Taming Wild Yeast: Potential of Conventional and Nonconventional Yeasts in Industrial Fermentations

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Abstract

Yeasts are the main driving force behind several industrial food fermentation processes, including the production of beer, wine, sake, bread, and chocolate. Historically, these processes developed from uncontrolled, spontaneous fermentation reactions that rely on a complex mixture of microbes present in the environment. Because such spontaneous processes are generally inconsistent and inefficient and often lead to the formation of off-flavors, most of today's industrial production utilizes defined starter cultures, often consisting of a specific domesticated strain of *Saccharomyces cerevisiae*, *S. bayanus*, or *S. pastorianus*. Although this practice greatly improved process consistency, efficiency, and overall quality, it also limited the sensorial complexity of the end product. In this review, we discuss how *Saccharomyces* yeasts were domesticated to become the main workhorse of food fermentations, and we investigate the potential and selection of nonconventional yeasts that are often found in spontaneous fermentations, such as *Brettanomyces*, *Hanseniaspora*, and *Pichia* spp.

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INTRODUCTION

Fermented foods and beverages have been part of the human diet for thousands of years. Much like civilization itself, these fermented products originated in early Neolithic times (67, 99), but despite these ancient roots, the biological principles underlying fermentation processes have been unraveled only recently. Initially, food fermentations were a spontaneous process, relying on naturally occurring microbes, mainly yeasts and bacteria that were coincidentally present in the raw material or equipment or introduced by insects. Soon, producers noticed that the speed, quality, and consistency of a fermentation process often increased when it was inoculated with a small sample of a finished fermentation product. It was not until the end of the nineteenth century that this natural microflora was gradually replaced by predefined microbial cultures consisting of only one or a few selected microbial strains.

Because of their abundance and dominance in many spontaneous fermentations, *Saccharomyces* spp. (especially *S. cerevisiae*) were selected for the majority of controlled fermentation processes, including the production of bread, beer, sake, and wine (32). These species combine several crucial characteristics, such as the production of desirable flavors, the absence of toxin production, and high ethanol production and tolerance (86). However, an increasing number of studies find that confining the microbial diversity limits the sensorial characteristics and reduces the complexity of the end product (31, 35). These drawbacks have inspired some producers, often small-scale artisans looking for authentic and special niche products, to rediscover the spontaneous fermentation process. Moreover, with an increasing demand for boutique-style products and products geared toward specific desires of the consumers, large-scale producers are now also showing an increased interest in using nonconventional (i.e., non-*Saccharomyces*) yeasts in controlled fermentation processes to increase the flavor diversity, control microbial spoilage, and/or alter other key characteristics, such as the final alcohol content.

In this review, we describe the historical context of the development of microbial starter cultures for controlled fermentations, and we discuss how evolution shaped the *Saccharomyces* genome for these highly selective, synthetic environments. Next, we summarize the microbial diversity encountered in the spontaneous fermentation of wine, beer, and chocolate (the latter being one of the few remaining products that is almost exclusively produced through spontaneous fermentation). In a last section, specific key features of nonconventional yeasts that may help to increase fermentation efficiency, health-related properties, and/or sensorial quality of fermented foods and beverages are reviewed.

TAMING *SACCHAROMYCES*, THE MAIN WORKHORSE OF INDUSTRIAL FERMENTATIONS

Although spontaneous fermentations have been used for thousands of years to produce fermented products, they have several drawbacks. Most notably, spontaneous fermentations tend to be variable and unpredictable, because even slight changes in the number and kind of microbes present can have far-reaching effects on fermentation efficiency or product quality (41, 88). Therefore, the majority of today's industrial bread, beer, and wine production relies on defined starter cultures. The most-used species is *S. cerevisiae*, followed by close relatives, such as *S. pastorianus* for lager fermentations and *S. bayanus* for low-temperature wine or cider fermentations (32). In this section, the origins of the modern starter culture and the suitability of *Saccharomyces* spp. in these starters are addressed.

History of Domesticated Starter Cultures for Industrial Fermentations

Despite the millennia-long association of fermentations with human activity, the underlying principles remained largely unknown until pioneers like Van Leeuwenhoek, Pasteur, and Hansen started unraveling the microbial underpinnings of fermentation. However, even before these scientists revealed how microbes contribute to food fermentations, people realized that exposing fruits and grains to the environment changed the characteristics of these products. This process was later named fermentation, deducted from the Latin word *fervere* (to boil), based on the observation that during the process, bubbles were formed in the fermenting medium (1). Skilled artisans soon understood that it paid to keep a small sample of fermented dough or beer sediment and to mix this sample with a new, unfermented batch. In this way, without realizing it, they were in fact using (impure) starter cultures by carrying over the microbes from one fermentation cycle to the next. Moreover, this practice likely also led to the gradual adaptation of the microbes to the man-made conditions, thereby initiating the domestication of industrial microbes. However, it was not until the mid-1600s that scientists started to unravel the mechanism of the fermentation process, thereby opening the door to the development of pure starter cultures and a more direct selection and breeding of superior strains and variants.

The world of microbes remained concealed until the invention of the microscope. Robert Hooke and (more famously) Antonie van Leeuwenhoek were the first to visualize and describe microorganisms, e.g., from samples of the bottom of a beer vat. Interestingly, the first steps in unraveling the basic principles of fermentation were taken by some of the world's most famous chemists, such as Antoine Lavoisier and Joseph Gay-Lussac, who formulated the basic chemical reaction that occurs during fermentations (grape must becomes carbonic acid and alcohol). Next, three scientists (Cagniard-Latour, Kützing, and Schwann) independently posed the (at that time controversial) statement that "ferments," or yeasts, are living organisms, an observation later

confirmed by Louis Pasteur and described in a seminal manuscript entitled "Mémoire sur la Fermentation Alcoolique" (5–7).

These insights into the fermentation process paved the way for the isolation of pure, defined starter cultures. In the early 1880s, Emile Christian Hansen, an employee of the Carlsberg Laboratory in Copenhagen, described several techniques to isolate single microbial cells. Using these techniques, he was the first to succeed in isolating pure yeast strains (8). In November 1883, the Old Carlsberg Brewery started using one of Hansen's isolates, Unterhefe Nr. 1 (*S. carlsbergensis*, now reclassified as *S. pastorianus*), for the production of their lager beers. This is the first reported use of a yeast starter culture consisting of one well-defined and characterized microbial strain. Given the extraordinary consistency and efficiency of controlled fermentations with an appropriate starter culture, this technique was quickly adopted by other breweries and other industrial branches, resulting in the widespread use of pure-culture *Saccharomyces* fermentation starters. For example, in 1888, Auguste Joseph François de Bavay, a Belgian brewer employed in Melbourne, isolated and introduced Australian No. 2, probably the world's first pure culture used in ale brewing (84). In 1890, Müller-Thurgau introduced the application of pure yeast cultures for wine making (88).

Domesticated *Saccharomyces* Strains as the Ultimate Fermentation Specialists

It is interesting to note that *S. cerevisiae* is often the yeast of choice in controlled industrial fermentation processes. This species possesses several features that explain its current status as preferred industrial microbe. First and foremost, *S. cerevisiae* produces high concentrations of ethanol, which, apart from exerting interesting side effects on human physiology, helps to protect beverages against microbial spoilage. Moreover, fermentation is the preferred mode of metabolism in *S. cerevisiae*; it is a natural fermenter. Specifically, glucose represses respiration (Crabtree effect) so that even at the beginning of fermentation, when oxygen is often still available, the cells favor fermentation over respiration. On top of the higher carbon flux and faster production of energy that can be achieved by fermentation compared to respiration, the Crabtree effect fits a "make-accumulate-consume" strategy, where ethanol is first produced to inhibit the growth of other microbes and later consumed again when all fermentable sugars have been converted (106). In keeping with this ecological strategy, *S. cerevisiae* evolved a high tolerance to ethanol and several other environmental stresses (such as high temperatures), a very high glycolytic flux, and the ability to grow in both aerobic and anaerobic conditions (26, 48, 86). Whereas these individual properties are also present in other yeasts, they are uniquely combined and tuned in *S. cerevisiae* and its closest relatives, enabling these species to outcompete other wild yeasts in most fermentation processes (86).

Several studies addressed the genetics underlying these unique properties and tried to unravel the evolutionary path *Saccharomyces* strains have undergone to become the specialized fermentation organisms they are today. It was shown that duplication of several key genes, such as those encoding alcohol dehydrogenase (56, 106), hexose transporters (71), and enzymes linked to glycolysis (26), as well as global rewiring of the transcriptional network after whole genome duplication (60), may contribute to the suitability of *S. cerevisiae* as a driver of industrial fermentations. Interestingly, all these adaptive genetic events happened approximately 80–150 million years ago, far before human production of fermented foodstuffs, probably as an adaptation to the new niche created by the emergence of fruit-bearing plants (86, 113).

Apart from this more general adaptation of *Saccharomyces* spp. to sugar-rich, oxygen-limited environments, several other adaptations seem to increase the yeasts' performance in specific man-made fermentations. During these fermentations, several novel superior yeast mutants and variants emerged through (mainly unintentional) breeding and directed evolution. This resulted in

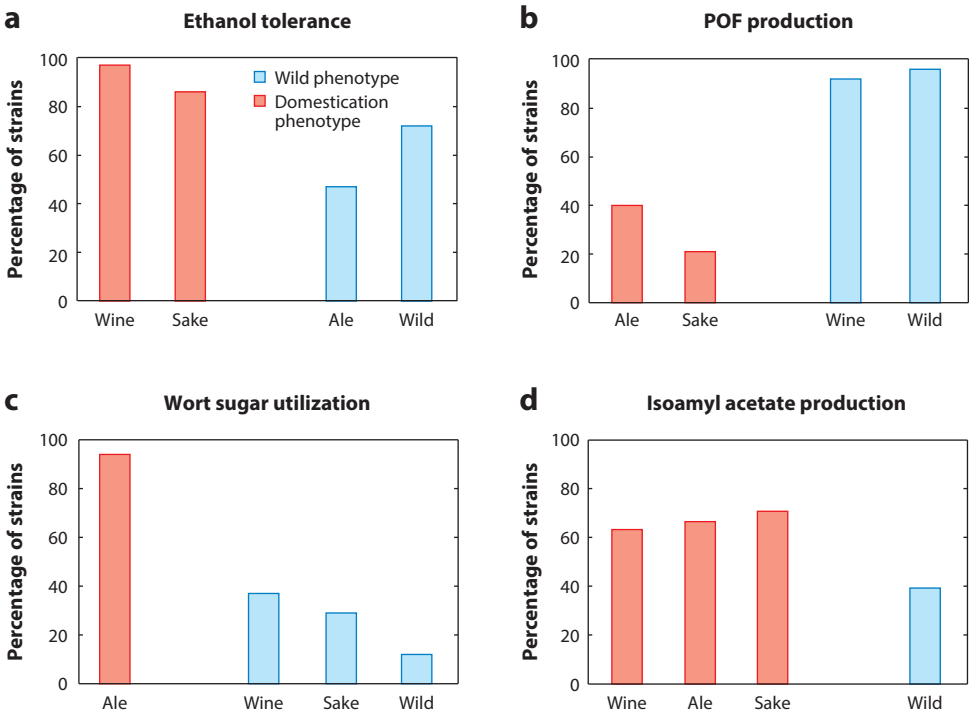

Figure 1

Phenotypic behavior of 230 domesticated (industrial) and nondomesticated (wild) *Saccharomyces cerevisiae* strains. This illustrates the selection for specific industrially relevant phenotypic traits, the so-called domestication traits. (*a*) Tolerance to alcohol (tested by growth on medium containing 12% ethanol) has been selected for in wine, sake, or bioethanol production strains, whereas production strains of lower-alcohol beverages such as beers are more sensitive for this stressor. (*b*) Phenolic off-flavors (POF) production, a consequence of the yeast's capability to produce 4-vinylguaiacol in a ferulic acid–containing growth medium, is counterselected for in production strains of beer and sake, where the production of these flavors can have a marked negative effect on product quality, whereas most other industrial and wild strains test positive for this trait. (*c*) Whereas 94% of the tested ale yeasts could ferment at least 50% of all sugars available in beer wort within a seven-day fermentation at 20°C, only 12% of wild strains and 36% of strains from other industrial branches were able to do this. (*d*) General production of fruity aroma compounds, here illustrated by isoamyl acetate production in rich growth medium (>1 ppm is scored as positive), is greater in production strains of alcoholic beverages compared with wild strains (unpublished data).

organisms specialized in specific fermentation environments but behaving suboptimally in most other, more natural environments (41, 72, 99), making *S. cerevisiae* one of the oldest domesticated organisms on the planet. Because of these specific adaptations, industrial strains often carry genetic signatures, characteristic for each type of industrial fermentation process, e.g., brewing, baking, and wine making (14, 91, 101, 117, 120) (**Figure 1**). Interestingly, much of this selection and domestication happened haphazardly, and in cases where selection was intentional, it was often not documented. Still, there are a few clear examples that demonstrate just how domestication shaped today's industrial yeasts (**Table 1**).

Multiple adaptations to the beer-making process have been described. For example, brewers' strains show a duplication of genes involved in the utilization of maltose and maltotriose, the prime carbon sources in beer fermentations (15, 17, 113). In addition, beer yeasts show signs of

Table 1 Examples of (suspected) domestication traits of *Saccharomyces cerevisiae*. Domestication traits are characteristics that have diverged between the domesticate and the wild ancestors (34). However, it is important to note that some of these trait divergences might be due to genetic drift and in fact predate or coincide with the emergence of synthetic fermentation environments (as suggested in 117)

Trait	Industry
Stress tolerance	
Copper	Wine (42, 72, 117)
Ethanol	Wine[a], sake[a], biofuel[a]
Molasses toxin	Beer (14), distillery (79)
Sulfite	Wine (85)
Nutrient utilization	
Fructose	Wine (47, 81)
Maltose/maltotriose	Beer (15, 17, 37, 113)
Xylose	Wine (119)
Sensorial quality	
Acetate ester production	Wine (59), beer[a], sake[a]
General wine aroma production	Wine (59)
Reduced phenolic off-flavor production	Beer[a], sake[a]
Other	
Flocculation/flor-forming behavior	Sherry (44), beer[a]
Lag phase	Wine (55)
Mesophilic behavior	Lager beer (37)
Vitamin biosynthesis	Biofuel (102)

[a]Unpublished results.

selection against the production of specific beer-related off-flavors, most notably 4-vinylguaiacol (4-VG), a clove-like flavor that is only desirable in a few specific beer styles, such as Belgian wheat ales and German Hefeweizen beers. Although this selection has not been documented, a screen of 230 different yeasts showed that about 96% of *Saccharomyces* strains not related to beer brewing (e.g., wine-related or nondomesticated strains) produce 4-VG, whereas only 40% of beer yeasts show 4-VG production (and these are mostly known as Hefeweizen yeasts) (**Figure 1*b***). Another example of domestication is the flocculation behavior of yeast. Flocculation is the ability of cells to stick to each other and form aggregates that rapidly sediment, which is an important trait in the beer and champagne industry, because it provides an easy and cheap way to separate the yeast cells from the finished beverage (111). Flocculation is an extremely variable trait among different *S. cerevisiae* strains (19, 112), but brewers' yeasts have been selected to flocculate at the exact moment when all fermentable sugars have been converted into carbon dioxide and ethanol. Moreover, some reports suggest that brewers can (and have) fine-tune(d) the flocculation behavior of their yeast strain by selecting specific layers of yeast sediment for reinoculation of a next fermentation batch (87).

Similarly, there are several reports on adaptive mechanisms in wine yeasts (90). An interesting example is the acquisition through horizontal gene transfer of a high-affinity fructose/H^+ symporter, *FSY1*, in the industrial wine yeast EC1118. This gene may improve fructose utilization, a

vital feature for wine yeasts (47, 81). Furthermore, in many wine yeasts, the capacity to ferment fructose is further enhanced by a mutation in the *HXT3* transporter allele (54). In addition, as a consequence of the frequent use of sulfites as a preservative, many wine yeasts show increased expression of *SSU1* (a gene involved in sulfite resistance). Similarly, duplications of the *CUP1* gene (a metallothionein affecting copper resistance) offered increased resistance to the copper-based antifungals (the so-called Bordeaux mixture) often used in vineyards (85, 117). Last but not least, the production of desirable flavors and the absence of off-flavors have also been selected for in commercial wine strains (59).

It is interesting to note that although the main mechanism of reproduction of *Saccharomyces* yeast is asexual proliferation (94), unintentional breeding through sexual hybridization, within or even between species, also occurred and has a marked effect on the current phenotypic landscape of domesticated *Saccharomyces* yeasts. Hybridizing different strains can combine beneficial traits from both parents or allow the hybrids to better adapt to fluctuating conditions (90). Interestingly, *S. cerevisiae* biodiversity seems to be defined by only a few well-defined lineages, which are the genetic foundation for a plethora of mosaic strains, representing the majority of currently applied industrial strains (72, 116). Moreover, although some of these main lineages fully correspond to a geographical origin (such as the West Africa or North America lineage), many genetically closely related (industrial) strains are sampled from widely separated locations, a phenomenon not encountered in nondomesticated species, such as *S. paradoxus* (72). These findings suggest that the close association of *S. cerevisiae* with human activity facilitated crossbreeding of geographically isolated lineages and thus generation of new, phenotypically divergent variants that were in turn spread across the globe. For example, it has been suggested that most current bakers' strains arose from relatively recent sexual crosses between other lineages (e.g., a tetraploidization event of an ale and wine strain) that had appropriate fermentation properties, such as stress resistance and high growth rates under carbon-limiting conditions (91).

Furthermore, sexual reproduction in the *Saccharomyces* sensu stricto group is usually not confined to species boundaries. For example, the widely used lager yeast, *S. pastorianus*, is an interspecific hybrid of *S. cerevisiae* and *S. eubayanus* (68). Interestingly, the current biodiversity of this new species is believed to be the result of two independent hybridization events that gave rise to the so-called Frohberg and Saaz lager yeasts (37). The success of this species in lager fermentations, which are typically performed at low temperatures to ensure the typical flavor profile, can be explained by the combination of *S. eubayanus*–derived cold tolerance with the general superior fermentation characteristics of *S. cerevisiae*. Similarly, several ale and wine strains were recently identified as hybrids of *S. cerevisiae* and the cryotolerant, strong-aroma-producing species *S. kudriavzevii* (52).

In conclusion, *S. cerevisiae* combines several natural features that allow it to thrive in industrial fermentation processes. Moreover, these features were further enhanced during domestication. The repeated used of the strains selected for adaptive mutations and the capacity of *S. cerevisiae* to reproduce sexually and even hybridize with other species facilitated the combination of desirable traits into domesticated industrial strains. Hence, because of their abundance in and positive contribution to spontaneous fermentations (see below), it is perhaps not a surprise that *Saccharomyces* strains were selected for the majority of starter cultures.

SPONTANEOUS FERMENTATIONS—A RICH SOURCE FOR NEW INDUSTRIAL YEASTS

The use of starter cultures has become a standard practice in today's large-scale fermentation industry, where consistency and process efficiency are prime goals. However, there are several industrial processes where the use of defined starter cultures is not (yet) common practice. One

of the most striking examples of this is the fermentation of cocoa beans for the production of chocolate. Furthermore, for some processes, including the production of beer and wine, where starter cultures are widely used, some artisans are revisiting the practice to rely on the local microflora in an attempt to introduce more character, complexity, and/or authenticity in their products. In this section, we describe the microbial populations encountered in spontaneous wine, beer, and chocolate fermentation processes.

Wine

Although yeast starters for controlled wine fermentations have been available since 1890 (cf. section History of Domesticated Starter Cultures for Industrial Fermentation), many of today's boutique wineries prefer to rely on spontaneous fermentation driven by local microflora to achieve stylistic distinction, vintage variability, and *terroir* (the link between a wine and its particular vineyard) (88). Because of the popularity and economic success of these boutique wines, the underlying microbiomes are intensively studied.

Spontaneous wine fermentations involve a complex ecological and biochemical process, induced by microbes present on the surface of grape skins and the indigenous microbiota associated with winery surfaces (**Figure 2*a***). Whereas yeasts are the most predominant microbial subgroup, other microbes, such as lactic acid bacteria (LAB), acetic acid bacteria (AAB), epiphytic fungi, mycoviruses, and bacteriophages can be isolated from berries or fermenting must (12, 13, 46). However, these microbes only occur at low frequencies, with the notable exception of *Oenococcus oeni*, the predominant species in the malolactic fermentation process (88).

Generally, the fermentation is initiated by fast glucose-utilizing (mostly Crabtree-negative) non-*Saccharomyces* yeasts, such as *Hanseniaspora uvarum*, *Candida stellata*, *C. zemplinina*, and *Metschnikowia pulcherrima*, most of which are frequently associated with the grape surface (88). Whereas many of these species never reach very high cell counts in the must, their ability to synthesize volatile flavor-active compounds can influence the wine's bouquet (cf. section Bioflavoring). Generally, these early fermentation yeasts grow to approximately 10^6–10^7 CFU/mL but start to decline and eventually die off as the ethanol concentration increases (45). Subsequently, more ethanol-tolerant species, mostly *S. cerevisiae*, *S. bayanus* (in the case of cold fermentations), or in some rare cases *Pichia kudriavzevii* (114), become the predominant yeast species, reaching densities up to 10^8–10^9 CFU/mL and performing the main alcoholic fermentation. Interestingly, these species are rarely isolated from grapes, with the incidence of isolation reported for *S. cerevisiae* as low as 1/1,000 intact berries (78). Their presence on damaged berries and winery surfaces (13, 45) and/or transport by insect vectors (49) are therefore most likely the primary sources of these species in spontaneous wine fermentations.

Apart from the yeast species diversity associated with spontaneous wine fermentations, several studies describe the intraspecific diversity of the main fermentation organism, *S. cerevisiae*, during the fermentation (28, 33, 89, 97, 114). This diversity can be substantial, with one study reporting as many as 43 different *S. cerevisiae* strains within one fermentation batch (97). Moreover, the introduction of a single-strain *S. cerevisiae* starter culture does not automatically imply a fully controlled fermentation process, given that indigenous *S. cerevisiae* strains might be better adapted to the fermentation environment than the introduced strain, so that the native yeasts coexist with or even outcompete the starter culture (9, 38).

Lambic-Style Beers

Although the vast majority of today's beer is produced by inoculated fermentations, some breweries produce beers that rely on spontaneous fermentations. Some of these traditional beers are produced

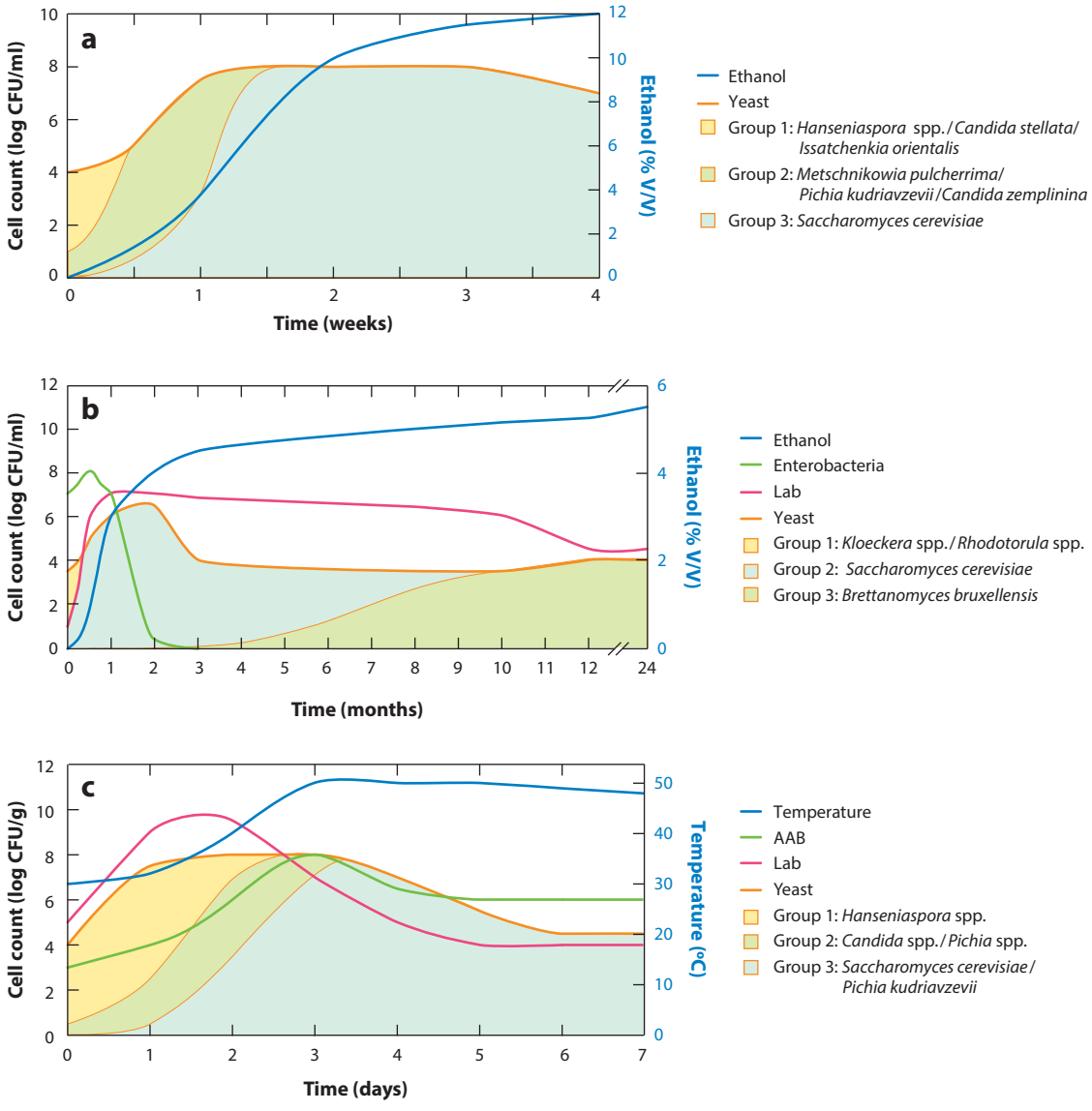

Figure 2

General trends of the microbial population in three different spontaneous fermentation processes. The most-encountered yeast species and other microbial subgroups [lactic acid bacteria (LAB) and acetic acid bacteria (AAB)] at different stages of the fermentations are indicated. Despite differences in substrate and environmental conditions between these food fermentations, the microbial profiles show striking similarities. The first fermentation phase is usually dominated by fast glucose-consuming (often apiculate) yeasts. In a second phase, more stress-tolerant (heat and/or ethanol) species (mostly *Saccharomyces cerevisiae* or *Pichia kudriavzevii*) become dominant, and these yeasts are responsible for the main fermentation. This trend is also apparent in the fermentation process of several other products that are not discussed in this review, such as cachaça or cider. (*a*) General microbial profile of spontaneous wine fermentation. The fermentation time varies and depends on many factors: temperature, SO_2 concentration, juice composition, etc. (*b*) General microbial profile of lambic-style beer fermentations. (*c*) General microbial profile of spontaneous cocoa fermentations.

using the so-called back-slopping approach; i.e., serial reinoculation of the yeast slurry from the previous batch (74), but the fermentation process of the most famous style of traditional beers, the Belgian lambic beers, is still initiated without any microbial starter inoculum.

In lambic-style beer brewing, the fermenting medium (called wort) is first boiled, after which it is cooled overnight in large, shallow open tanks (coolships) where the wort is in contact with the open air. The wort is subsequently transferred to fermentation vessels (typically oak casks), where it ferments for up to three years before packaging (110). Because preparation of the wort includes a boiling step, microbes originating from the raw material are, unlike in wine production, seldom relevant for the fermentation. However, microbes present in the air and on surfaces in the brewery find their way into the wort during the cooling, transfer, and fermentation steps. Whereas most previous studies investigated lambic-style beers in Belgium, a recent study provided a detailed analysis of the microbiome in American coolship ales, an American descendant of the Belgian lambic style (11). This latter study showed that the fermentation process involves a complex, multiphase succession of several yeast and bacterial species. Moreover, a core microbial profile that is consistently present in different fermentation batches was identified, suggesting the presence of resident brewhouse microbiota that helps to increase consistency between successive fermentations. Additionally, at higher taxonomic levels, the core profile of the American coolship ale fermentation displayed some remarkable similarities to the microbial profile of Belgian lambics, suggesting that the shared production methods create a common selective niche environment for spontaneous beer fermentations (11, 109).

Typical spontaneous beer fermentations generally show three distinct phases, dubbed initial phase, main fermentation, and maturation (**Figure 2*b***). Similar to spontaneous wine fermentations, the initial phase is characterized by a broad microbial diversity mainly consisting of enterobacteria and non-*Saccharomyces* yeasts, such as *Kluyveromyces*, *Rhodotorula*, and *Pichia* spp. Next, due to physical and chemical changes in the fermentation environment (most notably increased ethanol concentrations and decreased pH), *Saccharomyces* spp. outcompete the non-*Saccharomyces* spp. and carry out the main alcoholic fermentation. In this phase, most fermentable sugars in the medium are converted to ethanol, a process that can last 3–4 months. Coexisting with these *Saccharomyces* spp. are LAB and (in lower numbers) AAB (110). When all sugars that *Saccharomyces* spp. can ferment (generally mono-, di-, and trisaccharides such as glucose, fructose, sucrose, maltose, and maltotriose) have been used, the *Saccharomyces* cell count declines. This niche is subsequently filled by *Brettanomyces* spp., mainly *B. bruxellensis*, which reach 10^4–10^5 cells/mL and remain the dominant microbial subgroup during maturation, which lasts several months or even years. *Brettanomyces* spp. combine high tolerance to ethanol with the ability to superattenuate the fermenting wort, meaning that they can utilize more complex carbohydrates, such as maltotetraose and maltopentaose, that are normally not fermented (or attenuated) by *Saccharomyces* spp. (64) (cf. section Carbon Metabolism).

Cocoa

With an annual production of more than 4×10^6 tons of beans, cocoa fermentation is one of the largest industrial spontaneous fermentation processes (62). Although often referred to as cocoa bean fermentation, it is actually the pectinaceous, sugar-rich pulp surrounding the cocoa beans that is fermented into a liquid that drains away, leaving the beans without the pulp for further processing. This fermentation process is crucial for the final chocolate flavor and is also responsible for the killing of the seed embryo of the cocoa bean. These fermentations are usually carried out close to the site of harvest in equatorial regions throughout the world. Whereas the production of chocolate from cocoa beans can be tracked back to the Mayan civilization in Mesoamerica

(600–400 BCE) (108), West Africa and Southeast Asia are currently the main cocoa producing regions (62). After the fermentation, the cocoa beans are sun dried and shipped to the chocolate production sites, often located in the Western world.

Several research groups analyzed the species diversity and community dynamics of spontaneous cocoa fermentation. Together, these studies reveal yeasts, LAB, and AAB as the key players, each fulfilling a specific role in the fermentation process (3, 16, 75, 80, 83). LAB proliferate primarily in the initial phases of the fermentation, at cell densities up to 10^8–10^9 CFU/g. These bacteria metabolize sugars and citric acid to produce lactic acid, acetic acid, and ethanol. Yeasts, mainly present at 10^7–10^8 CFU/g, but sometimes absent at final stages of the process, produce pectinolytic enzymes that break down the pectin-rich cocoa pulp and convert the available sugars into ethanol, organic acids, and (precursors of) aroma compounds. Aerobic AAB usually increase in number (up to 10^8 CFU/g) in a second phase of the fermentation, after yeasts produce the ethanol that serves as a carbon source for AAB and after turning of the beans, a standard practice that introduces air into the fermenting mass. This aeration is necessary because conversion of ethanol into acetic acid by AAB is an aerobic process (16). The exothermic oxidation of ethanol into acetic acid causes a steep temperature increase that contributes to the inactivation of the plant embryo and also contributes to the decline of nonthermotolerant microbes.

Interestingly, whereas the bacterial population is very consistent between different production batches and regions, with a dominance of *Acetobacter pasteurianus*, *Lactobacillus fermentatum*, and *L. plantarum*, the diversity of yeast species is substantial, even between fermentation batches carried out at the same cocoa farm in the same season. More than 50 species have been detected in spontaneous cocoa fermentations, belonging to 16 genera, most notably *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, and *Wickerhamomyces* (61, 70, 75). However, despite this diversity, the succession of yeasts during the fermentation process generally shows a consistent pattern (**Figure 2*c***). Similar to spontaneous wine fermentations, fast-growing, mostly Crabtree-negative yeasts (mainly *Hanseniaspora* spp.) dominate early in the fermentation process. Because the concentration of ethanol generally does not exceed 2–3% v/v (70), it is more likely that the high temperature [sometimes >50°C (98)], mainly induced by the AAB metabolism, is the major selective factor at later stages of the fermentation, thereby favoring the growth of thermotolerant yeast species (such as *P. kudriavzevii* and/or *S. cerevisiae*) that outcompete these initial species after one or two days.

TAMING NONCONVENTIONAL YEASTS—THE POTENTIAL OF NEW YEASTS IN STARTER CULTURES

Despite the clear advantages of *Saccharomyces* spp., their dominant position as starters in industrial fermentations limits the spectrum of fermentation characteristics, and more generally bioconversion processes, available to producers. Moreover, it is unclear whether the currently used *S. cerevisiae* strains are optimally suited for their industrial tasks, as many of today's production strains show specific shortcomings (e.g., the production of off-flavors, suboptimal fermentation performance).

Several research groups are trying to overcome these limitations by creating superior variants of *S. cerevisiae*, either by genetic modification (GM) or non-GM techniques, such as selective breeding or adaptive evolution (103). In addition, an increasing number of teams are exploring the idea of using non-*Saccharomyces* species in starter cultures, either as pure cultures or as an addition to more traditional *Saccharomyces* starter cultures.

Spontaneous fermentations provide an obvious source for nonconventional yeasts that could be used as starter cultures. However, not all of the yeasts isolated from spontaneous processes are

equally suited as starters. Below, several industrially relevant key traits characteristic for specific nonconventional yeasts are discussed, underscoring their potential role in industrial fermentation.

Bioflavoring

The sensorial characteristics of a fermentation product are a key determinant of consumer preference (82). Many non-*Saccharomyces* yeasts are able to produce a large range of aroma compounds that contribute to the general aroma profile. For example, *B. bruxellensis*, a species frequently encountered in spontaneous beer and wine fermentation processes, is now employed for the bottle conditioning (a second fermentation carried out after bottling) of several ale beers, including one of the classic Belgian Trappist ales. The *Brettanomyces*-specific production of volatiles, most notably phenolic compounds, ethyl esters, and (fatty) acids, results in a complex sensorial perception that is described as (among others) clove, barnyard, smoke, humid leather, tropical fruit, and/or spices, but it is more generally called the Brett flavor (57, 69). Interestingly, *Brettanomyces* yeasts also produce mousy, animal-like flavors that are perceived as undesired in the wine industry (118), illustrating that starter cultures are product specific.

In the wine industry, numerous research groups investigated the possibility of using alternative starter cultures, often consisting of a mixture of non-*Saccharomyces* strains (often isolated from the vineyards) with a specific flavoring potential for increasing the fullness and complexity of the wine and a *Saccharomyces* strain with a high fermentation capacity that ensures complete fermentation of the grape must. These species can be inoculated together at the beginning of the fermentation process, or used in sequential fermentations, where the *Saccharomyces* strain is only added after several days, thereby enabling less-competitive species to dominate the first fermentation phase. For example, *P. anomala* (36, 92), *P. fermentans* (24), and *H. guilliermondii* (77, 92) have been investigated for the production of wine with a specific flavor profile, mainly enriched in volatile esters and/or higher alcohols. Similarly, the use of *P. kluyveri* to produce chocolate and beer with higher isoamyl acetate concentrations, accounting for a banana-like aroma, was recently proposed (27, 18).

Interestingly, yeasts not only are responsible for the direct production of aroma compounds but also can mediate the bioconversion of covalently bound, nonvolatile, and odorless flavor precursors into flavor-active compounds. This way, the choice of an appropriate strain can liberate this otherwise lost or hidden fraction of the flavor potential. In certain wine varieties, such as sauvignon blanc and scheubere, volatile thiols, such as 4-mercapto-4-methylpentan-2-one and 4-methyl-4-sulfanylpentan-2-one are essential contributors to the aroma. The conversion of the cysteinylated precursors into active flavor compounds during fermentation is mediated by a carbon-sulfur β-lyase produced by yeast cells (58), but the exact mechanism is yet to be elucidated. In recent studies, it has been shown that coinoculation with several indigenous non-*Saccharomyces* yeasts, such as *P. kluyveri* (2), *Metschnikowia pulcherrima* (95, 122), *Torulaspora delbrueckii* (95, 122), or *K. thermotolerans* (122), can help to increase the concentrations of desirable volatile thiols in wine.

Similarly, several flavor-active compounds, such as monoterpene alcohols, norisoprenoids, and aliphatic alcohols, can be covalently bound to a sugar moiety, often β-D-glucose, to form nonvolatile, odorless glycosides. These glycosides are present in several important ingredients of fermentation media, such as hops (beer), grapes (wine), and other fruits (50, 105). When these compounds are cleaved from the sugar residues, they can positively affect the aroma. Interestingly, some microbes produce glycosidases that catalyze the liberation of the volatile aroma-active aglycons (29). Although some industrial *S. cerevisiae* strains show glycosidase activity, the incidence is very low (93) and the activity relatively weak (30). However, several nonconventional yeasts, such as *Brettanomyces* spp. (29, 43), *Debaryomyces* spp. (93, 121), *Hanseniaspora* spp. (43, 4), and *Issatchenkia terricola* (53), can produce high levels of β-glycosidase. These strains are therefore

interesting candidates to use in (mixed) fermentations for beverages where a more intense and expanded natural flavor profile is desired.

Carbon Metabolism

There is an increasing demand from both consumers and producers for fermented beverages with lower ethanol levels that do not compromise product quality (66). This is mainly driven by health-related concerns and government policies that discourage the production of high-alcohol beverages with high taxes. Moreover, it has been argued that the climate change in several wine-producing areas resulted in an increase in grape sugar content, leading to an undesirable increase in wine ethanol concentration (63). Given that approaches implementing additional processing steps, such as postfermentation removal of alcohol, usually yield beverages with inferior organoleptic quality and introduce extra processing costs, careful selection of the appropriate yeast strains can provide a useful alternative.

Several approaches using GM *S. cerevisiae* strains have been described (66, 103), but these strains are, because of legislature and/or public perception, often not applied on an industrial scale. Hence, nonconventional yeasts that produce less ethanol because of their alternative carbon metabolism pathways can be interesting alternatives (51). In the beer industry, *S. ludwigii*, a species unable to ferment maltose and maltotriose (the main, nonsweet carbon sources in beer wort), is sometimes used to produce low-alcohol beer. Alternatively, several studies describe wine fermentations with (mixed) starter cultures containing oxidative species, such as *C. zemplinina* (73), *Williopsis saturnus* (39), and *C. stellata* (= *Starmerella bombicola*) (22), to reduce ethanol formation by rerouting carbon flux to glycerol or to the TCA cycle and respiration.

Apart from (too) high ethanol production, inefficient or incomplete utilization of all available carbon sources can also be problematic. For example, a high relative fructose-to-glucose ratio in must or apple juice can reduce the fermentation efficiency of wine or cider yeasts and lead to incomplete (stuck or sluggish) fermentations, yielding unappealing sweet end products with low alcohol and flavor levels (47, 115). A possible solution is to perform fermentations with mixed starter cultures consisting of both *S. cerevisiae* and a fructophilic nonconventional yeast. Several indigenous *Candida* spp. isolated from wine fermentations, most notably *C. stellata*, have been shown to reach high levels of attenuation in wine fermentations because of the efficient consumption of fructose (23, 76).

Similarly, although industrial *S. cerevisiae* strains are incapable of fermenting complex sugars such as maltotetraose and other higher dextrins, nonconventional strains with amylase activity can be applied to lower the amount of residual sugars in beer. This yields highly attenuated products, a prerequisite for the production of low-carb, light, or diet beers. *S. cerevisiae* var. *diastaticus*, formerly known as *S. diastaticus*, contains three genes (*STA1*, *STA2*, and *STA3*) encoding glucoamylases that facilitate the breakdown of complex sugars (104). However, these strains additionally produce strong phenolic off-flavors, limiting their use in beer fermentations. Nevertheless, several strategies, including selective breeding and genetic engineering, are relying on *S. cerevisiae* var. *diastaticus* to construct novel dextrin-degrading brewing yeasts. Alternatively, dextrin-degrading non-*Saccharomyces* species, such as *B. bruxellensis*, responsible for the superattenuation in lambic beers by expressing α-glucosidase (65), could be used to obtain highly attenuated beverages.

Spoilage Control

One of the prerequisites of good starter cultures is that they are able to dominate the fermentation. This is usually not a problem when a sterile medium is fermented in sterile containers, as is the

case in beer fermentations. However, the fermentation of nonsterile raw materials (e.g., grapes) or fermentations carried out in nonsterile environments (e.g., in cocoa fermentations) can result in the growth of undesirable microorganisms that may come to dominate the starter culture. Yeasts that are able to produce killer toxins that kill certain other yeasts can be used as biological antimicrobial agents in starter cultures. Killer-active yeasts produce and secrete these mycotoxins (mostly viral dsRNA toxin-antitoxin systems) (96). The phenomenon was initially described in 1963 for an *S. cerevisiae* spoilage yeast in the brewery, and since then, *Saccharomyces* killer yeasts have been commonly used in controlled fermentations (10). However, the narrow activity spectrum of *Saccharomyces* killer toxins, mostly restricted to *Saccharomyces* strains, often limits their effectiveness in industrial settings. For example, in the wine industry, apiculate yeasts (in early stages) and *Brettanomyces* yeasts (in later stages) are two of the most important yeast spoilage groups, and these species are not sufficiently affected by *S. cerevisiae* killer toxins (20). To control proliferation of apiculate yeasts, *Tetrapisispora phaffii* was suggested as an adjunct to the starter culture, whereas antimicrobial activity against *B. bruxellensis* was described in two species, *K. wickerhamii* and *P. anomala* (21, 25).

CONCLUSION AND PERSPECTIVES

The omnipresence of *S. cerevisiae* and a few related species or hybrids in industrial fermentations is not a coincidence. These yeasts combine many desirable properties, including fermentation performance, stress tolerance, the production of desirable aromas, and the absence of toxic metabolites. However, the increased use of pure *Saccharomyces* starter cultures also comes with some disadvantages and limits the possibilities to develop novel products with specific features.

Nonconventional yeasts possess several traits that are of industrial interest, including the production or liberation of aroma-active compounds, atypical carbon metabolisms, and antimicrobial activities. Despite significant progress in the isolation and characterization of these yeasts, there are still plenty of opportunities to further explore the available biodiversity by isolating new strains from diverse ecological niches and to look for applications of these yeasts by intensive screening for industrially relevant features. However, despite their interesting features, non-*Saccharomyces* yeasts often display an inferior fermentation efficiency compared with *S. cerevisiae*. Mixed cultures consisting of *Saccharomyces* and non-*Saccharomyces* yeasts are therefore an interesting option to combine fermentation efficiency with specific non-*Saccharomyces* features. Moreover, directed evolution, selective breeding, and perhaps also genetic modification can be employed to further tune these nonconventional yeasts for industrial use, similar to the domestication of industrial *S. cerevisiae* strains (103).

One major challenge when exploring the potential of nonconventional yeasts, especially for food production, is to assure biosafety. Our current knowledge of nonconventional yeasts is rather limited, and the list of yeast species approved by the US Food and Drug Administration or the European Food Safety Authority for the production of food is therefore extremely restricted (40, 107). It seems reasonable to assume that species that are often encountered in traditional spontaneous fermentation processes, the products of which have been consumed without specific health issues, are generally safe to use in controlled fermentations. However, some yeasts isolated from spontaneous fermentations actually produce potentially harmful compounds, and this effect could be aggravated when the yeasts are inoculated and dominate the fermentation process. For example, many yeast strains are able to produce biogenic amines, which are neurotoxins that can induce drastic physiological effects when absorbed at high concentration, especially in sensitive consumers (100). Moreover, the production of other health-threatening by-products, such as methanol (derived from pectin) or urea (which can spontaneously react with ethanol to form ethyl

carbamate, a suspected carcinogen) must be considered before industrial implementation. Still, many compounds are only produced in trace amounts (if at all), and given the necessary testing and precautions, the use of nonconventional yeasts provides interesting opportunities for artisans to diversify their product palette or to create innovative, high-quality fermented foodstuffs.

DISCLOSURE STATEMENT

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