Chapter 4

Saccharomyces and Non-*Saccharomyces* Starter Yeasts

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Additional information is available at the end of the chapter

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Abstract

This chapter describes the importance of yeast in beer fermentation. Initially, the differences between *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* in the production of "ale" and "lager" beers are analyzed. Then, the relationships between beer nutrients and yeast growth are discussed, with special emphasis on the production of the flavor compounds. The impact of the wort composition on flocculation is also discussed. Furthermore, conventional approaches to starter yeast selection and the development of genetically modified microorganisms are analyzed. Recent discoveries relating to the use of *S. cerevisiae* strains isolated from different food matrices (i.e., bread and wine) and the potential for the use of non-*Saccharomyces* starter strains in beer production are discussed. A detailed review of the selection of starter strains for the production of specialty beers then follows, such as for gluten-free beers and biologically aged beers. Yeast recovery from top-cropping and bottom-cropping systems and the methodologies and issues in yeast propagation in the laboratory and brewery (i.e., re-pitching) are also analyzed. Finally, the available commercial preparations of starter yeast and the methods to evaluate yeast viability prior to inoculation of the must are analyzed.

Keywords: yeast, *Saccharomyces*, non-*Saccharomyces*, fermentation, aroma

1. Introduction

Beer is a very old biotechnology, with its origins dating back to around 10,000 years ago [1]. Due to this ancient history, this alcoholic beverage has undergone three particularly important revolutions: (i) the practice of the *inoculum* of a selected yeast culture; (ii) the craft and home brewing movement from Europe to USA; and (iii) the influence of the genomic era and "big data" that now allow comparative genomic analyses that have provided new knowledge about yeast. The interest in comparative genomics of the yeast genome is in part motivated

by the rationale of "beer *du terroir*": beers produced with local raw materials and local yeast. In this context, yeast appear to be a relatively unexplored tool for the diversification of local beers, as much of the characteristics of such beers are due to the yeast strains used. Indeed, the transformation of wort into beer essentially represents the yeast-driven conversion of sugars into ethanol, CO_2, and many other secondary products that provide specific aromas and flavors.

All brewers know that during the fermentation of the wort, the yeast have to complete two major tasks. The primary task is, as indicated, to convert the sugar into ethanol, CO_2, and the aromas for the production of quality beer, while they also need growth to produce biomass that will be re-pitchable into new brews. Thus, it is imperative to satisfy the physical and nutritional requirements of the yeast. The growth rate of yeast can be modulated by manipulation of some of the fermentation parameters, such as the supply of nutrients, the dissolved oxygen, and the temperature. Furthermore, to end up with a high quality beer, there must be a balance between the nutrients absorbed and the products released.

Beer production is essentially a two-phase process: primary fermentation and secondary fermentation (or maturation). Primary fermentation is a short and vigorous step, during which almost all of the sugars are fermented, accompanied by the production of the secondary compounds that result from the yeast metabolism, most of which are associated with the final beer aroma. At the end of this stage, most of the yeast biomass is collected and separated from the "green" beer, which then undergoes secondary fermentation. During this second phase, the yeast completes the fermentation of the residual sugar, with undesirable compounds removed, and the final taste of beer defined. Among the fermentative yeast, *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* are the most frequently used in starter cultures in breweries. As the species of the genus *Saccharomyces*—and particularly *S. cerevisiae*—dominated during spontaneous fermentation, they were selected over the years across different cultures and from distinct environments. In particular, in the beer industry, the goal of the use of inoculated yeast is to increase the fermentation efficiency, to develop new beers, and especially to enhance the sensory complexity of the beer that is produced.

2. Yeast in beer

2.1. Physical and nutritional requirements of brewing yeast

Brewing yeast are mainly classified as the top-fermenting or ale yeast of *S. cerevisiae*, and the bottom-fermenting or lager yeast of *S. pastorianus*. These two yeast species can be differentiated in terms of their temperature of fermentation, sugar assimilation, genomic organization, evolutionary domestication, and phylogenesis. *S. cerevisiae* ferments between 18 and 24°C, with a maximum growth temperature of 37°C or higher; in contrast, *S. pastorianus* often ferments at lower temperatures, of between 8 and 14°C, although with a maximum growth temperature of 34°C. In general, brewing yeast requires high water activity, and in high sugar-containing wort they can overcome this stress condition through the overproduction of osmolytes, such as glycerol and trehalose, to protect their cell membranes. These osmolytes can replace water,

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to restore cell volume and osmotic pressure, and thus to allow regular yeast metabolism. For their pH requirements, yeast cells grow well between pH 4.5 and 6.5. During nutrient transport, yeast cells acidify their environment through a combination of proton secretion, direct secretion of organic acids, and CO_2 dissolution. Oxygen is required as a growth factor for the biosynthesis of their membrane fatty acids and sterols. As well as oxygen, yeast cells require the macronutrients (i.e., those needed at millimolar concentrations in the medium) of sulfur, free amino nitrogen, phosphorous, potassium, and magnesium. The micronutrients required by yeast cells (i.e., those needed at micromolar concentrations in the medium) are calcium, copper, iron, manganese, and zinc. *S. cerevisiae* requires low concentrations of growth factors, such as vitamins (e.g., biotin can be limiting), pyrimidines, purines, nucleotides, nucleosides, amino acids, sterols, and fatty acids. Generally, the malt wort provides growth factors, although in certain cases it can be necessary to supplement the wort with commercial yeast growth factors, as a mix of yeast extract, ammonium sulfate, and minerals (e.g., magnesium and zinc in particular). Barley malt wort is rich in maltose (50–60% total sugars), maltotriose (15–20%), glucose (10–15%), fructose and sucrose (1–2%, each), and dextrins (20–30%). These are derived from the hydrolysis of the starch by malt amylases during malting. However, *S. cerevisiae* cannot ferment dextrins, while *S. cerevisiae* and *S. pastorianus* show differences in terms of their melibiose fermentation, as only *S. pastorianus* can use this sugar (**Figure 1**).

The uptake of nutrients by yeast depends on the nutrient type, the yeast species, and the fermentation conditions. Generally, glucose is transported through the cell membrane into the cell by facilitated diffusion, and maltose by active transport. A high glucose concentration

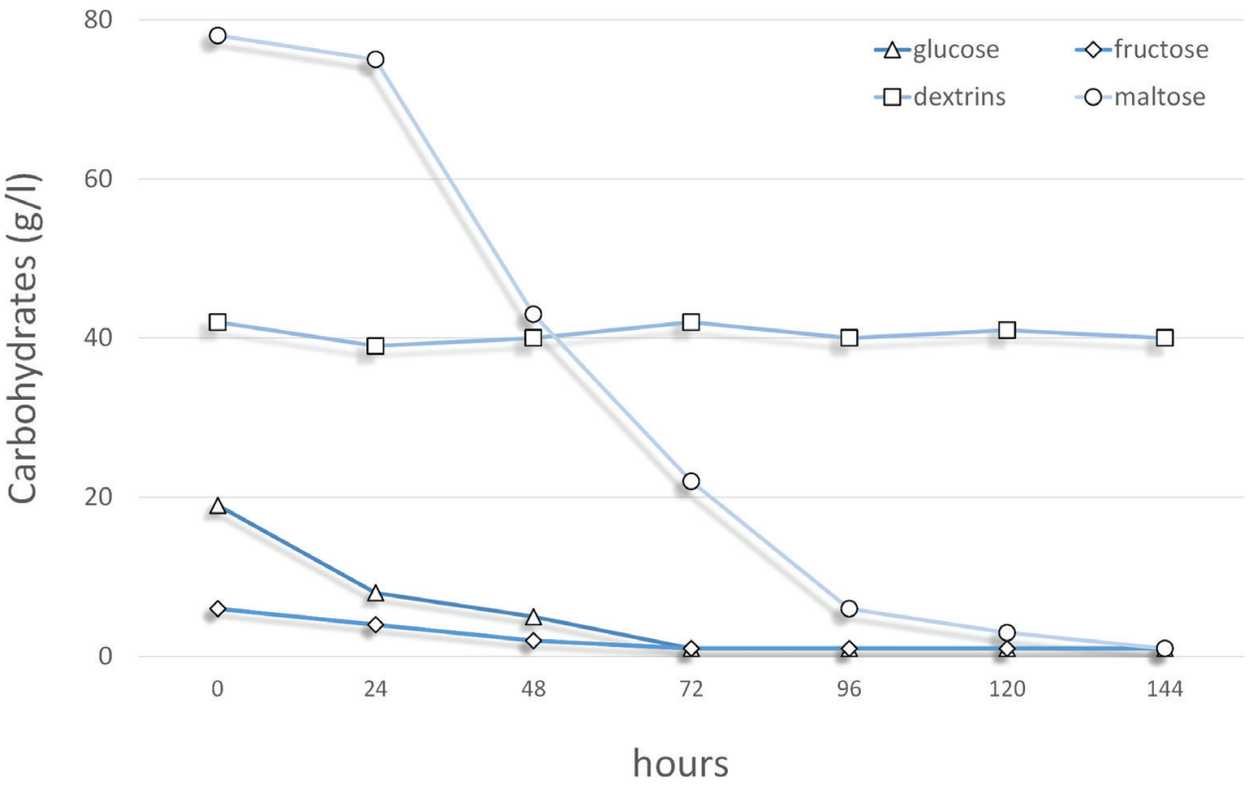

Figure 1. Carbohydrate uptake during wort fermentation (adapted from Ref. [2]).

in the wort can suppress the assimilation of maltose and other sugars (i.e., sugar catabolite repression). As a source of nitrogen, brewing yeast require assimilable organic (e.g., amino acids) and inorganic (e.g., ammonium salts) nitrogen for growth and fermentation. Again, high levels of ammonium ions in the wort can suppress the uptake of amino acids (i.e., nitrogen catabolite repression). Amino acid uptake occurs through two transport systems: general amino acid permease (GAP) and specific transporters for the different amino acids. The dissimilation of amino acids (i.e., decarboxylation, transamination, and fermentation) produces ammonium, glutamate, and higher alcohols (i.e., the fuel oils).

2.2. Genomic features of *S. cerevisiae* and *S. pastorianus* brewing strains

Over time, there was gradual domestication and selection of yeasts [3]. Moreover, there are two counteracting forces that act on yeast selection: yeast research needs to homogenize biological systems and to refer different yeast strains to species, while brewers need to differentiate and select yeast strains based on their fermentation characteristics. *S. cerevisiae* and *S. pastorianus* have followed different paths in their domestication that can now be read in their genome.

Ale strains of *S. cerevisiae* show wide heterogeneity, with differences in ploidy, genomic structure, and phenotypic behavior. *S. cerevisiae* has 5780 protein-encoding genes on 16 chromosomes (i.e., in haploids cells). However, the strains of *S. cerevisiae* that have been sequenced since 1996 represent laboratory yeast strains, and although these represent a well-characterized cell system, most of the known data are not adequate to understand and analyze the differences that characterize the diverse strains of ale yeast. *S. cerevisiae* ale strains can have different levels of ploidy, with some being aneuploid (i.e., with an abnormal chromosome number), and others being polyploid (i.e., with multiple complete genomes). Brewers have selected yeast strains over the centuries for the stability of their traits, and this has resulted in low spore viability and yeast that are deficient in sexual recombination.

Brewing strains belonging to *S. pastorianus* have been strictly linked to lager beer production for centuries, as seen by the fermenters and barrels of the central European brewers (e.g., Germany, Denmark, and Czech Republic). The origin of these strains has only recently been discovered through comparative genomic analysis, which revealed that *S. pastorianus* is a hybrid between *S. cerevisiae* cool-adapted strains and *Saccharomyces eubayanus* strains that have only been isolated from two possible wild reservoirs in Patagonia and Tibet. The production of lager beers started in central Europe around the end of the fourteenth century.

Recently, it was shown that on the basis of the sequence of different isolates of lager beer strains, *S. pastorianus* can be divided into two lineages [4]. One of these is associated with breweries in Denmark (i.e., Saaz-type, formerly *Saccharomyces carlsbergensis* triploid), which is characterized by a genome that is composed of most of the *S. eubayanus* genome (2n) and a partial *S. cerevisiae* genome (1n). The other lineage is from Germany (i.e., Frohberg-type, tetraploid) and is composed of equal genomes from *S. eubayanus* (2n) and *S. cerevisiae* (2n). Furthermore, the present-day *S. eubayanus* strains can also be hybrids themselves, with their

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genomes including portions of the genomes from *Saccharomyces uvarum*, the old *S. eubayanus*, and *S. cerevisiae*. In European wild environments, *S. eubayanus* has never been isolated, and this supports the hypothesis that *S. eubayanus* is a product of the lager-brewing environment, where these different species can be found growing together. Cold-tolerant yeast hybrids adapted well through serial passages during lager production, and these have become the dominant strains. Indeed, *S. pastorianus, Saccharomyces bayanus*, and *S. uvarum* have only been isolated from human-associated fermentation environments (**Figure 2**).

2.3. *Brettanomyces* species

Other species can contribute to wort fermentation and beer quality, including wild strains and species in open and uncontrolled fermentations. *Brettanomyces bruxellensis* is characteristic of the fermentation of Belgian lambic and geuze beers. Recently, genomic differences were reported for the *B. bruxellensis* contaminant yeast in wine (i.e., spoilage yeast) and the *B. bruxellensis* isolated from Belgian beers (i.e., brewing yeast). This study revealed that 20 genes in the spoilage strain genomes have been deleted in the brewing strains, many of which are involved in carbon and nitrogen metabolism. DNA fingerprinting has revealed that brewing strains have a unique profile, which means that they can be distinguish from spoilage strains. Thus, as for *Saccharomyces* strains, the selection undergone by *Brettanomyces* spp. also appears to have been influenced by adaptive modifications to brewing processes.

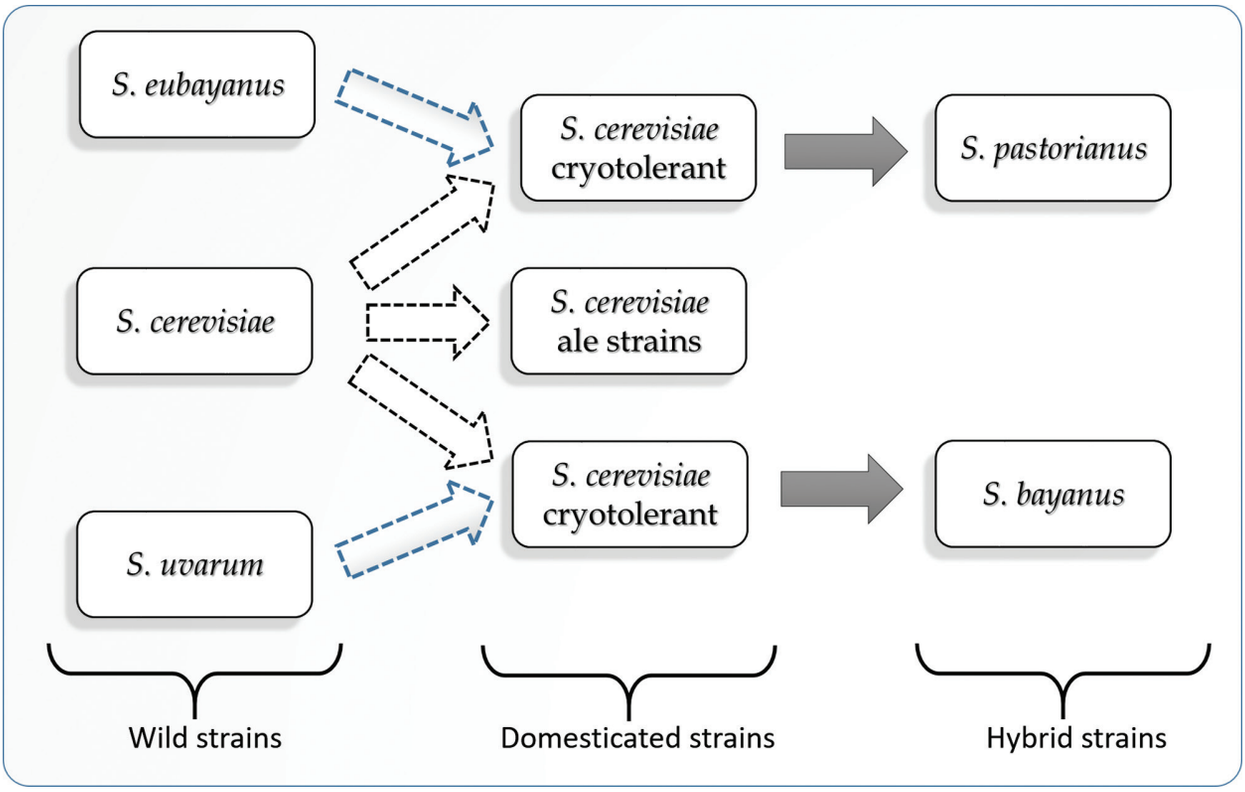

Figure 2. Source and selection of brewing yeast species (adapted from Ref. [5]).

3. Yeast management for aroma production

Generally, at the end of the boiling, the wort contains all of the nutrients that are required for yeast growth and fermentation. Thus, from a microbiological perspective, the main aspects to consider are not whether the wort is suitable for yeast growth, but rather what is the balance of the flavor compounds that will be produced by the yeast. Mathematical models have been developed to predict the final concentrations of some of these volatile compounds from the (known) quantities of their precursor(s) in the wort [6]. However, the application of these methods requires particularly deep knowledge of the wort composition, while brewers usually conduct very basic measurements in their evaluation of wort quality. For example, there are indications that small changes in the spectrum of the wort amino acid composition can result in dramatic changes in the final beer aroma. The most important metabolites synthesized by yeast and related to beer quality are sulfur compounds, organic and fatty acids, carbonyl compounds, higher alcohols, and esters.

3.1. Sulfur compounds

Sulfur compounds, such as hydrogen sulfide, methional, and dimethyl sulfide (DMS), are active flavor components of the beer that is generated during mashing and fermentation. During fermentation, through the metabolizing of amino acids and vitamins, and through the use of the inorganic components of the wort (e.g., sulfates), *S. cerevisiae* produces sulfur compounds, which include hydrogen sulfide. Sulfur compounds impart specific flavors to the beer, which have been defined as onion, rotted vegetables, or cabbage flavors, among others. While the over accumulation of these sulfur compounds is often undesirable, under specific circumstances, sufficient sulfite levels are necessary in the beer, to maintain flavor stability. Moreover, the sulfidic taste of dimethyl sulfide (below 100 μg/l) is an essential part of the flavor of lager beers and some ale beers. The major source of DMS in beer is the enzymatic conversion of dimethyl sulfoxide (DMSO) by yeast reductases. In particular, it has been shown that the disruption of the genes coding for methionine sulfoxide reductase abolished formation of DMS from DMSO in both *S. cerevisiae* and *S. pastorianus* [7, 8]. Methionine has a key role in the production of sulfur compounds by yeast. Indeed, when there are sufficient levels of methionine in the wort, this can cause inhibition of sulfite uptake, and the production of hydrogen sulfide [9].

3.2. Organic and fatty acids

The organic acids in beer are derived mainly from the yeast, as they are produced during the tricarboxylic acid, or Kreb's cycle (e.g., succinate and malate), from the catabolism of amino acids, and from redox reactions. Other organic acids, such as citrate and pyroglutamate, derive directly from the wort, and the yeast do not affect their concentrations in the beer. Overall, more than 100 organic acids have been identified in beers. These contribute to the reduction in pH during fermentation, and to the "sour" or "salty" taste of the beers. Fatty acids are of particular interest here, because of their involvement in the synthesis of esters. Yeast can incorporate saturated fatty acids and unsaturated fatty acids (UFAs) from the wort,

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or they can synthesize these from acetyl-CoA. However, the lack of sufficient oxygen in the later phases of fermentation makes the synthesis of UFAs impossible, and as a consequence, medium-chain fatty acids (MCFAs) are released into the medium [10]. These MCFAs are powerful detergents, and they can influence yeast vitality, beer taste, and foam stability. In particular, the typical flavor that is characteristic of MCFAs is defined as a "rancid goaty" flavor, and hence is often described as a "caprylic" flavor.

3.3. Carbonyl compounds

The presence of aldehydes and vicinal diketones is considered undesirable for beer quality. Acetaldehydes have unpleasant "grassy" flavors that are reminiscent of green apples and dry cider. In some circumstances, such as when there is excessive wort oxygenation and high pitching rates, aldehydes can accumulate in concentrations above the flavor threshold [11]. The vicinal diketones are important off-flavors of lager beers, which include diacetyl. During fermentation, yeast cells excrete an intermediate of valine biosynthesis, α-acetolactate, that is, spontaneously decarboxylated to diacetyl. Diacetyl has a strong aroma of toffee and butterscotch, and at concentrations above 0.05 ppm, it is considered as undesirable in lager beers. During conditioning, diacetyl is assimilated by yeast, and thus reduced to acetoin and 2-3 butandiol, which have much lower impact on beer quality. Traditionally, the rate-determining step of diacetyl accumulation in beer has been considered as the spontaneous decarboxylation of acetolactate, with yeast assimilation left with a marginal role. However, the physiological conditions of yeast are essential for diacetyl production and the time necessary for its reduction. High concentrations of valine and isoleucine in the wort inhibit vicinal diketone production by yeast. High assimilation rates have been observed at higher fermentation temperatures and when yeast is grown under aerobic de-repressed conditions. On the contrary, at higher pitching rates, the elevated concentrations of vicinal diketones produced by yeast require longer standing times [12].

3.4. Higher alcohols

The higher alcohols are also known as "fusel alcohols", and these are the most abundant organoleptic compounds in beer. Isoamyl alcohol, n-propanol, isobutanol, 2-phenyl-ethanol, and triptothol are important flavor and aroma components in terms of their concentrations. Below 300 mg/L, these compounds add complexity to the beer, by conferring refreshing, flower, and pleasant notes, and imparting a desirable warming character. On the contrary, above these concentrations, these compounds can have unpleasant heavy solvent-like odors. The formation of higher alcohols by brewing yeast involves different complex pathways, and a lot of progress has been made in the determination of the roles of the key genes involved in their biosynthesis [13]. The predominant idea for many years was that the higher alcohols are produced *via* the Ehrlich pathway. In this scheme, the yeast absorbs and deaminates the amino acids in the wort, with the resulting α-keto acids decarboxylated to aldehydes, and then reduced to higher alcohols. While this pathway can correctly explain the relationships between leucine and the corresponding isoamyl alcohol, it fails to explain why some fusel alcohols (e.g., n-propanol) do not correspond to any known amino acid, and that in wort

containing low amino acid levels, there is no significant correlation between amino acids and higher alcohol composition of the beer. Indeed, in complex media such as the wort, most higher alcohols are formed following the glycolytic pathway. By fermenting the wort sugars, yeast not only produce ethanol, but also a number of long-chain alpha-acids that can subsequently be transformed into amino acids such as aspartate and glutamate. Finally, the choice of yeast strain can have great impact on higher alcohol production, and ale strains are considered to be higher producers than lager strains [13].

3.5. Esters

Esters are chemical compounds derived from a carboxylic acid and an alcohol, and they are of major industrial interest because they have very low thresholds and define the fruity aroma of the beer. Two main classes of esters are of particular interest for brewers: acetate esters and MCFA esters. Acetate esters have concentrations above threshold levels in most lager beers (e.g., isoamyl acetate is responsible for the banana-like aroma) and ale beers (e.g., ethyl acetate gives a solvent-like aroma). It is generally believed that the acetyl-CoA that is necessary for formation of acetate esters derives from oxidation of acetaldehyde. The acyl-CoAs required for the synthesis of MCFA esters originate from intermediates in the synthesis of fatty acids. Among the MCFA esters, ethyl hexanoate (i.e., an apple-like aroma) is an important flavoring compound, with levels above threshold in ale beers. The biosynthesis of esters requires acetyl-CoA or acyl-CoAs esterification with ethanol or higher alcohols by the specific alcohol acetyltransferase enzymes ATF1 and ATF2. Different studies have focused on the manipulation of fermentation conditions and the wort composition in ways that favor the availability of these factors and that lead to increased production of higher alcohols and esters.

4. Fermentation conditions

It is generally accepted that any condition that stimulates yeast growth will increase the production of higher alcohols and their acetate esters during fermentation. In this respect, increasing fermentation temperatures leads to an accumulation of acetate esters, with no significant differences in the levels of the MCFA esters. In particular, increasing the temperature from 10 to 12°C, increases ester production by up to 75% [14]. This phenomenon is dependent on increased alcohol acetyltransferase activity and stimulation of higher alcohol synthesis, which results from greater amino acid turnover. In addition, it has been suggested that higher fermentation temperatures increase the synthesis of a specific permease, Bap2p, that is, involved in import of the branched-chain amino acids valine, leucine, and isoleucine, which are known precursors of the higher alcohols [15].

Oxygen has an ambiguous role here. Indeed, oxygenation of the wort provides for better yeast growth, and consequently increased higher alcohol production. However, it is well-known that oxygenation leads to lower levels of esters in the beer. Oxygen acts in two different ways. First, availability of oxygen allows the biosynthesis of UFAs that is required to sustain yeast growth during fermentation. UFAs are esterified to glycerol to form membrane lipids, and

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thus, less acyl-CoA is available for synthesis of MCFA esters. Second, oxygen inhibits transcription of the alcohol-acetyltransferase-encoding gene *ATF1*, and consequently it reduces synthesis of acetate esters [13]. Thus, correct management of wort oxygenation at the time of pitching is essential to produce quality beers.

Another fermentation parameter that can affect ester synthesis is the hydrostatic pressure on the yeast cells. While in small craft breweries this is not a problem, the use of big cylindroconical reactors in industrial beer production substantially increases the concentration of carbon dioxide dissolved in the beer. This has a double effect on ester synthesis. First, it inhibits yeast growth by lowering intracellular pH, and second, it directly reduces decarboxylation reactions, such as acetyl-CoA synthesis from pyruvate. As a consequence, large fermenters have been successfully used for the reduction of esters in beer, in particular during the fermentation of high gravity wort.

Stirring of the medium modulates the effects of the oxygen and carbon dioxide that are dissolved in the beer, to provide better oxygen distribution and decrease carbon dioxide supersaturation. Consequently, the synthesis of higher alcohols is stimulated, while that of MCFA esters is reduced, and the beer will have a less fruity aroma.

5. Wort composition

The amounts and types of sugars in the wort can influence the aromatic profile of the beer. Beers obtained from worts with higher percentages of glucose and fructose have higher ester levels than those obtained from maltose-rich worts. It has been suggested that glucose and fructose stimulate the glycolytic pathway and eventually lead to high levels of cytoplasmic acetyl-CoA, while maltose-rich wort only weakly induces acetyl-CoA formation [16]. Moreover, glucose induces *ATF1* and *ATF2* expression [13]. Similarly, high gravity brewing leads to the production of disproportionate amounts of ethyl acetate and isoamyl acetate, which give the beer over-fruity and solvent-like aromas, even after dilution to the standard ethanol content (i.e., 5% vol/vol).

It has been suggested that high nitrogen wort induces the transcription of *BAT1* and *ATF1*, thus, increasing both higher alcohols and acetate ester synthesis [16]. This effect is even more pronounced in the presence of elevated concentrations of valine, isoleucine, and leucine, which are known precursors of higher alcohol synthesis *via* the Ehrlich pathway.

The effects of free UFAs on beer aroma have been well documented. Similar to oxygen, low UFA levels in the wort increase ester synthesis, by recovering optimal yeast growth. However, at higher concentrations, UFAs relieve the need for the yeast to produce acetyl-CoA for lipid biosynthesis. This in turn induces lower MCFAs production, and lower production of their respective esters. Moreover, UFAs can directly repress *ATF1* transcription through the low oxygen response element [13]. Considering these effects, addition of UFAs has been proposed to modulate the final aroma of the beer obtained from a high gravity wort.

Finally, zinc stimulates the breakdown of α-keto acids to higher alcohols, thus, increasing their concentrations, and those of their corresponding esters.

5.1. Flocculation

At the end of the primary fermentation, yeast cells must be removed from the "green", or immature, beer. Flocculation is the process by which yeast cells aggregate and form "flocs" consisting of thousands of cells. *S. cerevisiae* ale strains rapidly separate from the beer by rising to the surface of the fermenter, probably adsorbed to carbon dioxide bubbles. On the contrary, *S. pastorianus* lager strains form flocs that sediment in the bottom of the fermenter. Yeast flocculation is an off-cost process of cell separation that does not require energy input. Given the importance of flocculation for the brewing industry, and to increase the efficiency of this process while avoiding premature flocculation, several mechanisms have been proposed to explain the physiological mechanisms involved in flocculation. In particular, the lectin-like mechanism is that generally accepted [17]. In this model, flocculent cells express lectin-like proteins that once activated by calcium ions, can recognize and bind to mannans in the cell wall of adjacent cells. This interaction is further stabilized by hydrogen bonds and hydrophobic interactions. In particular, positive correlation between cell hydrophobicity and flocculation has been demonstrated [18]. Transcriptional regulation and structural characteristics of the flocculation (*FLO*) genes that encode for lectin-like proteins are essential to produce yeast cells that can undergo flocculation. In particular, *FLO1* causes flocculation of a Flo1 phenotype, while a *FLO1* homolog, called Lg-*FLO1*, is responsible for the NewFlo phenotype observed in lager and ale brewing strains [19]. The NewFlo phenotype is modulated by sugars (i.e., maltose, glucose, mannose, and sucrose), extreme pH, oxygen availability, cations (e.g., Ba^{2+}, Sr^{2+}, and Pb^{2+}), and temperature. Sugars inhibit flocculation by competing with the binding sites of lectins, and by affecting the expression of the *FLO* genes. A pH outside the optimal range of pH 3–5 can affect yeast flocculation by interfering with the lectin conformation, by antagonizing the calcium activity, and by modifying the net electrical charge of the cell. Oxygenation of the medium represses expression of the anerobic cell wall mannoproteins and flocculation lectins [20].

Thus, on this basis, the nutritional and physiochemical conditions of sweet wort inhibit yeast flocculation. Indeed, the *FLO* genes are repressed and the yeast cell wall has a net negative charge that prevents cells from interacting with each other, and thus from aggregating. During fermentation, the decrease in pH, the prolonged anerobiosis, and the reduced availability of nutrients induce the NewFlo phenotype. In particular, lack of sugars (i.e., glucose, fructose, and maltose), or of nitrogen sources and lipids, can trigger flocculation of ale and lager yeast strains, respectively [21]. In addition, the increased ethanol concentration has a positive effect on yeast flocculation, by acting on the expression of the *FLO* genes and causing decreased cell–cell electrostatic repulsion. Finally, at the end of fermentation, triggering of the NewFlo phenotype strains occurs, because strains at the end of their exponential phase of growth are more flocculent [21].

As well as sugars, lipids, metal ions, and nitrogenous compounds, other minor wort components can influence the flocculation of yeast. In particular, complex polysaccharides that can induce premature flocculation have been identified. These polysaccharides can act as a bridge between cells by interacting with yeast lectin-like proteins. It has been suggested that the binding between these polysaccharides and yeast cells is mediated by cationic antimicrobial

peptides [22]. These compounds are produced by barley to protect against microbial attack, or in response to fungal contamination during the malting process. Premature flocculation leads to incomplete attenuation of the wort, as aggregated cells cannot ferment the residual sugars of the wort.

Another major industrial problem is that flocculent strains can gradually lose their ability to flocculate, and thus eventually they will not form aggregates. It has been suggested that this phenomenon can be ascribed to genetic alterations during yeast multiplication and subsequent re-pitching [20]. In the case of insufficient flocculation, the yeast cells need to be removed at the end of fermentation by centrifugation or other separation techniques. However, yeast cells exposed to the stress associated with centrifugation have lower viability and vitality. This, in turn, can negatively affect beer quality and stability.

6. Novel starters for novel beer

The use of *Saccharomyces* strains in controlled fermentations over decades is essentially based on three main features: (a) high and efficient ethanol production; (b) use of fermentation as the preferential metabolic pathway, combined with the positive Crabtree effect (i.e., repression of respiration by glucose); and (c) higher tolerance to ethanol and other environmental stresses. The domination of the fermentation processes by the inoculated yeast (i.e., first fermentation and re-fermentation in craft beers) is fundamental to the aromatic profile of the final beer produced.

Over time, there has been gradual domestication and selection of yeasts [3]. Selected *Saccharomyces* strains are used for various purposes because of their plasticity for the assimilation of different substrates, which are usually not incorporated by *S. cerevisiae*. Indeed, choice of the yeast strain to use in the brewing process is also crucial to achieve a product that is valued by consumers and that has the required distinctive features and flavors. Also, yeast strains isolated from fermented foods other than beer can produce distinctive fermentative aroma profiles in beer [23, 24]. They can transform flavor precursors of the raw materials into more flavor-active compounds, which thus contribute to the final aroma of the beer [13, 25]. In this context, genetic strain-improvement strategies to enhance the fermentation efficiency and aromatic profiles of *S. cerevisiae* have been proposed [26].

6.1. Genetic improvement of brewing strains

High quality sequencing, *de-novo* assembly, and extensive phenotyping of 157 *S. cerevisiae* strains used for industrial production of beer and other fermented beverages (in their natural ploidy) have revealed that industrial yeast are genetically and phenotypically distinct from wild strains [27]. On this basis, there are many genetic approaches to the design of a superior yeast that can ferment and provide a particular style of beer. To obtain this, genetic modification approaches have mainly been applied to *S. cerevisiae*, such as rational metabolic engineering and inverse metabolic engineering. The aims here have been to increase the favored aromatic compounds or reduce undesired molecules. The nongenetic modification technique

of hybridization can be used to increase the yeast fermentation fitness, ethanol tolerance, flocculation, and dextran degradation. Evolutionary engineering methods have mainly been applied to *S. pastorianus*, to improve fermentation capacity and flavor formation [28].

The positive role of *S. eubayanus* in the brewing process was only recently discovered through the use of hybridization tools [29]. Indeed, the natural interspecies *S. pastorianus* (i.e., *S. cerevisiae* × *S. eubayanus*) hybrid yeast is responsible for global lager beer production and is one of the most important industrial microorganisms [30]. Its success in the lager-brewing environment is due to a combination of traits that are not commonly found in *S. cerevisiae* yeast, as mainly the low temperature tolerance and maltotriose use. However, the hybrid origin of *S. pastorianus* and the presence of more genomes in one strain might affect the genetic improvement of these strains. To overcome this inconvenience, *de-novo S. cerevisiae* × *S. eubayanus* hybrids have been shown to outperform their parent strains in a number of respects, including, but not restricted to, fermentation rate, sugar use, stress tolerance, and aroma formation.

6.2. Non-*Saccharomyces* yeast

In the last few years, the selection of starter strains has also been carried out within non-*Saccharomyces* species that have been isolated and characterized as having distinctive aromatic and flavor components [11]. Indeed, the production of quality beer depends on the activity of the fermenting yeast that are selected not only for their good fermentation efficiency, but also for the characteristic aroma and flavors that they can give to the final product. In particular, the worldwide growth of craft beer has reinforced and encouraged the selection and use of different yeast genera, with pronounced impacts on aroma and flavor [31]. Indeed, non-*Saccharomyces* yeast represents a large source of biodiversity for the production of new beer styles, and they have the potential for wider application to other beverages and for other industrial applications. Within non-*Saccharomyces* yeast that can be used as pure starter cultures for the wort fermentation, different genera and species have been proposed, including *B. bruxellensis, Torulaspora delbrueckii, Candida shehatae, Candida tropicalis, Zygosaccharomyces rouxii, Lachancea thermotolerans, Saccharomycodes ludwigii*, and *Pichia kluyveri*.

7. Spontaneous fermentation

It is already known that some of the above mentioned yeast take part in the spontaneous fermentation processes of specialty beers. A typical example of this spontaneous process is the lambic beers. The fermentation of these beers is driven by brewery-resident microorganisms that are self-inoculated by exposing the wort in open tanks during the overnight cooling, before transferring it to wooden barrels for fermentation and aging. The fermentation of such Belgian lambic beers involves *Saccharomyce* spp. and *Brettanomyces* spp., with contributions from lactic acid bacteria and acetic acid bacteria [32]. Natural mixed fermentations are also used in the production of some German style "weiss" beers. Different yeast have been isolated during the maturation of acidic ale beers, including those that belong to the *Candida, Torulopsis, Pichia, Hansenula*, and *Criptococcus* genera, although their contributions to the aroma composition

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have not been well investigated. American coolship ale beer is another example of spontaneous fermentation, where the process can be divided into various steps: *Enterobacteriaceae* and oxidative yeast in the first phase, which are replaced by *Saccharomyces* spp. and *Lactobacillales*, and then *Dekkera bruxellensis*, which prevails through the final process [33]. Other nonconventional beers, such as Tchapalo, are brewed using *C. tropicalis* and *S. cerevisiae* cultures that have been selected for their fermenting of sorghum wort. In Africa, different types of beers are made, and one of these is known as "*Tchoukoutou*", which is a Beninese sorghum beer. The microbial ecology of the starter used to produce this beer demonstrated the presence of different species of non-*Saccharomyces* yeast, including *Pichia kudriavzevii*, *Candida ethanolica*, and *Debaryomyces hansenii* [34].

8. Nonconventional yeast

Recently, growing attention has been given to the possible contributions of nonconventional yeast to beer production. The success of craft beers has induced brewers to look for new alternatives for fermentation, such as nonconventional yeast, to impact on the aroma and flavor, and thus to generate differentiated products. The production and increase in the aroma compounds through biological methods exploits the metabolic pathways of the yeast for the promotion of the so-called bioflavor. This approach includes microbial bioconversion of the flavor precursors, use of strains that produce the required compounds, and genetic modification of the yeast [35]. In this regard, although still poorly investigated in the brewing sector, the use of nonconventional yeast might enhance the analytical and aromatic profiles of the final product and reduce the alcohol content [36].

Among the nonconventional yeast that can potentially be used in brewing, *T. delbrueckii* has received attention due to its fermenting of maltose, to produce ester compounds, and its biotransformation of the monoterpenoid flavor compounds of hops [25, 31, 36, 37]. More recently, it was studied the involvement of *T. delbrueckii* strains in the production of wheat style ("weiss") beers. In this investigation, it was demonstrated that *T. delbrueckii* can consume maltose more slowly than the *S. cerevisiae* commercial starter strain, while on the other hand, the nonconventional yeast give more intensity and complexity to the product [37]. In a recent study of the use of *T. delbrueckii* in wort fermentation Canonico and colleagues [36] looked at its influence on the analytical and aromatic profile of the beer, and the potential to produce low alcohol beer. These authors, after a preliminary screening among 28 strains, evaluated the use of a strain of *T. delbrueckii* in wort fermentation in pure and mixed cultures. The influence on the analytical and aromatic profile of beer, as well as the potential of producing a low alcohol beer using this strain of *T. delbrueckii* was evaluated. Results indicated that *T. delbrueckii* in mixed fermentation with a *S. cerevisiae* commercial starter can fully convert the fermentable sugars exhibiting distinctive analytical and aromatic profiles.

Moreover, Michel and colleagues [31] screened 10 *T. delbrueckii* strains on several brewing features, such us sugar use, hops, ethanol resistance, propagation, amino acid metabolism, and phenolic off-flavor-forming production, which revealed overall good fermentation of the

wort and the production of a desirable fruity aroma. *L. thermotolerans* was also investigated for fermentation of the wort. Domizio and colleagues [38] tested three *L. thermotolerans* strains for important traits for beer production, including pitching rate, generational capacity, foam stability, hop tolerance, vicinal diketone production, oxygen requirement and flocculation, suggesting that *L. thermotolerans* may be a good choice for producing sour beers in a single fermentation step without the use of lactic acid bacteria.

8.1. Specialty beers

In response to increased consumer demand, the brewing industry has devoted much research effort to the development of new technologies and innovations for expansion of the assortment of specialty beers. Five types of specialty beers of particular interest have been described: low calorie beer, low alcohol or nonalcohol beer, novel-flavored beer, gluten-free beer, and functional beer [39]. Beers with a low calorie content have achieved great interest due to the problem of obesity, especially in Western populations, which accounts for a growing market segment.

This type of beer can be made by special mashing and collection of the wort with large amounts of fermentable sugars, or by inoculating microorganisms that can hydrolyze the more complex sugars, to reduce the concentrations of residual sugars in the final product. This is the case for *Brettanomyces*/*Dekkera*, the use of which leads to low calorie, but slightly more alcoholic, beers. Concerning this last aspect, new methods have been studied to produce beverages with lower alcohol contents, while providing superior sensory quality [40]. In this sense, non-*Saccharomyces* yeast represent a very attractive alternative, both for null processing of extra costs, and for the advantage of avoiding involuntary extraction of flavor compounds. *Wickerhamomyces subpelliculosus* (formerly *Pichia subpelliculosa*) and *Cyberlindnera saturnus* (formerly *Williopsis saturnus*), for example, have shown interesting results in the production of low alcohol beers with acceptable flavor profiles. *S. ludwigii* and the osmotic tolerant *Z. rouxii* are good examples of the novel use of non-*Saccharomyces* yeast to produce low alcohol or alcohol-free beers. *Pichia kluivery* was also recently proposed to produce positive flavor compounds from different hop varieties, thus indicating its potential application to the production of alcohol-free beer. Also, the use of *T. delbrueckii* strains that cannot degrade maltose, maltotriose, and other complex carbohydrates might be an interesting way to produce lower alcohol content in beers. The alcohol content here is usually close to 0.9% (v/v), with the advantage that many of these strains impart rich fruity flavor and aroma to the beer. The concept of low alcohol beer is necessarily linked to a functional product, and to a matrix with substantial amounts of fiber, vitamins, minerals, and polyphenols.

9. Functional beers

Functional beers are defined as beers with health benefits for those who consume them moderately. These are based on the use of nonconventional yeast that can produce or transform some

beneficial compounds. This is the case for melatonin, which is a sleep-regulating hormone in mammals that has antioxidant properties and that can be produced in beer during alcoholic fermentation by the appropriate yeast [41]. Within functional beers, there are gluten-free beers for consumers with the condition known as coeliac disease, which is a gluten-sensitive and immune-mediated enteropathy. Recently, there has been increased demand and consumer interest to develop gluten-free beers from alternative cereals, such as sorghum and maize. In addition to those mentioned above, other yeast are promising candidates for the production of specialty beers. Indeed, yeast from the genera *Hanseniaspora, Pichia, Torulaspora, Wickerhamomyces*, and others, can offer diversified enzymatic and bioconversion, which are allowing brewers to work with new concepts that include bioflavoring and beers with reduced calorie and alcohol contents, or even functional beers.

10. Yeast handling in the brewery

One of the common and efficient cost-reduction measures in beer production is serial re-pitching of the yeast at the end of the fermentation. The type of fermenter and yeast used define the procedures needed for the recovery of the yeast biomass. Generally, by the time a crop has formed, the yeast should be removed as soon as possible, as it has no further positive role in the fermentation. The optimum time for yeast removal is usually decided by the brewers, by considering the different parameters, such as full attenuation of the wort, or the reduction of vicinal diketones to optimal levels.

Top-fermenting (ale) yeast are removed using specific skimming systems in the fermenters. Not all of the yeast heads that are cropped are retained for re-pitching. Only a fraction of the yeast head is used, as that composed of middle to young yeast cells and relatively free from trub. When using bottom-fermenting (lager) yeast, the yeast slurry on the base of the fermenting vessel is obtained by simply removing the overlying beer. As in the case of top-fermenting yeast, for subsequent use it is necessary to retain the yeast cells that are less enriched in trub and are of a middle age. This fraction can be identified in the middle of the sediment, as the lowest layers are enriched in trub. Furthermore, some authors have suggested that the more flocculent portion of the cells settle in the middle, while the less flocculent cells are in the top portion of the cone. In the majority of breweries, pitching yeast is usually converted to a liquid slurry by adding water or by leaving enough entrained beer to facilitate yeast transport to the storage vessels via pumping. Alternatively, yeast cakes can be obtained by recovering the yeast from the entrained beer through filtering.

10.1. Yeast storage

During storage, yeast quality decreases as a function of storage conditions and procedures. This can lead to aberrant fermentation, which can be seen as slow attenuation rates, poor flocculation performance, and undesired flavor development. In particular, it is necessary to avoid yeast contamination with bacteria, wild yeast, or other starter yeast (cross-contamination). A

common procedure to reduce the bacterial load is to treat the yeast slurry with a chemical disinfectant at low pH (i.e., acid washing). Usually citric and phosphoric acids are used at low temperatures (2–4°C) with continuous gentle stirring [42]. The correct procedures during acid washing allow the removal of bacteria without affecting the yeast performances in the subsequent fermentation.

Another aspect of primary importance is the avoidance of excessive stress to the yeast cells during cropping and storage, to minimize any changes in their physiological conditions. In particular, the intracellular concentrations of storage carbohydrates (i.e., glycogen and trehalose) and sterols and other lipids are of primary importance for the duration of storage [43]. Indeed, the storage phase is a period of starvation, and the yeast need to rely on nutritional reserves that were accumulated during fermentation. Glycogen is synthesized during mid-fermentation, and its dissimulation is directly correlated with the storage temperatures. Furthermore, supplementation of cropped yeast with linoleic acid before pitching has been suggested as a convenient way to improve the yeast physiology without affecting the yeast growth, fermentation rate, and production of volatile compounds during the subsequent beer production [44].

10.2. Continuous re-pitching

The influence of serial cropping and re-pitching on the consistency of fermentation performance and beer composition poses technological questions about the number of generations that can be allowed to elapse before introducing new yeast. This decision is usually made by the individual brewers, as there are no pre-determined rules. In breweries with high hygiene standards, serial re-pitching can continue for 15–20 generations, while in microbreweries, even 5–10 generations is considered excessive. Continued serial re-pitching of yeast can be associated with gradual deterioration in the yeast conditions, which can result in decline in fermentation performance. Indeed, the aging process in yeast is associated with gradual disruption of many of their metabolic processes. On the other hand, there is an economic cost to propagation, and if a re-pitched yeast is performing satisfactorily, there is less need to introduce a newly propagated yeast. Indeed, the first generation fermentation using new yeast lines is atypical, as the yeast cells are less adapted to the wort and fermentation conditions, particularly for high gravity brewing. To assist brewers in the decision of when to introduce a new yeast line, several methodologies have been developed. The most widely used assay to evaluate yeast viability involves microscopic observation of the yeast cells stained with methylene blue. While this method is economic and simple to perform, it is also subject to operator error and known to overestimate viability [45]. Recently, flow-cytometric methods have been developed to assist brewers in the evaluation of their yeast viability and vitality. In particular, analysis of the yeast cells stained with the fluorescent dye oxonol allows automatic detection of yeast viability without interference from the wort trub [46]. Moreover, flow cytometry assays are not limited to viability tests, but can also be used for vitality tests that are related to the yeast fermentation performance, which can be implemented using specific fluorophores.

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