

Molecular and biochemical aspects of *Brettanomyces* in brewing

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***Brettanomyces* is a semi-domesticated yeast that is a crucial component of lambic beers and is increasingly attracting the attention of the brewing industry. *Brettanomyces* display *Saccharomyces*-like features, such as a positive Crabtree effect, ethanol synthesis and tolerance to harsh environments. Additionally, *Brettanomyces* exhibit β -glucosidase and esterase activities, the production of phenolic compounds and tetrahydropyridines, together with the ability to ferment dextrans and breakdown cellobiose from wooden casks. Although the importance of *Brettanomyces* species is documented in the production of different beer styles, the molecular and biochemical features of these species required for brewing are poorly understood. Therefore, this work reviews the current knowledge of the molecular biology and biochemistry underlying the performance of *Brettanomyces* in the brewing industry. © 2019 The Institute of Brewing & Distilling**

Keywords: *Brettanomyces*; brewing; yeasts; fermentation; volatile compounds; stress tolerance

Introduction

Beer, one of the oldest biotechnological products, has significant nutritional, social, scientific and economic impact. Beer combines cereal malt, hops and/or different herbs, and water to create wort that is fermented by indigenous yeast/bacteria or, more typically, by pure cultures of *Saccharomyces* species. According to archaeological data, beer can be traced back to the first agricultural societies ~10,000 years ago, coinciding with cereal domestication (1). Currently, the consumption of the beer is generally increasing worldwide and the brewing industry is showing broad growth. Hence, scientific research in the brewing process and raw materials remains an important activity to support advances in knowledge and development.

Various yeast species were only discovered to be responsible for beer fermentation in the 1860's as a consequence of Louis Pasteur's work (2). Recognition of yeast and its domestication allowed better control of the fermentation process and an improvement in the quality of the final product, leading to the selection of a plethora of yeast strains used in brewing (3). These yeast strains include *Saccharomyces cerevisiae*, *Saccharomyces pastorianus* and semi-domesticated unconventional species (4,5). A non-conventional brewing yeast genus that is attracting attention owing its unusual features is *Brettanomyces* (6,7). Niels Hjelte Claussen first mentioned this genus in 1904 while searching the Carlsberg Brewery for an explanation for the peculiar characteristics of English stock ales (e.g. copious and lasting foam, acid and volatile substances) (8). *Brettanomyces* and its teleomorph form *Dekkera* are mainly associated with wine spoilage (9). *Brettanomyces* can also negatively affect beers as a contaminant during fermentation, conditioning and dispense of draught beer, producing compounds that are considered to be off-flavours (10,11). On the other hand, the positive contributions of *Brettanomyces* to flavour, aroma and attenuation are well recognised in Belgian beers such as lambic and gueuze (12,13). Additionally, this genus has an important role in the secondary conditioning of Trappist beer, English stock ales and American coolship ales (8,14,15).

Brettanomyces possess a high esterase activity, responsible for the biosynthesis of fruit-like esters (16). Additionally, *Brettanomyces* release flavour-active compounds in response to β -glucosidase activity, which degrades glycosides from hops or fruits to aglycones (e.g. linalool) (17). Moreover, *Brettanomyces* species produce volatile phenols such as 4-vinylguaiacol (clove flavour) and tetrahydropyridines (mousy/cracker biscuit-like flavour) (Figures 1 and 2) (18–23).

Although the importance of *Brettanomyces* species in wine, beer and bioethanol fermentation is acknowledged (9,24–26), the molecular and biochemical features of these species required for brewing are poorly understood. Thus, the aim of this work is to review the current knowledge of the molecular and biochemical pathways, as well as the biotechnological potential of these yeasts in the brewing industry, with a particular focus on aromatic compound biosynthesis.

Brettanomyces taxonomy

The name *Brettanomyces* is derived from the Greek meaning 'British fungus' (8). However, it was not the first name given to this genus, and it was included as a *Torula* species (27,28). Likewise, species belonging to the genus *Brettanomyces* have undergone many reclassifications over the years and its taxonomy remains poorly defined. Currently, the genus *Brettanomyces* includes six species recognised within the anamorphic (asexual) form and two species within the teleomorph (sexual) form. The anamorphic forms are *B. bruxellensis*, *B. anomalus*, *B. custersianus*,

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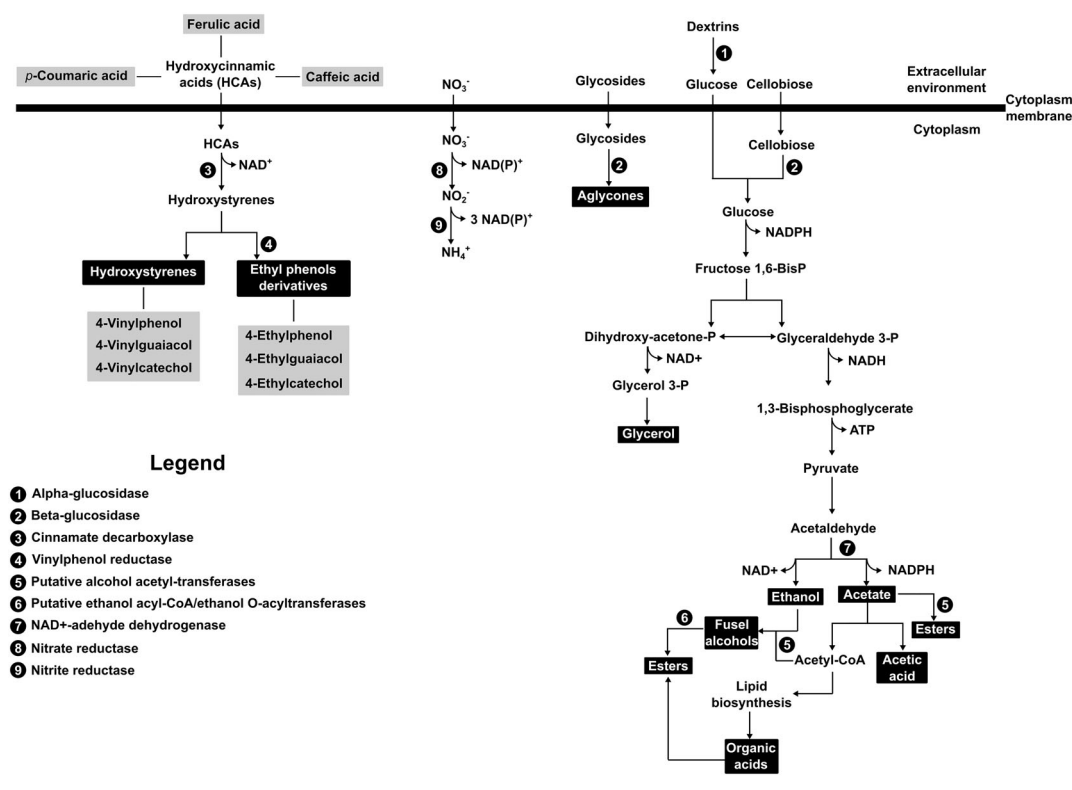


Figure 1. Schematic overview of the main metabolic pathways in *Brettanomyces* species during beer fermentation, focusing on the key enzymes linked to flavour active compound biosynthesis and the regulation of the redox balance (NAD⁺/NADH) associated with the Custers effect. The flavour active compounds are indicated in the figure by grey and black boxes. The main enzymes responsible for the generation of flavour active compounds are indicated by black circles and by the inset legend in the figure.

B. naardenensis, *B. nanus* and the newly proposed species *B. acidodurans*. In turn, teleomorphic forms include *Dekkera bruxellensis* and *Dekkera anomala* (29–32). *Brettanomyces* and *Dekkera* are often used as synonyms but are described here as *Brettanomyces*.

Several biochemical and molecular features have been used to reconstruct *Brettanomyces* phylogeny. The data include cellular morphology, physiological comparisons (i.e. metabolism of different carbon sources), single nucleotide polymorphisms in the coenzyme Q gene, G+C content and DNA similarities (e.g. rDNA 26S), isoenzymes and type of conidiogenesis (29,33–36). Currently, next generation genome sequencing provides an easier and faster method for comparing species through an analysis of orthologous genes, thus facilitating distinctions among species (37,38).

Current phylogeny places this genus within the clade of the methylotrophic species *Komagataella (Pichia) pastoris*, *Kuraishia capsulata* and *Ogataea polymorpha*, thus forming an ‘intermediate’ evolutionary group between the Saccharomycetaceae and CTG clade (defined by all yeast species that translate the codon CTG as serine instead of leucine) (37). However, a multigenic phylogeny analysis positioned *K. pastoris* outside of the clade that contains *Brettanomyces* (39).

The classification and species nomenclature of the *Brettanomyces* genus is confusing, as yeast manufacturers have applied other species names that are incorrect and belong to an older nomenclature. For example, *B. lambicus*, which is an important microorganism in the spontaneously fermented lambic beers and Kombucha (40). However, rather than *B. lambicus*, the yeast is a strain of the species *B. bruxellensis* (41). Other synonyms present in the literature for this species are *B. abstinens*, *B. custersii* and *B.*

intermedius (42–44). *Brettanomyces anomalus* only has one alternative name in *B. claussenii* (42). Furthermore, the teleomorph form, *D. bruxellensis* has one synonym, which in some studies is reported as *D. intermedia* (36). Since *B. bruxellensis* is the best known species within this genus, the majority of molecular/biochemical data reported here relate to this species.

Brettanomyces vs. *Saccharomyces*

Although the genus is phylogenetically separated from *S. cerevisiae* by 200 million years, *Brettanomyces* species share numerous phenotypes with *S. cerevisiae* that are of interest to the brewing industry, including biochemical (Crabtree effect and biosynthesis of flavour active compounds; Table 1, Figures 1 and 2) and molecular aspects (transcriptome plasticity to deal with stress inducing environments) (45). Both yeast species have converged to similar ecological niches (i.e. fruit peels, beer fermentation vessels and casks etc.) and use carbon sources through fermentation (45,46). While probably relying on different biochemical and molecular mechanisms, both species fall within the scope of interest for the beer industry, as they produce large amounts of ethanol by anaerobic fermentation (up to 14% ABV (w/v)), grow in anaerobic, acidic environments, tolerate high osmotic pressures and environments with low levels of nutrients (45,47).

Crabtree effect and ethanol yield

Like *S. cerevisiae*, *Brettanomyces* species display the Crabtree effect. Here, under aerobic conditions respiratory development is repressed (‘catabolite repression’) in the presence of a fermentable

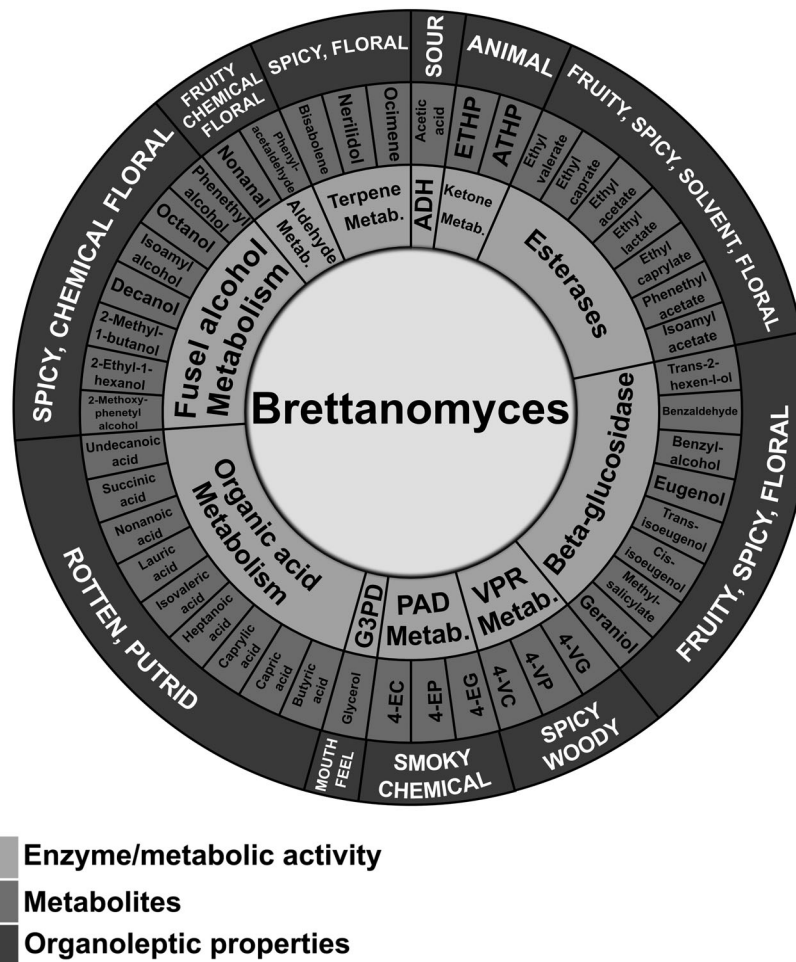


Figure 2. Aroma/flavour wheel containing the major metabolic pathways, enzymes and metabolites produced by different *Brettanomyces* species during beer fermentation. The aroma/flavours, enzymes/metabolic pathways and metabolites are indicated in the wheel by different grey shadows, defined in the legend below the wheel. Abbreviations: ADH, NAD⁺-aldehyde dehydrogenase; ETHP, 2-ethyltetrahydropyridine; ATHP, 2-acetyl tetrahydropyridine; VPR, vinylphenol reductase; 4-VG, 4-vinylguaiaicol; 4VP, 4-vinylphenol; 4-VC, 4-vinylcatechol; PAD, phenylacrylic acid decarboxylase; 4-EG, 4-ethylguaiaicol; 4-EP, 4-ethylphenol; 4-EC, 4-ethylcatechol; G3PD, glycerol 3-phosphate dehydrogenase; metab., metabolism (116).

Table 1. An overview of the major genetic, phenotype and metabolic characteristics of brewing strains of *Brettanomyces* species compared with *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*

Characteristic	<i>Brettanomyces</i> species	<i>S. cerevisiae</i> (ale yeast)	<i>S. pastorianus</i> (lager yeast)
Polyploidy (aneuploidy/euploidy) genome	Yes	Yes	Yes
Nitrate metabolism	Yes	No	No
Pseudohyphae formation (pellicle/biofilm)	Yes	Yes	No
Crabtree effect	Yes	Yes	Yes
Custer effect	Yes	No	No
α -Glucosidase activity	Yes	Yes	No
Sucrose consumption	Yes	Yes	Yes
Glucose metabolism	Yes	Yes	Yes
Fructose metabolism	Yes	Yes	Yes
Maltose metabolism	Yes	Yes	Yes
Maltotriose metabolism	Yes	Yes	Yes
Dextrin metabolism	Yes	Yes ^a	No
Cellobiose metabolism	Yes	No	No
Galactose metabolism	Yes	Yes	Yes

^aDiastatic *S. cerevisiae* brewing yeasts

carbon source at concentrations $>0.3\%$ (w/v; Table 1) (48). The Crabtree effect allows the yeast to rapidly assimilate glucose and generate ethanol, thereby inhibiting the growth of competing microorganisms. The Crabtree effect is part of the 'make–accumulate–consume' strategy used by microorganisms, where – under aerobic conditions – ethanol is consumed through respiration after glucose depletion (45,48). The Crabtree effect also provides more ATP than aerobic metabolism when high concentrations of glucose are available owing to the fast breakdown of glucose through glycolytic/fermentative pathways (48).

Genes linked to rapid growth (encoding enzymes involved in rRNA biosynthesis, the formation of pyrimidines, RNA helicases and proteins linked to RNA biogenesis and transport), respiration (encoding mitochondrial ribosomal proteins) and proteins necessary for the mitochondrial respiratory complex and ion transport to cytochrome oxidase) have a fixed promoter motif (AATTTT) in closely related species of *S. cerevisiae* and *B. bruxellensis* (i.e. *Kluyveromyces lactis*, *Ashbya gossypii*, *Candida albicans*, *Debaryomyces hansenii* and *K. waltii*). Nevertheless, *S. cerevisiae* and *Brettanomyces* underwent promoter restructuring, resulting in a loss of this motif in those genes associated with respiration. The AATTTT motif is absent in a permanent position in genes linked to respiration in *S. cerevisiae* and *B. bruxellensis* (~90% of genes). Thus, a significant decrease in respiration associated gene expression has been observed during cell growth in a medium containing fermentable carbon sources, as the fermentation associated genes are expressed at higher levels than genes associated with respiration (48,49).

The Crabtree effect is an important characteristic in emergent unconventional yeasts used in the brewing industry, as it confers the ability to produce ethanol in appreciable amounts (5–15% ABV). Ethanol yield can be $>14\%$ (v/v) in fermentations using *B. bruxellensis* (48). Therefore, although there will be an impact on flavour, *Brettanomyces* can be employed in the manufacture of high gravity beers that contain a high concentration of ethanol (49–51).

Custer effect

Anaerobiosis in *Brettanomyces* species inhibits the fermentation of glucose to ethanol (52). Glucose fermentation is stimulated in the presence of oxygen or organic (H^+) acceptors (e.g. acetone, acetoin and dihydroxyacetone; Figure 1) (52,53). The inability to ferment sugars in the absence of oxygen was termed the 'negative Pasteur effect' or the Custers effect (54,55). The biochemical and molecular mechanisms that drive the Custers effect are still not fully understood. However, the continuous production of acetate from acetaldehyde promotes the accumulation of NADH, causing a redox imbalance that inhibits glycolysis and fermentation. This imbalance prolongs a lag phase when cells switch from an aerobic to an anaerobic environment, which can be ameliorated by the addition of H^+ acceptors. In the presence of oxygen/ H^+ acceptors, NADH and NADPH are oxidised during aerobic metabolism, restoring the redox balance (56). Additionally, *Brettanomyces* cells express NADH ubiquinone reductase (part of mitochondrial complex I) at high levels when growing in semi-anaerobic medium (57). Thus, in semi-anaerobic environments, more NADH generating enzymes are expressed than NAD^+ generating enzymes, which explains why the NAD^+ /NADH imbalance occurs (58). Nevertheless, some pathways partially and slowly restore the NAD^+ /NADH balance. These mechanisms involve reoxidation of NADH, thereby providing NAD^+ for the metabolism of

glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate during glycolysis. One of these features is the ability of the yeast to utilise nitrate as a sole nitrogen source, since nitrate assimilation and metabolism require NADH and NADPH as electron donors (Figure 1). Interestingly, nitrate metabolism abolishes the Custers effect, therefore improving fermentation in anaerobic environments (59). Moreover, reactions involving NADH/NADPH reoxidation include the metabolism of hydroxycinnamic acids (*p*-coumaric and ferulic acids) present in beer (19).

Saccharomyces cerevisiae does not display the Custers effect, suggesting that the fermentation associated characteristics of *Brettanomyces* evolved in a different way. The *Brettanomyces* phenotype is strictly oxygen linked, and therefore high levels of dissolved oxygen in wort should be considered to encourage growth and metabolism, particularly when *Brettanomyces* is chosen for primary fermentation (50,60).

Acetic acid synthesis

Brettanomyces species may synthesise considerable quantities of acetic acid and potentially use this compound as a non-fermentative carbon source (Figure 1) (61–64). Acetic acid acidifies the medium, inhibiting the growth of potential microbial competitors. *Brettanomyces bruxellensis* can grow at pH 2.3, compared with *S. cerevisiae*, which is limited to pH 3.2 (45). High acetic acid yields in *Brettanomyces* are associated with fermentative metabolism. Acetaldehyde is produced from pyruvate and enzymatically oxidised to acetate in response to NAD^+ -aldehyde dehydrogenase activity (Figure 1). Since acetyl-CoA synthetase activity is strongly repressed in sugar rich environments in response to the Crabtree effect, excess quantities of acetic acid are generated once the acetaldehyde is channelled towards acetate biosynthesis in place of acetyl-CoA (Figure 1) (65). Acetate biosynthesis is induced in *B. anomala* IGC 5153 in the presence of 2% (w/v) glucose, while acetic acid is not synthesised in culture medium with low sugar concentrations (65). In contrast, acetogenic *B. abstinens* (currently *B. bruxellensis*) reportedly shows NAD^+ -aldehyde dehydrogenase activity even in the presence of low glucose concentrations, i.e. 0.3% (w/v) (52).

The presence of acetic acid is considered a positive characteristic in some types of beer, particularly in spontaneously fermented barrel aged beers such as lambic, gueuze, Flanders and Coolship ales. The amount of acetic acid produced is related to how the process is managed, particularly the choice of yeast strain and initial wort oxygenation. A high oxygen concentration stimulates the growth of *Brettanomyces* and the synthesis of acetic acid, and accordingly wort with high initial levels of oxygen will contain higher concentrations of acetic acid and form more acetate dependent esters (60,66).

Volatile esters and Brettanomyces

Esters are one of the main flavour compounds in top-fermented (ale) and bottom-fermented (lager) beers and are important in spontaneously fermented lambic beers (67–70). During beer production, several esters are produced in a yeast strain dependent manner, and their presence impacts beers either positively (fruity aroma) or negatively (solvent aroma, excessively fruity). The initial conditions of beer fermentation, such as temperature, wort composition and oxygenation, directly affect the overall concentration of esters (67–69,71).

Two groups of volatile esters are present in beer: the acetate esters and medium chain fatty acid (MCFA) ethyl esters. *Saccharomyces cerevisiae* has four enzymes that are responsible for acetate and MCFA ester formation. However, ester synthesising enzymes in *Brettanomyces* species have yet to be studied. In *S. cerevisiae*, acetate ester production depends on two enzymes: alcohol acetyl-transferase I and II (AATases I and II). MCFA ester production requires ethanol acyl-CoA/ethanol O-acyltransferase (AEATase) activity (Figures 1 and 2) (68,72–80).

While biochemical information about ester biosynthesis in *Brettanomyces* is unavailable, the data suggest that *B. bruxellensis* is capable of producing large amounts of acetate and MCFA esters (Figure 2). These esters include ethyl acetate, ethyl lactate, isoamyl acetate and phenethyl acetate (Figure 2), which are mainly found in lambic beers and American coolship ales (16,67,81). In addition, *Brettanomyces* accumulates fatty acids including octanoic (C8) to dodecanoic acid (C12) and converts them to their respective esters, suggesting elevated β -oxidation activity. The ester levels present in beer are influenced by the (possible) presence of acetic and lactic acid bacteria, whose fermentation by-products are substrates for ester synthesis (15,67). Although the formation of acetate esters was experimentally quantified utilising commercial beers supplemented with maltooligosaccharides for fermentation by eight strains of *Brettanomyces bruxellensis*, little is known about the *Brettanomyces* ester composition in pure culture fermentation (82).

***Brettanomyces* and the synthesis of aromatic phenolic compounds**

Volatile phenols comprise a group of aromatic molecules that are often found in fermented alcoholic beverages, including beer (83). Their presence arises from the metabolism of barley and hop derived hydroxycinnamic acids during fermentation by bacteria and yeast. Like esters, volatile phenols contribute to aroma (spicy, clove and smoky) and off-flavours (phenolic, medicinal, stable and barnyard; Figure 2). Aromatic phenols are an important part of the organoleptic properties of different beer styles, such as American coolship ale, Flanders red ale, lambic, fruit lambic and Oud Bruin (all containing *Brettanomyces*) (60,84).

Brettanomyces species have the ability to produce these strong aromatic compounds using cinnamate decarboxylase and vinylphenol reductase (VPR) (Figures 1 and 2) (19,85–87). The synthesis of volatile phenols occurs in two enzymatic sequential steps: (a) decarboxylation of *p*-coumaric and ferulic acids to their corresponding hydroxystyrenes (4-vinylphenol and 4-vinylguaiacol) by cinnamate decarboxylase; and (b) reduction of these molecules to 4-ethylphenol and 4-ethylguaiacol by vinylphenol reductase (Figure 1). In addition, 4-ethylcatechol is formed from caffeic acid in low amounts (Figure 1) (18,22). Notably, some *S. cerevisiae* strains also form hydroxystyrenes from hydroxycinnamic acids but are unable to further transform these compounds to the phenols. Hydroxystyrene synthesis arises from both phenyl acrylic acid decarboxylase (*PAD1*) and a putative ferulic acid decarboxylase (*FDC1*), which is a cinnamate decarboxylase (Figure 1) (88). The phenotype of *S. cerevisiae* strains that contain enzymes responsible for hydroxystyrene synthesis is POF⁺ (phenolic off-flavour).

Although the genes in *Brettanomyces* required for the phenolic biosynthesis have not been fully identified, two key enzymes, *DbPAD* and *DbPAD2*, with phenylacrylic acid decarboxylase activity are responsible for producing 4-vinylphenol from *p*-coumaric acid

(89,90). In order to better understand the biosynthesis of phenolic compounds in *Brettanomyces*, it is still necessary to identify all enzymes that transform hydroxystyrenes to their ethyl derivatives.

The production of ethylphenols strongly depends on the strain and environment (91). As shown by Kosel *et al.* (21) in a pure culture fermentation, hydroxycinnamic acids are quickly and completely converted to vinylphenols. However, a 30% decrease in the conversion to ethylphenols was obtained in mixed cultures with *Brettanomyces* and *S. cerevisiae*. Thus, the authors concluded that *Brettanomyces* have a metabolic preference for hydroxycinnamic acids instead of direct uptake of vinylphenols synthesised by *S. cerevisiae*. This hypothesis was corroborated by showing that VPR gene was expressed at lower levels in mixed fermentation cultures, where smaller amounts of 4-vinylphenol and 4-vinylguaiacol were available (21). In a recent study variations of 0.28–1.13 mg/L of 4-ethylphenol and 0.52–5.8 mg/L of 4-ethylguaiacol in lambic beers (92) were found.

***Brettanomyces*-associated α - and β -glucosidase activity and flavour-active aglycones**

Numerous plant sensorial molecules have been identified and many of those compounds are glycosylated (e.g. flavonols, anthocyanins, monoterpenes and norisoprenoidic compounds) and flavourless (93). On the other hand, the degradation of glycosylated molecules in aglycones is directly linked to fruity and/or floral aromas and flavours in beer (Figures 1 and 2) (93). Some *Saccharomyces* strains metabolise glycosides to aglycones using *exo*- β -glucanase (e.g. Exg1p). However, the metabolism of glycosides apparently occurs at a higher rate in *Brettanomyces* species. Daenen *et al.* identified a cell associated β -glucosidase with a broader activity in a lambic isolated *Brettanomyces custersii* strain LD72 (17). The β -glucosidase enzyme of *B. custersii* LD72 releases different aglycones, such as *trans*-2-hexen-1-ol, benzaldehyde, benzyl alcohol, eugenol, *trans*- and *cis*-isoeugenol, methyl salicylate and geraniol from the conversion of glycosides present in sour cherries (94). This study also provided preliminary evidence that amygdalin hydrolysis, resulting in the production of benzaldehyde, benzyl alcohol and benzyl acetate, occurs in response to the activity of glycoside hydrolase in some *Brettanomyces* species (Figure 2) (94).

With regard to new characteristics in beer, the biological transformation of glycosides from hops and fruits to aroma active aglycones could be offered by the use of *Brettanomyces* strains (60). Interestingly, extracellular β -glucosidase activity in *B. bruxellensis* is also associated with resveratrol production, a potential antioxidant, antimicrobial and anti-ageing compound (95). Additionally, the presence of β -glucosidase allows *Brettanomyces* species to use cellobiose – from the wood in oak barrels – as a carbon source. The last phase of lambic fermentation (13–24 months after the start of fermentation) is mainly dominated by *B. bruxellensis*, supported by the cellobiose released by wooden casks (14,96). The capacity to utilise cellobiose induces *Brettanomyces* species to form biofilms in the cask, allowing the breweries to use this *Brettanomyces* biofilm to contribute 'Brett' characteristics into the beer (50). Notably, the characteristic associated with the wort over-attenuating properties of *Brettanomyces* species is derived from the α -glucosidase activity, leading to the formulation of low calorie beers (97).

Genome organisation in *Brettanomyces* species

The genomes sequenced from different *Brettanomyces* species are currently few in number and this limits the assessment of the taxonomic diversity of the *Brettanomyces* genus. The major genome information that is available for researchers has been obtained from *B. bruxellensis* (strains AWRI1499, CBS2499, AWRI1608, AWRI1613, YV397, CBS2796, BioProject PRJEB11548 and PRJEB21262) (37,46,98). The genome sequences from *B. anomalus* (YV396) and *B. naardenensis* (CBS7540) (98) have also been reported. The lack of more genome sequences and especially a well defined sequence annotation for the *Brettanomyces* taxon has restricted other high throughput studies, including the transcriptome and proteome. Despite the lack of genome data, some initial studies have been performed by focusing on the genome structure and organisation.

B. bruxellensis has ~5400 genes with similar introns to *S. cerevisiae* and other hemiascomycetes (~4% of the genes) (37,99). Many of these genes encode enzymes and transporters related to nitrogen and lipid metabolism, allowing the yeast to survive in environments with low nutrients (37). Like *S. cerevisiae*, the *Brettanomyces* genus is able to form petite mutants resulting from mutations in the mtDNA that render them respiratory deficient (100). In terms of chromosome number, four to nine chromosomes have been identified in *B. bruxellensis* strains, with lengths from <1 to >6 Mbp (101). From the comparison of allele proportions at heterozygous sites for the five *B. bruxellensis* strains (AWRI1499, CBS2499, AWRI1608, AWRI1613 and YV397), a triploid genome has been suggested for AWRI1499 and CBS2499 and a diploid genome for AWRI1613 and YV397 (102). *B. bruxellensis* strains with a triploid genome harbour two copies of a common chromosome and an unusual set of other chromosomes (Table 1). The presence of the third chromosome copy is probably linked to sulphite resistance in wineries (102). Similarly, its occurrence provides selective advantages in nutrient-scarce and stressful environments, such as beer, where limited amounts of carbohydrates and amino acids are present, thus exerting a strong positive selection for the maintenance of polyploidy (103). Additionally, *Brettanomyces* polyploidy points to distinct hybridisation events that occurred at different geographical sites. Furthermore, the plasticity in the chromosomal structure with regard to unusual centromeres reinforces the occurrence of hybridisation (103). Avramova *et al.* reported three genetic clusters for *B. bruxellensis* strains through an analysis of 1488 isolates using micro-satellite genotyping: AWRI1499-like, AWRI1608-like and CBS 2499-like groups (103). Interestingly, *Brettanomyces* wine and beer strains have different chromosome structures that are probably linked to phenotypic differences related to adaptive advantages in wine and beer fermentation environments (103). Also, *B. bruxellensis* can be considered a diploid–triploid complex taxon with coexistence of sub-populations containing different numbers of ploidy (103).

Genes and transcription factors modulated under stress conditions in *Brettanomyces*

Brettanomyces species have been reported to tolerate more stress than *S. cerevisiae*. Indeed, *Brettanomyces* exhibits growth after primary fermentation by *S. cerevisiae* in both beer and wine, which contain high levels of ethanol and little or no dissolved oxygen

(104–108). The capacity of *Brettanomyces* species to survive such environments is linked to the cell wall structure/composition, and the presence of proteins involved in adhesion, cell wall budding and pseudohyphal growth (37,102). Moreover, *Brettanomyces* can use nitrogen sources more effectively than *S. cerevisiae* (109,110). Nitrate metabolism could be important in supporting *Brettanomyces* in beer environments as hops can provide substantial quantities of nitrate (up to 87 mg/mL) to the wort (102,111). However, not all *Brettanomyces* strains can use nitrate as their sole nitrogen source (112). The ability to use nitrate is due to the expression of genes that encode nitrate transporter (*YNT1*), nitrate reductase (*YNR1*) and nitrite reductase (*YNR1*), along with two transcription factors important for nitrate use (*YNA1* and *YNA2*).

Several genes encoding membrane associated proteins involved in alternative carbon metabolism are present in the genus, allowing *Brettanomyces* to use chitin, *N*-acetylglucosamine, galactose, mannose and lactose (37,112). Moreover, important genes involved in stress tolerance, such as *ATP1*, *ERG6* and *VPS34*, along with the stress regulators *MSN4*, *SNF1*, *HSP82* and *NTH1*, have been characterised in *B. bruxellensis* (47,113). The ability of *Brettanomyces* species to utilise trace amounts of nutrients provides some explanation for why this genus is able to survive in situations where *Saccharomyces* species are unable to survive (108). Importantly, *Brettanomyces* species have the capacity to tolerate sulphur derived compounds, particularly sulphur dioxide (101,114).

Conclusions

Brettanomyces is a genus that is attracting increased attention in the brewing world. The biochemical and molecular resources described here suggest that the potential of *Brettanomyces* species and strains exceeds our current knowledge. Consumer interest in sour, strong and highly hopped beers is increasing and *Brettanomyces* strains have the potential to contribute to production of these beer styles. The capacity of these species to tolerate environments with low nutrients, low pH and elevated stress-associated factors, such as high osmotic pressure, ethanol concentration and low levels of the nutrients, suggests their broad applicability in the brewing industry. Additionally, *Brettanomyces* species produce a diversity of phenolic and acid compounds. Furthermore, the ability of *Brettanomyces* to produce volatile compounds, such as esters and aglycones, could be explored to create a broad variety of biotransformation by-products from herbs and hop beers.

Finally, increasing interest in the biotechnological applications of yeast intra- and inter-specific hybridisation has been noted. Guided hybridisation has been performed under laboratory conditions to elucidate the evolutionary origins of yeast species and design tailor-made yeast strains for various biotechnology applications (115). *Brettanomyces*, which probably resulted from hybridisation owing to the occurrence of the triploid genome and chromosome abnormalities, might serve as a chassis to design new hybrids with biochemical and molecular resources that differ from other known yeast species.

Acknowledgements

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant number 302969/2016–0). The authors have no conflicts of interest to declare.

References

- Fagan, B.M. (1996) *The Oxford Companion to Archaeology* 2nd edition. Oxford: Oxford University Press. <https://doi.org/10.1093/acref/9780195076189.001.0001>
- Barnett, J.A. (1998) A history of research on yeasts 1: Work by chemists and biologists 1789–1850, *Yeast* 14, 1439–1451. [https://doi.org/10.1002/\(SICI\)1097-0061\(199812\)14:16<1439::AID-YEA339>3.CO;2-Z](https://doi.org/10.1002/(SICI)1097-0061(199812)14:16<1439::AID-YEA339>3.CO;2-Z)
- Boulton, C., and Quain, D. (2001). *Brewing Yeast & Fermentation*. Oxford: Blackwell. pp. 6–11. <https://doi.org/10.1002/9780470999417>
- Priest, F. G., and Campbell, I. (2003). *Brewing Microbiology*. New York: Springer Science + Business Media. <https://doi.org/10.1016/C2014-0-03102-4>
- Bokulich, N.A., and Bamforth, C. W. (2013) The microbiology of malting and brewing, *Microbiol. Mol. Biol. Rev.* 77, 157–172. <https://doi.org/10.1128/MMBR.00060-12>
- Michel, M., Meier-Dörnberg, T., Jacob, F., Methner, F.-J., Wagner R.S., and Hutzler, M. (2016) Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications, *J. Inst. Brew.* 122, 569–587. <https://doi.org/10.1002/jib.381>
- Gibson, B., Geertman, J.-M., Hittinger, C. T., Krogerus, K., Libkind, D. Louis, E. J., Magalhães, F., and Sampaio, J. P. (2017) New yeasts-new brews: Modern approaches to brewing yeast design and development, *FEMS Yeast Res.* 17, 1–13. <https://doi.org/10.1093/femsyr/fox038>
- Claussen N. H. (1904) On a method for the application of Hansen's pure yeast system in the manufacturing of well-conditioned English stock beers, *J. Inst. Brew.* 10, 308–331. <https://doi.org/10.1002/j.2050-0416.1904.tb04656.x>
- Loureiro, V., and Malfeito-Ferreira, M. (2003) Spoilage yeasts in the wine industry, *Int. J. Food Microbiol.* 86, 23–50. [https://doi.org/10.1016/S0168-1605\(03\)00246-0](https://doi.org/10.1016/S0168-1605(03)00246-0)
- Shimotsu, S., Asano, S., Lijima, K., Suzuki, K., Yamagishi, H., and Aizawa, M. (2015) Investigation of beer-spoilage ability of *Dekkera/Brettanomyces* yeasts and development of multiplex PCR method for beer-spoilage yeasts, *J. Inst. Brew.* 121, 177–180. <https://doi.org/10.1002/jib.209>
- Wiles, A. E. (1950). Studies of some yeasts causing spoilage of draught beer, *J. Inst. Brew.* 56, 183–193. <https://doi.org/10.1002/j.2050-0416.1950.tb01531.x>
- Van Oevelen, D., L'Escaille, F., and Verachtert, H. (1976) Synthesis of aroma components during the spontaneous fermentation of lambic and gueuze, *J. Inst. Brew.* 82, 322–326. <https://doi.org/10.1002/j.2050-0416.1975.tb06953.x>
- Roos, J., and Vuyst, L. (2018) Microbial acidification, alcoholization, and aroma production during spontaneous lambic beer production, *J. Sci. Food Agric.* <https://doi.org/10.1002/jsfa.9291>
- Vanderhaegen, B., Neven, H., Coghe, S., Verstrepen, K. J., Derdelinckx, G., and Verachtert, H. (2003) Bioflavoring and beer refermentation, *Appl. Microbiol. Biotechnol.* 62, 140–150. <https://doi.org/10.1007/s00253-003-1340-5>
- Bokulich, N. A., Bamforth, C. W., and Mills, D. A. (2012) Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale, *PLoS One* 7, e35507. <https://doi.org/10.1371/journal.pone.0035507>
- Spaepen, M., and Verachtert, H. (1982) Esterase activity in the genus *Brettanomyces*, *J. Inst. Brew.* 88, 11–17. <https://doi.org/10.1002/j.2050-0416.1982.tb04061.x>
- Daenen, L., Saison, D., Sterckx, F. R., Verachtert, H., and Derdelinckx, G. (2008, a) Screening and evaluation of the glucoside hydrolase activity in *Saccharomyces* and *Brettanomyces* brewing yeasts, *J. Appl. Microbiol.* 104, 478–488. <https://doi.org/10.1111/j.1365-2672.2007.03566.x>
- Cabrita, M.J., Palma, V., Patão, R., and Freitas, A. M. C. (2012) Conversion of hydroxycinnamic acids into volatile phenols in a synthetic medium and in red wine by *Dekkera bruxellensis*, *Cienc. Tecnol. Aliment.* 32(1), 106–111. <https://doi.org/10.1590/S0101-20612012005000024>
- Chatonnet, P., Dubourdieu, D., Boidron, J., and Pons, M. (1992) The origin of ethylphenols in wines, *J. Sci. Food Agric.* 60, 165–178. <https://doi.org/10.1002/jsfa.2740600205>
- Chatonnet, P., Dubourdieu, D., and Boidron, J. N. (1995) The influence of *Brettanomyces/Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines, *Am. J. Enol. Vitic.* 46, 463–468.
- Kosel, J., Čadež, N., and Raspor, P. (2014) Factors affecting volatile phenol production during fermentations with pure and mixed cultures of *Dekkera bruxellensis* and *Saccharomyces cerevisiae*, *Food Technol. Biotechnol.* 52, 35–45.
- Edlin, D. A. N., Narbad, A., Gasson, M. J., Llody, J. R. (1998) Purification and characterization of hydroxycinnamate decarboxylase from *Brettanomyces anomalus*, *Enzyme Microb. Technol.* 22, 232–239. [https://doi.org/10.1016/S0141-0229\(97\)00169-5](https://doi.org/10.1016/S0141-0229(97)00169-5)
- Snowdon, E. M. Bowyer, M. C., Grbin, P. R., and Bowyer, P. K. (2006) Mousy off-flavor: A review, *J. Agric. Food Chem.* 54, 6465–6474. <https://doi.org/10.1021/jf0528613>
- Blomqvist, J., and Passoth, V. (2015) *Dekkera bruxellensis* – Spoilage yeast with biotechnological potential, and a model for yeast evolution, physiology and competitiveness, *FEMS Yeast Res.* 15, fov021. <https://doi.org/10.1093/femsyr/fov021>
- Colomer, M. S., Funch, B., and Forster, J. (2018) The raise of *Brettanomyces* yeast species for beer production, *Curr. Opin. Biotechnol.* 56, 30–35. <https://doi.org/10.1016/j.copbio.2018.07.009>
- Joseph, L. C. M., Albino, E., and Bisson, L. F. (2017) Creation and use of a *Brettanomyces* Aroma Wheel, *Catalyst.* 1, 12–20. <https://doi.org/10.5344/catalyst.2016.16003>
- Gilliland, R. (1961) *Brettanomyces*. I. Occurrence, characteristics, and effects on beer flavor, *J. Inst. Brew.* 67, 257–261. <https://doi.org/10.1002/j.2050-0416.1961.tb01791.x>
- Custers, M.T.J., (1940). Onderzoekingen over het gistgeslacht *Brettanomyces*. Delft University, Delft.
- Boekhout, T., Kurtzman, C. P., O'Donnell, K., and Smith M. T. (1994) Phylogeny of the yeast genera *Hanseniaspora* (anamorph *Kloeckera*), *Dekkera* (anamorph *Brettanomyces*), and *Eeniella* as inferred from partial 26S ribosomal DNA nucleotide sequences, *Int. J. Syst. Bacteriol.* 44, 781–786. <https://doi.org/10.1099/00207713-44-4-781>
- Cocolin, L., Rantsiou, K., Iacumin, L., Zironi, R., and Comi, G. (2004) Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkera anomalus* in spoiled wines, *Appl. Environ. Microbiol.* 70, 1347–1355. <https://doi.org/10.1128/AEM.70.3.1347-1355.2004>
- Oelofse, A., Pretorius, I. S., du Toit, M. (2008) Significance of *Brettanomyces* and *Dekkera* during winemaking: A synoptic review, *South African J. Enol. Vitic.* 29, 128–144.
- Péter, G., Dlauchy, D., Tóbiás A. Fülöp L., Podgoršek, M., and Čadež, N. (2017) *Brettanomyces acidodurans* sp. nov., a new acetic acid producing yeast species from olive oil, *Antonie Van Leeuwenhoek* 110, 657–664. <https://doi.org/10.1007/s10482-017-0832-8>
- Meyer, S. A., Smith, M. T., and Simione, F. P. (1978) Systematics of *Hanseniaspora* Zikes and *Kloeckera* Janke, *Antonie Van Leeuwenhoek* 44, 79–96. <https://doi.org/10.1007/bf00400078>
- Van der Walt, J. P. (1984). *Dekkera* van der Walt, in *The Yeasts, a Taxonomic Study* (N. J. W. Kreger-van Rij Ed.), 3rd ed., pp. 146–150, Elsevier Science, Amsterdam.
- Yamada, Y., Takinami-Nakamura, H., Tahara, Y., and Smith, M. T. (1980). The coenzyme Q system in the classification of the ascosporogenous yeast genus *Dekkera* and the asporogenous yeast genus *Brettanomyces*, *Antonie Van Leeuwenhoek* 46, 595–599. <https://doi.org/10.1007/bf00394015>
- Smith M. T. H., Yamazaki M., and Poot G. A. (1990) *Dekkera*, *Brettanomyces* and *Eeniella*: Electrophoretic comparison of enzymes and DNA–DNA homology, *Yeast* 6, 299–310. <https://doi.org/10.1002/yea.320060403>
- Curtin, C. D., Borneman A.R., Chambers, P.J., and Pretorius, I. S. (2012) *De-novo* assembly and analysis of the heterozygous triploid genome of the wine spoilage yeast *Dekkera bruxellensis* AWRI1499, *PLoS ONE* 7, e33840. <https://doi.org/10.1371/journal.pone.0033840>
- Curtin, C. D., Pretorius, I.S., (2014). Genomic insights into the evolution of industrial yeast species *Brettanomyces bruxellensis*, *FEMS Yeast Res.* 14, 997–1005. <https://doi.org/10.1111/1567-1364.12198>
- Kurtzman, C. P., Robnett, C. J. (2013) Relationships among genera of the Saccharomycotina (Ascomycota) from multigene phylogenetic analysis of type species, *FEMS Yeast Res.* 13, 23–33. <https://doi.org/10.1111/1567-1364.12006>
- Ashrafi, A., Joker, M., and Nafchi A. M. (2018) Preparation and characterization of biocomposite film based on chitosan and kombucha tea as active food packaging, *Int. J. Biol. Macromol.* 108, 444–454. <https://doi.org/10.1016/j.ijbiomac.2017.12.028>
- Molina F. I., Shen, P., and Jong, S.C. (1993) Validation of the species concept in the genus *Dekkera* by restriction analysis of genes coding

- for rRNA, *Int. J. Syst. Bacteriol.* 43, 32–35. <https://doi.org/10.1099/00207713-43-1-32>
42. Barnett, J. A., and Lichtenthaler, F. W., (2001). A history of research on yeasts 3: Emil Fischer, Eduard Buchner and their contemporaries, 1880–1900, *Yeast* 18, 363–388. [https://doi.org/10.1002/1097-0061\(20010315\)18:4%3C363::AID-YEA677%3E3.O.CO;2-R](https://doi.org/10.1002/1097-0061(20010315)18:4%3C363::AID-YEA677%3E3.O.CO;2-R)
 43. Put, H., De Jong, J., Sand, F., and Van Grinsven, A., (1976). Heat resistance studies on yeast spp. causing spoilage in soft drinks, *J. Appl. Bacteriol.* 40, 135–152. <https://doi.org/10.1111/j.1365-2672.1976.tb04162.x>
 44. Verachtert, H., (1992). Lambic and gueuze brewing: Mixed cultures in action, *COMETT Course on Microb. Cont.*, Helsinki, pp. 243–262.
 45. Rozpedowska, E., Hellborg, L., Ishchuk, O.P., Orhan, F., Galafassi, S., Merico, A., Woolfit, M., Compagno, C., Piskur, J. (2011). Parallel evolution of the make–accumulate–consume strategy in *Saccharomyces* and *Dekkera* yeasts, *Nat. Commun.* 2, 302. <https://doi.org/10.1038/ncomms1305>
 46. Piškur, J., Ling Z, Marcet-Houben M., Ishchuk, O. P., Aerts, A. LaButti, K, Copeland, A., Lindquist, E., Barry, K., Compagno, C., Bisson, L., Grigorev, I. V., Gabald n, T., and Phister, T. (2012) The genome of wine yeast *Dekkera bruxellensis* provides a tool to explore its food-related properties, *Int. J. Food Microbiol.* 157, 202–209. <https://doi.org/10.1016/j.ijfoodmicro.2012.05.008>
 47. Nardi, T., Remize, F., and Alexandre, H. (2010) Adaptation of yeasts *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* to winemaking conditions: A comparative study of stress genes expression, *Appl. Microbiol. Biotechnol.* 88, 925–937. <https://doi.org/10.1007/s00253-010-2786-x>
 48. De Deken, R. H. (1966) The Crabtree effect: A regulatory system in yeast, *J. Gen. Microbiol.* 44, 149–156. <https://doi.org/10.1099/0021287-44-2-149>
 49. Galafassi, S., Merico, A., Pizza, F., Helborg, L., Molinari, F., Piškur, J., and Compagno, C. (2010) *Dekkera/Brettanomyces* yeasts for ethanol production from renewable sources under oxygen-limited and low-pH conditions, *J. Ind. Microbiol. Biotechnol.* 38, 1079–1088. <https://doi.org/10.1007/s10295-010-0885-4>
 50. White, C. and Zainasheff, J. (2010) *Brettanomyces*, in *Yeast: The Practical Guide to Beer Fermentation*. 1st ed., pp. 61–64. Brewers Association, Boulder, CO, USA.
 51. Stewart, G. G. (2017) Stress effects on yeast during brewing and distilling fermentations: High gravity effects, in *Brewing and Distilling Yeasts. The Yeast Handbook*. (Stewart, G.G. Ed.) 1st ed., pp.199–240, Springer, Cham. <https://doi.org/10.1007/978-3-319-69126-8>
 52. Carrascosai, J. M., Viguera, M. D., de Castro, N. I., and Scheffers, W. A. (1981). Metabolism of acetaldehyde and Custers effect in the yeast *Brettanomyces abstiniens*, *Antonie Van Leeuwenhoek* 47, 209–215. <https://doi.org/10.1007/bf00403392>
 53. Wijsman, M. R., van Dijken, J. O., van Kleeff, B. H. A., and Scheffers, W. A. (1984) Inhibition of fermentation and growth in batch cultures of the yeast *Brettanomyces intermedius* upon a shift from aerobic to anaerobic condition (Custers effect), *Antonie Van Leeuwenhoek* 50, 183–190. <https://doi.org/10.1007/BF00400180>
 54. Wik n, B. J. A., and Entian, K. D. (2005). A history of research on yeasts – 9: Regulation of sugar metabolism, *Yeast* 22, 835–894. <https://doi.org/10.1002/yea.1249>
 55. Wik n, T., Scheffers, W., and Verhaar, A. (1961). On the existence of a negative Pasteur effect in yeasts classified in the genus *Brettanomyces* Kufferath et van Laer, *Antonie Van Leeuwenhoek* 27, 401–433. <https://doi.org/10.1007/bf02538468>
 56. Gaunt, D. M., Degn, H., and Lloyd D. (1988) The influence of oxygen and organic hydrogen acceptors on glycolytic carbon dioxide production in *Brettanomyces anomalus*, *Yeast* 4, 249–255. <https://doi.org/10.1002/yea.320040403>
 57. Tiukova, I. A., Petterson, M.E., Tellgren-Roth, C., Bunikis, I., Eberhard, T., Petterson, O. V., and Passoth, V. (2013) Transcriptome of the alternative ethanol production strain *Dekkera bruxellensis* CBS 11270 in sugar limited, low oxygen Cultivation, *PLoS ONE* 8, e58455. <https://doi.org/10.1371/journal.pone.0058455>
 58. Steensels, J., Daenen, L., Malcorps, P., Derdelinckx, G., Verachtert, H., Verstrepen, K. J. (2015) *Brettanomyces* yeasts – From spoilage organisms to valuable contributors to industrial fermentations, *Int. J. Food Microbiol.* 206, 24–38. <https://doi.org/10.1016/j.ijfoodmicro.2015.04.005>
 59. Galafassi, S., Capusoni, C., Muktaduzzaman, M., and Compagno, C. (2013) Utilization of nitrate abolishes the ‘Custers effect’ in *Dekkera bruxellensis* and determines a different pattern of fermentation products, *J. Ind. Microbiol. Biotechnol.* 40, 297–303. <https://doi.org/10.1007/s10295-012-1229-3>
 60. Tonsmeire, M. (2014) 100% *Brettanomyces* fermentations, in *American Sour Beer: Innovative Techniques for Mixed Fermentations*. 1st ed., pp. 181–195. Brewers Association, Boulder, CO.
 61. Gamero A., Ferreira V., Pretorius I.S., Querol A. (2014) Wine, beer and cider: unravelling the aroma profile, in: *Molecular Mechanisms in Yeast Carbon Metabolism* (Piškur J., and Compagno C. Eds.). 1st ed., pp. 261–297, Springer, Berlin. https://doi.org/10.1007/978-3-642-55013-3_10
 62. Freer, S. N. (2002). Acetic acid production by *Dekkera/Brettanomyces* yeasts, *World J. Microbiol. Biotechnol.* 18, 271–275. <https://doi.org/10.1023/A:1022592810405>
 63. Freer, S. N., Dien, B., and Matsuda, S. (2003) Production of acetic acid by *Dekkera/Brettanomyces* yeasts under conditions of constant pH, *World J. Microbiol. Biotechnol.* 19, 101–105. <https://doi.org/10.1023/A:1022592810405>
 64. Castro-Martinez, C., Escudero-Abarca, B.I., Gomez Rodriguez, J., Hayward-Jones, P.M., and Aguilar-Uscanga, M.G., (2005) Effect of physical factors on acetic acid production in *Brettanomyces* strains, *J. Food Process Eng.* 28, 133–143. <https://doi.org/10.1111/j.1745-4530.2005.00393.x>
 65. Ger s, H., Azevedo, M. M., and C ssio, F. (2000) Biochemical studies on the production of acetic acid by the yeast *Dekkera anomala*, *Food Technol. Biotechnol.* 38, 59–62.
 66. Sparrow, J. (2005) Wild fermentation, in *Wild Brews: Beer beyond the Influence of Brewer’s Yeast*. 1st ed., pp. Brewers Publications, Boulder, CO.
 67. Spaepen B. M., Oevelen, D. V., and Verachtert, H. (1978) Fatty acids and esters produced during the spontaneous fermentation of lambic and gueuze, *J. Inst. Brew.* 84, 278–282. <https://doi.org/10.1002/j.2050-0416.1978.tb03888.x0>
 68. Pires, E. J., Teixeira, J. A., Br nyik, T and Vicente A. A. (2014) Yeast: the soul of beer’s aroma – A review of flavour-active esters and higher alcohols produced by the brewing yeast, *Appl. Microbiol. Biotechnol.* 98, 1937–1949. <https://doi.org/10.1007/s00253-013-5470-0>
 69. Verstrepen, K. J., Derdelinckx, G., Dufour, J. P., Winderickx, J., Thevelein, J. M., Pretorius, I. S., and Delvaux, F. R. (2003) Flavor-active esters: Adding fruitiness to beer, *J. Biosci. Bioeng.* 96, 110–118. [https://doi.org/10.1016/S1389-1723\(03\)90112-5](https://doi.org/10.1016/S1389-1723(03)90112-5)
 70. Xu, Y., Wang, D., Hong Li, Hao, J., and Jiang, W. (2017) Flavor contribution of esters in lager beers and an analysis of their flavor thresholds, *J. Am. Soc. Brew Chem.* 75, 201–206. <https://doi.org/10.1094/ASBCJ-2017-3007-01>
 71. Hiralal, L., Olaniran, A. O., and Pillay, B. (2013) Aroma-active ester profile of ale beer produced under different fermentation and nutritional conditions, *J. Biosci. Bioeng.* 117, 57–64. <https://doi.org/10.1016/j.jbiosc.2013.06.002>
 72. Yoshioka, K., and Hashimoto, N. (1981) Ester formation by alcohol acetyltransferase from brewer’s yeast, *Agric. Biol. Chem.* 45, 2183–2190. <https://doi.org/10.1080/00021369.1981.10864861>
 73. Malcorps, P., and Dufour, J. P. (1992) Short-chain and medium-chain aliphatic- ester synthesis in *Saccharomyces cerevisiae*, *Eur. J. Biochem.* 210, 1015–1022. <https://doi.org/10.1111/j.1432-1033.1992.tb17507.x>
 74. Fujii, T., Nagasawa, N., Iwamatsu, A., Bogaki, T., Tamai, Y., and Hamachi, M. (1994) Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene, *Appl. Environ. Microbiol.* 60, 2786–2792.
 75. Nagasawa, N., Bogaki, T., Iwamatsu, A., Hamachi, M., and Kumagai C (1998) Cloning and nucleotide sequence of the alcohol acetyltransferase II gene (ATF2) from *Saccharomyces cerevisiae* Kyokai No. 7, *Biosci. Biotechnol. Biochem.* 62, 1852–1857. <https://doi.org/10.1271/bbb.62.1852>
 76. Yoshimoto, H., Fujiwara, D., Momma, T., Ito, C., Sone, H., Kaneko, Y., and Tamai, Y. (1998) Characterization of the ATF1 and Lg-ATF1 genes encoding alcohol acetyltransferases in the bottom fermenting yeast *Saccharomyces pastorianus*, *J. Ferment. Bioeng.* 86, 15–20. [https://doi.org/10.1016/S0922-338X\(98\)80027-5](https://doi.org/10.1016/S0922-338X(98)80027-5)
 77. Verstrepen, K. J., Van Laere, S. D., Vanderhaegen, B. M., Derdelinckx, G., Dufour, J.P., Pretorius, I. S., Winderickx, J., Thevelein, J. M., and Delvaux, F. R. (2003) Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters, *Appl. Environ. Microbiol.* 69, 5228–5237. <https://doi.org/10.1128/aem.69.9.5228-5237.2003>

78. Molina, A. M., Swiegers, J. H., Varela, C., Pretorius, I.S., and Agosin, E. (2007) Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds, *Appl. Microbiol. Biotechnol.* **77**, 675–687. <https://doi.org/10.1007/s00253-007-1194-3>
79. Dekoninck, T., Verbelen, P. J., Delvaux, F., Van Mulders, S. E., Delvaux, R. F. (2012) The importance of wort composition for yeast metabolism during accelerated brewery fermentations, *J. Am. Soc. Brew Chem.* **70**, 195–204. <https://doi.org/10.1016/j.cervis.2013.09.026>
80. Zhang, C-Y., Liu Y-L., Qi, Y-N, Zhang, J-W, Dai, L-H, Lin, X, Xiao, D-G (2013) Increased esters and decreased higher alcohols production by engineered brewer's yeast strains, *Eur. Food Res. Technol.* **236**, 1009–1014. <https://doi.org/10.1007/s00217-013-1966-1>
81. Yakobson, C. M., (2009). *Pure Culture Fermentation Characteristics of Brettanomyces Yeast Species and their Use in the Brewing Industry*. School of Life Sciences, Heriot-Watt University, Edinburgh.
82. Crauwels, S., Opstaele, F. V., Jaskula-Goiris, B., Steensels, J., Verreth, C., Bosmans, L., Paulussen, C., Herrera-Malaver, B., Jonge, R., Clippeleer, J., Marchal, K., Samblanx, G., Willems, K. A., Verstrepen, K. J., Aerts, G., and Lievens, B. (2017) Fermentation assays reveal differences in sugar and (off-) flavor metabolism across different *Brettanomyces bruxellensis* strains, *FEMS Yeast Res.*, **17**, 1–10. <https://doi.org/10.1093/femsyr/fow105>
83. Vanbeneden, N., Gils, F., Delvaux, F., and Delvaux, F. R. (2008) Formation of 4-vinyl and 4-ethyl derivatives from hydroxycinnamic acids: Occurrence of volatile phenolic flavour compounds in beer and distribution of Pad1-activity among brewing yeasts, *Food Chem.* **107**, 221–230. <https://doi.org/10.1016/j.foodchem.2007.08.008>
84. Holt, S., Mukherjee, V., Lievens, B., Verstrepen, K. J., and Thevelein, J. M. (2018) Bioflavoring by non-conventional yeasts in sequential beer fermentations, *Food Microbiol.* **72**, 55–66. <https://doi.org/10.1016/j.fm.2017.11.008>
85. Heresztyn T. (1986) Metabolism of volatile phenolic compounds from hydroxycinnamic acids by *Brettanomyces* yeast, *Arch. Microbiol.* **146**, 96–98. <https://doi.org/10.1007/BF00690165>
86. Tchobanov, I., Gal, L., Guilloux-Benatier, M, Remize, F., Nardi, T., Guzzo, J., Serpaggi, V., and Alexandre, H. (2008) Partial vinylphenol reductase purification and characterization from *Brettanomyces bruxellensis*, *FEMS Microbiol. Lett.* **284**, 213–217. <https://doi.org/10.1111/j.1574-6968.2008.01192.x>
87. Harris, V., Ford, C. M., Jiranek, V., and Grbin, P. R. (2009) Survey of enzyme activity responsible for phenolic off-flavour production by *Dekkera* and *Brettanomyces* yeast, *Appl. Microbiol. Biotechnol.* **81**, 1117–1127. <https://doi.org/10.1007/s00253-008-1708-7>
88. Mukai, N., Masaki, K., Fujii, T. Kawamukai, M., and Iefuji, H. (2010) PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*, *J. Biosci. Bioeng.* **109**, 564–569. <https://doi.org/10.1016/j.jbiosc.2009.11.011>
89. Godoy, L., García, V, Peña, R., Martínez, C., and Ganga, M. A. (2014) Identification of the *Dekkera bruxellensis* phenolic acid decarboxylase (PAD) gene responsible for wine spoilage, *Food Control* **45**, 81–86. <https://doi.org/10.1016/j.foodcont.2014.03.041>
90. González, C., Godoy, L., and Ganga, M. A. (2017) Identification of a second PAD1 in *Brettanomyces bruxellensis* LAMAP2480, *Antonie Van Leeuwenhoek* **110**, 291–296. <https://doi.org/10.1007/s10482-016-0793-3>
91. Lentz, M., and Harris, C. (2015) Analysis of growth inhibition and metabolism of hydroxycinnamic acids by brewing and spoilage strains of *Brettanomyces* yeast, *Foods*, **4**, 581–593. <https://doi.org/10.3390/foods4040581>
92. Witrick, K. T., Duncan, S. E., Hurley, K. E., O'Keefe, S. F. (2017) Acid and volatiles of commercially-available lambic beers, *Beverages*. **3**, 51 <https://doi.org/10.3390/beverages3040051>
93. Sarry, J-E., and Günata, Z. (2004) Plant and microbial glycoside hydrolases: Volatile release from glycosidic aroma precursors, *Food Chem.* **87**, 509–521. <https://doi.org/10.1016/j.foodchem.2004.01.003>
94. Daenen, L., Sterckx, F., Delvaux, F. R., Verachtert, H., and Derdelinckx, G. (2008) Evaluation of the glycoside hydrolase activity of a *Brettanomyces* strain on glycosides from sour cherry (*Prunus cerasus* L.) used in the production of special fruit beers, *FEMS Yeast Res.* **8**, 1103–1114. <https://doi.org/10.1111/j.1567-1364.2008.00421.x>
95. Kuo, H-P., Wang, R., Huang, C-Y., Lai, J-T., Lo, Y-C., and Huang, S-T. (2018) Characterization of an extracellular β -glucosidase from *Dekkera bruxellensis* for resveratrol production, *J. Food Drug Anal.* **26**, 163–171. <https://doi.org/10.1016/j.jfda.2016.12.016>
96. Verachtert, H., and Dawoud, E. (1984) Microbiology of lambic-type beers, *J. Appl. Bacteriol.* **57**, R11–R12.
97. Kumara, H. M. C. S., De Cort, S., and Verachtert, H. (1993) Localization and characterization of α -glucosidase activity in *Brettanomyces lambicus*, *Appl. Environ. Microbiol.* **59**, 2352–2358.
98. Vervoort, Y., Herrera-Malaver, B., Mertens, S. Guadalupe Medina, V., Duitama, J., Michiels, L., Derdelinckx, G., Voordeckers, K., and Verstrepen, K. J. (2016) Characterization of the recombinant *Brettanomyces anomalus* β -glucosidase and its potential for bioflavouring, *J. Appl. Microbiol.* **121**, 721–733. <https://doi.org/10.1111/jam.13200>
99. Woolfit, M., Rozpdowska, E., Piškur, J., and Wolfe, H. K. (2007) Genome survey sequencing of the wine spoilage yeast *Dekkera* (*Brettanomyces*) *bruxellensis*, *Eukaryot. Cell* **6**, 721–733. <https://doi.org/10.11128/EC.00338-06>
100. McArthur, C. R., and Clark-Walker, G. D. (1997) Mitochondrial DNA Size diversity in the *Dekkera/Brettanomyces* yeasts, *Curr. Genet.* **7**, 29–35. <https://doi.org/10.1007/BF00365677>
101. Hellborg, L., and Piškur, J. (2009) Complex nature of the genome in a wine spoilage yeast, *Dekkera bruxellensis*, *Eukaryot. Cell* **8**, 1739–1749. <https://doi.org/10.1128/EC.00115-09>
102. Borneman, A. R., Zeppel, R., Chambers, P. J., and Curtin, C. D. (2014) Insights into the *Dekkera bruxellensis* genomic landscape: comparative genomics reveals variations in ploidy and nutrient utilisation potential amongst wine isolates, *PLoS Genet.* **10**, e1004161. <https://doi.org/10.1371/journal.pgen.1004161>
103. Avramova, M., Cibario, A., Peltier, E. Coton, M., Coton, E., Schacherer, J., Spano, G., Capozzi, V., Blaiotta, G., Salin, F., Dols-Lafargue, M., Grbin, P., Curtin, C., Albertin, W., and Masneuf-Pomarede, I. (2018) *Brettanomyces bruxellensis* population survey reveals a diploid-triploid complex structured according to substrate of isolation and geographical distribution, *Nature* **8**, 4136. <https://doi.org/10.1038/s41598-018-22580-7>
104. Curtin, C. D., Bellon, J. R., Coulter, A., Cowey, G., Robinson, E., de Barros Lopes, M. A., Godden, P. W., Henschke, P. A., Pretorius, I. S. (2005) The six tribes of 'Brett' in Australia – Distribution of genetically divergent *Dekkera bruxellensis* strains across Australian winemaking regions, *Aus. Wine Ind. J.* **20**, 28–36.
105. Curtin, C.D., Bellon, J.R., Henschke, P.A., Godden, P.W., and De Barros Lopes, M.A. (2007) Genetic diversity of *Dekkera bruxellensis* yeasts isolated from Australian wineries, *FEMS Yeast Res.* **7**, 471–481. <https://doi.org/10.1111/j.1567-1364.2006.00183.x>
106. Curtin, C., Kennedy, E., and Henschke, P.A. (2012) Genotype-dependent sulphite tolerance of Australian *Dekkera* (*Brettanomyces*) *bruxellensis* wine isolates, *Lett. Appl. Microbiol.* **55**, 56–61. <https://doi.org/10.1111/j.1472-765X.2012.03257.x>
107. Curtin, C.D., Langhans, G., Henschke, P. A., Grbin, P. R. (2013). Impact of Australian *Dekkera bruxellensis* strains grown under oxygen-limited conditions on model wine composition and aroma, *Food Microbiol.* **36**, 241–247 <https://doi.org/10.1016/j.fm.2013.06.008>
108. Smith B. D., and Divol, B. (2016) *Brettanomyces bruxellensis*, a survivalist prepared for the wine apocalypse and other beverages, *Food Microbiol.* **59**, 161–175. <https://doi.org/10.1016/j.fm.2016.06.008>
109. Conterno, L, Joseph, C. M. L., Arvik, T. J., Henick-Kling, T., and Bisson, L. F. (2006) Genetic and physiological characterization of *Brettanomyces bruxellensis* strains isolated from wines, *Am. J. Enol. Vitic.* **57**, 139–147.
110. de Barros Pita, W., Leite F. C., de Souza Liberal, A. T., Simões, D. A., and de Moraes, M. A. Jr. (2011) The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes, *Antonie Van Leeuwenhoek* **100**, 99–107. <https://doi.org/10.1007/s10482-011-9568-z>
111. Kippenberger, M., Hanke, S., Biendl, M., Stettner, G., and Lagemann, A. (2014) Transfer of nitrate and various pesticides into beer during dry hopping, *Brew. Sci.* **67**, 1–9.
112. Crauwels, S., Zhu, B., Steensels, J., Busschaert, P., Samblanx, G. D., Marchal, K., Willems, K. A., Verstrepen, K. J., and Lievens, B. (2014) Assessing genetic diversity among *Brettanomyces* yeasts by DNA fingerprinting and whole-genome sequencing, *Appl. Environ. Microbiol.* **8**, 4398–4413. <https://doi.org/10.1128/AEM.00601-14>
113. Vigentini, I., Romano, A., Compagno, C., Merico, A., Molinari, F., Tirelli, A., Foschino, R., and Volonterio, G. (2008) Physiological and oenological traits of different *Dekkera/Brettanomyces bruxellensis* strains under wine-model conditions, *FEMS Yeast Res.* **8**, 1087–1096. <https://doi.org/10.1111/j.1567-1364.2008.00395.x>

114. Vigentini, I., Lucy Joseph, C. M., Picozzi, C., Foschino, R., and Bisson, L.F. (2013) Assessment of the *Brettanomyces bruxellensis* metabolome during sulphur dioxide exposure, *FEMS Yeast Res.* *13*, 597–608. <https://doi.org/10.1111/1567-1364.12060>
115. Krogerus, K., Magalhães, F., Vidgren, V., and Gibson, B. (2017) Novel brewing yeast hybrids: Creation and application, *Appl. Microbiol. Biotechnol.* *101*, 65–78. <https://doi.org/10.1007/s00253-016-8007-5>
116. Joseph, C. M. L., Albino, E. A., Ebeler, S. E., and Bisson, L. F. (2015) *Brettanomyces bruxellensis* aroma-active compounds determined by SPME GC-MS olfactory analysis, *Am. J. Enol. Vitic.* *66*, 379–387. 1 <https://doi.org/10.5344/ajev.2015.14073>