellular aggregates generally referred to as flocs. In *S. cerevisiae*, where such aggregates have been extensively studied, a group of proteins called flocculins is responsible for the phenotype [8]. While most ascomycetous yeasts are distributed in the subphylum Saccharomycotina, a few unicellular or dimorphic fungi in which the unicellular form is restricted to specific environmental conditions also exist in the subphylum Pezizomycotina [5].

Multicellularity improves yeast access to complex substrates, allows for efficient nutrient uptake, and enhances the stress and toxin resistance [3, 8]. Despite these benefits, most yeasts maintain a long-term single-celled lifestyle. With this morphology and limited dispersal, most yeasts have evolved adaptive mechanisms that allow them to thrive in liquid environments containing concentrated simple sugars (*e.g*., plant-derived liquids, such as fruit juices, honeydew, and nectar), where they have a fitness advantage over prokaryotes [1, 2]. Such adaptations are explained by many genetic features that have undergone multiple rounds of modifications to endow different species with traits that allow for niche specialization. These genetic signatures and their associated phenotypes will be discussed in detail in subsequent sections.

MOLECULAR DRIVERS OF EVOLUTION

Gene losses, expansions and concomitant fine-tuning were the important drivers in the switch of yeast from their multicellular origins to single-celled lifestyle; these and additional modifications have also contributed significantly to yeast evolution and species diversification. Mainly, these modifications include Whole-Genome Duplication (WGD), Large Scale Genome Rearrangements (LSGR), Horizontal Gene Transfer (HGT), Copy Number Variations (CNV). WGD is a process by which additional copies of the genome are generated due to nondisjunction during meiosis. Through this process, organisms can acquire more than two complete sets of chromosomes, leading to a change in ploidy. Acquisition of genome copies can arise through interspecies hybridization, resulting in allopolyploids or intraspecies hybridization, leading to autopolyploidization. WGD is typically followed by inter-chromosomal rearrangements and the loss of one of the gene duplicates [9]. Large-scale genome rearrangements may occur through chromosome duplications or aneuploidy, thus creating copy number variations that may change gene dosage [10]. CNVs refer to

CHAPTER 2

Ecology: Yeasts on their Natural Environment

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Abstract: Yeasts are prevalent in most habitats on Earth, where they often reach high abundance and establish species-rich communities. To date, most research efforts have focused on cataloging the prevalence and diversity (at the phylogenetic and/or physiological level) of yeasts in different habitats and searching for reservoirs of novel yeast taxa. However, little is known regarding the ecological roles that yeasts play in their natural habitats or the relationships that they maintain with other coexisting organisms. This chapter provides a general overview of yeast habitats, with attention to the response of yeasts to diverse abiotic and biotic factors. Furthermore, the chapter presents a detailed description of some relevant systems where yeasts interact with other macro- and microorganisms, namely the insect microbiome, phylloplane, decaying cactus tissues, angiosperm flowers, human microbiome, and industrial processes. Future challenges in the study of yeast ecology are briefly discussed.

Keywords: Anthropogenic environment, Aquatic habitat, Atmosphere, Cactus, Community, Dispersal, Diversity, Ecology, Environmental factor, Evolution, Floral nectar, Flower, Human mycobiome, Industrial process, Insect microbiome, Multipartite interaction, Phylloplane, Soil, Symbiosis, Yeast.

INTRODUCTION

Virtually, all ecosystems on Earth contain yeasts. These taxonomically and phylogenetically diverse unicellular fungi colonize most terrestrial and aquatic habitats, including those most inhospitable, and can also be found in the atmosphere [1 - 5]. Furthermore, many yeast species are integral to human society as they are involved in the production of diverse food products, beverages, and industrial chemicals, and may act as human or animal pathogens. In addition, they provide excellent study models for use in cell biology and other disciplines. However, the ecology of most known yeast species is still poorly understood, and even the natural habitats of renowned model yeasts such as *Saccharomyces cerevisiae* are far from being fully characterized [6, 7].

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An important hurdle in the study of yeast ecology is that, until recently, most studies of yeast presence in natural and anthropogenic environments have utilized culture-based approaches, which tend to be biased toward the most abundant members of a community and often neglect low-abundance and slow-growing species [4, 8, 9]. Additionally, the use of different sampling strategies, culture media, and incubation conditions has made it difficult to compare the results obtained in different studies. Fortunately, recent developments in next-generation sequencing and other DNA-based culturing-independent methods have improved our knowledge regarding the diversity and habitat distribution of yeasts and other fungi in nature [10, 11].

This chapter provides a general overview of yeast habitats, with a special focus on the response of yeasts to diverse environmental factors. Subsequently, it delves into a more detailed description of some systems where yeasts interact with other macro- and microorganisms, often participating in multipartite interactions. Finally, future challenges in the study of yeast ecology are briefly discussed.

YEAST HABITATS

Yeast abundance and diversity in natural and anthropogenic habitats are determined by a variety of abiotic and biotic factors that frequently exhibit spatial heterogeneity and temporal variation (Table **1**). In addition, such growth-limiting factors usually come into force together and simultaneously, mutually influencing each other, so that the outcome of these interactions may be difficult to predict [2, 3]. Moreover, there are large-scale phenomena, such as climate and biogeography, which manifest themselves through changes in abiotic and biotic factors (*e.g.*, temperature, humidity, solar radiation, soil composition, vegetation, and animal vectors) [2].

Table 1. Overview of the main environmental factors that influence the metabolic activity, growth, and survival of yeasts [2, 3, 5].

Factors	Short Description
Temperature	Temperature influences yeast growth and generation time. Most yeasts are mesophilic, and grow best between 20 and 30 °C. Some species, mostly pathogens of warm-blooded animals, can grow at 37 ºC. The few yeast species capable of growing at 48–50 ºC are considered thermotolerant, rather than truly thermophilic. Temperatures >50 °C are usually lethal for vegetative yeast cells. The lower temperature limit of growth for some psychrotolerant species may extend below 0 °C, if water remains fluid (*e.g.*, in salty seawater).
Light and solar radiation	Yeasts are not photosynthetic organisms, so illumination is not a requirement for their existence. However, ultraviolet radiation can be lethal.

(Table 1) cont.....

Factors	Short Description
Pressure	Under natural conditions, the normal atmospheric pressure does not affect yeast growth. However, in the deep sea and some industrial processes, yeast cells must withstand high pressure. The viability of yeast cells decreases with increasing pressures above 100 MPa, and the cells are destroyed between 200 and 300 MPa.
Water activity	Water availability, generally expressed as water activity (a_w), is an important factor affecting yeast growth. Most yeasts can grow well at water activities 0.95–0.90. Only a few yeast species require reduced water activity and are considered truly xerophilic. Nevertheless, many yeast species can grow at high sugar and/or high salt concentrations and are classified as xerotolerant.
Oxygen dependence	Yeasts are basically aerobic organisms. Fermentative yeasts, which represent around half of the species described to date, are only facultative anaerobes.
pH	In general, yeasts prefer a slightly acidic medium and have an optimum pH between 4.5 and 5.5, but most species tolerate a wide range of pH values (generally between 3 and 10). Some species can grow at a strongly acidic pH (≤1.5). The tolerance to low pH depends on the type of acidulant, with organic acids possessing a stronger inhibitory effect than inorganic acids. Although acidic conditions are better tolerated than alkaline ones, numerous yeast species can thrive at pH above 10.
Nutrient availability	Yeasts require some sources of carbon, nitrogen, mineral salts, and certain vitamins and growth factors. Differences among yeast species in their ability to assimilate specific nutrients play a major role in habitat specificity. In general, cosmopolitan yeast species are generally the most heterogeneous in their nutritional abilities, whereas yeasts that have specialized habitats exhibit narrower nutritional potentials.
Presence of toxic compounds	Ethanol, which is the main product of alcoholic fermentation, exerts a toxic effect on various yeast species. *Saccharomyces cerevisiae* can tolerate 13–15% ethanol, and some strains even >18%. Carbon dioxide (CO_2), which is the second product of alcoholic fermentation, rarely accumulates at inhibitory concentrations under natural conditions, but yeasts living in the intestinal tract of animals may be subjected to high CO_2 concentrations. CO_2 can dissolve in water and, depending on the pH, form bicarbonate ions that inhibit yeast growth. Acetate, lactate, and other weak organic acids widely used as preservatives in the food industry (*e.g.*, benzoic and sorbic acid) exert specific inhibitory effects on yeasts. Plant and animal tissues contain diverse compounds that may inhibit yeast growth.
Interaction with other organisms	In their natural habitats, yeasts often interact with different macro- and microorganisms. Such interactions can be facultative or obligate, mutual or unidirectional, and they may have a positive (+), negative (-) or neutral (0) effect on the partners involved. The following modalities are possible: mutualism (+/+ interaction), competition (-/-), commensalism (+/0), amensalism (-/0), and predation/parasitism (+/-).

Among all the yeast species found in any habitat, it is important to distinguish those that are essential components of the community from those that are transient members [4]. Moreover, while some yeast species are ubiquitous generalists that occupy a wide geographic range and can dwell in different habitats, other species seem to have a more restricted distribution [5]. Determining whether a given yeast species is an essential or transient member of the community, or is a habitat

CHAPTER 3

Yeast Taxonomy

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Abstract: The massive parallel sequencing technology, applied to the taxonomy of microorganisms, has been affecting the traditional phenotypic and molecular phylogenies based on the sequence of a single gene or a small handful of genes. The exponential accumulation of new, entire genome sequences of microorganisms in public databases in recent years, especially in the fields of taxonomic and biotechnology, is driving a conceptual revolution in the way of understanding the concepts of species in microorganisms in general and fungi in particular. The problems of drawing species boundaries, reclassification of species, discovering new taxa and clades, recognizing synonyms, and new species for science can now be addressed with genomic approaches. Derived from all this, much more robust high-resolution phylogenies, based on core genomes or broad collections of genes and their deduced proteins, are currently being reconstructed. Although this effort is still far from being a canon in the taxonomy of yeasts, it will gradually turn into a change and challenge that researchers are taking into account due to the great power and reliability of these genomic approaches and bioinformatics tools. Likewise, the complete sequence of the genomes of the strains of microorganisms of industrial or biotechnological interest will allow limiting biopiracy, help protect patents, recognize the appellation of origin, discourage violations of intellectual property rights, and resolve conflicts over the rights of the commercial exploitation of microorganisms. In this chapter, an effort is made to compare conventional taxonomy techniques with the latest work involving genomic sciences as a key tool in yeast taxonomy.

Keywords: Bioinfomatics, Orthologues, Phylogenomics, Yeast, Yeast Taxonomy.

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INTRODUCTION

Phenotypic Taxonomy of Yeast

Formerly, the techniques used in yeast taxonomy were mainly based on phenotypic traits and physiological characteristics [1]. The identification and description of new species depended on the comparison of morphological features as well as on biochemical and physiological profiles with previously described species [2]. Fermentation and/or assimilation of several sugars, organic acids, alcohols, sugar alcohols, starch, and different nitrogen sources, as well as growth differences, were used for both identification and formal description of new species. Gradually, new biochemical tests, such as polysaccharide composition assays of the cell wall and capsule, mycocin susceptibility, and electrophoretic comparisons of enzymes, are being used to determine subtle differences among closely related strains [3]. However, these approaches require large numbers of tests, chemical standards and substrates, substantial equipment, sensitive techniques, and type strains to identify an isolate or describe a new species; such studies were generally only performed by specialists in yeast taxonomy. Consequently, only a few laboratories in the world had the capacity to fully identify or describe new yeast species. The time-consuming phenotypic characterization of pure cultures and the formal description of the new species became a bottleneck for non-specialist scientists, who resigned themselves to reporting their isolates and strains only at the genus level. In retrospect, we can see that many new yeasts of industrial or ecological interest that were reported before the mid-90s were only characterized to the genus level due to difficulties in analyzing all evidence for proper identification and because of the low number of species formally described.

However, as more strains of each species were isolated and phenotypically characterized, it became evident that a great phenotypic intraspecific diversity was universal to many yeast species, which complicated their adequate identification, species limits, and species concepts [4, 5].

MOLECULAR TAXONOMY OF YEAST

At the global level, the importance of the identification of yeasts with the use of molecular methods is remarkable. Genetic characterization of ascomycete yeasts began with the determination of DNA base composition, traditionally expressed as CG content, which could provide information on the dissimilarity of two organisms but not on their similarity since two organisms that are not phylogenetically related often have a similar CG content [6]. Among yeasts, a

ratio between 28 and 50% GC was calculated, whereas, in basidiomycete yeasts, it ranged between 50 and 70% GC. The wide margins of these intervals were insufficient and useless as elements for the identification of species [7].

Later, genetic relatedness using nuclear DNA-DNA reassociation or hybridization of DNA-DNA techniques impacted the taxonomy of fungi in general and that of yeasts in particular because it revealed a clearer picture of similarity and allowed to recognize synonymy of many species, providing a quantitative value as a percentage of the differences [8]. Although DNA-DNA hybridization is a laborious method, the technique had a marked impact on yeast recognition, even if it did not solve the issue of the genetic differentiation of closely related species [9]. An arbitrary minimum cutoff value of 70% of the DNA association of two species was considered sufficient to recognize two strains as belonging to the same species [10]. The DNA-DNA reassociation values were subsequently related to the percentage of similarity of the bacterial 16S rRNA gene. Thus, a DNA-DNA reassociation value of 70% was equivalent to a 97% percent similarity of the 16S rRNA gene [11]. In recent years, an alternative measurement has been adopted: the mean nucleotide identity (ANI), which is calculated by computational comparison of two sequences of complete genomes. An ANI percentage of 95–96% generally corresponds to the threshold of 70% in DNA-DNA hybridization [12, 13].

However, it was not until the advent of sequencing gene and non-coding DNA fragments that molecular biology methods became important due to their ease of performance, economy and universality. Gene sequencing was a relatively easy, quick and powerful method of identifying until species level many microbial groups by phylogenetic reconstruction and nucleotide similitude supported by bioinformatics software tools. As seen in Fig. (**1C**), the yeast ribosomal regions offer several possibilities for sequencing, but domain 2 of large subunit 26S ribosomal RNA (26S r RNA or 26S/28S LSU) was initially used as a molecular marker because it apparently contained sufficiently variable information to distinguish between closely related species [14]. However, the sequencing of both D1 and D2 domains (D1/D2) of 26S rRNA yeast genes (~600 bp) quickly became a popular tool for Ascomycota yeast identification, and the sequence databases suddenly became enriched.

CHAPTER 4

Saccharomyces: The 5 Ws and One H

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Abstract: The monophyletic *Saccharomyces sensu strictu* genus is composed of 8 species and several interspecies hybrids. Strains of this genus have been used in various processes that form a significant part of human culture and history. These include brewing, baking, production of wine and several other fermented beverages, and more recently, the production of biofuels, drugs, and chemicals. They can be found in the most diverse environments on almost all continents worldwide. A prominent example is the species *S. cerevisiae*, which has a remarkable history with humankind. In the present chapter, we illustrate the habitats of the *Saccharomyces* species and their long-lasting domestication process, as well as the hybridization that occurs between various species of this genus and their underlying industrial applications. We then finalize the text with an emblematic case study of its application in industrial sugarcane-based ethanol production, as performed in Brazil.

Keywords: Diversity, Domestication, Ethanol, Fermented beverages, Fermentation processes, Habitat, Hybridization, Stress, Sugarcane, *Saccharomyces*, yeast.

INTRODUCTION

The monophyletic *Saccharomyces sensu strictu* genus is actually composed of 8 species: *S. arboricola*, *S. cerevisiae*, *S. eubayanus*, *S. jurei*, *S. kudriavzevii*, *S. mi-*

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katae, *S. paradoxus*, *S. uvarum*, and several interspecies hybrids, including *S. bayanus* and *S. pastorianus* [1]. *Saccharomyce*s is considered a ubiquitous genus as it can be found in the most diverse natural environments. For example, non-domesticated *Saccharomyces* species have already been isolated on all continents on Earth, except for one (Antarctica). *Saccharomyces* species have been identified both in freshwater and seawater as well as in soil, fruits, and the gastrointestinal tracts of various animals [1 - 5]. The domestication of *Saccharom yces* began even before the domestication of animals by humankind, spanning for thousands of years, during the course of winemaking, brewing, baking, and more recently, the production of fuels and chemicals [6].

Natural or artificial hybridization between strains or species is a very common phenomenon that occurs in almost all sexually reproducing groups of organisms. Moreover, hybrids normally provide a selective advantage in a given environment [7]. Their chimeric genomes usually exhibit unique phenotypic traits that are not necessarily intermediate between those present in the progenitors. Therefore, *Saccharomyces* hybrids have found important industrial applications across industries, including the ones mentioned above.

Humans have consistently exploited one particular species, *S. cerevisiae*, for a myriad of bioprocesses [8, 9]. Specifically, in bioethanol production, this yeast species performs pivotal roles in Brazil, enabling the country to produce renewable and green fuel for transportation, contributing to sustain its renewable energy matrix, which constitutes an interesting case study for yeast industrial biotechnology. In this aspect, Brazil has the most economical and sustainable ethanol fermentation process in the world, with a very favorable energy balance.

SACCHAROMYCES HABITATS: WHERE?

Although *Saccharomyces* yeasts are widely recognized for their biotechnological applications through which they have co-existed with humans for approximately 10,000 years, they can be found in the most diverse natural environments. Due to thousands of years of domestication of the species of this genus (please, refer to the next section), which began even before the discovery of microorganisms [10], the distribution of *Saccharomyces* around the planet is a two-way street with its domesticators: on the one hand, archaeological records and analysis of genetic sequencing point out that similar fermentative processes were conducted by the polyphyletic strains of *Saccharomyces* in different places on the planet, demonstrating that fermentations started by naturally occurring yeasts [11 - 16], while on the other, there are indications that human beings may have transported these yeasts to regions where they were not previously found. Given the close millennial relationship between *Saccharomyces* and humans, the presence of

some species in certain places on Earth has been attributed to the migratory movements of humanity [13, 15], as recently reported for the presence of Holarctic strains of *S. uvarum* in Patagonia [17]. As a matter of fact, this region of South America harbors two genetically differentiated populations of *S. uvarum*, with one of them being closely related to the North-hemisphere strains. It is believed that this Holarctic-derived population is a result of the introduction of apple trees by European immigrants in the 16th century [17].

To the best of our knowledge, non-domesticated *Saccharomyces* species have already been isolated on all continents on Earth, with the exception of Antarctica [16, 18, 19]. The City of Fairbanks, in central Alaska, US, accounts for the northernmost point (65°59′ N), where a wild strain of *Saccharomyces* (of the species *S. ellipsoideus*) was found [20]. In the southern hemisphere, the Martial Glacier in Ushuaia (Tierra del Fuego, Argentina) appears as the place of isolation of a wild member of *Saccharomyces* (of the species *S. uvarum*) of greater south latitude (54°77' S) [21]. Finally, Auckland, New Zealand is the most easterly point (174°46' E) and the Island of Hawaii, US (155°50' W), the most westerly point, where non-domesticated representatives of this genus (of species *S. cerevisiae* and *S. paradoxus*, respectively) have been reported [22, 23]. However, at the opposite ends of longitude, both places are Pacific islands relatively close to each other (~7200 km). In addition to their wide distribution on the planet, interestingly, a strain of the main species of this genus —*S. cerevisiae*— even surpassed the limits of our biosphere and survived on a 40-day space flight in the Russian space station Mir, despite having presented mutation frequencies up to three times higher than those observed in their parental counterpart strain that stayed on the ground [24].

Therefore, these yeasts are also versatile concerning different temperature conditions. Despite being considered mesophilic microorganisms, in the extreme South and North regions where these yeasts have been found, the average annual temperature is between –1 °C and 2 °C, reaching up to –20 °C in winter (data from CLIMATE-DATA – www.climate-data.org). One of the warmest places where wild *Saccharomyces* strains have been found may be the Amazonian Forest biome. In this environment, Barbosa and co-workers [25] found these yeasts when they carried out a survey of wild *Saccharomyces* populations in the Brazilian state of Roraima, which is cut by the Equator. In this region of Brazil, the average minimum and maximum temperatures range between 22 °C and 34 °C (data from the Weather Forecast and Climate Studies Center of Brazil – www.cptec.inpe.br). These data demonstrate the adaptive diversity within a single genus and the consequent facilitation of its domestication processes.

CHAPTER 5

Candida

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Abstract: The genus *Candida* represents a huge repertoire of fungal species with diverse functions. These unicellular yeasts possess great clinical significance because of the high level of pathogenicity exhibited by some members of the genus. Moreover, several species of *Candida* are highly valued industrially as microbial platforms to produce commercial products. Therefore, there is a persistent need to describe the genus as a whole, considering its immense applications in both medical and biotechnological grounds. The genus is being continuously explored, with new species regularly emerging as pathogenic. However, since most of the in-depth research has been focused on a few species of these yeasts, therefore, only the pathogenic species have been described in this chapter, reviewing the underlying characteristics that label their pathogenicity, which include their incidence of occurrence among the population, spread of infection, factors affecting the host immune system, and disease control. Further, the major studies identifying the biotechnological potential of the species have been discussed.

Keywords: Adhesin, Biodegradation, Biofilm, Bioremediation, Biosurfactants, Candidiasis, CTG clade, Drug resistance, Enzymes, Hyphae, Non-albicans *Candida*, Parasexual, Pathogenic, Phagocytes, Pseudohyphae, Single-cell oil, Single-cell protein, Transcription factor, Virulence, Yeast.

INTRODUCTION

The genus *Candida* contains a mixed population of unicellular yeasts, which are difficult to group by their morphological or physiological characteristics. The species belonging to this genus do not share similar types of distinctive features. The term '*Candida*' loosely refers to imperfect fungi as the members do not exhibit a clearly defined sexual cycle. However, the name originates from the Latin word 'candidus' meaning white, as pigmentation is usually absent in these yeasts [1]. In 1923, the Danish microbiologist Christine Berkhout first classified

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nine yeast species, originally assigned to the *Monilla* genus, to this new taxon based on certain morphological, biochemical, and physiological characteristics [2]. Since then, the genus was reassigned several times, and finally, with the aid of molecular biology tools, a definite classification has been possible. Different species of these yeasts are widely distributed in natural ecosystems and are usually found in human microflora as saprophytes. However, some of them can become opportunistic pathogens given a congenial host environment.

(I). *CANDIDA*: A POLYPHYLETIC GENUS

The genus currently comprises around 200 species of ascomycetous yeasts (kingdom: Fungi, division: Ascomycota, subdivision: Saccharomycotina) grouped under Hemiascomycetes class and Saccharomycetaceae family [1]. The cell shape and size vary from species to species and also on the environmental conditions, but usually range from ovoid to elongate, and (1-8) μm x (1-6) μm [3, 4]. Cells may or may not form mycelium; pseudohyphae and occasionally true hyphae are observed [1, 5]. The usual mode of reproduction is budding, but the sexual cycle is observed in some cases [6]. Typically, *Candida* species are aerobic, glucose-fermenting yeasts, unable to assimilate nitrate or inositol, and do not synthesize carotenoids [1, 4]. A small number of *Candida* species are efficient pentose-utilizers that have been exploited for the effective use of hemicellulosic wastes. Numerous studies also focused on these yeasts metabolizing plant byproducts and subsequent bioconversion to important biotechnological compounds such as antibiotics, vitamins, complex alkanes, and biofuel [7]. Several species like *C. tropicalis*, *C. maltosa*, *C. famata*, *C. guilliermondii,* and *C. krusei* have been extensively employed for the industrial production of value-added metabolites [8]. Despite such immense biotechnological potential, the genus has gained its importance because of the pathogenesis and clinical significance of many species, the foremost being *C. albicans*, closely followed by *C. tropicalis, C. parapsilosis,* and *C. glabrata*.

Despite the numerous attempts at reclassification, a common evolutionary origin has not been established among the members of this genus; hence the genus *Candida* appears to be a polyphyletic group posing a substantial problem in characterizing the species. A breakthrough came in 1989 when Kawaguchi *et al.* described a different codon usage for CTG (or CUG) codon in *C. cylindraceae* (currently known as *C. rugosa*), which was found to code for serine instead of the usual leucine [9]. Further investigation by Sugita and Nakase identified 67 such species exhibiting alternative codon usage [10]. Currently, phylogenetic clustering based on whole-genome sequencing has divided the Saccharomycotina yeasts into two major groups- 1) **CTG clade** with species exhibiting non-classical translation

of CUG into serine (Fig. **1**), and 2) non-CTG clade including **whole-genome duplication (WGD) clade** comprising yeasts which have undergone genome duplication, including *Saccharomyces* species and the pathogenic *C. glabrata*. While many of the *Candida* species belong to the CTG clade, including *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. maltosa*, *C. famata*, *C. guilliermondii*, *C. lusitaniae*, *C. oleophila*, and *C. rugosa,* which are mostly pathogenic, the non-*Candida* yeast *Pichia stipitis* also falls in this group [11 - 14]. Such reassignment of codon has been correlated with the length of the isoprene chain present in coenzyme Q9 (Co-Q9), the predominant ubiquinone found in these species [10].

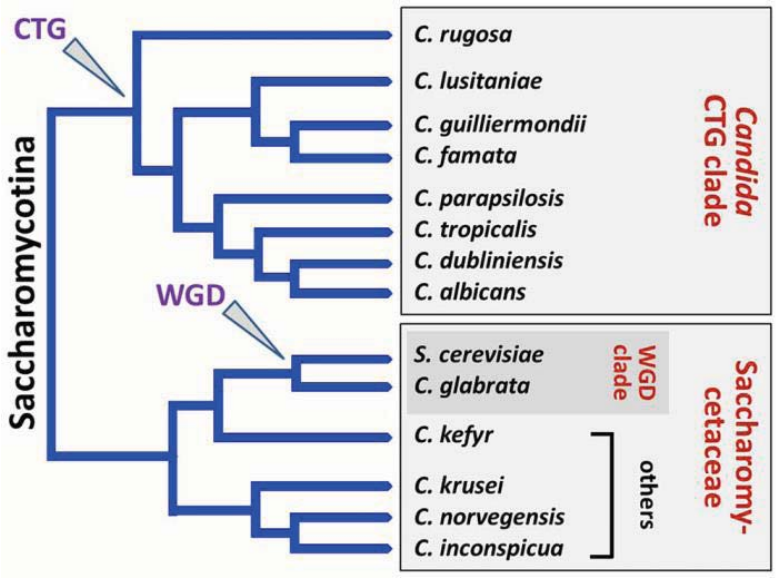

Fig. (1). Phylogenetic distribution of *Candida* species of yeasts. Most of the important pathogens of the genus fall into the CTG clade translating the CUG codon into serine, except for the highly frequent *C. glabrata,* which falls in the WGD clade and is more closely related to *S. cerevisiae*. The figure is taken from Papon *et al*., 2013 [15].

Normally mistranslation of codons is seen in organisms as an adaptive response to various stresses. For example, the mistranslation of UGA stop codon to selenocysteine in response to oxidative stress in neurons has been reported [16]. Likewise, the mistranslation of CUG in *C. albicans* is an example of stress-induced response, although 3% of codons are still translated as leucine in the yeast following standard codon usage pattern [17]. Misincorporation of serine initiates a cascade of morphological changes in the organism, altering its pathogenicity and susceptibility to the host immune system and morphogenic transition. Miranda *et al*. reported that CUG mistranslation dramatically changes

CHAPTER 6

Pichia: From Supporting Actors to the Leading Roles

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Abstract: *Pichia pastoris* are heterotrophic yeasts able to use many carbon sources such as glucose, glycerol, and methanol; they are unable, however, to metabolize lactose. Their methylotrophic properties, high yield, efficient post-translational modifications, and secretion of recombinant proteins, alongside a lack of hyperglycosylation, a post-translational process similar to that of mammals, and low maintenance costs for large-scale applications, make this yeast a promising alternative to produce recombinant proteins. The main recombinant products obtained from *P. pastoris* include vaccines and other biopharmaceuticals, enzymes, proteins, and pigments. *Pichia* spp. are also used in ethanol production and many other foods such as fermentation of coffee, cocoa, and olives, as well as alcoholic beverages. The use of *Pichia* yeasts in wastewater treatment and in fungal control of stored grains and fruit has also been reported. This chapter will discuss the environmental diversity of many species of *Pichia*, especially *P. pastoris*. Furthermore, the main uses of *Pichia* spp. in many bioprocesses will also be explored.

Keywords: Alcoholic beverages, Alcoholic beverages, Bioprocesses, Biocontrol systems, Carotenoids, Cocoa fermentation, Ethanol, Enzymes, Environmental diversity, Fermentation, Hyaluronic acid, Isobutanol, Pharmaceuticals, *Pichia pastoris*, Recombinant proteins, Ricinoleic acid, Vaccines, Wastewater treatment, Xylitol, Yeast.

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INTRODUCTION

The methylotrophic yeast *Pichia pastoris,* also known as *Komagataella pastoris,* has been commercialized by the Phillips Petroleum Company as a source of single-cell protein (SCP) destined for animal feed. It grows using methanol as a carbon source, causing overexpression of alcohol oxidase enzyme (AOX1) [1]. However, the increase in oil prices around 1970 negatively affected the use of *P. pastoris* as SCP [2]. Later on, Phillips Petroleum contacted Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA) seeking to develop a *Pichia* strain that could be used as a host cell for recombinant protein production [3, 4]. Based on the success this strain has shown as host, many different companies and research groups refined the initial protein expression system seeking to improve the recombinant protein expression rate. Its potential applications now include synthetic biology and whole-cell biotransformation.

The first record of protein production through biological systems for human use was a protein-based smallpox vaccine developed by Edward Jenner in 1796. From 1990 onwards, the biotechnology industry has been using microbial fermentation techniques to obtain products to be used in many different areas, such as the production of cleaning agents, fabrics, medicines, plastics, and even nutrition supplements. With the advent of recombinant DNA, it is now possible to use cultures of yeast, mold, bacteria, mammal cells, and even bugs in recombinant protein production (RPP) [5].

Escherichia coli is one of the most commonly used microorganisms in recombinant protein research, mostly due to its quick duplication time, high cell density, fully mapped genome, and low cost. However, the use of *E. coli* also has disadvantages, such as the lack of post-translational processing (glycosylation), reduced yield of recombinant products, presence of inactive proteins, and potential production of cytotoxic compounds [6, 7]. Many proteins are not able to be expressed in *E. coli* strains, as they require exact levels of post-translational maturity and, as such, must be produced by methylotrophic yeasts [8].

In this regard, yeasts such as *Pichia pastoris*, *Saccharomyces cerevisiae*, *Hansenula polymorpha,* and *Kluyveromyces lactis* are the most prominent [9, 10]. These yeasts tend to be applied in the production of heterologous proteins, mostly due to their high yield, strain stability, rapid growth, high cell density, and post-translational processing similar to that of mammals [11]; however, their glycosylation pattern remains different from that of human cells [12, 13]. The use of non-conventional yeasts has become a promising alternative, merging microbial advantages and eukaryotic protein processing while displaying several

advantages over *S. cerevisiae* regarding pathway requirements, product profiles, and overall cell physiology [6, 14].

Regarding the protein expression system using recombinant DNA techniques, *P. pastoris* displays improved productivity rates, more efficient post-translational modifications, better secretion of recombinant proteins, lack of hyper-glycosylation, reduced costs for large-scale production and maintenance, as well the ability to grow under high cell density conditions (up to 130 g/L) when compared to *S. cerevisiae* [4, 15, 16]. *P. pastoris* also secretes low amounts of endogenous proteins, which helps with the purification process, and adapts well to genetic manipulation, which allows the use of advanced genetic modification tools (*i.e.,* CRISPR/Cas9). These factors, in tandem with *P. pastoris*' low production costs [17, 18], make it a versatile option for biotechnological expression systems.

Pichia pastoris has been successfully used in the production of many recombinant heterologous proteins [4, 19 - 21] and multiple enzymes, such as α-amylase [19], β-mannanase [22], and β-glucosidase [23] to be used in chemical, pharmaceutical, and food industries. Furthermore, the increase in knowledge of *P. pastoris*' properties, the availability of genome data, and the development of new tools for cloning multiple genes have expanded its applications in industrial processes and in the production of important chemical compounds *via* metabolic engineering. These compounds and processes include xanthophylls [24], carotenoids [25], hyaluronic acid [26], ricinoleic acid [27], ethanol [28, 29], isobutanol [30], xylitol [31 - 33], cocoa fermentation [34], vaccine production [35 - 42], biocontrol systems [43 - 45], and removal of dyes and colorings from wastewaters [31 - 33].

PICHIA: A DIVERSITY OF ENVIRONMENTS

More than 100 species of *Pichia* have been discovered, most of them found in rotting plants or symbiotically with insects; some, however, can be found in the necrotic tissues of some cacti (*Pichia cactophila*) [46], in cured cheeses (*P. membranifaciens*) [47, 48], raw milk and fresh cheese (*P. anômala*) [49], and even some citruses [50]. Many of these *Pichia* species might even be present in many foods, beverages, and products with high sugar content, as undesirable contaminants. Some of these species are able to utilize organic acids present in many foods, causing spoilage, usually as a thin film on the surface of pickles [51], beer, and wines. Among all yeasts, *Pichia*, *Candida*, *Saccharomyces*, and *Rhodotorula* are the genera most commonly associated with spoilage of juices and wines, as these products contain microflora naturally resistant to the product's acidity. The main effects of the spoilage brought by these microorganisms include

CHAPTER 7

Brettanomyces: Diversity and Potential Applications in Industrial Fermentation

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Abstract: Although mainly known for their role in wine spoilage, *Brettanomyces* yeasts have been increasingly recognized as having beneficial effects on fermented beverages. These microorganisms can, for instance, increase flavor complexity, a property that can be controlled by understanding the physiological, genetic, and biochemical traits of *Brettanomyces* species in fermentation processes. Moreover, their genetic diversity, exceptional stress and low-pH tolerance, and peculiar metabolism suggest great potential for bioethanol production. This chapter summarizes the most notable features of *Brettanomyces*, briefly highlights recent insights into their genetic characteristics, and discusses potential applications in industrial fermentation processes, such as for the production of specialty beers, wines, and bioethanol.

Keywords: Acetic acid, Aroma, Beer, Bioethanol, Bioprocess, Crabtree effect, Custers effect, Fermentation, Oak barrel, Off-flavor, Spoilage yeast, Volatile phenol, Wine.

INTRODUCTION

Usually considered no more than a spoilage yeast, *Brettanomyces* isolated from ale beer was in fact, the first microorganism to be patented in history. Unique flavors produced by the fungus have become associated with British beers, hence the genus name *Brettanomyces*, derived from the Greek words brettano (British) and myces (fungus). Since the first description in 1904 [1], *Brettanomyces* species have been isolated in wineries and breweries all over the world [2]. The taxonomy of the genus has gone through several reclassifications over the years. In 1940, Custers [3] performed the first systematic study of *Brettanomyces* yeasts. Initially, the classification was based solely on asexually reproducing (anamorphic)

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variants. In 1960, the genus *Dekkera* was proposed as a teleomorphic (sexually reproducing) counterpart of *Brettanomyces* after the observation of ascospores in some strains. More recent molecular DNA techniques revealed no differences between anamorphic and teleomorphic forms, and currently, there is no separation between these groups. Both terms (*Brettanomyces* and *Dekkera*) are commonly used in wine research, but the term *Brettanomyces* is preferred in industrial settings [4 - 6].

Molecular analysis of the genus identified five species, the anamorphs *B. bruxellensis*, *B. anomalus*, *B. custersianus*, *B. naardenensi*s, and *B. nanus*, the first two of which also occur as teleomorphs, known as *Dekkera bruxellensis* and *Dekkera anomala* [5]. The species primarily associated with winemaking is *B. bruxellensis* (or *D. bruxellensis*), although recent wine-related investigations often include *D. anomala* along with *D. bruxellensis*, as current methods have had difficulty in differentiating between these two species [6].

Brettanomyces is a controversial yeast that has gained increasing attention in recent years because of its association with wine spoilage and the production of ethyl phenols. The yeast grows slowly; therefore, it usually imparts intense flavors (volatile phenols) in aged beverages, the so-called Brett flavors and odors, described as strong, smoky, or aromatic [7, 8]. Such descriptors may be either negative or positive depending on compound concentration and consumer expectation [9, 10]. This chapter presents a summary of the major phenotypic characteristics, growth patterns, and roles of *Brettanomyces* in fermentation processes, encompassing from beverage off-flavors to future perspectives in the production of spontaneously fermented beer, wine, and bioethanol.

DIVERSITY OF *BRETTANOMYCES* HABITATS

Brettanomyces is ubiquitous in nature. The yeast can be isolated from fermented food products, particularly during post-fermentation processing and aging of alcoholic beverages such as wine, beer, and cider, and is scarcely found outside these environments [6]. *Brettanomyces* niches comprise spontaneous alcoholic fermentation media with high ethanol concentrations, low pH, absence of readily fermentable nitrogen and carbon sources, and low oxygen [11]. Once alcoholic fermentation is completed, the remaining traces of residual sugars are sufficient for the proliferation of this slow-growing yeast [4]. *Brettanomyces* shows a high preference for fermented media, but it usually occurs at low concentrations and is, therefore, not considered a contaminant. Contamination only occurs when other microorganisms have been inhibited, evidence of the yeast's exceptional resistance to low-nutrient conditions, which allows it to adapt to harsh environments and outcompete other microorganisms [6, 11]. Malolactic

fermentation and aging in used oak barrels are recognized as the most critical stages of wine production for *Brettanomyces* contamination. Low concentrations of free sulfur dioxide (SO_2) and residual sugars, yeast autolysis with the nutrient release, presence of cellobiose (the main disaccharide in wood), and difficulty in sanitizing used barrels are factors that favor *Brettanomyces* growth and wine contamination [4].

Microbial contamination is an inevitable, undesired, complex event. Knowledge of yeast growth requirements and awareness of natural occurrence in raw ingredients may facilitate the identification of unwanted spoilage microorganisms. *Brettanomyces* is the most monitored yeast, particularly in winemaking [12]. The prevalence of *Brettanomyces* on grape skins is remarkably low, as previously assessed with the aid of an enrichment medium [13]. *Brettanomyces* has not yet been detected in the air during the first stages of harvesting [12], but it was identified in air samples of crush pads, tanks, barrels, and bottling rooms of a winery [14, 15]. Dweck *et al*. [16] observed that flies, known to be vectors of *Brettanomyces,* react to the yeast's smell. As expected, it was not difficult to detect the yeast in washing water and winery equipment used at advanced stages of vinification, oak aging, and bottle aging [12, 17, 18]. Differences in yeast population levels throughout winemaking can be attributed to production stage, time of year, degree of cleanliness, and cleaning protocols [12, 17]. Barrel sanitation is a very difficult task, and the use of sanitizing agents, such as SO_2, may contribute to the development of tolerant strains, as will be discussed in the following paragraphs [4]. Chemicals, ozone, biofilms, and sonication are alternative methods recommended for barrel and equipment sanitation to decrease *Brettanomyces* populations and volatile phenol production, but the effectiveness of such methods is debatable [4, 19]. Fig. (**1**) shows some factors associated with the presence of *Brettanomyces* during winemaking.

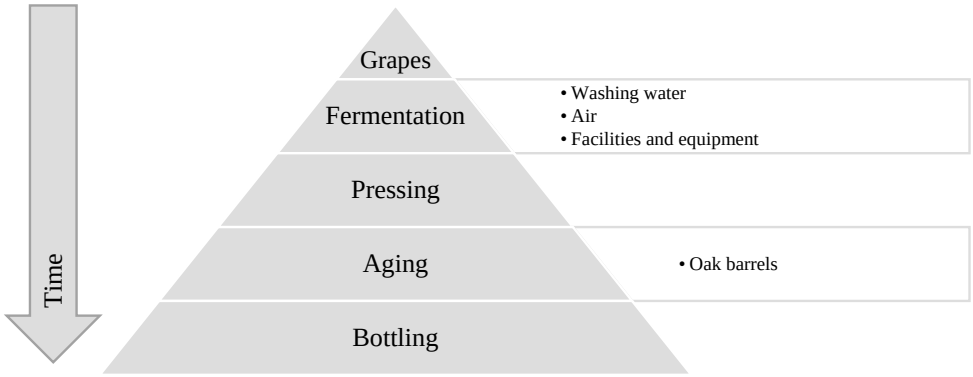

Fig. (1). Factors promoting the growth of Brettanomyces populations during winemaking.

CHAPTER 8

Spathaspora and *Scheffersomyces*: Promising Roles in Biorefineries

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Abstract: Currently, biotechnologies that aim to optimize the residual lignocellulosic biomass are receiving widespread attention, mainly when it comes to developing integrated systems that allow the generation of multi-products in industrial plants, especially for ethanol production. One of the main bottlenecks for efficient conversion of lignocellulosic biomass into ethanol is the limitation of *Saccharomyces cerevisiae*, the most widely used yeast in bioethanol production, in metabolizing xylose. This pentose is the main constituent of the hemicellulose fractions in plant cell walls and the second most abundant monosaccharide in lignocellulosic biomass. This challenge is being overcome by the isolation and intense molecular evaluation of new yeast species, mainly members of the genera *Spathaspora* and *Scheffersomyces*, since they have shown high capacities for xylose assimilation, which has been corroborated through studies aimed at improving ethanol production and other products *via* the association of these yeasts with improved fermentation capacity. In this sense, this chapter addresses the recent advances in the identification of novel isolates of the genera *Spathaspora* and *Scheffersomyces*, particularly emphasizing the applications of these genera in ethanol and xylitol production.

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Keywords: Atlantic rainforest, Biofuel, Biotechnology, *Candida*, Cellobiose, Ethanol, Fermentation, Fungi, Genomic analysis, Gut of insects, Hexoses, Lignocellulosic biomass, Pentoses, *Pichia*, Rotting wood, *Sp. passalidarum*, *Sc. stipitis*, Sugarcane hydrolysate, Xylitol, Xylose.

INTRODUCTION

The current model of society is characterized by increased consumption of biomass, which puts the environmental systems under pressure to meet the growing demand. Unchecked development and limited resources make waste management a key component to sustaining the current consumption pattern. The current linear thinking model, "take-make-dispose," is no longer sustainable, necessitating the shift to a circular economy, based on the use of waste as raw materials to acquire new products [1, 2].

Due to the necessity to change production systems, the biorefinery concept has been highlighted as a more holistic view of the exploration of biomass, ensuring the maximum use of the structure of the raw material by generating different products in the same industrial plant. This configuration enables the lignocellulosic biomass conversion in energy, chemicals, or biomaterials, thus adding value to waste, minimizing the impacts of production activities, and ensuring the sustainability of these activities [3].

The conversion of lignocellulosic biomass is dependent on fractionating the complex structure formed by different polymers, such as cellulose, hemicellulose, lignin, and pectin, in varying concentrations depending on the type of biomass [4]. Among the sugars released from the lignocellulosic biomass, glucose is released from cellulose. This hexose is a high-affinity substrate for various microorganisms, showing high conversion rates into ethanol. The primary sugar released from hemicellulose hydrolysis is xylose, which is the most abundant in xylan. However, unlike glucose, xylose offers low conversion efficiency by microorganisms [5]. The successful conversion of lignocellulosic biomass into bioproducts depends on the efficient use of the released sugars, such as glucose, D-xylose, cellobiose, galactose, rhamnose, arabinose, and mannose, by the microorganisms suitable to produce value-added products [6, 7].

Second-generation ethanol is one of the leading products resulting from the biotechnological processes involving the use of lignocellulosic biomass, and is a fundamental fuel to meet the demand and change the current energy matrix. Besides ethanol, other products of high added value can be obtained from these residues by biological routes, such as xylitol, a sugar-alcohol with great industrial applicability (*e.g.,* pharmaceutical and food), which is currently obtained *via* expensive chemical processes. This further emphasizes the importance of finding

economically and environmentally viable alternatives to get these products as well as the importance of the biorefinery approach [8].

Traditionally, yeasts of *Saccharomyces cerevisiae* and *Zymomonas mobilis* species are used industrially, but they either metabolize the pentoses found in the hydrolysates with low efficiency or do not metabolize them at all [9]. New approaches to prospect strains capable of acting widely in the fermentation process of complex substrates using different sugars have gained strength, presenting underutilized and unexploited bioresources with relevant characteristics for industrial exploration in biorefineries. In this scenario, yeasts of the genera *Spathaspora* and *Scheffersomyces* are highlighted as some species that can convert hexose and pentose sugars to ethanol and xylitol, which are isolated from different environments and which have shown a wide spectrum of industrial applications.

The performance evaluation of these yeast species is being explored as a valuable bioresource for the configurations of multi-product industrial plants. The metabolic capacity for pentoses and hexoses and the possibility of changes in the fermentative conditions to obtain different products have been intensely explored. In this scenario, this chapter aims to address the characteristics of yeasts of the genera *Spathaspora* and *Scheffersomyces*, emphasizing the environments from which they can be isolated and the primary studies on their application in fermentation systems, emphasizing the production of ethanol and xylitol.

SCHEFFERSOMYCES AND *SPATHASPORA* PHYLOGENY AND TAXONOMY

Scheffersomyces and *Spathaspora* yeasts have also demonstrated the potential for converting mixed sugars, which is interesting for biorefineries as products, such as xylitol, enzymes, and ethanol, can also be obtained [7, 10]. The relationship between these genera was described by Kurtzman e Suzuki [11] as being relatively closely related due to the fermentative capacity of D-xylose present in the species of both genera.

The genus *Spathaspora* was first described in 2006 by Nguyen and co-workers [12] to accommodate the single species *Sp. passalidarum*. The yeast was isolated from the intestine of *Odontotaenius disjunctus* (beetle) in Louisiana (USA) and highlighted for its characteristics of fermenting xylose as well as its distinct morphology, containing a single ascospore with curved ends, unlike any other known yeast [12, 13]. *Spathaspora* means a type of wide sword (*spatha*) and seed (*spora*), which was also named in honor of Joseph W. Spatafora for his contributions to the field of insect-fungus interactions, which were extremely relevant to the discovery of this yeast [12].

CHAPTER 9

Engineered *Saccharomyces* or Prospected non-*Saccharomyces*: Is There Only One Good Choice for Biorefineries?

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Abstract: Biorefineries require residual biomass as a raw material for their processes. Among all the possible products, 2G ethanol is undoubtedly the most studied and is probably the most desired in environmental terms. Carbohydrate-rich feedstocks used in biorefineries are mainly composed of polysaccharides, cellulose and hemicellulose (xylan), which initially require the action of hydrolytic enzymes to release their constituent monosaccharides, mostly glucose (from cellulose) and xylose (from hemicellulose). The conversion of glucose into ethanol is carried out by the yeast *Saccharomyces cerevisiae* with an efficiency close to the theoretical maximum yield (> 90%). Although it is the most widely used yeast in alcoholic fermentation processes, *S. cerevisiae* cannot metabolize xylose unless it undergoes genetic or evolutionary engineering. However, in recent decades, wild yeasts with an innate capacity to ferment this pentose and even hydrolyze the polysaccharides from lignocellulosic biomasses have been isolated and characterized from natural environments. Facing this duality, we conducted a major literature review and presented the data both in favor of engineering *S. cerevisiae* and the prospective use of wild yeasts in this chapter. To analyze the strengths of each strategy, this chapter also highlights the applications of integrated hydrolysis and fermentation processes and the possibility of simultaneously generating xylitol as the second product in biorefineries.

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Keywords: Bioprospection, *Candida*, Ethanol, Evolutionary engineering, Fermentation, Hydrolysis, Lignocellulosic biomass, *Meyerozyma*, *Pichia*, *Saccharomyces*, *Scheffersomyces*, *Spathaspora*, *Sugiyamaella*, *Wickerhamomyces*, Xylan, Xylanases, Xylitol, Xylooligosaccharide, Xylose.

INTRODUCTION

In terms of maximum production volume, fuel ethanol processing is the most extensive process to employ yeast as a fermenting microorganism. The world production of biofuel exceeds 100 billion liters per year. In this scenario, the USA and Brazil are the two largest producers, with ~60 and ~30 billion liters/year, respectively. These data place both countries at the forefront of bioethanol, although they have significantly different first-generation processes. In US production, yeasts ferment corn starch hydrolysates, whereas in Brazilian production, these microorganisms primarily ferment sucrose from the juice and molasses obtained from the milling of sugarcane [1].

Almost all ethanol production in both countries relies on the yeast *Saccharomyces cerevisiae*. This species is one of the best-studied eukaryotes, and its presence in fermentation processes dates back to the Neolithic revolution. Over thousands of years of coexistence with humanity, this yeast suffered different selective pressures that ended up domesticating it and generating a true workhorse microbe for the fermentation industry [1]. Although strains of *S. cerevisiae* differ genetically and phenotypically depending on the industrial sector in which they are found, some common characteristics make the species the preferred one in alcoholic fermentation, such as the ability to ferment sugars efficiently even in the presence of oxygen and to tolerate: (i) high concentrations of ethanol in the final stages of fermentation, (ii) the low pH levels of the medium, (iii) the osmotic stress caused by the high concentrations of sugars, and (iv) the hydrostatic pressure caused by the large volume of liquid contained in the fermentation tanks [2 - 4]. This yeast also stands out for being among the best glucose fermenters [5], which is the most abundant sugar in lignocellulosic residues, a biomass rich in cellulose and hemicellulose used as raw material in biorefineries [6 - 8]. From these residues, biorefineries can, separately or concomitantly, produce different fermentation products, including xylitol and second-generation ethanol (2G ethanol) [9 - 13]. Taking Brazil as an example, and considering only the sugarcane residue from the first-generation production of the fuel, it would be possible to increase the volume of ethanol produced in Brazil by up to 50%. To this end, however, it would be necessary for alcoholic fermentation to occur with a degree of efficiency of ~90%, similar to what already occurs for 1G ethanol [14, 15].

However, to achieve the production increase mentioned above, the fermenting microorganism must convert into ethanol the second-most abundant monosaccharide in lignocellulosic residue hydrolysates: xylose [16, 17]. In the fermentation of this pentose, one of the main obstacles of 2G ethanol is found, given that wild and industrial strains of *S. cerevisiae* are incapable of fermenting it [1, 15]. As a result, second-generation production is still in its infancy, representing less than 1% of the total volume of ethanol produced annually worldwide [18]. Thus, especially in the last two decades, research groups worldwide have made efforts to overcome the xylose-fermentation obstacle, either by engineering industrial strains of *S. cerevisiae* or by bioprospecting of wild non-*Saccharomyces* yeasts. In the present chapter, we address these different approaches to verify if there is a better choice for biorefineries.

FEEDSTOCK STRUCTURE AND FERMENTATION CHALLENGES

As an abundant source and for not serving as food for animals and humans, the great effort from scientists to replace the output of oil with ethanol is not unexpected [19]. The need for a transition from fossil fuels to renewable energy sources is evident on account of the climatic changes caused by modern times, mainly due to undesirable effects on atmospheric carbon balance and its disastrous effects on global warming. The development of transportation, for example, has influenced the environment in which emissions from internal combustion engines used in automobiles are the major source of air pollution in many urban areas [20]. The reduction of CO_2 emissions significantly contributes to minimizing environmental impact, and the use of a sugarcane ethanol system, for instance (like the Brazilian one), may offset 86% of CO_2 emissions compared to oil use [14].

Lignocellulosic biomass is found in several raw materials, ranging from urban and industrial waste, wood, and agricultural residues such as corn straw, wheat straw, rice straw, and sugarcane bagasse [21]. This material is derived from the cell wall of plants and is a rich source of inspiration for biotechnology, biofuels, and industrial biomaterials. The plant cell wall is a structure characterized by a mesh of polysaccharides, structural proteins, and phenolic compounds that protect the plant cell against external attacks and provide structural and mechanical support to the plant tissue, making it highly compact and treatment-resistant structure. It consists essentially of cellulose microfibrils, representing 30–60% of the total composition, as well as hemicellulose and lignin, representing between 20–40% and 10–20%, respectively. The chemical composition variation is due to various factors, such as climatic variability [7, 8, 22].

CHAPTER 10

Yeasts in the Beverage Industry: Patagonia Gets Wild

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Abstract: Yeasts are intimately involved in the production of fermented alcoholic beverages being the most popular examples of beer, cider and wine. The present chapter reviews the impact of yeasts in the production of these three fermented beverages and focuses on recent innovation trends regarding the use of non-conventional yeasts for the increase of flavour complexity and/or the development of novel special products that better meet current customer's demands. The granting of regional identity by using locally sourced yeast strains is also revised, and the experience gathered in the region of Andean Patagonia (Argentina) related to the isolation, screening, selection, improvement (in some cases) and all the way to the industrial application is described. North-western Patagonia natural forests harbour yeasts species of great scientific and fundamental relevance, among which the cryotolerant species *Saccharomyces uvarum* and *Saccharomyces eubayanus* are the most important for this chapter. The successful cases reviewed here of the study and application of Patagonian cold-adapted wild *Saccharomyces* yeasts for beer, cider, and wine innovation demonstrate that the laborious journey from nature to industry application is feasible and advantageous.

Keywords: Andes, Argentina, Beer, Cider, Craft industry, Euby, Hybridization, Microbe domestication, Native starter, Native yeast, Natural environments, Patagonia, *Saccharomyces cerevisiae*, *Saccharomyces eubayanus*, *Saccharomyces uvarum*, Selective isolation, Wild yeast, Wine, Yeast bioprospection, Yeast isolation.

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INTRODUCTION

Yeast, alone or in consortia with other microorganisms, has a profound role in the industrial and traditional production of many beverages. These are generally recognized as fermented and usually contain alcohol. Humans have consumed fermented beverages since the Neolithic period (*c*.10 000 BC [1];), however, it is still unclear whether, in ancient times, our ancestors accidentally stumbled across fermented beverages like wine or beer, or was it a product intended as such. Undoubtedly, alcoholic beverages have been part of the diet and culture of many of the civilizations that have preceded us and are among the most popular products consumed today. Fermented alcoholic beverages are complex solutions of thousands of chemical compounds that originate from the metabolism of yeasts and other microorganisms from a sugar substrate during fermentation, and from later stages that include secondary fermentations and / or chemical reactions during aging. The most popular non-distilled fermented beverages are obtained from cereal starches (by enzymatic pre-hydrolysis) in the case of beer (barley and wheat) and / or from fruits (which do not require pre-hydrolysis) in the case of wine (grapes) and cider (apples and pears). Yeasts of the genus *Saccharomyces* are the most prevalent microorganisms in the production of these fermented beverages [2]. The genus is composed of eight natural species, namely *Saccharomyces cerevisiae*, *S. paradoxus*, *S. uvarum*, *S. mikatae*, *S. kudriavzevii*, *S. arboricola*, *S. eubayanus* and *S. jurei* [3] (Fig. **1**). *S. cerevisiae* is by far the most recognized and ubiquitous species in the production of fermented foods and beverages. Nevertheless, the cryophilic species *S. uvarum* and *S. kudriavzevii,* and the hybrid species *S. bayanus* and *S. pastorianus*, play a fundamental role in the production of beverages such as beer and wine [2]. Furthermore, *S. paradoxus* and the latest additions to the genus *S. eubayanus* and *S. jurei*, are also being studied for their application in the fermentation industry [4 - 6].

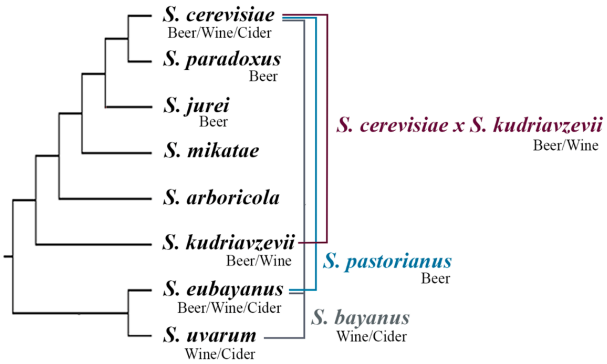

Fig. (1). Phylogenetic relationships of biological recognized species *Saccharomyces* species (black font), along with the most industrial relevant hybrids (non-black font), and their participation in the fermented beverages reviewed in this chapter.

During fermentation, yeasts produce alcohol, carbon dioxide and a range of secondary metabolites, such as esters, volatile fatty acids, higher alcohols, organic acids, volatile sulphur compounds and volatile phenols, that contribute significantly to the flavour and aroma of the final product [7, 8]. Certain strains of these yeasts, like many other microorganisms associated with man-made niches, have gone through a domestication process referred to as the artificial selection and breeding of wild specimens to obtain more fitted cultivated variants that better meet human or industrial requirements. Domesticated strains show improved adaptation to sugar-rich, oxygen-limited environments and high tolerance to ethanol, as well as other novel phenotypes, which can be specified for each fermentation environment. For instance, some beer yeasts can metabolize maltotriose (a beer-specific sugar), while wine yeasts can withstand the predominant sterilization agents in the winery (sulfite) and vineyard (copper sulphate) (for a review, see Steensels *et al*. [9]). These new domesticated traits result from the accumulation of defined genetic and genomic changes that only recently began to be elucidated and that may include inter-species gene introgressions (*i.e*. *S. uvarum* [10];) as well as inter-species hybridizations (*i.e*. *S. pastorianus* and *S. bayanus* [11];). Several non-*Saccharomyces* yeasts that are typically associated with early stages of spontaneous man-made fermentations are also relevant in the production of industrial and traditional fermented beverages. These include species of the genera *Brettanomyces*, *Torulaspora*, *Schizosaccharomyces*, *Metschnikowia, Hanseniaspora*, *Pichia*, *Lachancea* and *Kluyveromyces,* among others [12]. Because of their ability to modify the sensory quality of the final products, they are normally considered contaminants. However, due to new market trends in favor of products with differential and more complex organoleptic characteristics, they began to gain relevance in the beverage industry, contributing positively to the sensory quality of wine [13] and for bioflavouring purposes in brewing [14]. Another source of non-conventional yeasts with productive potential are natural environments and, even though these non-domesticated (wild) yeasts typically show inadequate fermentative traits for their implementation in the industry, exceptions exist mostly within the genus *Saccharomyces*. This approach has the advantage of providing additional regional identity and exclusivity to the beverages produced with local wild yeasts. Thus, the study and application of wild *Saccharomyces* for alcoholic beverage innovation have gained special attention in the last few years. In some cases, genetic improvement of wild yeasts was achieved using either genetic engineering or editing, mutagenesis or using non-GMO producing techniques such as directed experimental evolution and/or hybridization [4, 15, 16]. The whole process of isolating a wild yeast and inserting it into the market of fermented beverages is long and involves different research and development stages; initiating with yeast search and isolation and ending in scale-up trials and the technology transfer to

CHAPTER 11

Yeasts and Breadmaking

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Abstract: The earliest known evidence of leavened bread comes from Egypt and China in the second and first millennia BC, although records of unleavened breads and potential flour production date back tens of thousands of years. In the 19th century, the discovery of yeast fermentation led to the development of industrial bakeries in parallel with traditional sourdough bakeries. While strains of *Saccharomyces cerevisiae* were selected for and became the primary yeast used in industrial breadmaking, some artisanal bakeries continued to use natural sourdough. The maintenance of these two types of bakery practices led to the evolution of two genetically and phenotypically distinct clades of *Saccharomyces* bakery yeast. In addition to *S. cerevisiae*, other yeast species are regularly found in sourdoughs, in particular yeasts of the genus *Kazachstania*. In the sourdough ecosystem, these yeasts interact with each other and with bacteria in a positive or negative way, depending on the species and strains involved. In both sourdough and yeasted industrial dough, traits of interest include aroma production, efficient maltose utilization, osmotolerance, desiccation resistance, and freeze tolerance. These traits have largely been explored in *S. cerevisiae*, but there is abundant diversity in these traits even amongst strains of *S. cerevisiae*, and in the handful of yeast species that have been surveyed outside of *Saccharomyces*. The new interest in sourdough breadmaking and the societal desire to develop more sustainable and biodiversity-friendly bakeries is now leading bakers and scientists to explore the genetic and metabolic diversity of other yeast species.

Keywords: Aroma, Baking, Bread, Domestication, Evolution, Fermentation, Genomic, *Kazachstania*, Lactic acid bacteria, Maltose, Microbial community, Microbial interaction, Ploidy, *Pichia*, *Saccharomyces*, Sourdough, Stress tolerance, *Torulaspora*, *Wickerhamomyces*, Yeast.

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INTRODUCTION

Bread has long been a staple food, perhaps starting when humans still persisted as hunter-gatherers. The art of making bread developed in the Neolithic, with the emergence of agriculture and the domestication of cereal grasses. Since then, yeast has been used unconsciously and consciously to make bread rise. In this chapter, after presenting the milestones in the history of bread, we will present the knowledge on the evolution, ecology, and metabolism of yeasts associated with the making of traditional sourdough bread and industrial bread.

HISTORY OF BREADMAKING

The origins of bread remain largely unknown. Some of the earliest records of the human processing of wild cereal grasses date back to 23,500 to 22,500 years ago in what is now Israel [1], and evidence of grinding of other starches suggests that vegetal food processing and possibly flour production was occurring in what is now known as Europe and Australia 14,000-30,000 years ago [2 - 4]. The first known breads were of a flatbread-like form made from wild grasses and tubers 14,400 years ago, recently identified in present-day Jordan [5]. Bread-like finds became more common in Neolithic sites in Europe and southwest Asia as cereals like wheat and barley were domesticated around 9000 years ago, and dome-like ovens were identified at sites in Turkey beginning in the late 8th millennium [6].

Ancient writings and artistic representations document the centrality of grain to many societies. Indeed, the control of grains becomes synonymous with power and control of people, as depicted in this quotation from a Sumerian text, “Whoever has silver, whoever has jewels, whoever has cattle, whoever has sheep shall take a seat at the gate of whoever has grain, and pass his time there.” Throughout the Bronze Age, Mesopotamian households were paid with barley rations, and consumed many types of beers, porridge, cakes, and breads forming the central part of their daily diet [7]. We can speculate that some of these breads were leavened, as fermentation of beer using malted barley was practiced, and exchange between brewing and baking likely passed yeast back and forth. For example, brewing in both Mesopotamia and Egypt relied on a bread-like substance, which was only partially baked, presumably to preserve yeast viability, which was used to inoculate beer fermentation with yeast [8].

Baking in Ancient Egypt has received quite a lot of attention due to the prolific documentation of baking and brewing in art, writings, and actual remains of bread and storage vessels, especially during the period of 2500-2100 BC [9]. Egyptian breads were quite varied in size and texture, although all recovered loaves were made primarily from emmer wheat and had a dense crumb, some were flavored with coriander and fig [8, 10]. Some scholars point to the origin of what we now

refer to as "sourdough starter" to this time period in Egypt, in which some dough was kept and maintained with flour and water to be used in baking the next day [11].

The first direct evidence of the presence of yeast in bread comes from Egypt and China in the second and first millennia BC [10, 12]. Scanning electron microscopy of bread and brewing remains from central Egypt 1500-1300 BC identified budding yeasts [10]. In China, the first records of beer brewing using barley date between 3400-2900 BC [13], although barley did not become an important subsistence crop there until the Han dynasty (206 BC-AD 220). Proteomic analysis of food materials resembling sourdough bread from 500–300 BC identified Saccharomycetaceae yeasts and lactic acid bacteria [12].

The rise of bakeries is often attributed to the Roman empire. As the population of the city of Rome outpaced the ability of the surrounding regions to supply food to the populace, the Roman empire began importing grain and providing grain allotments at free or subsidized prices to its poor citizens [14]. Grain would have been taken to a mill and then the flour used to bake breads in home or communal ovens, or in a bakery. Pliny the Elder records that professional bakers appeared in Rome beginning in 168 BCE [15]. Grains were traditionally ground using a rounded stone pressed manually against a flat stone bed, until milling technology originated in Greece in the 5th century BCE and the Greeks and Romans advanced mill technology to include animal-driven and water-driven mills in the centuries following [16]. Starting in the 3rd century AD, grain allotments were replaced with bread [17], which persisted until the end of the Roman empire in the 6th century AD. Water-mills spread through the Roman and Byzantine empires across Eurasia in the centuries following.

It was not until the 1700s that yeast began to be produced for the purpose of use in bread and beer. At this time, yeast was recognized as "the ferment put into drink to make it work; and into bread, to lighten and swell it," but it was not recognized as a living organism [18]. Fermentation in the late eighteenth century and into the nineteenth century was studied exclusively by chemists, but with improvements in the microscope, yeast became recognized as a living organism by several scientists beginning in 1827. In 1837, a German physiologist named Theodor Schwann published his observations that yeast consume sugar and excrete ethanol, and reproduce by budding, linking fermentation to yeast. It was at this time that Schwann consulted with the mycologist Franz Julius Ferdinand Meyen who coined the term "*Saccharomyces*," based on the Greek words for "sugar" and "mushroom." Controversy over whether yeast was a living organism persisted for several decades, but as this idea became more accepted, fermentation became increasingly studied by biologists instead of chemists. In 1860, both Louis Pasteur

CHAPTER 12

Biotechnological Applications of Oleaginous Yeasts

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Abstract: Oleaginous yeasts are potential renewable sources of alternative biofuels due to high lipid contents and fatty acid profiles similar to those of plant oils. To increase the biotechnological potential of oleaginous yeasts, strategic cultivation of them using a wide variety of low-cost materials as substrates has been investigated intensively. Their metabolisms toward various substrates for the synthesis of lipids through *de novo* and *ex novo* processes have been described. In addition, direct transesterification processes that combine cell disruption, lipid extraction, and biodiesel production into a single step are proposed. This chapter thoroughly reviews recent research into the broad characteristics of oleaginous yeasts, the utilization of promising low-cost materials as substrates for yeast cultivation, and direct processing for biodiesel production from yeast lipids.

Keywords: Direct transesterification, Lipid synthesis, Low-cost materials, Oleaginous yeasts.

INTRODUCTION

Oleaginous microorganisms, including bacteria, yeasts, molds and microalgae, are able to accumulate lipids to over 20% of their biomass [1-3]. Microalgae can produce large amounts of lipids and hydrocarbons, but they require sunlight and carbon dioxide from fuel gas [2], a large cultivation area, and a long cultivation period [3]. Most bacterial species are not lipid producers, and they accumulate complex lipoids such as polyhydroxyalkanoates that are difficult to extract because these lipoids are generated in the outer membrane [4, 5]. Filamentous fungi can accumulate high intracellular lipid contents composed of triacylglycerols and specific polyunsaturated fatty acids [6], but fungi grow much slower than yeasts and form mycelia that cause high viscosity of the culture medium and decrease oxygen dispersion in the culture [7]. Unlike these micro-

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organisms, oleaginous yeasts (*i.e.*, *Yarrowia*, *Candida*, *Cryptococcus*, *Rhodotorula*, *Rhodosporidium*, *Trichosporon*, *Lipomyces*) have numerous advantages such as fast growth rate and high lipid content with high triacylglycerol fraction [8]. They typically contain a variety of lipids like triacylglycerol, diacylglycerol, monoacylglycerol, fatty acid, steryl ester, free sterol, glycerophospholipid, cardiolipin, sphingolipid, glycolipid, hydrocarbon, long-chain alcohol, wax, polyprenol, and isoprenoid quinone. Besides, four types of lipids can be found in the cytoplasmic membrane, namely glycerophospholipid, sphingolipid, steryl ester from ergosterol, and mono and diacylglycerols [9]. The oleaginous yeasts contain 80–90% triacylglycerol and a minor fraction of steryl esters, accumulated in special cell compartments known as lipid droplets (LDs) or lipid bodies (LBs) [10].

Yeast lipid costs are much higher than those of plant and animal oils because of the cost of nutrient media, and this is one of the major obstacles to large-scale yeast oil deployment [11, 12]. However, yeasts are able to utilize a wide variety of low-cost materials such as nutritional residues from agriculture and industry [13, 14]. The lipid production by oleaginous yeasts using wastes from agro-industry or industry as substrates has been extensively investigated for a variety of candidate substrates, including food waste, chicken tallow, durian peel, sorghum stalk, switchgrass, waste office paper, and crude glycerol, in order to reduce costs and make yeast lipids production economically viable [8, 15 - 20].

Yeast lipid-based biodiesel production through transesterification reactions has been investigated. The conversion of yeast lipids through transesterification reactions comprises numerous steps, including the drying of cells, the disruption of cells, lipids extraction, separation, and transesterification. In fact, several drawbacks directly affect conversion efficiency, such as long processing duration, need for large amounts of solvent, and high total costs [11]. To overcome these drawbacks, direct transesterification has been proposed. This approach excludes the cell drying step and combines the lipid extraction step with the transesterification step. Therefore, the overall production costs of biodiesel from yeast lipids could be reduced, making this process economically feasible [10 - 12]. Recently, many strategies for the direct transesterification of yeast lipids have been successfully demonstrated [13 - 16]. This article first introduces the characteristics of a broad range of oleaginous yeast species and their derived lipids. Recent challenges in the utilization of low-cost materials and wastes as alternative substrates by oleaginous yeasts and their biochemical conversion platform to produce lipids in yeast cells are highlighted. Finally, this chapter provides a current overview of published results on the direct transesterification of yeast lipids as a viable approach to economically viable biodiesel processing.

CHARACTERISTICS OF OLEAGINOUS YEASTS

Oleaginous yeasts are normally nonpathogenic unicellular budding organisms that can accumulate lipids to over 20% of their cell dry weight [17, 18]. Over 70 of the approximately 1,600 yeast species are well-known to be oleaginous [9], in five orders of Ascomycota and Basidiomycota, namely Saccharomycetales, Sporidiobolales, Tremellales, Trichosporonales and Cystobasidiales [18]. The order Saccharomycetales contains the two well-explored oleaginous yeast genera *Yarrowia* and *Lipomyces* and the new oleaginous species *Schwanniomyces etchellsii*, originally *Debaryomyces etchellsii*. The order Sporidiobolales contains *Rhodosporidium* and *Rhodotorula*. The orders Tremellales and Cystobasidiales contain diversified genera such as *Cryptococcus*, *Naganishia*, *Saitozyma*, and *Vishniacozyma*. The order Trichosporonales contains *Cutaneotrichosporon* genus, formerly *Trichosporon* (Table **1**). Depending on the species and the cultivation conditions, the lipid content in yeast biomass can be much improved to as high as 80% of the cell dry weight (Table **1**). In contrast, non-oleaginous yeasts (such as the baker's yeast *Saccharomyces cerevisiae* and the food yeast *Candida utilis*) usually cannot accumulate lipids to exceed 10% of their biomass [19]. Most well-known oleaginous yeasts contain fatty acids in the C16-C18 range of carbon atom counts. For example, *Y. lipolytica* contains mostly palmitic, stearic, oleic, linoleic, linoelaidic, and linolenic acids [20]. Recently, Carranba *et al.* [21] reported that *Y. lipolytica* Po1dL is a good candidate for lipid production in a glucose-based medium under nitrogen-limited conditions. Its lipid content reached up to 61% (w/w). *L. starkeyi* is a sustainable lipid producer, which has the potential to convert a wide variety of carbon sources into lipids in the form of triacylglycerols (TAG) for more than 70% of its dry cell weight [22]. According to Juanssilfero *et al.* [23], *L. starkeyi* NBRC10381 achieves lipid contents as high as 79.6% (w/w) in nitrogen-limited mineral media with glucose as the carbon source.

Table 1. Lipid composition of various oleaginous yeasts.

Yeast Strains	Lipid Components (%)				References
	TAG	DAG	MAG	FFA	
Yarrowia lipolytica	9.33	0.89	-	1.38	[36]
Cryptococcus vishniaccii	63.4	19.63	1.07	-	[37]
Cryptococcus curvatus	91.4	3.3	4.9	0.5	[38]
Rhodosporidium toruloides	92.2	2.7	4.7	0.4	[38]

TAG, DAG, MAG, and FFA are triacylglycerides, diacylglycerides, monoacylglycerides, and free fatty acids, respectively

CHAPTER 13

Improvement of Organic Agriculture with Growth-Promoting and Biocontrol Yeasts

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Abstract: Organic agriculture has significantly expanded over the years, increasing population. The productive methodology adopted in globalized agricultural systems reinforced the need to develop technologies that reduce the problems caused by the excessive use of pesticides and synthetic fertilizers. Some progress is being made by applying yeasts in agriculture due to the advantages associated with their use, such as promoting plant growth, biological control, inhibition of pathogens, and production of phytohormones. This chapter discusses studies that demonstrate the potential of yeasts in agriculture for biocontrol and plant growth. Yeasts are widely disseminated in the soil, increase and promote biological control, and show positive and promising results in the management of various phytopathogens. The interactions of these organisms influence multiple processes, such as the mineralization of organic matter in the soil, nutrient cycle, disease and weed control, and ecological balance. Efforts must be made to enable the production and application of yeasts as control agents in agriculture. Considering the diversity of yeast species present in the soil, their morphological, physiological, and phenotypic properties, understanding interactions and environmental effects integrating an ecological scenario is the key to good agricultural practices in a more sustainable context.

Keywords: Biological control, Growth promotion, Organic agriculture, Sustainability, Yeasts.

INTRODUCTION

Intensive population growth and food production methods in global ecological systems under stress have aroused interest in developing productive, stable, resilient, and environmentally friendly agricultural systems that produce healthy

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food and guarantee environmental integrity for future generations [1]. In conventional agriculture, weed management is carried out using pesticides, such as herbicides. In addition, the intensive and prolonged use of chemical pesticides causes environmental impacts, such as water and soil contamination, and the emergence of weed-resistant herbicides, which have increased by approximately 30% in the last ten years worldwide [2 - 4]. Resistance to pesticides has led to increased applications, higher crop losses, and farmers' mounting costs [1].

In this scenario, organic agriculture offers approaches that reduce the dependence on pesticides. Organic agriculture has expanded in many countries over the last few years; between 1999 and 2017, it has increased six-fold. In 2017, approximately 1.4 percent of the world's agricultural land was organic. Fourteen countries corresponded to more than 10% of these areas, and Australia has been a substantial contributor to exponential growth over the ten years before 2017 [5]. This increase in organic agriculture reflects the growing concern about environmental issues in intensive agriculture, mainly related to problems with the use of pesticides and commercial fertilizers. In addition, there is an increase in consumer demand for organic products, which has been supported by research and funding funds in many countries [6].

There is an expanded interest in productive and ecologically sound agriculture. Global data on organic production and markets are highly relevant for organic agriculture, considering it to be a sustainable form of production and dependent on policymakers that contribute to expanding these crops [5].

The development of new technologies that sustainably enable food security is essential in agriculture and the development of products that can reduce the excessive use of chemicals in crops to minimize environmental impacts. Significant advances are being made using microorganisms as an alternative in growth-promoting and biological control in agriculture because of their advantages over synthetic compounds, such as biodegradability, reduced half-life, and environmental safety.

The use of plant growth-promoting (PGP) microorganisms offers an alternative to reduce chemical fertilizers, resulting in increased tolerance to abiotic stresses, nutrient assimilation, pest control, plant height, root length, dry matter, *etc* [7, 8]. These benefits result from different mechanisms that help directly, for example, by phytohormone production that stimulates plant development and improves nutrient absorption by solubilization of compounds or indirectly by preventing the adverse effects of phytopathogenic microorganisms [9, 10]. PGP microorganisms have a crucial role in management systems to reduce agrochemical rates and increase the focus on biological methods for agriculture [7].

In addition to direct benefits, the potential of microorganisms for disease control in agriculture is an alternative tool in organic and conventional agriculture. It is an essential element for pest management [1]. Biological control of crops with naturally occurring microorganisms is an alternative to chemical control. It is based on a natural interaction between yeast and filamentous fungi. By different mechanisms, bacteria can protect plant crops against diseases, such as toxin and volatile compound production and specific enzyme secretion [11 - 13].

Yeast is a single-celled fungus that is abundant in the soil, with rapid growth and excellent characteristics such as PGP and biological control [14, 15]. Although research on endophytic yeasts as biocontrollers has only gained prominence in recent years, these yeasts have great potential for inhibiting phytopathogens because these microorganisms can act through several action mechanisms, thus reducing pathogen resistance in the environment [8, 16].

Yeasts can produce hormones such as indol-3-acetic acid (IAA), indol-3-pyruvic acid (IPYA), cytokinins, and several biologically active compounds that stimulate plant growth and development and increase crop productivity. Studies have identified improvements in plant growth, germination, and length after inoculation of seeds with yeast strains. When applied to biocontrol, yeasts can improve the uptake of water and nutrients, such as nitrogen and potassium, and reduce the risk of phytopathogenic infections because of the production of antimicrobial substances [17, 18].

Yeast's role in agricultural ecosystems needs advances, mainly because it is not entirely understood, and research on these microorganisms as PGP and biocontrol is scarce [10]. In this context, this chapter aims to highlight works present in the literature on the potential use of yeasts in agriculture for biocontrol and plant growth.

YEASTS IN THE NATURAL ENVIRONMENT

Living organisms can be divided into prokaryotes and eukaryotes. In the prokaryote group, we have bacteria and archaea. In eukaryotic organisms, we find cellular microorganisms, such as fungi (yeasts and molds), protozoa, algae, and higher organisms, such as plants and animals.

The Fungi Kingdom includes single-celled yeasts with approximately 680 known species distributed in two phyla (Ascomycota and Basidiomycota), multicellular molds, and macroscopic species such as mushrooms. Yeasts are non-filamentous, usually spherical or oval with a cell diameter between 1 and 10 μm, a rigid cell wall made up of the polysaccharide's glycan and mannan, which can grow in environments with low humidity, high osmotic pressure, and a wide pH range

CHAPTER 14

Yeasts: From the Laboratory to Bioprocesses

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Abstract: Yeasts are important industrial platforms for the efficient production of foods, beverages, commodity chemicals, and biofuels. Although these yeasts usually have beneficial native phenotypes, it is often desirable to engineer these cell factories to increase yield, titer, and production rates, or even promote the production of new molecules. In the present chapter, we describe several classical genetic approaches to improve industrial yeast strains (mating, cell and protoplast fusion techniques, mutagenesis, genome shuffling, adaptive laboratory evolution, *etc.*), as well as methods to identify the genetic basis of phenotypic traits, including phenotypes controlled by quantitative trait loci (QTL), through bulk segregant analysis (BSA) and DNA sequencing. We then review modern technologies for industrial yeast strain improvement (genomic engineering through homologous recombination, CRISPR-Cas9, synthetic chromosomes, synthetic genomes and SCRaMbLE) both in conventional (mostly *Saccharomyces* strains) as well as non-conventional yeasts. Finally, we give several current examples (and ideas for the future) of yeast strains genetically modified in the laboratory to produce a range of commercial products and biofuels through industrial bioprocesses.

Keywords: CRISPR-Cas9, Genetic engineering, Genomic engineering, Homologous recombination, SCRaMbLE, Synthetic chromosomes, Yeast breeding.

INTRODUCTION

Yeasts have been utilized wittingly and unwittingly by humans as tiny bio-factories over many millennia, possibly starting with our ancestors (*Homo erectus* or *H. neanderthal* [1, 2]), by transforming sugars into ethyl alcohol and carbon dioxide for purposes of imbibing, and later on, baking. The processes and rituals surrounding the making of alcoholic beverages and leavened bread have played an important role in human civilization, both socially and economically [3 - 5]. In particular, the quest to create alcoholic beverages has often been an impetus for

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scientific progress, especially in the areas of chemistry, biochemistry and microbiology: the brewing industry has even been cited as the foundation for the field of biotechnology [6].

But what exactly are these yeasts that have been our longtime companions? The yeasts responsible for producing much of our leavened bread, as well as almost all of the alcoholic beverages consumed in the world -including beer, wine, sake and distilled spirits- are "budding yeasts": small single-celled fungi that belong to the genus *Saccharomyces* (Latin for "sugar fungus"). These unassuming organisms are the world champions at performing the process of alcoholic fermentation. During such fermentations, *Saccharomyces* yeasts earn their Latin name by consuming the sugars present in the starting material -grapes in the case of wine and wort in the case of beer- and converting them into ethanol and carbon dioxide gas. In addition, yeasts will convert some of the more complex molecules already present in grapes or beer wort into novel characteristic flavor and aroma molecules; the whole endeavor results in delicious alcoholic beverages. In the case of leavened bread, the yeast consumes sugars created by the breakdown of the starch found in flour to produce alcohol and carbon dioxide; the latter makes bubbles in the rising dough, while the alcohol evaporates during the baking process.

Within the *Saccharomyces* genus, there are eight closely related, naturally occurring species so far known, as described in detail in a recent review ([7]; also see Chapter 4). The life cycle of *Saccharomyces* yeasts includes both asexual and sexual phases. Their genomes, like ours, are organized into linear chromosomes contained within a nucleus. Yeasts also contain cytoplasmically-located mitochondria that have their separate genomes [8]. Unlike us, however, the presence of mitochondria is not essential for *Saccharomyces* yeasts to survive, although functioning mitochondria are important for optimal yeast performance in many industrial processes. All *Saccharomyces* species possess very similar genomes, with the same number of chromosomes and with most genes (and gene order) shared among all species; additionally, the genomes are very similar at the DNA level. Likewise, all *Saccharomyces* species share the same basic life cycle and mating systems, and yeast cells can exist freely in the haploid or diploid state, where the haploid and diploid genomes are defined as containing one copy, or two copies, respectively, of each of the 16 different chromosomes. Haploid cells can exist briefly within the sexual mating cycle, derived by sporulation of the diploid cell, or they can exist indefinitely as free-living cells if they are unable to mate successfully, for example, due to physical isolation or mutations in the mating system [8].

Yeast cells undergo mitosis (in other words, they continually divide asexually, also called "clonally") when sufficient nutrients are present. This occurs by a "budding" process, where a small bulge on the side of the mother cell grows larger and larger until almost the same size as the mother. At this point, a new nucleus with its own set of chromosomes, as well as cytoplasmic organelles and mitochondria, are transported into the daughter bud, and a new cell wall grows between the daughter bud and the mother. After this, the bud separates away and starts its own mitosis process. A single mother cell produces an average of ~20-30 buds in its lifetime [8, 9]. However, when nutrients, especially nitrogen, become limiting, a diploid cell (but not haploid) can progress through meiosis to produce haploid spores, which are specialized gamete cells that can survive harsh conditions. In *Saccharomyces* yeasts, meiosis results in 4 haploid spores: two spores each of two opposite mating types, called "**a**" and "α" (see also Chapter 4). These two spore mating types can be thought of as "egg" and "sperm", where "**a**" cells can only mate with "α" cells and vice versa. When the two haploid spores mate (fuse together), they create a new diploid cell that combines the nuclear genomes of each parent spore, receiving one set of chromosomes from each parent [8].

Interestingly -and importantly for industrial applications- all eight *Saccharomyces* species are able to mate with each other: *i.e.*, haploid spores of one *Saccharomyces* species are able to mate with haploid spores of the opposite mating type of any of the other *Saccharomyces* species to form an interspecific hybrid, similar to a mule which is an interspecific hybrid between donkey and horse; such hybridization occurs both in the wild and in human-related environments (reviewed by [10, 11]; also refer to Chapter 4 of this book). These interspecific hybrids can proceed through sexual division (meiosis), although this results in mostly inviable spore progeny and thus, like the mule, they are "sterile". However, they are able to indefinitely reproduce in the mitotic asexual (clonal) manner. Other mechanisms, such as multiple rounds of spontaneous genome duplication, or aberrant mating between diploids, can lead to polyploidy (more than 2 copies of each of the basic haploid set of 16 chromosomes) within a species and similar aberrant mating of higher ploidy cells between different species can give rise to interspecific hybrids of varying ploidy levels [12, 13].

Finally, it is important to note that any diploid or polyploid yeast cell can usually tolerate not only mutations in single genes, but (amazingly) the loss or gain of a single chromosome, or even several chromosomes, leading to a state called "aneuploidy", where different chromosomes are present at different copy numbers [14, 15]. Equally important, most aneuploid and higher ploidy strains -as well as interspecific hybrids- are "sterile": *i.e.*, they either cannot mate, cannot complete meiosis, and/or cannot produce viable spores, and thus they cannot be subjected to

CHAPTER 15

Are Yeasts "Humanity's Best Friends"?

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Abstract: The beginning of the relationship between humans and yeasts is commonly assigned to the Neolithic revolution. However, the role of these microorganisms as gut symbionts of humans and other animals cannot be disregarded. In this case, the timespan of this relationship should be measured in hundreds (not tens) of thousands of years. Evidently, the hypothesis that the aforementioned symbiosis began precisely with the domestication of yeasts during the Neolithic revolution period cannot be ruled out as well. In any case, the relationship between humans and yeasts has broadly developed from the moment humanity started to domesticate them to produce bread and beverages, which seems to coincide with the Neolithic revolution period. Since then, humanity has created novel bioprocesses with yeasts, even though the role of these microorganisms was only really understood in the 19th century, especially with the studies of Louis Pasteur. Today, yeasts drive a trillion-dollar global market, which most likely presents the highest value among all sectors of industrial microbiology. In this context, this book's last chapter addresses the importance of yeasts in our society, with positive impacts on the economy and the health of humans, animals, and plants. We also discuss the role of these microorganisms in maintaining the balance and diversity of species in the environment as a whole. Finally, we close the chapter by highlighting the effects of their environmental role on human well-being and outlining the potential of wild yeasts that can drift from nature to new bioprocesses.

Keywords: Beer, Beverage, Biocontrol, Biodiesel, Biotechnology, Bread, Cheese, Chocolate, Decomposition, Ethanol, Food industry, Growth-promoting, Microbial factory, Pharmaceuticals, Probiotic, *Saccharomyces*, Single-cell protein, Vaccine, VOCs, Wine.

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INTRODUCTION

This is not the first time yeast has been claimed as a man's best friend [1, 2]. Since the Neolithic revolution, yeasts have been employed as fermenting microorganisms and their relationship with humans facilitated the change from a gathering-hunting lifestyle to the establishment of permanent settlements. New archaeobotanical evidence reveals that the preparation of bread-like products occurred 4,000 years before the emergence of the Neolithic agricultural way of life, probably 14,400 years ago in northeastern Jordan [3]. Although at that moment breadmaking may have occurred without fermentation (the so-called flatbread), at least from 10,000 ya, yeasts and humans have become increasingly close as the millennia have passed due to the diversification of various bioprocesses.

Yeasts have a direct or indirect bond (or a potential bond) with at least 9 of the 21 industrial sectors established by the United Nations (UN) classification — International Standard Industrial Classification of All Economic Activities (ISIC) [4]. Yeasts can also be used for heterologous expression, making them an excellent choice to act as microbial factories [5 - 12], greatly expanding their biotechnological and industrial potentials. Moreover, if one considers the approaches of Yeast Synthetic Biology, Synthetic Genomics, *Saccharomyces cerevisiae* v.2.0 (Sc2.0), and Synthetic Chromosome Recombination and Modification by LoxP-mediated Evolution (SCRaMbLE), this potential becomes even more significant [13, 14].

Furthermore, as new biotech prospects have been identified in recent years, the potential use of yeasts as bio-based (greener) alternatives to the conventional techniques in the chemical and petrochemical industries has also been envisioned [15]. Indeed, these microorganisms have proven to be increasingly versatile; not withstanding the countless industrial processes in which these microorganisms are already employed, it seems that they can be applied in an even greater variety of bioprocesses in the future. This chapter will summarise the main biotechnological applications of yeasts and outline their ecological roles, which also positively impact human welfare.

THE THINGS WE LOVE THE MOST

The most traditional biotechnological products, whose processing involves the use of yeasts, are also the most profitable ones. Besides, these products are also related to the joy, happiness, sociability, and pleasure of individuals. Therefore, they are very likely the bioproducts that humans enjoy the most. Together these industrial segments of joy comprise a trillionaire market, surpassing US$ 1.3 trillion worth of value.

Alcoholic Beverages

Most alcoholic beverages are produced due to the fermentation capacity of yeast cells. In this scenario, the species *S. cerevisiae* stands out as the primary yeast used for the beverages with the highest production volume and the most extensive market sizes (Table **1**).

Table 1. Alcoholic beverages and their markets.

Dominant Yeast Species in the Processes	Beverage	Global Production[a]	Global Market Size	References/Sources
S. cerevisiae and *S. pastorianus*	Beer	194 billion L	US$ 623 billion	[17, 19]
S. cerevisiae	Wine	29.2 billion L	US$ 327 billion	[17, 18, 20]
S. cerevisiae	Whiskey	5.2 billion L	US$ 60 billion	[21 - 23]
S. cerevisiae	Vodka	3 billion L	US$ 45 billion	[24 - 26]
S. cerevisiae	Tequila	0.25 billion L	US$ 10 billion	[27 - 29]
S. cerevisiae	Sake	0.6 billion L	US$ 9 billion	[30 - 32]
S. cerevisiae	Cachaça	1.8 billion L	US$ 2 billion	[33, 34]

[a] Approximated values per year.

Beer is a non-distilled beverage obtained from the fermentation of a wort composed of malted cereals, hops, and freshwater. Besides its millenary history, beer is now the leading alcoholic product consumed in the world. Its production has increased gradually over the last decades [16], reaching 194 billion liters in 2018. This amount represented a 50% increase in the last two decades and was six times higher than the wine production in that same year (29.2 billion liters) [17].

Although wine has a significantly lower production volume than beer, it is a higher value-added product, in such a way that the global wine market size is worth half of beer's (Table **1**). As a result, wine accounts for nearly one-quarter of the global alcoholic-beverages market [18]. Together, wine and beer represent almost 90% of the total alcoholic beverages produced on earth [17].

Considering the market of some of the most conventional alcoholic beverages alone, yeasts contribute to more than US$ 1 trillion market revenue worldwide (see Table **1**). The notoriety of this sum becomes even more evident when compared to another segment of the food and beverage industry: dairy products. This is a very representative sector of the employment of bacteria (in more significant proportion) and fungi; however, the entire dairy market, having a value of US$ 489.74 billion [35], does not even correspond to half the market size of the alcoholic beverages listed in Table **1**. Such difference between dairy and

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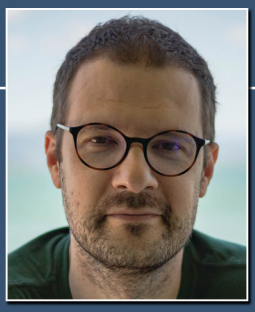
“Yeasts are the world’s foremost exploited microorganisms — from beer to biopharmaceuticals — but they also contribute immensely to our understanding of higher life processes. This book takes us on a fascinating journey through the wonderful world of the yeasts — from evolutionary aspects, to domestication, to industrial exploitation, including the emerging importance of non-Saccharomyces species.

Overall, this book represents a most worthwhile reference text that will be widely welcomed by yeast scientists and technologists.

Graeme Walker
Professor of Zymology, Abertay University, Dundee, Scotland.”



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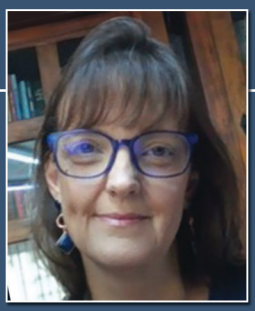

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