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The raise of *Brettanomyces* yeast species for beer production

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The adequate application of *Brettanomyces* species could raise a potential opportunity for the beer industry, generating new products and optimizing production processes. Several valuable properties like high ethanol yield, tolerance to low pH and production of unique flavors have brought this yeast species into the spotlight. Aroma and flavor production of *Brettanomyces* in beer is currently under discussion, and it can be adjusted if the mechanism insights are understood. This review summarizes the recent findings in physiological, genetic and biochemical traits related to the application of *Brettanomyces* species for brewing.

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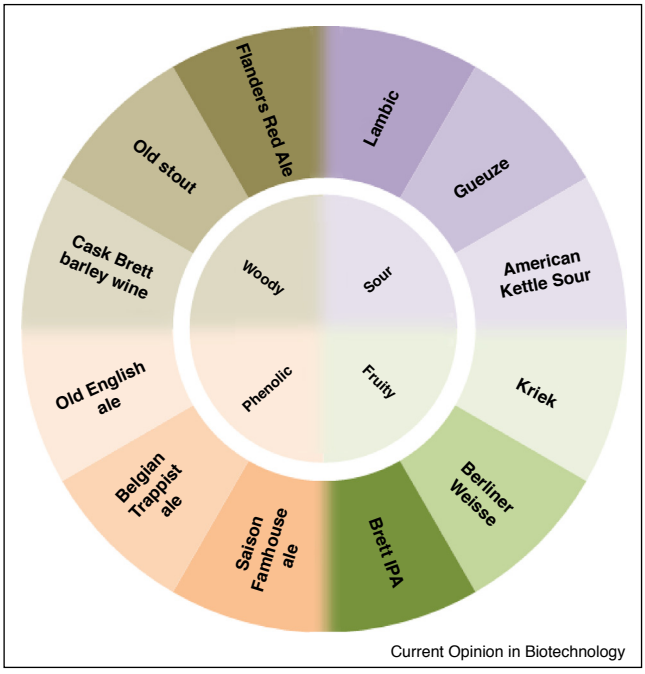
Introduction

In 1903, the first *Brettanomyces* yeast species was isolated by Hjelte Clausen at the Carlsberg Research Laboratory, and later referred to as *Brettanomyces clausenii*. *Brettanomyces* are non-conventional yeasts and can be isolated from different sources such as fruit peels, kombucha, kefir, tea, olives, sodas and wooden barrels, among others [1]. In breweries and especially wineries, *Brettanomyces* are typically recognized as a spoilage yeasts, being the cause of major economic losses. Its presence can completely change the organoleptic properties of the product, creating a controversial character, which is mainly due to the production of secondary metabolites when performing alcoholic fermentation. These metabollites have been associated to undesirable flavors, depicted as horse sweat, barnyard, medicinal or leathery [2]. However, applied in the right way *Brettanomyces* can contribute to exotic flavors (e.g. pineapple, mango, pear, grape) and today they are used in craft and specialty beers, and also finds application in natural wines. *Brettanomyces* are especially abundant in Belgian lambic and gueuze beers after spontaneous fermentation, being crucial for its particular taste [3]. The recent raise of the craft beer industry, along with the latest scientific discoveries have broadened *Brettanomyces* applications for novel flavors in unexplored beer styles (Figure 1). This review will give a concise view on the recent advances and contributions in understanding *Brettanomyces* species, including genomics, fermentation characteristics and flavor development, always with focus on beer production. Despite several *Brettanomyces* species being known, and the teleomorph form being called Dekkera [4], along this review the term *Brettanomyces* will be used to refer to the most common species *Brettanomyces bruxellensis* and *anomalus*.

Genomics and evolution

Recent advances in the development of high-throughput screening technologies and reduction of genome sequencing cost have strongly contributed to a better understanding and characterization of brewing yeast [5]. One of the most relevant discoveries is the sequencing of the lager yeast *Saccharomyces pastorianus* (syn. *S. carlsbergensis*) uncovering a hybridization event between *S. cerevisiae* and the wild Patagonian yeast *S. eubayanus* [6]. Humans have taken an important role in the development of today's brewer's yeast diversity, selecting for the most desirable phenotypical traits depending on the field of application [7,8]. Recently, a whole-genome sequencing survey of 1011 *S. cerevisiae* isolates suggested a one-common ancestor originating from China with several open reading frames (ORFs) being part of horizontal gene transfer from non-conventional yeasts [9•]. Modern sequencing techniques such as MinION or Illumina sequencing are providing new high-quality genomes of *Brettanomyces* as well [10]. Since two full-genome sequences [11,12] and full-transcriptome analyses [13] were released in 2012 and 2013, respectively, several meaningful studies came out. *Brettanomyces* display a greater diversity among strains than *S. cerevisiae*, both in chromosome number and ploidy [14]. Latest studies have revealed a robust correlation between ploidy (and genomic properties in general) and source of isolation. As wine isolates were found to be diploid and triploid, with the last one being more abundant, a hybridization event that confers phenotypical advantage was suggested [15]. In addition, beer strains were found to be mainly triploid, missing several ORFs present only in wine isolates [16]. A recent study of 1488 *B. bruxellensis* isolates using microsatellite analysis has confirmed a previous hypothesis and

Figure 1



Brettanomyces beer styles. Shown are the most common beer styles that *Brettanomyces* species can contribute to with its flavor properties. *Brettanomyces* beer styles have been arranged based on their most predominant flavor out of the 4 most common organoleptic properties woody, sour, phenolic and fruity found in *Brettanomyces* beers. Nonetheless every beer style holds substantial complexity and can exhibit characters from other categories.

determined a robust picture of the genetic diversity and population structure, confirming the complex diploid–triploid structure of *Brettanomyces* strains and showing a strong correlation between substrate of isolation and geographical origin [17••]. Curiously, the triploid wine-isolates show higher tolerance to SO_2 (used as wine preservative). This observation confirmed once more the human influence on the population structure of yeast, in this specific case in *B. bruxellensis*.

Recent evolution experiments co-culturing yeast and bacteria for several generations revealed that exposure to bacteria can lead to genome rearrangements in several non-conventional yeasts. *B. bruxellensis* and *B. anomalus* were among the ones showing highest reconfiguration [18••]. As *Brettanomyces* are commonly living organisms in lambic beer fermentations together with lactic and acetic acid bacteria, further studies could explain the influence of such community in the genomic set-up of lambic beer isolates.

Hardly any techniques for strain improvement have been reported in *Brettanomyces*. Although spore formation has been observed, there is still no evidence of mating types nor breeding partners. Nevertheless, mutant strains can be achieved by exposure to ultraviolet (UV) light or ethyl methanesulfonate (EMS) [19]. Although several genetic transformation methods have been established, targeted mutagenesis has not been achieved as none of the standard genome editing techniques such as CRISPR-Cas9 or homologous recombination has succeeded so far [14]. There is a clear need to develop advanced genomic tools for *Brettanomyces* species mutagenesis, to gain a better metabolic and physiological understanding and to facilitate strain development and implementation.

Fermentation and processing

Brettanomyces species differ from conventional brewer's yeast in several properties (Table 1). Both species display a 'Crabtree positive' phenotype. However, when switching to anaerobic conditions, a lag-phase occurs due to a redox imbalance in *Brettanomyces*, referred to as 'Custers effect'. *Brettanomyces* produce little to no glycerol, and can form considerable levels of acetic acid under aerobic conditions, compromising their own survival and contributing actively to the acidity in open-air fermentations [20]. Recent studies demonstrated a broad substrate range [21••] (Table 1). Despite glucose being the preferred carbon source, most *Brettanomyces* strains have the capability to metabolize a wide range of mono-saccharides, di-saccharides and trisaccharides and dextrins [22]. The latter constitute the majority of the residual sugar content in beer. Consequently, *Brettanomyces* can be used for the production of superattenuated and lower calorie beers [23]. Such degree of attenuation can vary depending on the pitching rate and the strain [24]. Under aerobiosis and nutrient depletion, *Brettanomyces* can use ethanol and acetic acid as a sole carbon source, causing a strong redox imbalance in the cell [14,21••]. In contrast, *Brettanomyces* turn into efficient ethanol producers under anaerobiosis, showing resistance to ethanol levels of up to 15% (v/v) and tolerance to pH as low as pH 3 [25,26].

The range of amino acids *Brettanomyces* can use as nitrogen source is wide, glutamine being the most preferred one [27]. However,*Brettanomyces* have developed an alternative strategy to survive in nitrogen-poor environments. An attractive trait that *Brettanomyces* possess is the capacity to assimilate nitrate from the media, which confers an advantage to conventional brewer's yeast in certain industrial fermentations [28]. The presence of nitrate in the media supports *Brettanomyces*' adaptation to anaerobic environments bypassing the 'Custers effect' and therefore increasing fermentation efficiency [29]. As hops are a significant source of nitrate in wort, both in boiling and dry hopping processes [30], this could explain the fast performance of *Brettanomyces* in highly-dosed hopped wort and also in maturation after dry hopping. This property builds upon the presence of a nitrate assimilation gene-cluster consisting of three ORFs, nitrate reductase (*YNR1*), nitrite reductase (*YNI1*) and a nitrate transporter (*YNT1*) [31]. However, this cluster is not present in all

Table 1

Main brewing features of *Brettanomyces*, *Saccharomyces pastorianus* (Lager) and *Saccharomyces cerevisiae* (Ale). This table represents a species generalization. However, there can be strong variability among strains of the same species. External conditions can also influence the described parameters

		Brettanomyces	*S. pastorianus*	*S. cerevisiae*
Consumption	Glucose	✓	✓	✓
	Maltose	✓	✓	✓
	Maltotriose	✓	✓	✓
	Dextrins	✓	✗	✗
	Cellobiose	✓	✗	✗[1]
	Nitrate	✓	✗	✗
Production	Ethanol	✓	✓	✓
	Glycerol	✗[2]	✓	✓
	Acetic acid	✓	✗[3]	✗[3]
	Phenolic Off-Flavor	✓	✗	✓
Fermentation	Crabtree	✓	✓	✓
	Custers	✓	✗	✗
	Optimal Brewing Temperature (°C)	21-25	14-16	24-28
	Attenuation	High	Normal	Normal
	Flocculation	Low	Low-High	Low-High
Strain improvement techniques	Mating/Breeding	✗	✓	✓
	UV sensitivity	✓	✓	✓
	Homologous Recombination	✗	✓	✓
	CRISPR-Cas9	✗	✓	✓

[1]Some strains have been reported to be capable of cellobiose utilization.
[2]Reported in very small amounts in few studies only.
[3]Typically produced. However, in much smaller amounts compared to *Brettanomyces*.

Brettanomyces strains, and no correlation with isolation niche has been established [32].

Volatile phenols

The range of aroma-active compounds produced by *Brettanomyces* is broad and complex, and has been newly allocated and classified in a *Brettanomyces* aroma wheel [33]. Perhaps the most prominent differential of *Brettanomyces* strains is the production of volatile phenols (VP), most notably 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG). The detection threshold for such compounds is very low, with diverse flavor and aroma descriptors comprising horse sweat, leathery, spicy, medicinal and smoky, among others [34,35].

The production of volatile phenols by *Brettanomyces* is a two-step conversion of ferulic and p-coumaric acid present in wort, comprising a decarboxylation and a reduction step [36]. Decarboxylation is mediated by a phenylacrilic acid decarboxylase (Pad1) [37•] while a vinylphenol reductase (Vpr) is responsible for the reduction step using NADH as a cofactor [38]. This last reduction step is exclusive to *Brettanomyces* and may be used to maintain the glycolytic flux of the cell under oxygen limited conditions [15]. However, a recent study has discarded this hypothesis as cell growth was not influenced by different exposures to stress, including the absence of p-coumaric acid [21••]. Furthermore, the expression of both *PAD1* and *VPR* varies under different conditions of pH, ethanol and SO_2 [39]. Interestingly, a recent study in *S. cerevisiae* has proposed the involvement of alternative genes (*ALD5*, *ATF1*, *ATF2*) in the production of phenolic compounds, alongside a novel conversion pathway [40•].

Several approaches have been used to reduce the sensory impact of VP, such as the addition of chitosans to red wine or the use of activated carbon [41,42]. Nonetheless, the selection of the *Brettanomyces* strain is crucial to control the VP content in the final beer. The source of isolation could have an influence in the metabolism of precursors, as beer isolates are more likely to metabolize ferulic acid and wine isolates, p-coumaric acid [43]. Lately, new methods facilitating selection of strains with reduced VP have been released, such as an absorbance-based method or resistance to γ-valerolactone [44,45].

Esters

Among the volatile compounds produced by *Brettanomyces*, esters are the most desired as they contribute with pleasant fruity flavor to beer. Ester production has also

been associated to a yeast survival and expansion strategy for attraction of insects [46]. Its formation is highly variable among *Brettanomyces* strains. Typically, compared to brewer's yeast, acetate esters such as isoamyl acetate (banana) and 2-phenylacetate (honey) are not produced and are even degraded by *Brettanomyces*. However, ethyl esters, such as ethyl acetate, hexanoate and octanoate, are present in high concentrations, contributing to tropical fruit and pineapple-like flavors [25]. This difference between acetate and ethyl esters could be due to the absence of essential genes for the production of acetate esters (e.g. *ATF1, ATF2)*, while several putative esterases such as *IAH1* have been reported in *Brettanomyces* [15]. Furthermore, *Brettanomyces* are capable of esterifying middle and long chain fatty acids (C9, C10, C12, C14, C16) commonly described as rancid and cheesy flavors, into its particular esters and consequently switching the beer flavor profile towards sweet, grape, apple, wine-like flavors. Recent advances focus on strain selection for improvement of ester production, either by enhancing fatty acid production, applying colorimetric screening methods or testing antibiotic resistance [47–49]. Additionally, a significant presence of ethyl lactate and ethyl acetate can be found in lambic and gueuze beers. The esters are formed from the lactic and acetic acid that is produced by a mixture of bacteria and yeast including *Brettanomyces* present in lambic and gueuze beer fermentation. Hence, using mixed cultures can be desirable to enhance flavor development.

β-Glucosidase activity and hop aromas

A remarkable feature of *Brettanomyces* is their β-glucosidase activity, which has a major impact on flavoring of the beer. Such activity has also been reported in *Saccharomyces* species but only in few cases, and with lower enzymatic activity [50]. β-Glucosidases allow *Brettanomyces* to breakdown cellobiose, a sugar present in wood, explaining the long-last survival of such species in wooden casks. Additionally, this enzyme has the capacity to release flavor-active compounds, which are odourless and non-volatile while bound to a sugar molecule. In beer fermentation, such glucosides are mainly derived from hops, and can result in a significant increase of several volatile compounds in the beer, including terpenes such as linalool [51]. Moreover, yeasts can convert monoterpenes further into β-citronellol or α-terpeniol, enhancing the floral citrusy flavor of the final beer [52]. Aglycones can also originate from fruits, like in the case of the traditional cherry beer Kriek, where *Brettanomyces* enhance the production of aromatic compounds such as benzaldehyde, linalool or eugenol [53]. *Brettanomyces*' β-glucosidase has been characterized for its potential use as a natural bioflavoring agent [54•]. It has also been studied for the production of resveratrol, a compound with antioxidant and antiaging properties [55]. Two genes encoding β-glucosidases have been reported in *Brettanomyces bruxellensis*. Curiously, most strains originating from beer possess only one of them, while strains isolated from wine have both ORFs [32]. Further research needs to be done to show the impact of such extra glucosidases on the consumption of β-glucosides and contribution to the overall flavor development. Several approaches for identifying strains with high β-glucosidase activity have been released, testing growth on agar with a β-glucoside as a substrate (cellobiose, salicin or esculin glycerol) being the most simple and cheapest one [54•,56].

Conclusions

Exploring *Brettanomyces* and other non-conventional yeast is highly attractive for the development of novel fermented beverages including craft and specialty beers. *Brettanomyces* may be used as a yeast for primary, secondary and bottle fermentation, facilitating the beer production process by adapting to extreme conditions. Furthermore, *Brettanomyces* yield natural flavors out of scope for conventional Saccharomyces species, and pair positively with other beer ingredients such as hops. *Brettanomyces* can be established if their undesirable flavors are avoided and the fermentation patterns are optimized. The wide range of existing *Brettanomyces* allows the straight-forward establishment of novel-to-beer flavors, and many studies are focusing on the application of selected *Brettanomyces* strains for the creation of new beverages. Advances in high-throughput technologies and next generation sequencing are accelerating the understanding of *Brettanomyces* biology. Within the last decade, the metabolic pathways responsible for flavor production in *Brettanomyces* have been elucidated. Understanding the genetics, physiology and biochemistry associated to flavor production will be fundamental in designing *Brettanomyces* with distinct flavor profiles, as novel methods and molecular tools for screening and identifying suitable strains for brewing are constantly being developed. Such advances could potentially propel the use of *Brettanomyces* species in the beer industry even further, resulting in new exiting products with novel attractive flavour compositions.

Conflict of interest

Nothing declared.

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