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# Use of Non-*Saccharomyces* Yeasts in Bottle Fermentation of Aged Beers

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Additional information is available at the end of the chapter

## Abstract

Bottle fermented and brewed beers are reaching more recognition in present days due to their high sensory complexity. These beers normally are produced by an initial tank fermentation to metabolize the sugars obtaining the typical alcoholic degree, and later the foam and $CO_2$ pressure is produced by subsequent bottle fermentation. The sensory profile is improved by the formation of some fermentative volatiles, but also by the ageing on lees, because beers are brewed during several months with the yeast cells that performed the fermentation. The use of non-*Saccharomyces* yeast is a trending topic in many fermentative food industries (wines, beer, bread, etc.). They open new possibilities to modulate flavor and other sensory properties during fermentation and biological ageing. This chapter review the effect of some non-*Saccharomyces* yeasts such as *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Saccharomycodes ludwigii*, and *Brettanomyces bruxellensis* in the bottle fermentation and brewing of beers analyzing their metabolic specificities and sensory contribution on beer taste.

**Keywords:** non-*Saccharomyces* yeasts, beer, bottle fermentation, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Saccharomycodes ludwigii*, *Brettanomyces bruxellensis*

## 1. Introduction

The beer-like beverages were already produced in the regions of Mesopotamia and Ancient Egypt since 5500 BC [1]. Home-made or small-scale trade brewing supplied an essential part of diet to a primarily agrarian population. The historical development of brewing and the

brewing industry is, however, linked with northern Europe where cold conditions inhibited the development of viticulture.

Although the main malted cereal for brewing is barley, other cereals such as oats, maize, rice, rye, sorghum, and wheat are malted for specific purposes, mainly for the production of peculiar beers. From the tenth century, adding of hops became usual from Germany across Europe to replace, or at least supplement, the variety of plants, herbs, and spices popular at that time. Not only the pleasing flavor and aroma of hop but perhaps, more importantly, their action in protecting the beer from being spoiled by the then unknown microbes, eventually led to their wide-scale adoption.

According with Anderson [2], in the medieval and early modern period the scale of brewing ranged from a few hectoliters annually in the average home to hundreds, or occasionally, thousands of hectoliters in the largest monasteries and country houses. Domestic brewing still accounted for well over half of the beer produced at the end of the seventeenth century. On the eighteenth and nineteenth centuries, the population of Europe's cities growth was accompanied by an increase in beer consumption. The leading European beer-drinking countries of nineteenth century were the United Kingdom, Germany, and Belgium. Large-scale production began on the nineteenth century with improvements of technology and scientific research on microbiology. First, little meaningful scientific research in brewing was carried out by Louis Pasteur whose investigations on wine and beer fermentations in the 1860s and 1870s showed the importance of eliminating deleterious bacteria [3]. Emil Christian Hansen became the first to isolate a pure yeast culture at Carlsberg in 1883.

The twentieth century saw the expansion in the beer industry. The industrial cooling allowed the introduction into the market of lager beers. Currently, there is a resurgence in craft or microbrewers [4], the market of craft beers in the United States grew 12.8% in 2015, and a significant strengthening of the sector has also been detected in several European countries, where growth from 2009 to 2014 outstripped 90–145% in Switzerland, the United Kingdom, France, Italy, and Slovenia; 333–400% in Slovakia, Czech Republic, Sweden, and Norway; and more than 1000% in Spain [5].

Brewing can be defined as the making of beer or related beverages by infusion, boiling, and fermentation. The various processes, inputs, and products of a brew house are shown in **Figure 1**. First part of the brewing process consist in converting the raw materials—water, malt, adjuncts, and hops—into a fermentable wort. Malt enzymes assist in the conversion of starch to fermentable sugars by mashing. For that reason, prior to the brewing process the malting process activates the natural enzyme systems of barley by controlled steeping, germination, and kilning. Mashing was simply the process of mixing warm water with ground malt, and cereal adjuncts if used, and after a period of standing, as much of the liquid as possible was recovered. The mash is then transferred to a mash filter (lauter tun) to produce bright wort and to collect the maximum amount of sugars (extract) from the residual solid materials ("spent" grain). Wort boiling satisfies a number of important objectives such as sterilization of the wort, extraction of the bittering compounds from hops, coagulation of excess proteins and tannins to form solid particles (hot trub) that can be removed later in the whirlpool, color and flavor formation, removal of undesirable volatiles such as dimethyl

sulfide (DMS), by evaporation, and the concentration of the sugars by evaporation of water. Hot trub does need to be removed if the beer stability is not to suffer. The wort is then cooled from almost boiling point to fermentation temperature through a heat exchanger using water as the main cooling medium. The temperature for fermentation (**Figure 2**) is different for ale
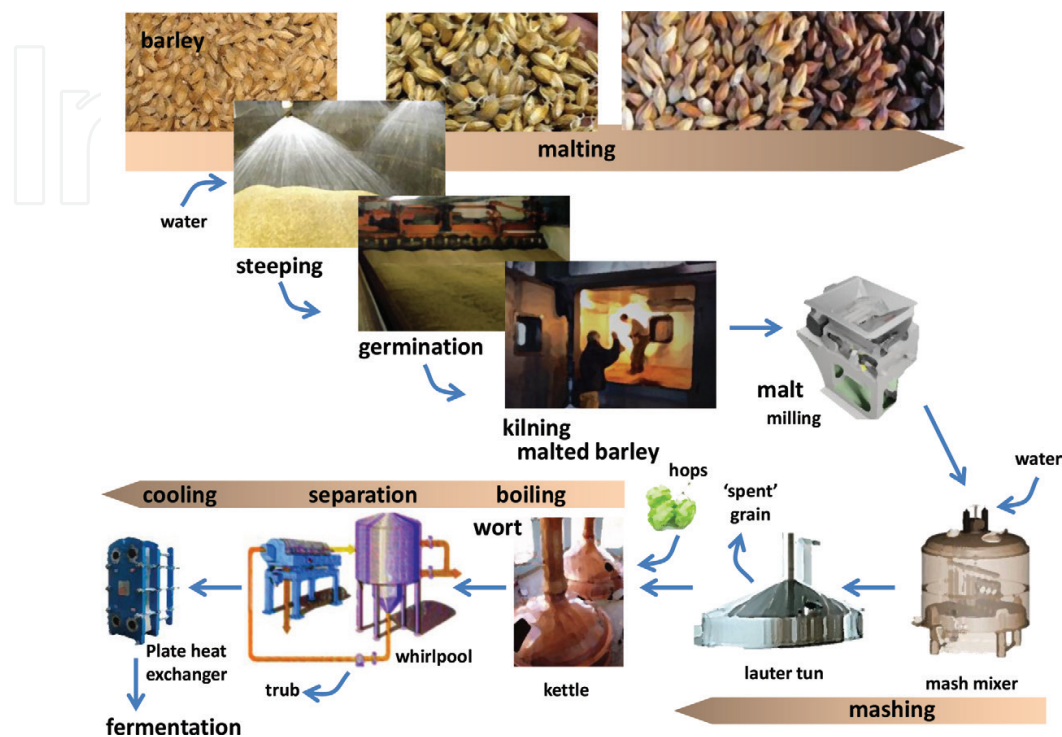
**Figure 1.** Malting and brewing process. First steps mashing and wort preparation.

**Figure 2.** Brewing process: fermentation and bottle fermentation.

or lager fermentation (typically 8–14°C for lager and 15–20°C for ale). Yeast is pitched into the cooled wort in the fermentation vessel. Once the fermentation of the wort is completed, it is important to remove the bulk of the excess yeast before maturation, generally by removing the beer from the settled yeast. Then green beer must be conditioned to produce a stable, quality product suitable for filtration, and packaging. This process is called aging (lagering in lager beers). The objectives of beer aging are chill haze formation, clarification, carbonation (to a limited extent), and flavor maturation (again to a limited extent).

Fermentations performed in closed cylindro-conicals vessels may be of either ale or lager type. At the end of primary fermentation, the bulk of the yeast is collected by the application of rapid chilling and we have a green beer. Maturation of green beer is needed to obtain flavor adjustment (diacetyl, $SO_2$, and DMS), yeast sedimentation, carbonation, and colloidal stability.

Typically, ale fermentations are fully attenuated and subjected to a short low-temperature conditioning in a separate tank to adjust carbonation, precipitate chill haze, and allow some loss of undesirable flavor volatiles trough gas purging. As lager beers ferment at much lower temperatures than ale yeasts (and therefore much slower), they also require an extended period of cellaring for maturation and development of the beer flavor. Conditioning is carried out at low temperature for no longer than a few days. As with chilled and filtered ales, this part of the process serves simply to adjust carbonation, develop chill haze protection, and clarify the beer. After conditioning the beers are filtered and packaged.

Other recovering old production methods for innovation are the production of cask- and bottle-conditioned beers. With bottle-conditioned beers a base beer is used. This base beer are as nearly as possible completely attenuated. This allows accurate control of addition of priming sugars. After bottling, beers are held in the brewery for a period from a few weeks to a few months depending on the temperature. Products of yeast metabolism are excreted into the beer providing distinctive flavor [4]. It is possible to apply this technique by adding pure cultures of different non-*Saccharomyces* yeast strains to increase the complexity of the final product.

## 2. Fermentation

From ancient times the elaboration of beer was carried out by spontaneous fermentations with exposure to ambient to air of the cereal slurry to induce "contamination." Bit-by-bit species of the genus *Saccharomyces* were intuitively selected from different raw materials. Nowadays, in most beers, the microorganisms that carry out fermentation belong to different *Saccharomyces* species and strains [6]. For 99% of the worldwide beers, *Saccharomyces* is the sole microbial inoculum.

According to Ref. [4], the use of *Saccharomyces* strains in controlled fermentations over decades is essentially based on three main features: (a) efficient production of high ethanol amounts; (b) the use of fermentation as the preferential metabolic pathway, combined to the positive

Crabtree effect (repression of respiration by glucose); and (c) higher tolerance to ethanol and other environmental stresses [7].

In brewing most of the strains of *Saccharomyces* are classified into the categories such as ale and lager yeasts. Although there are many different styles of beer, the main brewing classification criterion particularly relies on the selection of the yeast strain and type of fermentation. Ale yeasts or top-fermenting yeasts, which are *Saccharomyces cerevisiae* strains, rise up to the surface of the vessel with the escaping carbon dioxide gas bubbles and become entangled in the fermentation head, facilitating their collection by skimming. Fermentation temperature ranges between 15 and 20°C. Lager yeasts or bottom-fermenting yeast, do not rise and become entrapped in the foam but settle out at the end of the fermentation. The nomenclature of lager yeast has evolved as research has been developed. Successively, they have received different names such as *Saccharomyces carlsbergensis*, *Saccharomyces uvarum*, and *S. cereviseae* lager type. Now they are termed *S. pastorianus* [6]. Lager worts often ferment at lower temperatures (8–14°C) than ale yeasts and are therefore much slower.

The occurrence of other species different to *Saccharomyces* species and strains is commonly reported in some peculiar beer styles produced by spontaneous fermentations, as the Belgian acid beers (Lambic, Gueuze, and rodenbach), and the American Coolship Ales, an American descendant of the Belgian lambic style. In these processes, wort is spontaneously fermented by microbes present in the air and surfaces of the brewery. These microorganisms are introduced by exposing the wort in shallow tanks during the overnight cooling, before transferring it to wooden barrels for fermentation, and aging.

The non-*Saccharomyces* species can be used in the production of low-alcohol beer (0.5–1.2%, v/v) and alcohol-free beer (<0.5%, v/v) [8]. The suitability of yeast strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* for low-alcohol beer production has been analyzed by De Francesco et al. [9]. Most of the *Z. rouxii* strains were found unsuitable because of the production of high concentration of ethanol. The most successful genus used for the industrial production of alcohol-free beer is *S. ludwigii* due to its disability to ferment maltose and maltotriose, the prevailing fermentable sugars of all malt [10, 11].

Other application of non-*Saccharomyces* recently emerged is its use in controlled fermentations. This practice has been gaining popularity among brewers in order to obtain distinctive products, with distinctive aromatic and flavor components [12].

Although there are different options of innovation in the brewing process (using special malts or adjuncts, hop varieties, water quality, etc.), new and novel brewing yeasts strains can be discovered to enhance the aroma and flavor characteristics of beer and provide opportunities for developing new beers. The production of most aroma-active compounds is strictly dependent on the yeast strain chosen for the fermentation [13, 14]. This makes the selection of suitable strains the most important task to make good beer and opens new innovation opportunities to gain market especially in the market of craft-beer, to improve aroma profile after inoculation of non-*Saccharomyces* yeasts during bottle conditioning and ageing.

## 3. Use of non-*Saccharomyces* in beer fermentation and ageing

The use of non-*Saccharomyces* yeasts allows new possibilities in the improvement and innovation in sensory profile of beers but also technological advantages and new ways of ageing. Several articles review the importance of non-*Saccharomyces* yeasts in beer [4, 15] and other fermented beverages [16, 17].

The typical yeast species used in beer fermentation, and in most of food fermentations, is *S. cerevisiae* which is able to ferment monosaccharides like glucose, fructose, galactose, and mannose, and some disaccharides such as maltose and sucrose and also is able to use the trisaccharide rafinosse [18]. Some strains can metabolize more than 300 g/L of sugars by fermentation reaching 18% (v/v) of ethanol in some alcoholic beverages like wines. As nitrogen source one can use urea, ammonia, and several amino acids. Also phosphate, biotine, and other cofactors and micronutrients are also needed. *S. cerevisiae* has globous shape with multipolar budding and is teleomorphic yeast with tetrahedral sporulation that can be promoted in acetate agar.

Beer yeast can be classified in top and bottom-fermenting yeasts even when both belong to *S. cerevisiae* species. Ale strains ferment better at 15–20°C, a relatively warm temperature, and during fermentation they form a thick film on the surface formed by yeast cells. They are considered top-fermenting yeasts which forms high amounts of esters that produce the distinctive sensory profile of ale beers such as ales, porters, stouts, Altbier, Kölsch, and wheat beers. Lager strains works better at 8–14°C growing slowly and settling at the bottom of the tank, they are known as bottom-fermenting yeasts. Sensory profile depends a lot in the strain used, some famous lagers are Pilsners, Dortmunders, Märzen, Bocks, and American malt liquors.

The use of non-*Saccharomyces* yeasts open new possibilities compared with the traditional *S. cerevisiae* strains. In fact some traditional beers such as Lambic, are produced by spontaneous fermentations with the development of non-*Saccharomyces* yeasts specifically *Brettanomyces bruxellensis*. Their distinctive sensory profile is due to its metabolomic impact by the production and release of several esters with fruity smells such as ethyl acetate, ethyl caprate, ethyl caprylate, and ethyl lactate [4, 7, 15]. Moreover, the volatile acidity produces a sour taste typical in Lambic beer [19].

The main fermentative properties of interesting non-*Saccharomyces* yeasts reported as useful for beer and other fermented beverages are described in **Table 1**. *Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Schizosaccharomyces pombe* are currently produced at industrial level as dry yeast or liquid refrigerated starters by international biotechnological companies such as Chr. Hansen, Lallemand, Laffort, and Erbslöh [17].

One of the most studied non-*Saccharomyces* in modern food fermentations is *T. delbrueckii*, formerly known as *Saccharomyces delbrueckii* or *Saccharomyces rosei*. It is now being used to improve fermentation or technological properties in beer [4], wine [20], bread [21], and other food products, and it is possible to find commercial cultures as dry yeasts [17]. The morphology is ellipsoidal similar to *S. cerevisiae* (**Figure 3A**). *T. delbrueckii* is teleomorph yeast corresponding to the anamorph species *Candida colliculosa*. Typical sporulation form one or two

| Yeast species | Fermentative power (% v/v ethanol) | Able to ferment | Volatile acidity (g/L) | Volatile compounds | Effect on acidity | Ageing on lees | References |
|---|---|---|---|---|---|---|---|
| *S. cerevisiae* | 12–18 | Glucose Fructose Galactose (v) Sucrose Maltose | <0.5 | Higher alcohols Esters | Neutral | Depending on strains | [16] |
| *Torulaspora delbrueckii* | <9 | Glucose Fructose Galactose (v) Sucrose (v) Maltose (v) | <0.5 | Ethyl lactate 2-phenylethyl acetate 3-ethoxy propanol | Neutral | Moderate-high | [4, 23, 24] |
| *Lachancea thermotolerans* | <9 | Glucose Fructose Maltose (v) Galactose (v) | <0.5 | 2-phenylethyl acetate Ethyl lactate | Acidity enhancement lactic acid production | Depending on strains | [25] |
| *Schizosaccharomyces pombe* | 12–14 | Glucose Fructose Sucrose Maltose | 0.8–1.4 | Higher alcohols Esters | Maloalcoholic deacidification | Osmophilic high release of cell polysaccharydes | [16, 26, 27] |
| *Saccharomycodes ludwigii* | 12–14 | Glucose Fructose Sucrose | <0.5 | Diacetyl, acetoin | Neutral | Osmophilic high release of cell polysaccharydes | [9, 28] |
| *Dekkera (Brettanomyces) bruxellensis* | <2 | Glucose Fructose Sucrose Maltose Cellobiose | >1 | Ethylphenols Isovaleric acid Isobutyric acid Pyrazines | Acidity enhancement | Release of cell polysaccharydes | [4, 15, 19, 29–31] |

**Table 1.** Technological performance and features of non-*Saccharomyces* yeasts with potential advantages in brewing technology.
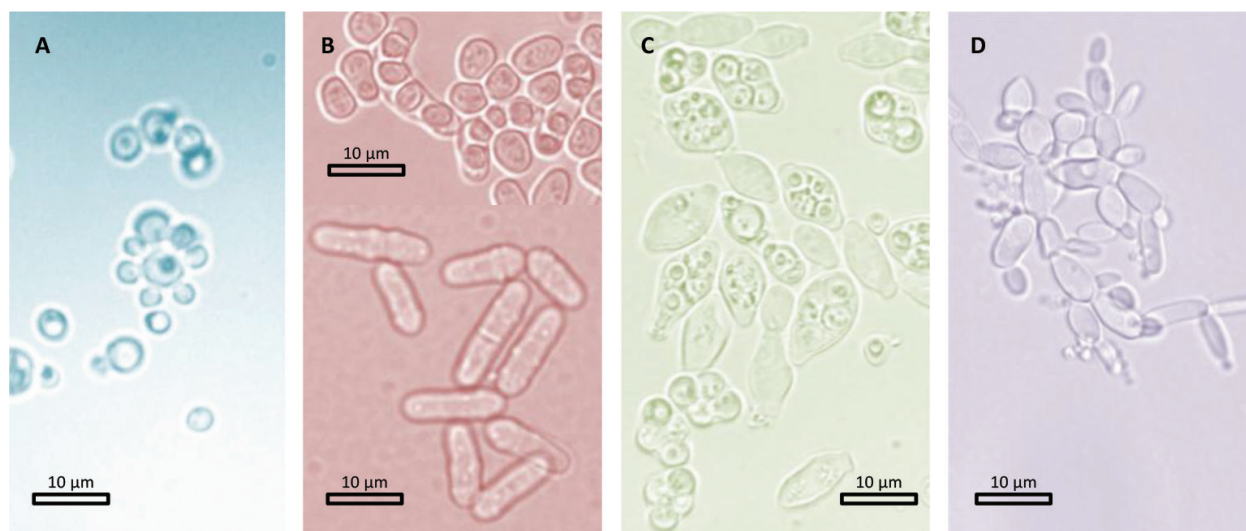
**Figure 3.** Optical microscopy of non-*Saccharomyces* yeasts: (A) *Torulaspora delbrueckii,* (B) *Schizosaccharomyces pombe,* (C) *Saccharomycodes ludwigii,* (D) *Dekkera* (*Brettanomyces*) *bruxelensis.*

spherical spores per ascus [22]. It has a low fermentative power reaching most of the strains a maximum of 6–9% (v/v) in ethanol. It has been described as osmotolerant [21] yeast with pure fermentation, with low production of volatile acidity and ethyl acetate.

It has also been described as a low producer of several volatile metabolites such as acetalde-hyde, acetoin, and $H_2S$ that can be unpleasant at high concentration [17, 25, 32]. Sequential fermentations with *S. cerevisiae* can be used to reduce volatile acidity [23]. *T. delbrueckii* also affects acidity balance by production of appreciable amounts of lactic and succinic acids [32]. Production of aromatic esters like ethyl lactate and 2-phenylethyl acetate is enhanced regarding single-culture *S. cerevisiae* fermentations. Some strains of *T. delbrueckii* have been described as stronger producers of 2-phenylethyl acetate in beers [33]. Ethyl lactate produces a coffee or strawberry smell that increases complexity also 2-phenylethyl acetate is the aromatic impact molecule of rose petals. 3-Ethoxy has been reported in sequential fermentations of *T. delbrueckii* and *S. cerevisiae* [24] with sensory repercussions for its either solvent of fruity (black currant) when combined with other flavors [34].

*L. thermotolerans* is getting attention in fermented beverages for its repercussion in sensory profile and the production of organic acids. Formerly it was known as *Kluyveromyces ther-motolerans. L. thermotolerans* appearance is quite similar in both shape and size, to *S. cerevi-siae* with an ellipsoidal morphology and impossible to be distinguish by optical microscopy. Asexual reproduction by multipolar budding also enables to sporulate with 1–4 spores in a dehiscent ascus. Its fermentation performance is medium and it can reach from 4 to 9% (v/v) in ethanol depending on the strain [25], but is unable to grow at higher contents than 9% [17]. Similar to *T. delbrueckii*, it shows good fermentation purity with low production of volatile acidity [35].

*L. thermotolerans* can be used to increase acidity during fermentation by production of lactic acid (**Table 1**). Concentrations of 9.6 g/L has been reported after fermentation [35].

This acidification is enough to decrease pH significantly [25] and to affect sourness taste [36]. Moreover, the ability to ferment from malt sugars producing significant amounts of lactic acid make *L. thermotolerans* suitable to be used in the production of acidic beers without the involvement of lactic bacteria and in a single-stage fermentation [37].

Enhance the production of 2-phenylethanol and glycerol during fermentation [25, 36], *L. thermotolerans* has been described as an osmophilic yeast probably with higher production of glycerol, which is a tool to balance osmotic pressure. The levels of acetaldehyde and higher alcohols can be controlled using *L. thermotolerans* [38].

*S. pombe* is peculiar yeast traditionally used in some areas from Africa in the production of mijo beer. Pombe means beer in Swahili language. This yeast was isolated initially by Lindner on 1893 in East Africa. *S. pombe* has a rod-shaped structure with 3–4 μm in diameter and 7–20 μm in length. Asexual reproduction is performed by fission being a main difference with *S. cerevisiae*. It forms an intermediate septum at the center of the cell that is clearly visible by optical micros-copy (**Figure 3B**). It is also teleomorph species having spherical spores in a linear organization with a typical amount of four per ascus (**Figure 3B**). The fermentative power is high being able to reach depending on the strain, 10–13% (v/v) in ethanol under anaerobiosis and 13–15 with slight aeration [39]. The production of acetic acid during fermentation is quite high (**Table 1**) being a main drawback in enology [20]; however, this parameter can be improved by selection, and also as the production depends on the amount of sugars metabolized in beer, the final lev-els are less conflictive. Other technological advantage is the resistance to sulfur dioxide.

Metabolism of organic acids is peculiar in *S. pombe* and different of its relative *S. cerevisiae*. *S. pombe* is a yeast that is able to degrade malic acid using the metabolic pathway call malo-alcoholic fermentation which yields as main products ethanol and $CO_2$. And the typical malic acid degradation done by lactic bacteria that also can be produced in beer or wine conditions uses other metabolic pathway call malolactic fermentation and produces lactic acid as main product. In the maloalcoholic fermentation, malate is decarboxylated to pyru-vate by malic enzyme and later it is decarboxylated to acetaldehyde and finally reduced to ethanol [40]. Under anaerobiosis the fermentation of 2.3 g/L of malic acid produces 0.1% (v/v) of ethanol [41]. Degradation of malic acid can be higher than 8 g/L [39]. The sen-sory effect of this process is a softening of the sourness when substrates rich in malic acid are metabolized by *S. pombe*. The levels of pyruvate released during fermentation are also higher than the average of *S. cerevisiae* probably because of an intermediate in MA fermen-tation. The production of pyruvate affects the formation of pyranoanthocyanin pigments (vitisin A-type) during red wine fermentation by *S. pombe* [42]. Also, it has been reported that *S. pombe* can metabolize gluconic acid contents [43]. The unusual metabolism of organic acids in *S. pombe* can help to get other sourness balance in beers.

*S. pombe* is an osmophilic yeast with a double-layered cell wall composed of glucose, galac-tose, and mannose polysaccharides. Ageing on lees is a biological ageing in which fermented beverages remains together with the fermentation lees for a long time, more than 9 months. After yeast autolysis, cell wall polysaccharides are released and affect the sensory perception normally softening the mouth fell of the fermented beverages [27]. The thickness of the cell wall in *S. pombe* facilitates and increases the release of polysaccharides during ageing on lees.

Concentrations of 10 times higher in *S. pombe* regarding *S. cerevisiae* has been measured after 2 months of ageing of lees [27].

Some repercussions of *S. pombe* in food security of fermented beverages have been reported. The urease activity of *S. pombe* can facilitate the reduction of urea levels in musts reducing the risks of ethyl carbamate formation [39]. Also, the consumption of yeast assimilable nitrogen (YAN) by *S. pombe* is lower than in *S. cerevisiae*. So, it can be fermented substrates with low contents of YAN [44], minimizing the risk of production of nitrogen metabolites like biogenic amines.

*S. ludwigii* is an apiculated yeast lemon shaped with bipolar budding (**Figure 3C**). The cells are rather large in size 3–5 μm in diameter and 10–20 μm in length. It is a teleomorph yeast showing a typical rhomboid distribution with 4 spherical spores per ascus.

*S. ludwigii* has a strong resistance to sulfur dioxide due to the formation of electrophilic adducts with acetaldehyde [45]. The production and release of acetaldehyde during fermentation increases according to the concentration of free $SO_2$. Some strains produce high volatile acidity [46] but most of them are moderated; however, the production of ethyl acetate is frequently high (400 mg/L in average) [28].

The influence in aromatic profile is because of the production of esters giving a fruity taste. *S. ludwigii* expresses an extracellular β-glicosidase that can affect the release of free terpenes. Production of acetoin and diacetyl are also enhanced in *S. ludwigii* [47], many strains are able to produce more than 100 mg/L of acetoin and also some of them can reach 300 mg/L during fermentation [48].

Also, it has being described as an osmophilic yeast with thick cell wall and is able to release higher amounts of polysaccharides from the external covering during autolysis. The release of polysaccharides is quite similar to *S. pombe* and about 10-fold higher than *Saccharomyces* after 2 months of over lees ageing [27].

Traditionally, it is considered as spoilage yeast in enology because of the production of off-flavors, especially excessive amounts of acetoin and diacetyl. However, new applications are open currently in beer fermentation because of the lower amount of fermentable sugars. *S. ludwigii* is unable or a weak fermenter of either maltose or maltotriose from wort, and is being useful to produce alcohol-free and low-alcohol (0.5–1.2%, v/v ethanol) beers [8, 9, 49].

*Dekkera bruxellensis* and its anamorph form *B. bruxellensis* are ogival in shape with elongated cells that in old cultures can be highly branched because of the incomplete separation of the cells (**Figure 3D**). Its characteristics are multipolar budding, hat-shaped spores, film formation in liquid surface, and ethanol tolerance of 13–15% (v/v) [50].

The production of acetic acid by *Brettanomyces* is high (**Table 1**) and strongly dependent on the aeration conditions and oxygen availability [51]. Traditionally used in the production of sour beers (Belgian Lambics and Gueuze). Lambic beers are produced by a heterogeneous mixture of bacteria and yeasts in several phases. Acidification starts with the activity of lactic bacteria and even acetic bacteria, and is followed by the prevalence of *Brettanomyces*, mainly *bruxellensis* replacing the yeasts *Saccharomyces* at the end, when alcoholic degree is 5–6%

(v/v) [15]. *Brettanomyces* overferment the wort and degrades complex carbohydrates. At this time the population of lactic bacteria decreases and the flavor is enriched in the "Brett" taste.

In wines it has been described as producer of several off-flavors "Brett taint" [29, 52], most important are ethylpehnols. *Brettanomyces* is able to transform hydroxycinnamic acids in ethylphenols by mean of two enzymatic activities hydroxycinnamate decarboxylase that produce and intermediate vinylphenol and later vinylphenol reductase yielding the later ethylphenol. Descriptors of 4-ethylphenol are band-aid, leather, and horse sweat.

*Brettanomyces* release high amount of cell wall polysaccharides in ageing on lees [31] and could be an interesting parameter to modulate beer taste during bottle fermentation and ageing.

Selection, isolation, and counting of non-*Saccharomyces* can be done using selective media. Most non-*Saccharomyces* yeast can be separated of *S. cerevisiae* by culture in synthetic lysine phosphate media. *S. cerevisiae* can be differentiated from most of the non-*Saccharomyces* species because it is able to grow at 39°C [24].

## 4. Sensory effects of the use of non-*Saccharomyces* in beer conditioning and ageing

There are several hundreds of flavor-active compounds in beer. The main fermentation products of the brewing yeast are ethanol and carbon dioxide. Other molecules produced in smaller concentrations by yeasts during fermentation as metabolic intermediates or byproducts have great impact on beer flavor and determine the final quality of beer.

Although non-*Saccharomyces* yeasts have been widely disregarded due to their possible overproduction of acetic acid and other flavor compounds, they can potentially exert positive influences on beer flavor through the synthesis of secondary metabolites and excretion of enzymes responsible for the bioconversion of nonvolatile precursors into desirable aroma compounds. Using pure culture of a specific yeast species it is possible to modulate beer flavor by a natural biological method. These bioflavoring processes allows obtaining beer with enhanced and differentiated sensory profiles.

Several factors affect yeast fermentation with considerable influence on sensory profile of beer, some of the most relevant are pitching rate, temperature, duration, aeration, and C/N ratio [6, 15]. Also important is the specific species or yeast strain used [15]. Some of the most important descriptors in sensory analysis of beers are described in **Figure 4**.

Secondary metabolites can be divided in several groups including fusel alcohols, esters sulfur-containing flavor compounds, undesirable carbonyl compounds, volatile phenols, organic acids, and monoterpene alcohols [8]. Higher (fusel) alcohols and esters are the most important flavor-active substances in beer. Higher alcohols contribute to alcoholic taste, spicy, vinous, pungent aroma, and esters to fruity aroma of beer. Isoamyl acetate is considered a major contributor to the fruitiness of beer. However, it is possible that the presence of different esters below their threshold levels can exert a synergistic effect and play a role in beer flavor [53].
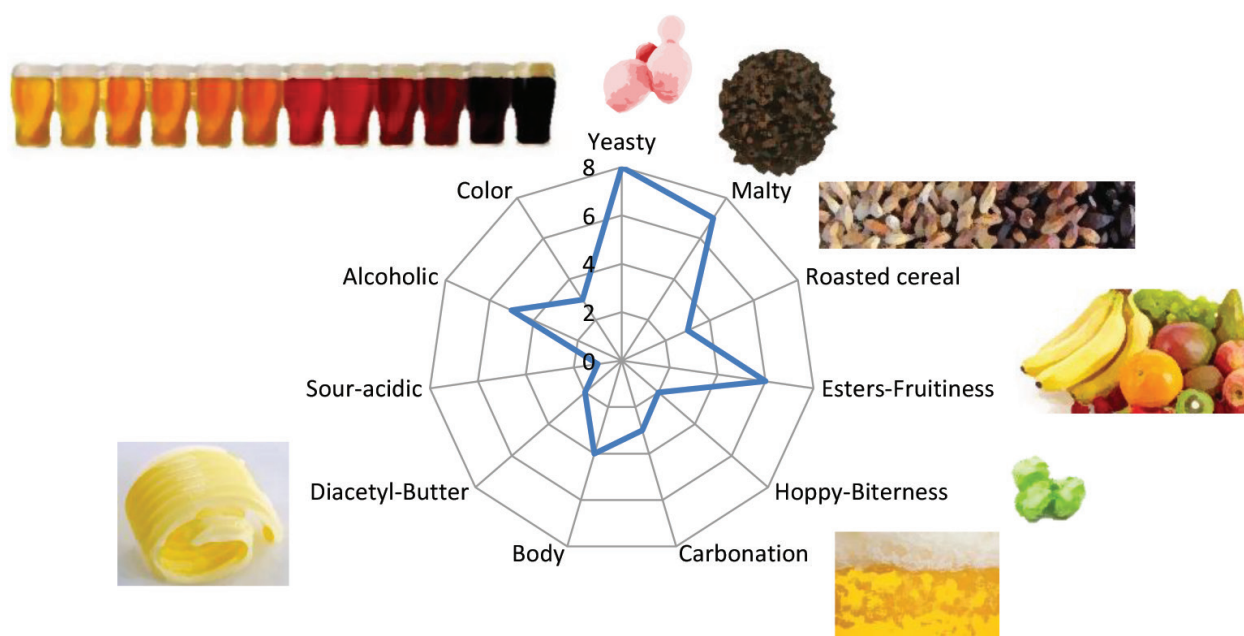
**Figure 4.** Spider net diagram for sensory profiling of beers.

The synthesis of fusel alcohols in beer fermentation is linked to the assimilation of the nitrogen sources by yeast and, therefore, typically the consumption and production of amino acids. Esters are formed later via reactions of alcohols (ethanol and fusels) and acids (AcylCoA compounds). These reactions are catalyzed by specific enzymes called acyl-alcohol transferases or esterases. When some non-*Saccharomyces* species fruity or floral ester production can be strongly enhanced, this biotechnology can be applied during main fermentation but also during second fermentation in the beers that are brewed and matured in bottle. *T. delbrueckii* and *L. thermotolerans* with medium fermentative power can be used to increase ester formation (**Table 1**).

Sulfur compounds are also produced by yeasts during fermentation, some of them behave as off-flavors and they have low sensory thresholds but others help to improve and modulate sensory profile of beers. Sulfur dioxide and hydrogen sulfide are included in these compounds [8], and they also have antioxidant properties and influence in flavor stability and increases shelf life. Hydrogen sulfide can be considered as a typical off-flavor in lager beers; however, at low concentrations it can be considered a typical aroma in some ale beers [54]. Generally, *Saccharomyces* strains used in ale fermentations are more prone to reduce sulfur dioxide to hydrogen sulfide than those used in lager beers.

Among carbonyl compounds the presence of diacetyl above the threshold levels is responsible of a buttery odor generally undesirable in lager beer but can be an interesting attribute in some beer styles such as English pale ales [53]. Diacetyl is reduced initially to acetoin and later to 2,3-butanediol, both with lower flavoring activity [54]. Malolactic fermentation by lactic acid bacteria (LAB) is a strategy widely used in wine making to enhance the production of diacetyl. In beer brewing, LABs are sensitive to hop bitter acids [53]. Increasing of temperature at the end of fermentation promotes the removal of diacetyl [54]. The levels of acetoin and diacetyl can

also be enhanced by using non-*Saccharomyces* yeasts like *S. ludwigii* during main or alternatively bottle fermentation (**Table 1**). Yeast-produced phenols are responsible of desirable flavors with descriptors like clove, smoky, spicy, medicinal, and burnt aromas traditionally noticeable in wheat beer styles such as Belgian White beers, German Rauch beers, and Weizen beers. Typical non-*Saccharomyces* yeasts with strong formation of ethylphenols from hydroxycinnamates are *Brettanomyces* and *Dekkera* genera by their specific enzymatic activities (**Table 1**).

The main volatile organic acids that occur in beer are acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic, caprylic, capric, and lauric acid. If they are present at high concentrations they contribute to sour and salty flavor to beer and can also contribute to off-flavors such as cheesy and sweaty [8].

Lastly, some terpenic compounds from hops and with positive repercussion on beer flavor can be released and enhanced by some enzymes expressed by yeasts [8]. *Pichia anomala* and *Kloeckera apiculata* species frequently express β-glucosidase activities.

## 5. Sensory aspects related with engineering and brewing process

Beer-making process with inputs, byproducts, and residues can be seen in **Figure 5**. Wort boiling is the most energy-consuming operation and different alternatives to reduce the consumption of thermal energy have been implemented. High-gravity brewing has been a common practice in many commercial breweries, especially in lager beers [13]. Worts with a very high specific gravity (HSG; >16°P) are fermented to obtain a very high ethanol content beer. This beer is then diluted to reach the normal ethanol content.

According to Lodolo et al. [55] elevated osmotic pressure and the dilution of other essential nutritional factors such as amino acids contributes toward poor fermentation performance. The use of worts with high specific gravity (HSG) results in an unbalanced flavor profile [13, 14] and severe overproduction of acetate esters [13].

Another aspect related with brewing process is the use of large lager fermentation vessels in big-scale production. Fementor design can lead great pressures inside and excessive top pressure leads to poor yeast growth [13, 14]. The higher hydrostatic pressure in tall fermenters increases the concentration of carbon dioxide dissolved in beer and affects the stratification and irregular distribution of $CO_2$ during fermentation. The excess in dissolved $CO_2$ inhibits yeast growth and metabolism [13, 14]: poor diacetyl reduction and, most importantly, low ester production. However, according to Verstrepen et al. [13], in high gravity brewing (HGW) or when high fermentation temperatures are applied, the excessive formation of esters could be reduced by the use of tall fermenters.

On the other hand, some innovative brewer rooms are equipped by horizontal tanks that can be refrigerated by cooling jackets. Horizontal geometry leads to control yeast growth, modulates the ratio between higher alcohols and esters, and affects the flocculation, being possible to keep more suspended yeasts at the end of fermentation. Moreover, this geometry improves cleaning by CIP systems, and promotes the formation of aromatic esters.
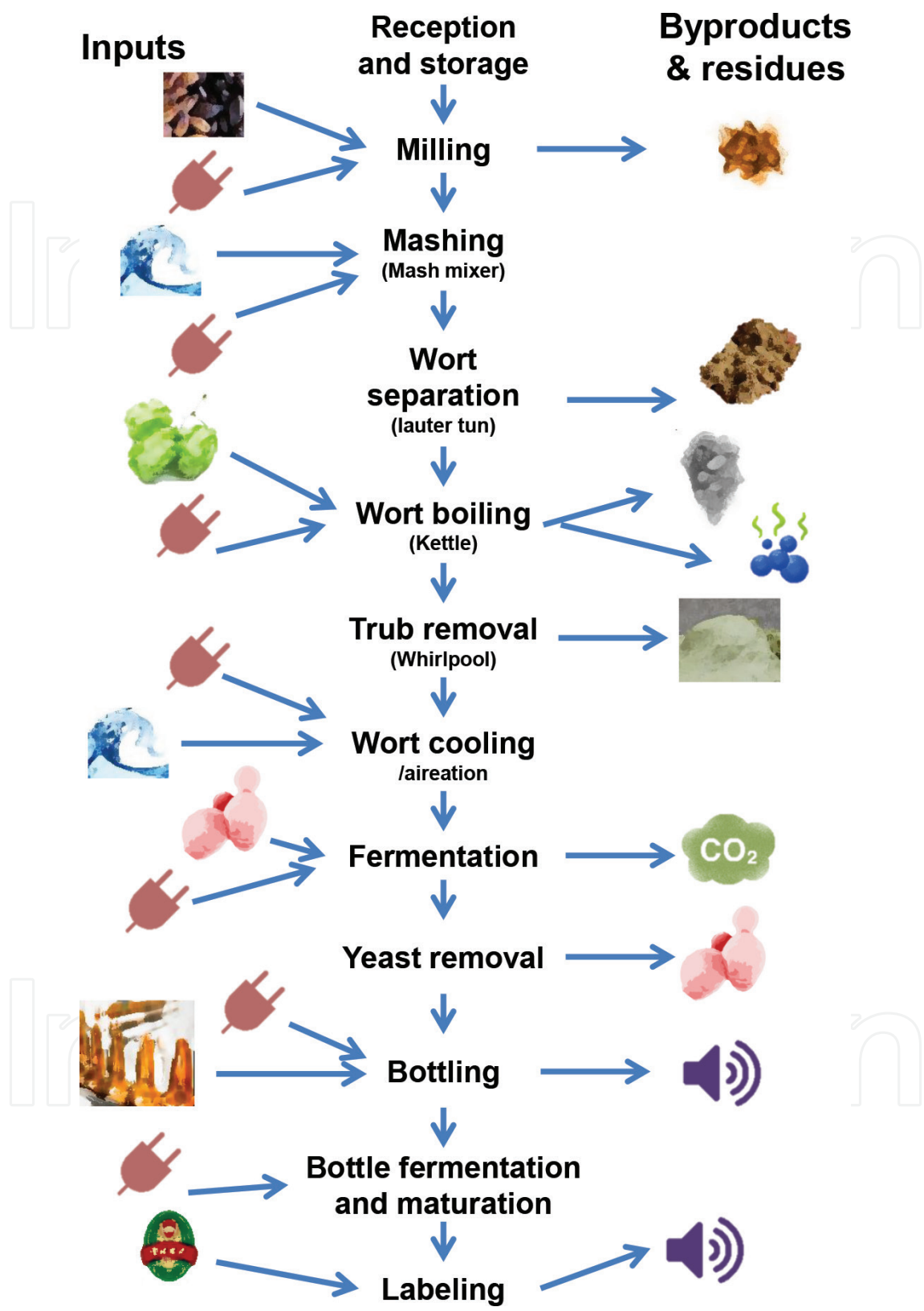
**Figure 5.** Inputs, byproducts, effluents, and residues in brewing process.

## 6. Conclusions and future trends

Non-*Saccharomyces* yeasts are a growing trend in new brewing biotechnology because of the improvements they can produce in sensory quality and differentiation, especially in craft beers. But also allows improved products as low-alcohol beers or implement new brewing processes. Probably in the future we will see new yeast species opening new possibilities of sensory and technological improvement. Bottle-conditioned beers are a good tool to obtain non-*Saccharomyces* bioflavored beers. Inoculation of new yeast species on a base beer completely attenuated with the addition of priming sugars allows creating optimal conditions to these yeasts.

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