Brettanomyces bruxellensis

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The *Brettanomyces bruxellensis* species plays various roles in both the industrial and food sectors. At the biotechnological level, *B. bruxellensis* is considered to be a promising species for biofuel production. Its presence in alcoholic beverages can be detrimental or beneficial to the final product; *B. bruxellensis* can contribute to spoilage of wine and beer, but can also produce good aromas.

Keywords: molecular biotechnology ; wine spoilage ; wine yeast ; Brettanomyces

1. Introduction

The *Brettanomyces* genus was first described by Kufferath and Van Laer in 1921, and its primary taxonomic framework was established in 1964 by van der Walt [1]. It is the anamorphic form of the *Dekkera* genus belonging to the *Pichiaceae* family [2][3]. The phylogenetic classification of *Brettanomyces* and *Dekkera* was first performed in 1987 by Clark–Walker *et al*., and then in 1993 by Molina et al. [2]. Subsequently, in 2011, Kurtzman et al. reclassified the genus to include five species: *D. bruxellensis*, *D. anomala*, *B. custersianus*, *B. nanus*, and *B. naardenensis* [4]. Recently, a new species named *B. acidodurans* was isolated from olive oil and has been characterized as a strongly acetic–acid–tolerant yeast [5]. In disagreement with the Melbourne Convention (International Code of Nomenclature for algae, fungi, and plants), which states that a single valid name must be assigned under the new code for a fungal species [6], both "*Brettanomyces*" and "*Dekkera*" have been used in the scientific literature. However, the designation "*Brettanomyces*" is more commonly applied in the food and biotechnology industries, wherein the species *B. bruxellensis* is immediately associated with wine by all the stakeholders of the sector [7][8]. Thus, we preferentially use *Brettanomyces* in this review, but also use the original names given in the literature cited. *B. bruxellensis* is the best–known species within the genus; it is a facultative anaerobic yeast that has been isolated from different sources including fruit peels, beer, wine, cheese, kombucha, kefir, tea, olives, sodas, and wooden barrels [9]. This species has a significant role in the production of Belgium–style beer, especially in the aromatic profiles of Lambic and Gueuze beers, via the release during its proliferation of different aromatic compounds such as fusel alcohols and esters (responsible for the floral, fruity, and spicy characteristics) [10][11][12][13][14][15][16]. In contrast, *B. bruxellensis* is considered to be a spoilage yeast in the wine sector, causing negative sensory properties for products aged in wood [17]. Contamination by this yeast causes the "Brett" character, consisting of unpleasant aromas that are perceived when specific thresholds are exceeded [18]. These compounds include secondary metabolites such as nitrogenous compounds (e.g., 2–acetyl–3,4,5,6–tetrahydropyridine, 2–acetyl–1,2,5,6–tetrahydropyridine, and 2–ethyl–3,4, 5,6–tetrahydropyridine) and/or volatile phenols (4–vinylphenol, 4–vinylguiacol, 4–ethyl phenol, and 4–ethylguiacol), the latter being associated with "horse sweat", "leather", "medicinal", "barnyard", and other similar descriptors [18][19][20].

Karyotype analyses, intron splice site amplification (ISS–PCR), and microsatellite genetic profiling performed on strains derived from different microbial collections worldwide have revealed that *B. bruxellensis* strains exhibit a high intraspecific variability, which is also supported by the chromosome number (from four to nine) and the ploidy state [21][22][23]. Karyotype, ploidy, and the source of isolation can be useful in classifying the different strains (e.g., dissimilar *B. bruxellensis* strains have been obtained from tequila/ethanol, wine, and kombucha environments). Similarly to other yeasts, changes in ploidy are believed to be a driver of adaptation [24]. *B. bruxellensis* isolated from wine shows specific adaptations, like tolerance to SO_2, which are linked to their diploid or triploid state [23][25]. This aspect deserves a thorough investigation; indeed, while gene duplication in *Saccharomyces cerevisiae* has been suggested to bring evolutionary and adaptive benefits to the species, recent studies have revealed that non–*Saccharomyces* yeast species (namely *Kluyveromyces marxianus*) face environmental stresses via only the up–/downregulation of multiple pathways (anti–osmotic, antioxidative, etc.) rather than a ploidy change [24][26].

B. bruxellensis strains possess all the characteristics required for a great metabolic potential exploitable in biotechnological applications, such as industrial fermentation processes (beer and wines) and biofuel productions (e.g., first– and second–generation ethanol) [27][28][29][30][31][32][33][34][35]. Despite the large amount of information concerning the

biotechnological peculiarities of *B. bruxellensis* in the literature, there have been no reviews focused on the molecular tools that can be used to modify strains for their biotechnological exploitation [27][28][29].

2. Development of Molecular Tools for the Genetic Modification of *B. bruxellensis*

To date, eight different *B. bruxellensis* strain genomes have been sequenced and deposited in the NCBI (National Center for Biotechnology Information) database. First, the DNA of the wine–isolated *B. bruxellensis* CBS2499 strain was partially sequenced by Woolfit et al. in 2007 [36]. Later, Piškur et al. (2012) completed its sequencing and discovered that it has the typical characteristics of the hemiascomycetes (gene number, intron size and number, intergenic length, and gene content) [37]. The genome analysis identified 5600 predicted genes, of which several were duplicated, suggesting a diploid form. In the same year, Curtin et al. (2012) fully investigated the genome of another wine–spoilage strain, the widespread triploid AWRI1499 [38], in which a high density of single–nucleotide polymorphisms (SNPs) and an enrichment in genes for membrane proteins and oxidoreductase enzymes were revealed. Thereafter, the genomes of other B. bruxellensis strains were sequenced: (i) CBS2796 (ATCC 52904) strain, isolated from sparkling wine, with a genome length of 11.77 Mb [36]; (ii) UMY321 strain (corresponding to L17 [22]) recognized to be diploid, with 82,632 SNPs revealing a high level of heterozygosity [39][40]; (iii) LAMAP2480 strain, with the largest genome found so far (26.99 Mb), has been the subject of study focusing on its spoilage role and biotechnological potential. The genome analysis showed the presence of several genes related to stress tolerance, nutrient uptake, ethanol production, and lignocellulose assimilation [41]; and (iv) UCD 2041 strain isolated from fruit wine, the DNA sequence of which was compared with strains belonging to different *Brettanomyces* species, such as *B. nanus* CBS1945, *B. anomala* CBS8139, *B. naardenensis* CBS6042, and *B. custersianus* CBS4805, revealing a large genetic distance within the genus. Moreover, relevant marker genes of domestic adaptation and fermentation were identified in UCD 2041, CBS1945, and CBS8139. Peculiar horizontal gene transfer events, which are probably responsible for the ability of these strains to utilize sucrose, were also detected [42]. Furthermore, CBS11270 is an example of another sequenced strain isolated from industrial ethanol production, with a genome size of 15.39 Mb across four chromosomes and a high level of SNPs (40.6% of which are in coding regions). Genomic analysis showed that this strain is diploid, with several genes replicated in chromosomes 1 and 4, highlighting interchromosomal gene duplications and loss of heterozygosity in some of them [43][44]. Lastly, the CRL–50 strain, which is one of the two oldest known *Brettanomyces* isolates, was collected from a Carlsberg beer sample in Denmark between the years 1904 and 1908 [44] and sequenced by Colomer et al. [45]. A comparative genome analysis was carried out in order to compare this strain with other *B. bruxellensis* strains isolated from beer, wine, kombucha, sodas, olives, and bioethanol production plants. The analysis indicated a higher genetic similarity among beer strains than among the wine–spoilage strains, probably resulting from different selection pressures linked with human activities [45]. Despite these recent studies, additional investigations are necessary to explore the great potential of *B. bruxellensis* strains in terms of bioengineering applications.

Drug sensitivity is one of the key factors impacting microbial manipulation and the construction of suitable molecular tools. Until now, the sensitivity of *B. bruxellensis* strains to antibiotics commonly used in molecular biotechnology approaches has not been deeply investigated. Recently, Di Canito *et al*. carried out experiments with drugs usually applied for the selection of transformed yeast cells (geneticin (G418), nourseothricin (NTC), hygromicin (Hyg), and canavanine (Can)) on six different *B. bruxellensis* strains (CBS2499, AWRI1499, UMY306, UMY308, UMY397, and UMY406) using the protocols described in the work of Vigentini et al. (2017) [46][47]. All strains were sensitive to G418, NTC, and Hyg at final concentrations higher than 400 µg mL^{-1}. In contrast, Can inhibited the growth of all strains at all tested concentrations. In 2019, Avramova *et al*. investigated the correlation between drug resistance and SO_2 tolerance in *B. bruxellensis*, demonstrating G418 and NTC to have significant effects on the growth of recombinant clones carrying the corresponding selectable markers [48]. In particular, the growth of AWRI1626 clones resistant to G418 decreased faster than that of those resistant to NTC when the concentration of SO_2 was around 0.6 mg L^{-1}, while the AWRI1499 and AWRI1608 transformants showed similar behavior in the presence of all the tested SO_2 concentrations, regardless of the antibiotic resistance [48]. Thus, considering this relevant strain–specific response, the influence of stress factors (such as those usually found in a wine–like environment) on the sensitivity to selectable markers useful for the generation of recombinant strains should be carefully considered in order to ensure reliable experimental results.

The current molecular tools for the genetic manipulation of *B. bruxellensis* face many issues, hindering the immediate implementation of this species in biotechnological applications. As mutagenesis through homologous integration is difficult to perform in many non–*Saccharomyces* yeasts [48], in 2013, Miklenic *et al*. first attempted and implemented the insertion of a specific genetic element into *B. bruxellensis* cells via nonhomologous recombination [49]. The authors transformed the CBS2499 strain with a heterologous DNA fragment of 1.9 kb, containing the kanMX4 sequence encoding for G418 resistance (G418R) and flanked with the regions 5′Sc and 3′Sc (220 bp of the 5′ end and 165 bp of the 3′ end of the

YMR224C ORF from *S. cerevisiae* [49]. A low transformation efficiency was detected, confirming the results obtained in S. cerevisiae following the nonhomologous integration of heterologous transforming DNA [50]. This could be attributed to a tolerance to DNA mismatches, a tendency to repair the DNA damage throughout the nonhomologous end–joining (NHEJ) pathway or the illegitimate recombination apparatus, or a lower efficiency of the short sequence recombination (SSR) system [51]. This result supports the phylogenetic proximity of *B. bruxellensis* to *P. pastoris*, which exhibits low transformation frequencies when homologous integration with ends–out vectors is carried out [51]. As a consequence, when proceeding with the genetic manipulation of a non–*Saccharomyces* yeast, and with the construction of associated molecular tools, the natural inclination towards nonhomologous recombination has to be considered. The first circular vector proposed for *B. bruxellensis* transformation was presented by Schifferdecker *et al*. in 2014. It contained CIGO1, 2, and 3 sequences to promote the autonomous replication of the plasmid [19]. Later, Ishchuck *et al*. (2016) inserted *CEN1* and *CEN2* sequences of *B. bruxellensis* in several vectors [51]. In this case, the transformation efficiency was lower than that obtained using the linearized plasmid P892. Although this outcome confirmed that integrating vectors have a higher stability than replicating vectors in non–*Saccharomyces* yeasts, the experiment suggested that the centromeric plasmids did not contain strong autonomously replicating sequences [51][52]. In her doctoral thesis, Avramova (2017) proposed other cassettes for *B. bruxellensis*, i.e., pMK–T–*TDH1*pr–*kan*MX and pMK–T–*TDH1*pr–*nat*MX cassettes, including the *B. bruxellensis TDH1* promoter (*BbTDH1*) and the *AgTEF2* terminator (derived from *Ashbya gossypii*) [53]. When these elements were integrated into the genomes of AWRI1499, AWRI1608, and AWRI1626 strains, they brought about antibiotic resistance without affecting the growth rates of the recombinants in comparison to the wild types [53]. The search for useful autonomously replicating vectors recently led to the construction of a series of pMA plasmids containing the aforementioned cassettes for G418, Nat, or Hyg as selectable markers [53][54]. The pMA–*TDH1*pr–*nat*MX vector was then modified to produce the fluorescent proteins GFP (green fluorescent protein) and TagBFP (blue fluorescent protein); both were flanked by the strong *S. cerevisiae* promoter ScFBA1p and its terminator Sc*PGK1*. The obtained plasmids were named pMA–*TDH1*pr–*nat*MX::GFP and pMA–*TDH1*pr–*nat*MX::BFP. Additionally, other plasmids containing the heterologous *AgTEF2* promoter instead of the homologous *BbTDH1* were constructed. However, the transformation experiments carried out with all the constructs revealed low efficiency [54]. This outcome is probably associated with the heterologous elements inserted into the vectors used, in which the *BbTDH1* promoter ensured an efficiency more than seven times higher than that of *AgTEF2*. In fact, the tailored cassette for *B. bruxellensis*, coupled with multiple–drug–resistant markers and fluorescent proteins, increased the transformation efficiency [54].

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