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Review

Molecular Tools to Exploit the Biotechnological Potential of *Brettanomyces bruxellensis*: A Review

Alessandra Di Canito, Roberto Foschino, Martina Mazzieri and Ileana Vigentini *

Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Via G. Celoria, 2-20133 Milan, Italy; alessandra.dicanito@unimi.it (A.D.C.); roberto.foschino@unimi.it (R.F.); martina9651615@gmail.com (M.M.)

* Correspondence: ileana.vigentini@unimi.it

Abstract: 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. However, little is known about its genetic characteristics and, despite the complete sequencing of several *B. bruxellensis* genomes and knowledge of its metabolic pathways, the toolkits for its efficient and easy genetic modification are still underdeveloped. Moreover, the different ploidy states and the high level of genotype diversity within this species makes the development of effective genetic manipulation tools challenging. This review summarizes the available tools for the genetic manipulation of *B. bruxellensis* and how they may be employed to improve the quality of wine and beer.

Keywords: *Brettanomyces bruxellensis*; *Dekkera bruxellensis*; molecular biology; molecular biotechnology; CRISPR/Cas9 approach

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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) [10–16]. 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 [18–20].

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 [21–23]. 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, such as 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 (Table 1) 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) [27–35]. 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 [27–29]. This information may be useful concerning the genetic mechanisms involved in the attractive physiological traits of this species, thus boosting its industrial application prospects. This review aims to provide a wide overview of the currently available tools for the manipulation of *B. bruxellensis* strains, from conventional to postgenomic era technologies.

Table 1. Physiological and metabolic features of *B. bruxellensis* species.

Features		References
Growth rate (μ, h^{-1})	0.037–0.114	[29]
Ethanol yield (g/g glucose)	0.44–0.46	[29]
Glycerol yield (g/g glucose)	0.0–0.026	[28]
Crabtree positive	yes	[9,20,28]
Custers effect	yes	[9,22,28,29]
Presence of respiratory complex 1	yes	[9,20,28]
Nitrate assimilation	strain specific	[9,22,28]
Ethanol tolerance	14%	[28]
Whole–genome sequencing	yes	[8,36,37]
AOX1	yes	[9,20,28]
Phenol metabolism	strain specific	[9,28]
Enzymatic activities (*VPR*; *CD*; β–glucosidase)	strain specific	[19,28]
SO_2 tolerance	strain specific	[37]

AOX1: Alcohol oxidase I; *VPR:* Vinyl Phenol Reductase; *CD:* cinnamate decarboxylase.

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 (some information can be seen in Table 2). First, the DNA of the wine–isolated *B. bruxellensis* CBS2499 strain was partially sequenced by Woolfit et al. in 2007 [38]. 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) [36]. 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 [37], 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 [38]; (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.

2.1. Drug Sensitivity

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 $\mu g\ mL^{-1}$. In contrast, Can inhibited the growth of all strains at all tested concentrations. The results related to the CBS2499 and AWRI1499 strains are reported in Table 3. 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.

2.2. Construction of Molecular Cassettes for B. bruxellensis Manipulation

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 ($G418^R$) 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 CIGO 1, 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–*kanMX* and pMK–T–*TDH1*pr–*natMX* cassettes, including the *B. bruxellensis TDH1* promoter (Bb*TDH1*) and the Ag*TEF2* 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–*natMX* 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 ScPGK1. The obtained plasmids were named pMA–*TDH1*pr–*natMX*::GFP and pMA–*TDH1*pr–*natMX*::BFP. Additionally, other plasmids containing the heterologous Ag*TEF2* promoter instead of the homologous Bb*TDH1* 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 Bb*TDH1* promoter ensured an efficiency more than seven times higher than that of Ag*TEF2*. In fact, the tailored cassette for *B. bruxellensis*, coupled with multiple–drug–resistant markers and fluorescent proteins, increased the transformation efficiency [54]. All the aforementioned constructed molecular cassettes for *B. bruxellensis* are described in Table 4.

Table 2. Complete sequenced and deposited *B. bruxellensis* strain genomes and their characteristics.

Strain	BioSample	Bioproject	Assembly	Size (Mb)	GC%	CDS		Ecological Origin	Geographical Origin	References
CBS 2499	SAMN00750237	PRJNA76499	GCA_000340765.1	13.36	40.3	5600		wine	France	[36]
AWRI1499	SAMN02261473	PRJNA78661	GCA_000259595.1	12.68	39.9	4861		wine	Australia	[37]
CBS 2796	SAMN05544770	PRJNA335438	GCA_001719535.1	11.77	39.8	–		sparkling wine	France	[38]
UMY321	SAMEA5744194	PRJEB33245	GCA_902155815.1	12.97	40.0	4666		wine	Italy	[22,39]
LAMAP2480	SAMN09981576	PRJNA231184	GCA_000688595.1	26.99	39.9	–		wine	Chile	[41]
UCD 2041	SAMN12257691	PRJNA554210	GCA_011074885.1	13.15	39.9	–		fruit wine	United States	[42]
CBS 11270	SAMEA104365571	PRJEB11548	GCA_900496985.1	15.39	41.6	4879		industrial ethanol	Sweden	[43]
CRL–50	SAMN13421994	PRJNA592329	GCA_012295375.1	17.82	39.8	–	2n	beer	Denmark	[45]

Table 3. Test for drug sensitivities on yeast nitrogen base (YNB) medium without amino acids supplemented with 5 g L^{-1} ammonium sulfate [$(NH_4)_2SO_4$] or 1 g L^{-1} L–glutamic acid (GLU) as a nitrogen source.

Drug Concentration (μg mL^{-1})	Geneticin (G418)				Nourseothricin (NTC)				Hygromicin (Hyg)				Canavanine (Can)			
	AWRI1499		CBS2499		AWRI1499		CBS2499		AWRI1499		CBS2499		AWRI1499		CBS2499	
	$(NH_4)_2SO_4$	GLU	$(NH_4)_2SO_4$	GLU	$(NH_4)_2SO_4$	GLU	$(NH_4)_2SO_4$	GLU	$(NH_4)_2SO_4$	GLU	$(NH_4)_2SO_4$	GLU	$(NH_4)_2SO_4$	GLU	$(NH_4)_2SO_4$	GLU
0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50	±	–	+	–	–	–	–	–	–	–	±	–	–	–	–	–
100	±	–	+	–	–	–	–	–	–	–	±	–	–	–	–	–
200	±	–	+	–	–	–	–	–	–	–	±	–	–	–	–	–
300	–	–	±	–	–	–	–	–	–	–	–	–	–	–	–	–
400	–	–	±	–	–	–	–	–	–	–	–	–	–	–	–	–
500	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
600	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

(+): presence of colonies; (±): few colonies; (–): no colonies.

Table 4. Available molecular cassettes for *B. bruxellensis* manipulation.

Vectors	Drug Resistance	Promotor	Terminator	Insertion	References
pMK–T–*TDH1*pr–*kanMX*	Kanamycin Geneticin	Bb*TDH1*	Ag*TEF2*	Bb*TDH1*, Ag*TEF2* Kan^R	[53,54]
pMK–T–*TDH1*pr–*natMX*	Kanamycin Nourseothricin	Bb*TDH1*	Ag*TEF2*	Bb*TDH1*, Ag*TEF2* Nat^R	[53,54]
pMK–T–*TDH1*pr–*hygMX*	Kanamycin Hygromicin	Bb*TDH1*	Ag*TEF2*	Bb*TDH1*, Ag*TEF2* Hyg^R	[53,54]
pMA–*TDH1*pr–*natMX*	Ampicillin Nourseothricin	Bb*TDH1*	Ag*TEF2*	Bb*TDH1*, Ag*TEF2* Nat^R	[53,54]
pMA–*TDH1*pr–*natMX*::GFP	Ampicillin Nourseothricin	Sc*FBA1*	Sc*PGK1*	Bb*TDH1*, Ag*TEF2* Nat^R GFP	[53,54]
pMA–*TDH1*pr–*natMX*::BFP	Ampicillin Nourseothricin	Sc*FBA1*	Sc*PGK1*	Bb*TDH1*, Ag*TEF2* Nat^R BFP	[53,54]

2.3. Transformation Protocols

Over the past 30 years, molecular tools and transformation protocols have been designed and exploited mainly for *S. cerevisiae* species. Nevertheless, these tools and protocols can be taken as models and applied to the manipulation of non–*Saccharomyces* yeasts (e.g., *Yarrowia lipolytica*, *Pichia pastoris* syn. *Komagataella phaffii*, *Hansenula polymorpha* syn. *Ogataea angusta*, *Kluyveromyces lactis*, *Candida albicans,* and *C. glabrata*) in order to study or modify metabolic traits that are useful in industrial processes [55]. In Table 5, a comparison of the different methods available for the transformation of *B. bruxellensis* strains is presented, considering that the well–known high genetic variability that exists in the species may create limitations. Various approaches have been proposed by a few research groups: the LiAc/PEG transformation protocol [49], electroporation treatment [49,56], the spheroplast transformation procedure, and the spheroplast intraspecific fusion methodology [49,57]. Lithium acetate treatment causes a transitional opening in the cell membranes of yeasts to permit the acquisition of genetic material from the outside; this method is highly efficient in *B. bruxellensis* when using up to 100 µg of DNA [49]. The electroporation method is quick and simple, whereby the exogenous DNA enters through pores in the cell wall and membrane created by a high–voltage pulse. Its efficiency significantly depends on the yeast species, cell growth phase, and final density of the culture, as well as the electroporation pulse parameters [49]. This protocol was optimized in 2015 in a study by Miklenić et al. [56], in which the electroporation pulse parameters (1.8 kV and 5 ms) and the optimal osmotic stabilizer (1M sorbitol) were established. Thereafter, this technique was utilized to transform several strains with cassettes containing genes encoding for fluorescent proteins to discriminate subpopulations by flow cytometry and fluorescent microscopy [56]. While spheroplast transformation was successfully applied in *S. cerevisiae* using 1 M sorbitol as the osmotic stabilizer, it resulted in a procedure unsuitable for *B. bruxellensis*, contrary to what was observed for the electroporation approach. Indeed, this chemical has a negative effect on this yeast species, inducing a state in which cells remain viable but become nonculturable, similar to what is observed after cell exposure to sulfite [49,58]. Another transformation technique that includes spheroplast formation is recursive intraspecific fusion based on genome shuffling. Genome shuffling is a feasible method for the rapid manipulation of complex phenotypes using whole cells and organisms. This procedure can be useful for generating stable changes in the genomes of *Brettanomyces* strains, and its application as an effective whole–cell engineering strategy could be advantageous for the rapid improvement of industrially important microbial phenotypes [58]. This overview shows that the most efficient strategy for *B. bruxellensis* transformation is the electroporation protocol proposed by Miklenić et al. in 2015 [56] and

Varela et al. in 2018 [54]. However, in order to improve the knowledge and efficiency of genetic manipulation methods for different *B. bruxellensis* strains, additional investigations are required.

Table 5. Available transformation approaches for *B. bruxellensis* manipulation.

Procedure	Medium	OD_{600nm}	Growth Temperature (°C)	Solution—Chemical Compounds	Transformation Efficiency	References
LiAc/PEG transformation	GYP; SCM	0.60–0.75	28 °C	M LiAc–50% PEG	16 transformants μg^{-1} DNA	[49]
Electroporation transformation	GYP	0.25–0.35	28 °C	M LiAc–0.5—1 M sorbitol	2.8×10^3 transformants μg^{-1} DNA	[49,56]
Spheroplast transformation	GYP; SCM	/	28 °C	Zymolyase–0.5 M–1 M sorbitol	plating efficiency * 75%; 7.4%	[49]
Spheroplast intraspecific fusion	YPD; MM	1.00	30 °C	Snailase–PE buffer–PEG 4000	**	[57]

* Alternative method to evaluate the transformation efficiency. ** Data are not available.

3. CRISPR/Cas9 System in *B. bruxellensis*

Genome editing using the CRISPR/Cas9 system is a valuable instrument for yeast genome manipulation that allows researchers to study the biochemical pathways or change the phenotypic characteristics of strains using metabolic engineering [47,55]. This breakthrough technique enables the creation of site–specific mutations in targeted locations and the integration of multiple DNA constructs in a single transformation event [59–62]. The great advantage of the CRISPR/Cas9 system is the use of a single Cas9 nuclease that can modify multiple alleles/genes simultaneously [62]. From this perspective, this method appears ideal for use in species such as *B. bruxellensis* that present different ploidy states [63]. Although the CRISPR/Cas9 system approach has been widely studied and applied in *S. cerevisiae* strains, its use in other yeast species is still challenging [64,65]. Several strategies have been developed for non–*Saccharomyces* yeasts (*Yarrowia lipolytica*, *Pichia pastoris* syn. *Komagataella phaffii*, *Kluyveromyces lactis*, *Candida albicans*, and *C. glabrata*), but the reported efficiencies are still lower than in *S. cerevisiae* strains [55]. Indeed, the crucial point of the CRISPR/Cas9 system is the insertion of double–strand breaks (DSB) and the subsequent recombination strategy; in fact, nonhomologous end joining (NHEJ) is the main route for most yeasts other than *S. cerevisiae*, which applies the homologous recombination (HR) strategy [65]. These two phenomena occur with different efficiencies: HR achieves precise mutations or alterations in the target locus, whereas NHEJ results in genetic mutations, deletions, and translocations [66]. Regrettably, NHEJ is the preferred DNA–repair approach in *B. bruxellensis*, which may prevent the possibility of overcoming the translation or deletion of genes induced by CRISPR/Cas9, limiting the survival of the transformed clones [66]. An interesting method was proposed by Weninger et al. [67] in 2017, in which the NHEJ–impeded *P. pastoris* CBS 7435 mutant Δku70 and the CRISPR/Cas9 system were combined to perform transformation experiments, increasing the HR efficiency. This strategy allowed them to obtain a CRISPR/Cas9–mediated integration of markerless donor cassettes with an efficiency of 100%, and, even though the transformation efficacy was lower than that achieved using conventional strategies, all the transformants demonstrated correct integration [67]. Subsequently, in 2020, Varela et al. described the application of the CRISPR/Cas9 system in *B. bruxellensis* [66]. In that study, several experiments were performed to obtain an expression–free CRISPR/Cas9 system to promote HR–based gene deletion in combination with *B. bruxellensis* gene transformation cassettes (described in the work of Varela et al. in 2018 [54] and listed above) for the deletion of the *SSU1* gene responsible for sulfite tolerance [68–70]. This method was previously investigated for pathogenic *Candida* species (other than *C. albicans*) by Grahl et al. (2017). These authors bypassed the inefficient heterologous expression of the CRISPR/Cas9 system components

in these species, delivering the ribonucleoprotein particle complex (RNPs) [71]. RNPs consist of a gRNA in complex with purified Cas9 protein. This complex is assembled in vitro and can be delivered using standard electroporation or transfection techniques [49,70]. Varela et al. obtained RNPs by co–incubating the crRNA designed to target ORF and tracrRNA. The authors achieved a targeted gene deletion of the *SSU1* gene in the haploid strain AWRI2804, demonstrating growth inhibition of the mutants in the presence of SO_2. Furthermore, they evaluated the possibility of using the same method in polyploid strains. They transformed the diploid strain AWRI1613 in order to delete both the *URA3* genes, combining the *natMX* cassette, containing long–flanking regions (1 kb) and RNPs. Two Δ*URA3::natMX* mutants were obtained; their genome analysis revealed the integration of a single copy of the cassette and the concomitant deletion of the two different alleles, one for each mutant, thus showing the impossibility of deleting both alleles in a single step. Accordingly, a second deletion was attempted by transforming one of the mutants to combine the *kanMX* cassette, which contained long–flanking regions (1 kb) and RNPs. The resulting Δ*URA3::kanMX* showed the deletion of the second copy of the *URA3* gene and the insertion of a single copy of the *kanMX* cassette [66]. Therefore, these outcomes support the use of this method, which combines an expression–free CRISPR/Cas9 system and tailored *B. bruxellensis* gene transformation cassettes and is potentially useful for polyploid strains in a stepwise transformation system.

4. Conclusions

A great amount of genetic information has been obtained about certain *B. bruxellensis* strains using whole–genome sequencing. However, more knowledge is required in order to develop the genetic tools necessary to efficiently engineer the species [65]. Indeed, as a result of its genomic characteristics and intraspecific heterogeneity, *B. bruxellensis* remains a challenge to manipulate. However, recent findings confirm that the CRISPR/Cas9 system is a promising technology for yeast engineering, as demonstrated by the rapidly increasing number of publications and the availability of many web–based bioinformatic tools for the design of gRNA [66,71,72].

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