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Construção e caracterização de espécies híbridas entre *Saccharomyces cerevisiae* e *Saccharomyces kudriavzevii* com possível aplicação na indústria cervejeira

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"We live in a society exquisitely dependent on science and technology, in which hardly anyone knows anything about science and technology."

- Carl Sagan

Agradecimentos

Agradeço a todas as pessoas que tornaram esse trabalho possível. Acima de tudo, sou grato aos pesquisadores brasileiros, que em tempos de tormenta, encontram força para continuar o que é imprescindível para o crescimento de qualquer nação: a Ciência.

Estrutura da dissertação

Essa dissertação de mestrado é dividida em introdução geral, um capítulo redigido em forma de artigo, discussão geral, conclusões gerais e conclusões específicas. Ainda, nos adendos, foi incluído um artigo de revisão redigido ao longo do período de mestrado e que já está publicado.

Na introdução geral é apresentado um breve histórico da cerveja e a sua importância, bem como uma descrição sucinta das matérias-primas e processos empregados na indústria cervejeira. Além disso, o tópico de leveduras cervejeiras é abordado, onde são descritas as cepas disponíveis para indústria, além da busca por alternativas. O seguinte tema é o estudo de cepas híbridas interespecíficas e a sua aplicação em pesquisas básicas. Por fim, as cervejas de fazenda norueguesas (*Norwegian farmhouse ales)* são descritas.

Os resultados discorrem sobre a geração e a caracterização dos híbridos interespecíficos entre *S. cerevisiae* e *S. kudriavezevii* gerados nesse trabalho, na forma de artigo com a formatação original em que será submetida ao periódico *Yeast.* Cabe ressaltar que o artigo foi escrito em conjunto com Grupo de Estudos de Leveduras Cevejeiras (GELC), uma vez que os dados fazem parte de uma pesquisa mais abrangente envolvendo a geração de híbridos entre leveduras usadas na fabricação de *farmhouse ales* norueguesas e belgas com *S. kudriavzevii.*

Finalmente, há uma discussão geral acerca dos resultados, bem como suas conclusões gerais e específicas, e perspectivas.

Nos adendos está inserido o artigo publicado no periódico *Journal of the Institute of Brewing* intitulado *Molecular and Biochemical aspects of Brettanomyces in Brewing*.

Sumário

Lista de abreviaturas

°P- Unidade de medida de densidade em graus Plato (g/100 mL)

***ATF1* –** Gene codificante para a enzima *alcohol acetyltransferase 1*

IBU - *International Bitter Units*

ITS1 - Região ITS1 (*Internal Transcribed Spacer*)

ITS2 - Região ITS2 (*Internal Transcribed Spacer*)

Kg/cm³ - Unidade de medida de densidade em quilogramas por centímetro cúbico

KKH1 - Híbrido 1 entre a cepa de *Saccharomyces cerevisiae* Voss Kveik e a cepa tipo de *Saccharomyces kudriavzevii*

KKH2 - Híbrido 2 entre a cepa de *Saccharomyces cerevisiae* Voss Kveik e a cepa tipo de *Saccharomyces kudriavzevii*

KKH3 - Híbrido 3 entre a cepa *de Saccharomyces cerevisia*e Voss Kveik e a cepa tipo de *Saccharomyces kudriavzevii*

KWH1 - Híbrido 1 entre a cepa de *Saccharomyces cerevisiae* Wallonian III e a cepa tipo de *Saccharomyces kudriavzevii*

KWH2 - Híbrido 2 entre a cepa de *Saccharomyces cerevisiae* Wallonian III e a cepa tipo de *Saccharomyces kudriavzevii*

KWH3 - Híbrido 3 entre a cepa de *Saccharomyces cerevisiae* Wallonian III e a cepa tipo de *Saccharomyces kudriavzevii*

Lys- - Mutantes auxotróficos para lisina

NCYC - *National Collection of Yeast Cultures*

NCYC2889 - Cepa tipo de *S. kudriavzevii*

Ura- - Mutantes auxotróficos para uracila

WLNIII - Cepa de *Saccharomyces cerevisiae* Wallonian III

YPM - Meio composto com extrato de levedura, peptona e extrato de malte

ATF2 - Gene codificante para a enzima *alcohol acetyltransferase 2*

PAD1 - Gene codificante para a enzima *phenylacrylic acid decarboxylase*

FDC1 - Gene codificante para a enzima *ferulic acid decarboxylase*

4-VG - 4-vinilguaiacol

GC-FID - Cromatografia Gasosa com Detector por Ionização de Chama

FLO1, FLO5, FLO8, FLO9, FLO10* e *FLO11 - Genes codificante para as floculinas

STA1 - Gene codificante para a enzima *glucoamylase*

Resumo

Leveduras híbridas intra e interespecíficas vem sendo construídas em laboratório visando ampliar a compreensão acerca das características fenotípicas e genotípicas de diferentes híbridos e o fornecimento de novas cepas ao mercado cervejeiro. Na presente pesquisa, foram gerados dez híbridos únicos entre a espécie criotolerante *Saccharomyces kudriavzevii,* que é relacionada à fermentação de vinhos e cervejas, e a espécie termotolerante *S. cerevisiae*, isolada de um ambiente relacionado às *farmhouse ales* do município de Voss, Condado de Hordaland, Noruega. As cepas híbridas resultantes possuem heterose em relação as parentais, possuindo boa performance fermentativa em baixas e altas temperaturas (17 °C, 25 °C e 37 °C) e alcançando altos valores de atenuação aparente. As cervejas obtidas com as cepas híbridas possuem um pH final de 3,70-4,4 após a fermentação nas temperaturas de 17 °C, 25 °C e 35 °C. Ainda, as cepas híbridas possuem a capacidade de gerar compostos aromáticos frutados como os ésteres pentanoato de etila (que remete à maçã vermelha e melão), hexanoato de etila (maçã e anis-estrelado), cinamato de etila (frutado) e acetato de feniletila (doce, mel e floral) que foram produzidos acima do limiar de percepção apenas pelos híbridos na temperatura utilizada para esse teste (25 °C), além de decanoato de etila (*brandy*, frutado e uva) que foi produzido também em níveis perceptíveis pelas cepas parentais. Os resultados dessa pesquisa indicam que a cepa Voss Kveik (*S. cerevisiae*) pode servir como chassi para a geração de novos híbridos interespecíficos e a possibilidade de uma ampla aplicação dos híbridos caracterizados (KKH1-KKH3) na indústria vinícola e cervejeira.

Abstract

Intra and interspecific hybrid yeasts have been built in the laboratory to broaden understanding of the phenotypic and genotypic characteristics of different hybrids and the supply of new strains to the beer market. In the present work, 10 unique hybrids were generated between cryotolerant wine and beer-associated *S. kudriavzevii* along with the heat-tolerant *S. cerevisiae* isolated from farmhouse brewing-related environment in Voss, Hordaland County, Norway. The resulting hybrid strains have heterosis in comparison to parental, good fermentative performance at low and high temperatures (17 ° C, 25 ° C and 37 ° C) and achieve high apparent attenuation values. Beers obtained with hybrid strains have a final pH of 3.70-4.4 after fermentation at temperatures of 17 ° C, 25 ° C and 35 °C. Further, the hybrids demonstrated capacity to generate pleasant fruity volatile compounds like the esters ethyl pentanoate (red apple and melon flavours), ethyl hexanoate (apple and aniseed), ethyl cinnamate (fruity) and phenethyl acetate (sweet, honey and floral) that was produced above the threshold only by the hybrids at the temperature utilized in the fermentation tests (25 °C) along with ethyl butanoate (pineapple, mango, tropical fruit), diethyl succinate (fruity) and ethyl decanoate (brandy, fruity and grape) that was also produced in perceptible levels by the parental strains. The results of this research indicate that the Voss Kveik strain (*S. cerevisae*) could serve as chassis for generation of new interspecific hybrids and a possibility for a broad application of the characterized KKH1-KKH3 in the industry, including those of bioethanol, wine and beer.

1. Introdução

1.1 Breve histórico e importância da cerveja

A possibilidade de estabelecer-se em localidades que permitiam o cultivo de alimentos transformou o modo de vida do homem no período do Neolítico, que migrou do nomadismo para o sedentarismo. Foi no Crescente Fértil que grãos como o trigo e a cevada passaram a ser plantados e estocados para a alimentação de povos residentes no que antes correspondia ao Antigo Egito e a Mesopotâmia. Além de compor a base para a fabricação de pão, esses ingredientes também eram utilizados para a produção de cerveja, bebida que resulta da fermentação dos açúcares extraídos dessas matérias-primas (HORNESEY, 2003).

A cerveja apresenta valores nutricionais, socioeconômicos e políticos desde sua origem, sendo indicadora de complexidade social em sociedades antigas (HORNESEY, 2003). Além de importante fonte nutricional, também contribuiu para a reorganização da agricultura, mobilização de trabalho, distribuição centralizada e como símbolos de poder das elites detentoras dos poderes políticos, econômicos e religiosos (JOFFE, 1998).

Por se tratar de uma bebida segura do ponto de vista microbiológico, devido ao seu processo de fabricação que envolve a fervura, a adição de ervas com potencial bacteriostático como *gruit* e lúpulo, baixos valores de pH (próximos de 5,0), etanol e gás carbônico, a cerveja é considerada uma importante fonte de água potável ao longo de toda Idade Média. Nesse período, o consumo de água era a principal via de transmissão de doenças, já que não havia saneamento básico, o que causava a contaminação dos lençóis freáticos (UNGER, 2004).

Atualmente, a produção de cerveja compõe uma significativa parcela na economia mundial e geração de empregos, com produção de 1,94 bilhões de hectolitros registrados no ano de 2018 (https://www.statista.com/statistics/270275/worldwide-beer-production/). No contexto mundial, o Brasil é o terceiro maior fabricante mundial de cervejas, com produção de aproximadamente 140 milhões de hectolitros registrados no ano de 2016, perdendo apenas para a líder China (460 milhões de hectolitros) e EUA (221 milhões de hectolitros). Com esses valores, o Brasil apresenta maior produção do que países relevantes no cenário cervejeiro mundial como Alemanha (95 milhões de hectolitros) e Rússia (78 milhões de hectolitros) (BARTH-HASS, 2016; CERVBRASIL, 2016). Além disso, o número de cervejarias no Brasil vem aumentando exponencialmente

nos últimos dez anos, apresentando, no ano de 2018, cerca de 889 cervejarias (MARCUSSO, 2018).

1.2 Cerveja: matérias-primas e processo

A cerveja é composta, principalmente, por quatro ingredientes, que são: água, malte, lúpulo e levedura. A água compõe cerca de 95% das cervejas (sendo o restante composto majoritariamente por etanol) e precisa atender determinadas características físico-químicas (composição salina, pH, dureza e alcalinidade) e microbiológicas (ausência de enterobactérias) para ser utilizada na fabricação cervejeira, podendo sofrer ajustes como adição de sais e ácidos, destilação, osmose reversa, entre outros processos usados na cervejaria (KUNZE, 2014).

O malte, por definição, é o resultado da modificação sofrida por cereais como a cevada, o trigo e a aveia durante o processo de malteação. A Malteação subdivide-se, basicamente, em 3 etapas: maceração, germinação e secagem. Durante a maceração, o grão é umedecido, induzindo sua germinação. Nesse passo, ocorre a ativação dos mecanismos de crescimento do grão, em especial, a ativação de α- e β-amilases que degradam o amido presente no endosperma em carboidratos de menor peso molecular, como glicose, frutose, maltose e maltotriose e dextrinas. Além disso, há a indução de proteases que degradam proteínas presentes no grão, gerando aminoácidos e peptídeos. Os carboidratos serão subsequentemente fermentados parcial ou totalmente no processo de fermentação, utilizando, para tanto, aminoácidos, peptídeos, compostos nitrogenados, íons metálicos, vitaminas e lipídeos oriundos do malte. Durante o processo de secagem, a germinação do grão é interrompida a temperaturas que variam de aproximadamente 20 °C a 85 °C. A temperatura de secagem interfere diretamente nas características organolépticas do malte, de modo que temperaturas baixas favorecem o desenvolvimento de maltes claros, como, por exemplo, o malte Pilsen e temperaturas altas, a produção de maltes de coloração mais escura, como no processo de produção de maltes torrados. A malteação é imprescindível para a fase quente do processo de fabricação cervejeira, denominada brassagem (descrição abaixo), etapa em que o amido tem sua conversão em carboidratos fermentescíveis a partir das amilases desenvolvidas na germinação. (KUNZE, 2014).

O lúpulo (*Humulus lupulus*) é uma planta utilizada na cerveja para conferir amargor, aroma e sabor às cervejas, e por apresentar características bacteriostáticas, conservantes

e antioxidantes. Sua implementação ocorreu apenas no século IX da Era Comum (EC) como alternativa ao *gruit*, mistura de ervas utilizada antes do lúpulo (HORNESEY, 2003). O lúpulo apresenta três grupos de compostos essenciais à cerveja: (i) resinas, (ii) óleos essenciais e (iii) polifenóis. As resinas são subdivididas em macias e duras, com destaque para as macias que contém α- e β-ácidos. Desses, os principais são os α-ácidos (humulona, cohumulona e adhumulona), compostos que são isomerizados durante a fervura em iso-humulona, iso-cohumulona e iso-adhumulona, compondo a maior parcela do amargor da cerveja. Os óleos essenciais compõem a porção volátil do lúpulo e são divididos em hidrocarbonetos, compostos oxigenados e sulfurados. Por serem facilmente volatizados, sua principal função na cerveja é fornecer aroma e sabor que podem remeter a notas cítricas, florais, resinosas, frutadas, terrosas, entre outras. Estima-se que exista aproximadamente 1000 óleos essenciais no lúpulo, sendo que desses, mais ou menos 440 já foram descritos. Os polifenóis, por sua vez, são importantes para a estabilidade coloidal da cerveja, uma vez que se ligam a proteínas em baixas temperaturas, formando a turvação a frio (*chill haze*) (ALMAGUER *et al*., 2014).

Por fim, as leveduras são os agentes responsáveis pela fermentação cervejeira e pelo fato de serem organismos microscópicos unicelulares, sua origem era desconhecida até o século XVIII EC (BOULTON & QUAIN, 2001). Nesse sentido, as informações sobre leveduras e as suas características que as tornam únicas para a fabricação de cervejeira serão aprofundadas no item 1.3.

Resumidamente, a fabricação de cervejas subdivide-se, em duas principais etapas: fase quente, onde ocorre a produção de mosto (brasagem) e fria, etapa de fermentação do mosto e posterior maturação (Figura 1). A fabricação de mosto (solução aquosa composta majoritariamente por açúcares simples extraídos do malte) tem seu início na etapa da mosturação. Durante esse passo, que se dá pela mistura de água quente (aproximadamente 65 °C) e malte moído, ocorre a degradação de proteínas pela ação das proteases e de amido pelas enzimas desenvolvidas durante a malteação, em especial α-amilase e β-amilase, resultando em carboidratos fermentescíveis como glicose, maltose e maltotriose e não fermentescíveis, denominados dextrinas. Posteriormente, no processo de clarificação, essa mistura de grãos e água é recirculada por meio da utilização de uma bomba, e o mosto filtrado é então fervido, momento onde ocorre a adição do lúpulo. Após a fervura, o mosto é resfriado e separado do aglomerado de proteínas coaguladas (*trub*) e ocorre a oxigenação do mosto, assim como o inóculo de leveduras. Durante a

fermentação, os açúcares dissolvidos no mosto são metabolizados a etanol, gás carbônico e compostos aromáticos, diminuindo a densidade até o momento onde ela é estabilizada. A partir desse momento, inicia-se a maturação, processo que ocorre a baixas temperaturas e é responsável pela estabilidade coloidal e aprimoramento das características organolépticas da cerveja (KUNZE, 2014).

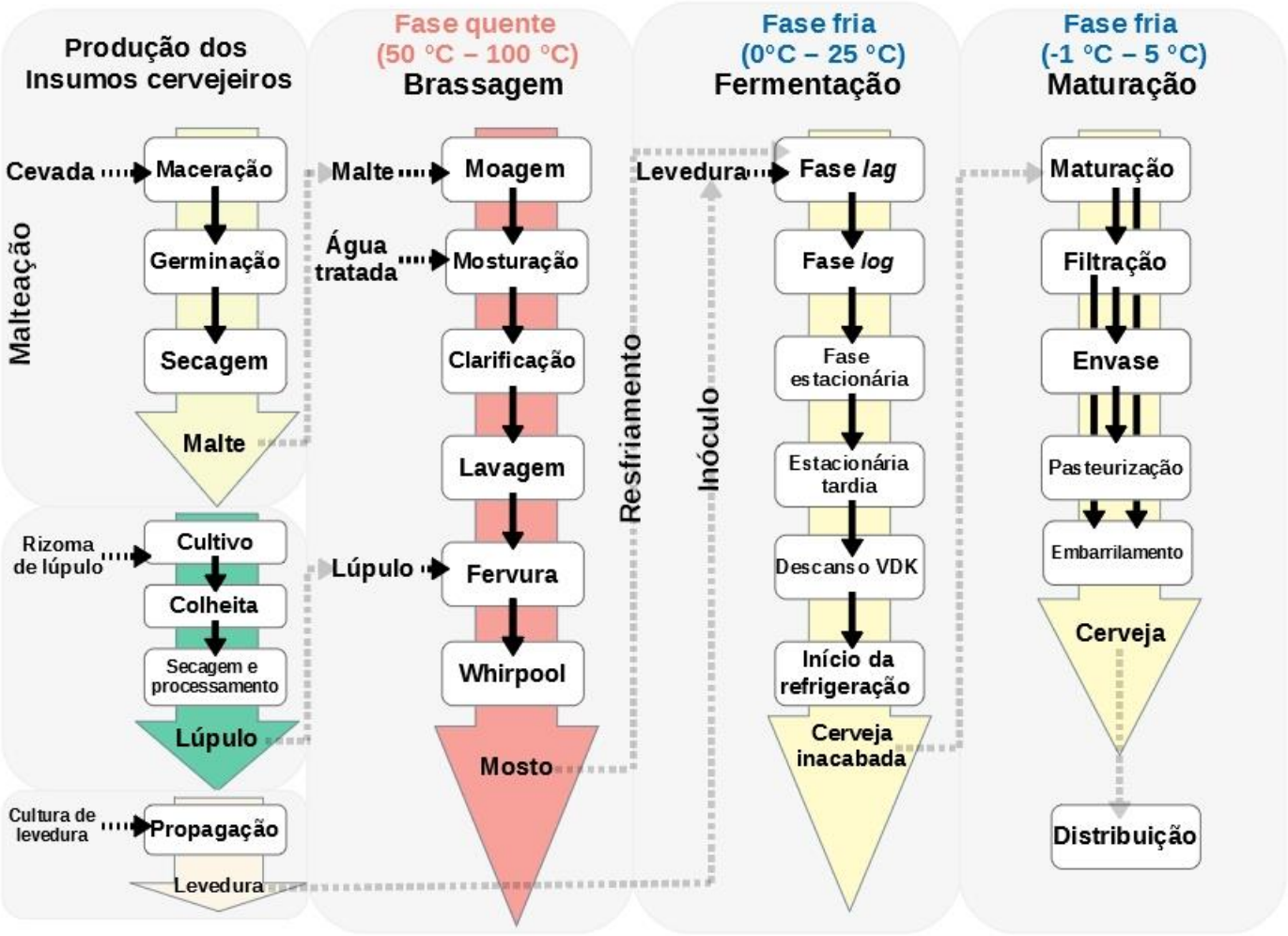

Figura 1. Fluxograma do processo convencional de fabricação cervejeira.

1.3 Leveduras cervejeiras: cepas disponíveis e a busca por alternativas

A domesticação de leveduras cervejeiras está diretamente atrelada à atividade humana. Aparentemente, bateladas que agradavam ao cervejeiro e aos consumidores tinham suas leveduras reutilizadas e, por vezes, compartilhadas, ao passo que lotes desagradáveis resultaram no descarte do fermento. Esse processo de domesticação, que vem ocorrendo ao longo de milhares de anos, está, também, intimamente associado ao fato de que fermentações espontâneas são geralmente demoradas, inconsistentes e ineficientes, resultando em produtos fermentados incompletos (muitos açúcares remanescentes após a fermentação, tornando a cerveja excessivamente doce), com aromas indesejáveis (os denominados *off-flavours*, que podem remeter a aromas como vômito, no caso do ácido isobutírico). Cabe ressaltar que existem cervejarias (como aquelas que produzem, por exemplo, *lambics* e *gueuzes*) que aprimoraram esse método de fermentação com leveduras e bactérias selvagens e continuam executando-o até os dias atuais. (BOKULICH *et al*., 2012; STEENSELS & VERSTREPEN, 2014 GALLONE *et al*., 2016). Nesse sentido, em geral, parece haver existido uma preferência na seleção de traços fenotípicos específicos para a fabricação cervejeira, favorecendo aquelas leveduras que: (i) apresentavam altas taxas de atenuação, ou seja, elevada capacidade de assimilação e fermentação de carboidratos simples (como os monossacarídeos glicose e frutose e dissacarídeos como a sacarose e maltose) e complexos (como o trissacarídeo maltotriose e, em alguns casos, dextrinas, como maltotetraose e moléculas com mais de 4 monômeros de glicose) em decorrência da duplicação dos genes *MAL*, (ii) ausência na formação de compostos fenólicos, em especial 4-vinilguaiacol, devido a mutações nos genes *PAD1* e *FDC1* (há exceções à regra onde estilos de cerveja requerem esses compostos, como cervejas de trigo do estilo Weiss e Witbier, por exemplo) e (iii) capacidade de flocular, que favorece o agrupamento das leveduras, clarificando naturalmente a cerveja e facilitando a retirada da biomassa de fermento para posterior utilização (MCMURROUGH *et al*., 1996; BROWN *et al*., 2010; STEENSELS & VERSTREPEN, 2014; GALLONE *et al*., 2016; GONÇALVES *et al*., 2016).

Sabe-se que as atuais cepas de leveduras cervejeiras disponíveis para indústria originaram-se de inúmeras cepas ancestrais, formando os seguintes clados: *Beer 1*, que contém a maioria das leveduras provenientes da Alemanha, Inglaterra e Estados Unidos e *Beer 2*, que contém cepas relacionadas às leveduras viníferas e não apresentam uma estrutura geográfica bem definida (GALLONE *et al*., 2016).

Atualmente, figuram como principais leveduras disponíveis para o mercado cervejeiro, as cepas pertencentes às espécies *Saccharomyces cerevisiae* e *Saccharomyces pastorianus. S. cerevisiae* é a levedura utilizada para fermentar as cervejas de alta fermentação, ou *ales*. Por outro lado, *S. pastorianus*, é responsável pela fermentação de cervejas de baixa fermentação, ou *lagers*. As cepas que pertencem à família *ale* contém maior número de exemplares, assim como uma maior diversidade de traços fenotípicos como formação de ésteres, compostos fenólicos e sulfurados, cepas com alta e baixa taxa de atenuação, ácidos orgânicos, álcoois superiores, entre outros (WHITE & ZAINASHEFF, 2010). Embora também existem inúmeras cepas de leveduras *lager*, estas apresentam menos cepas popularizadas para utilização na fabricação de cervejas, assim como menos traços fenotípicos descritos e observados. Suas principais representantes estão presentes nos dois principais grupos de *S. pastorianus*: *Saaz* e *Frohberg.* Em contrapartida, é a levedura mais usada atualmente (90% da produção mundial de cervejas utiliza essa espécie como agente fermentativo), uma vez que é responsável pela fermentação das cervejas de massa, mais conhecidas como *Pilsen* ou *Standard Lite Lager*. Além das cervejas massificadas, *S. pastorianu*s também é empregada na fermentação de cervejas artesanais, englobando uma série de estilos, como, por exemplo, Bock e Baltic Porter (WHITE & ZAINASHEFF, 2010).

Embora, juntas, *S. cerevisiae* e *S. pastorianus* apresentem uma ampla variedade de cepas atualmente disponíveis para o mercado cervejeiro, existe a demanda por microrganismos que apresentem características distintas das presentes nessas variedades, visando o desenvolvimento de novas cervejas (KROGERUS *et al.*, 2015; KROGERUS, 2017). Aplica-se a essa prospecção o recente uso de microrganismos semi-domesticados ou selvagens (não convencionais), como as espécies pertencentes ao gênero *Brettanomyces (Dekkera)* (vide apêndice): *B. anomalus, B. bruxellensis, B. custersianus, B. nanus* e *B. naardenensis,* além de *Torulaspora delbrueckii*, *Candida shehatae*, *Candida tropicalis*, *Saccharomycodes ludwigii*, *Zygosaccharomyces rouxi*, *Pichia kluyverii*, entre outros (MICHEL *et al*., 2016).

Por outro lado, a construção de novas cepas em laboratório também é uma alternativa para a geração de leveduras diferenciadas para a indústria. Nesse sentido, existem distintas técnicas moleculares, as quais incluem a geração de organismos geneticamente modificados (OGM) (DONALIES *et al.*, 2008). Entretanto, a legislação não permite a utilização de tais organismos na indústria cervejeira, além disso, outro fator

impeditivo do uso de organismos OGM é a baixa aceitação do público (SAERENS, DUONG & NEVOIGT, 2010). Assim sendo, técnicas capazes de gerar cepas que não envolvam engenharia genética tem se mostrado valiosas no que diz respeito à geração de novas alternativas de leveduras cervejeiras, assim como aceitação e utilização por parte das indústrias e do consumidor. Nesse sentido, destacam-se os métodos de hibridização interespecífica por formação de esporos e hibridização rara (KROGERUS *et al.*, 2017).

1.4 Surgimento de híbridos na natureza e aplicação em laboratório

Os estudos recentes demonstraram que algumas cepas cervejeiras agrupadas nas espécies *S. cerevisiae* e *S. pastorianus* são, ao contrário do que se pensava, híbridos interespecíficos. O exemplo mais significativo reside na descoberta de que os dois grupos de *S. pastorianus, Saaz* e *Frohberg*, são híbridos compostos por *S. eubayanus* e *S. cerevisiae* (KROGERUS *et al*., 2015). Além disso, cepas incluídas na espécie *S. cerevisiae*, como aquelas responsáveis pelas fermentações de cervejas de abadia ou trapistas são, na verdade, híbridos de *S. cerevisiae* e *S. kudriavzevii* (GONZÁLEZ *et al*., 2008).

O surgimento e estabelecimento de híbridos no ambiente cervejeiro provavelmente ocorre devido a vantagens adaptativas dos híbridos quando comparados aos seus progenitores. Nesse sentido, a criotolerância de *S. eubayanus* e a capacidade de metabolizar açúcares complexos, como a maltotriose de *S. cerevisiae*, possivelmente conferiram a *S. pastorianus* benefícios nos ambientes fermentativos que deram origem às cervejas *lager* (KROGERUS *et al*., 2015). Da mesma maneira, *S. kudriavzevii* parece ter incorporado aos híbridos de *S. cerevisiae* e *S. kudriavzevii* o recurso de metabolismo a baixas temperaturas, importante para a fermentação em regiões temperadas da Europa (GONZÁLEZ *et al.*, 2008).

A descoberta da origem de algumas cepas como sendo híbridas gerou subsídios para recriação dessas espécies em laboratório, tornando-se uma importante ferramenta biotecnológica na construção de cepas cervejeiras alternativas e não-GMO (KROGERUS *et al.*, 2017). Além disso, auxiliou na compreensão das complexas origens evolutivas de diferentes espécies cervejeiras, caso das leveduras *lager*. Nesse ponto de vista, pesquisadores geraram novas cepas de *S. pastorianus* com características de rápida fermentação, alta atenuação, produção de aromas frutados complexos e agradáveis, além de tolerância aos diferentes estresses impostos no ambiente cervejeiro, que apresentaram

performances melhores que as cepas empregadas atualmente na indústria (MERTENS *et al.*, 2015).

Uma vez estabelecidos os métodos de hibridização interespecíficas em laboratório, tornou-se possível extrapolar para outras cepas e espécies de leveduras, visando a aplicação na indústria cervejeira. Dessa forma, foram testadas hibridizações a partir de diversas cepas parentais, como *S. cerevisiae* (*ale*) x S. cerevisiae (*sake*), S. cerevisiae (*ale*) x *S. cerevisiae* var. *diastaticus*, *S. cerevisiae* (*ale*) x *S. bayanus* (criotolerante), *S. cerevisiae* (*ale*) x *S. cerevisiae* (vinífera) x *S. eubayanus*, entre outros (KROGERUS, 2017). O resultado desses cruzamentos foi a geração de inúmeros híbridos com recursos fermentativos de interesse industrial, como a maior formação de compostos aromáticos (ésteres, fenólicos, ácidos orgânicos e álcoois superiores), alta floculação, alta taxa de atenuação, termotolerância e capacidade de lidar com ambientes indutores de estresse (baixos pHs e altas concentrações etanol, por exemplo) (KROGERUS, 2017).

1.5 Cervejas de fazenda norueguesas (*Norwegian farmhouse ales*) e suas leveduras (Kveiks)

Embora a domesticação de leveduras cervejeiras tenha sido encorajada e efetuada mais rapidamente devido à produção massificada de cervejas, a cultura de compartilhamento de biomassa de leveduras também era uma prática comum desde antes da industrialização, especialmente no que diz respeito às *Farmhouse Ales*, mais conhecidas na região norte da Bélgica, com o estilo *Saison* e também presentes na região da Noruega, compondo as fermentações das *Norwegian farmhouse ales* (como as representantes *maltøl*, *kornøl* e *vossaøl*) (NORDLAND, 1969; MARKOWSKI, 2004; PREISS *et al*., 2018). A produção de *farmhouse ales* perdeu força a partir do século XIX em decorrência de melhorias no transporte e especialização econômica. Com isso, as leveduras Kveiks utilizadas para a produção das *Norwegian farmhouse ales* foram parcialmente substituídas por aquelas comercializadas, fazendo com que muitas linhagens dessas leveduras desaparecessem (NORDLAND, 1969). Em contrapartida, muitas cervejarias da região oeste da Noruega mantiveram a tradição de produção de *farmhouse ales* com as leveduras Kveiks.

As *Norwegian farmhouse ales* tem despertado interesse da indústria cervejeira devido à biodiversidade de leveduras empregada em suas fermentações e às particularidades do processo de fabricação de mosto (como a utilização de zimbro, por

exemplo) e inóculo das leveduras e fermentações a altas temperaturas (caracteristicamente de 28 °C a 40 °C), apresentando variações significativas (Figura 2) em relação a outras escolas cervejeiras, que empregam os métodos descritos acima (GARSHOL & PREISS, 2018). Embora pouco se conheça sobre as leveduras Kveiks, uma pesquisa recente utilizou as ferramentas de biologia molecular PCR interdelta com os primers δ12/21 e δ2/12 e posterior *fingerprinting*, além de sequenciamento de DNA dos seus principais representantes e caracterização fenotípica das cepas em mosto cervejeiro. Os dados dessa pesquisa indicam que Kveiks (i) apresentam traços de domesticação, (ii) são um grupo geneticamente inter-relacionado, (iii) formam um grupo filogeneticamente distinto das demais leveduras cervejeiras (Figura 3), com espécies que incluem *S. cerevisiae* e um híbrido interespecífico (*S. cerevisiae/eubayanus/uvarum*), (iv) contém características positivas do ponto de vista de biossíntese de compostos aromáticos e de sabor como ésteres e (v) apresentam tolerância aos diversos estresses fermentativos (como o térmico, demonstrando crescimento até 43 °C e o etanólico, com desenvolvimento em ambientes com até 16 % (v/v) de etanol). Essas características as tornam potenciais leveduras para uma gama de aplicações industriais, como as indústrias vinícola, a de destilados e, em especial, a cervejeira (NORDLAND, 1969; RASMUSSEN, 2016; PREISS *et al.*, 2018).

Em relação às características moleculares, a pesquisa de Preiss *et. al* demonstrou que as diferentes cepas sequenciadas (representantes de 6 grupos de Kveiks) são tetraploides, onde 4/6 apresentam aneuploidias e 3/6, uma cópia adicional do cromossomo IX. Ainda, as Kveiks apresentam altos níveis de heterozigosidade, com números de SNPs que variam de ~54.000 a 68.000. Além disso, apresentam viabilidade de esporos acima de 40 % para a maioria de suas representantes que tiverem o DNA sequenciado, como Granvin 1, Hornindal 1, Hornindal 2, Laerdal 2 e Voss 1, à exceção de Stordal Ebbegarden 1, que apresentou 6,9 % de viabilidade de esporos (PREISS *et al.*, 2018). Mutações pontuais (SNPs) nos genes *PAD1* e *FDC1* também foram demonstrados nas seis cepas sequenciadas, o que explica a perda de função das enzimas codificadas por esses genes (cinamato descarboxilase) e que é responsável pela formação do composto fenólico 4-vinilguaiacol. Amplificações no *loci MAL3x* (*MAL31* permease, *MAL32* maltase, os fatores de transcrição *MAL33* e *YPR195W*), explicam, por sua vez, a capacidade das Kveiks fermentarem os açúcares maltose e maltotriose, atenuando o mosto cervejeiro até valores próximos de 90 %.

Culturalmente, as leveduras Kveiks são armazenadas em anéis de madeira (anéis de Kveik) que são embebidos em cerveja durante a fermentação e posteriormente secos sendo, portanto, usados para estocá-las por períodos de até um ano (NORDLAND, 1969; RASMUSSEN, 2016). Cabe ressaltar que as culturas de Kveiks são normalmente uma mistura de mais de uma cepa. Isso implica que muitas características de cepas diferentes podem ser combinadas ao longo do processo de fermentação, por exemplo, cepas que apresentam baixa floculação podem ser "compensadas" por aquelas altamente floculantes (PREISS *et al*., 2018). As fermentações caracterizam-se por serem rápidas (24-48 horas) mesmo com densidades originais altas (~1,080 kg/cm³/19,25 °P), gerarem compostos aromáticos agradáveis, sobretudo ésteres frutados, como acetato de etila, propil acetato e decanoato de etila, apresentarem altas taxas de atenuação e um espectro de floculação de baixa a elevada (GARSHOL, 2014; GARSHOL, 2015; GARSHOL, 2016; RASMUSSEN, 2016; PREISS *et al*., 2018).
Entre as espécies identificadas e isoladas nos anéis de Kveik destacam-se as cepas de *S. cerevisiae*, que vem sendo utilizadas, especialmente, por microcervejarias e cervejeiros caseiros (GARSHOL & PREISS, 2018).

Os recursos apresentados por cepas de *S. cerevisiae* como *Sigmund's Voss* Kveik (The Yeast Bay), isolada a partir da *farmhouse ales* produzidas na cidade de Gjernes, na região oeste da Noruega, incluem fermentações rápidas, altas taxas de atenuação (78-83%), notas terrosas e de especiarias, acidez acentuada e perfil esterificado único que remete a laranja. No que diz respeito à floculação, dependendo das condições (pH, densidade, temperatura do mosto e número de células) essa cepa é capaz de formar grandes flocos, notavelmente maiores que outras cepas cervejeiras de *S. cerevisiae*, e assim mesmo, mantém altas capacidades atenuativas, diferente do que costuma-se observar em variedades altamente floculantes. Além disso apresenta uma extensa faixa de temperaturas de fermentação (20 °C – 38 °C) sem que altere drasticamente o perfil sensorial da cerveja, ou seja, não forma compostos desagradáveis como álcoois superiores, como o isobutanol, e compostos fenólicos, como o 4-vinilguaiacol, favorecendo-se de temperaturas mais altas, uma vez que resulta em maiores taxas de atenuação e formação de ésteres (acessado em http://www.theyeastbay.com/brewers-yeast-products/sigmunds-voss-kveik).

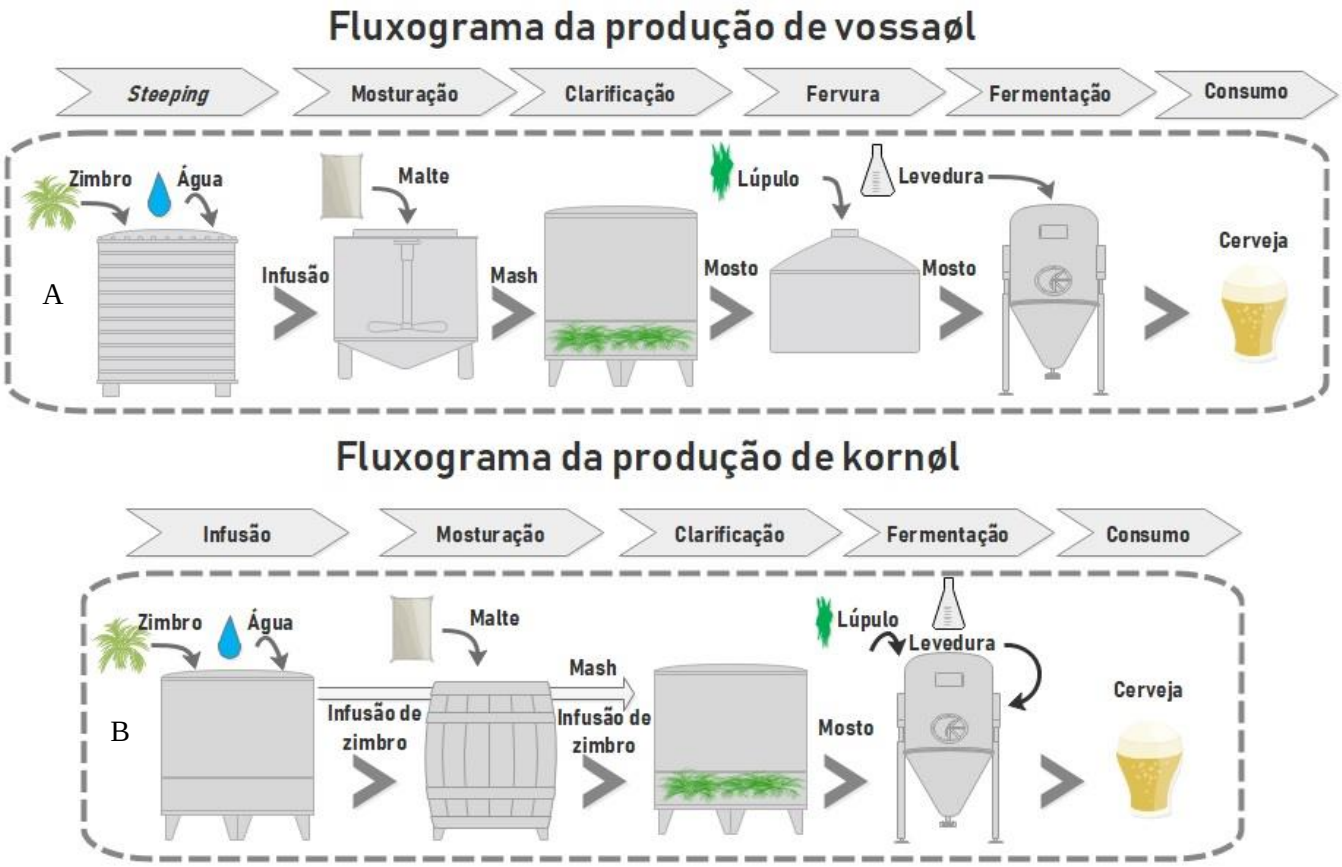

Figura 2. Fluxograma do processo de produção das *Norwegian farmhouse ales* (A. vossaøl e B. kornøl), exemplificando diferenças fundamentais em comparação com o processo tradicional de fabricação cervejeira.

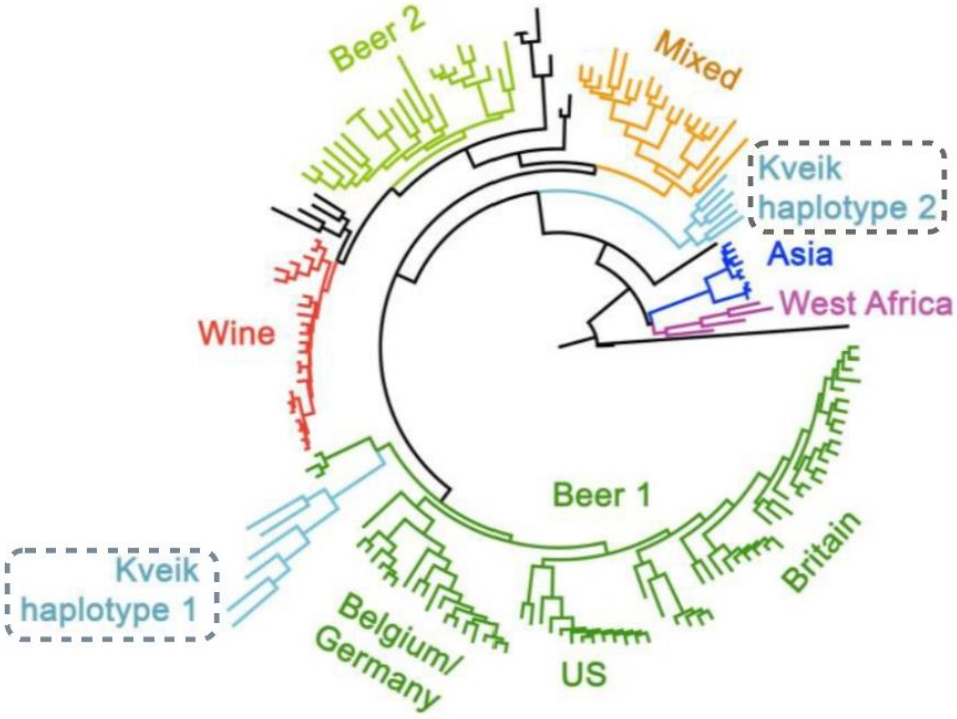

Figura 3. Dendograma simplificado demonstrando a posição das leveduras Kveiks em perspectiva com as outras leveduras cervejeiras domesticadas. Imagem adaptada de GARSHOL, L. M. & PREISS, R. How to brew with Kveik. *Master Brewers Association of the Americas*, 55 (4): 76-83, 2018.

2. Objetivos

2.1 Objetivo geral

Gerar novas cepas híbridas cervejeiras entre *Saccharomyces cerevisiae* provenientes de *Norwegian farmhouse ales* (Kveik) e *Saccharomyces kudriavzevii*.

2.2 Objetivos específicos

1. Gerar cepas híbridas de *S. cerevisiae* (Kveik) e *S. kudriavzevii* pelo método de hibridização rara.
2. Confirmar a ocorrência das hibridizações pelos métodos de análises fenotípicas por crescimento em meio mínimo e com o uso de ferramentas de biologia molecular, tais como PCR, RFLP e sequenciamento da região ITS1/ITS2.
3. Avaliar a formação de compostos aromáticos e capacidade atenuativa dos híbridos em mosto cervejeiro lupulado de pequena escala.

3. Resultados

Farmhouse ale yeast strains from Belgium and Norway as chassis for interspecific hybridizations

Artigo em fase inicial de redação

Farmhouse ale yeast strains from Belgium and Norway as chassis for interspecific hybridizations

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Abstract

The utilization of alternative interspecific brewing yeasts is growing in response to new industry demands such as fast fermentation and broad variety of pleasant volatile compounds production. In the present work, we generated six unique hybrids between cold and stress-tolerant wine and beer-associated *S. kudriavzevii* and two strains of heat and stress-tolerant *S. cerevisiae* isolated from farmhouse brewing-related environments, Walloon (Belgium) and Voss (Norway). All hybrids display an accumulation of the parental characteristics like good performance at low and high temperatures, fermenting complex carbohydrates from malt-derived brewing wort and reaching high attenuation apparent degree, sometimes fermenting faster than the parental strains, in an indication of heterosis. Tests in beer wort exhibited final pH values expected for regular beers after fermentation in the temperatures of 17 °C and 25 °C. Further, it was identified in beer fermented with the hybrids, pleasant fruity volatile compounds like the esters ethyl pentanoate (red apple and melon), ethyl hexanoate (apple and aniseed), ethyl cinnamate (fruity) and phenethyl acetate (sweet, honey and floral), compounds that were produced above the threshold exclusively by hybrids at the temperature utilized in the fermentation tests (25 °C) along with other compounds such ethyl butanoate (pineapple, mango, tropical fruit), diethyl succinate (fruity) and ethyl decanoate (brandy, fruity and grape) that was also produced in perceptible levels by the parental strains. The results of this research indicate a possibility for a broad application of KKH1-KKH3 and KWH1-KWH3 hybrids in the industry, including those of bioethanol, wine and beer.

Introduction

The diversity of fermented beverages currently available reflects the nutritional resources that early humans had (e.g. wheat and barley) along with the indigenous microorganisms that had eventually fermented sugars from these grains (yeasts and bacteria). The domestication process of yeast is strictly associated with the diversity of fermented beverages. In this sense, beer is a beverage with a high variety of yeast associated with its fermentation, and is highly appreciated and consumed worldwide. Two major species are used in brewing industry, *Saccharomyces cerevisiae* (top-fermented, ales) and *Saccharomyces pastorianus* (bottom-fermented, lagers). Two examples of farmhouse-shared yeasts are those responsible for fermentation of Belgian Saison originated in the region of Wallonia, Belgium, and Kveik yeast, which is responsible for the fermentation of the Norwegian farmhouse ales (Markowski, 2004; Preiss, Tyrawa, Krogerus, Garshol, and Van Der Merwe, 2018; Preiss *et al*., 2017; Rasmussen, 2016). In a recent work, Kveiks were considered a distinct group of domesticated *S. cerevisiae* brewing yeast, and many strains were identified as interspecific hybrids (Gallone et al., 2016; Garshol and Preiss, 2018; Preiss *et al*., 2018, 2017). The main example is the yeasts responsible for the most worldwide consumed beer style Lite Lager *S. pastorianus* which is a hybrid between *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* (De Barros Lopes, Bellon, Shirley and Ganter, 2002; Dunn and Sherlock, 2008; Libkind *et al*., 2011). Moreover, it was found that some of the Belgian Trappist yeast strains are hybrids between *S. cerevisiae* and *Saccharomyces kudriavzevii* (González, Barrio and Querol, 2008). Furthermore, in brewing, it was recently found a Kveik hybrid yeast among *S. cerevisiae* and *Saccharomyces uvarum* (Krogerus, Preiss and Gibson, 2018). It is known that hybrids use to perform better than the parental strains in brewing environment, a condition termed "heterosis" (Krogerus, 2017). In heterosis, hybrids aggregate resources from both parental species, resulting in a strain with more increased adaptability. For example, lager yeast *S. pastorianus* thrive in low temperature fermentations (*S. eubayanus*-linked feature) and, at the same time, has a good capacity in fermenting mono-, di-, and tri-saccharides (*S. cerevisiae*-linked resource). Similarly, some Trappist Belgian yeast strains display cold-tolerance capacity due to effective protein translation and stress-related mechanisms from *S. kudriavzevii* along with fermentation of complex sugars from *S. cerevisiae.* In the present study, we have constructed interspecific hybrids between *S. cerevisiae* strains from farmhouse-associated brewing (Norwegian farmhouse

ale and Belgian Saison) and *S. kudriavzevii* type strain. Looking for relevant traits towards brewing industry, we performed fermentation tests of six chosen hybrids in hopped 12 °P beer wort provided from a local brewery. Parameters such as fermentation capacity (% attenuation rate), temperature range (15-35 °C), generation of main beer-associated volatile compounds (alcohols, vicinal diketone, acetaldehyde and esters) and final pH indicates a promising application of this set of unique hybrids in the brewing industry.

Material and Methods

Yeast strains

The *S. cerevisiae* yeast strains used in this work was chosen for its fermentative characteristics (high attenuation degree and biosynthesis of pleasant esters and phenols) and *S. kudriavzevii* for its cryotolerance and biosynthesis of phenols. The yeasts are summarized in Table 1. The cultures are stored in -80 ° C freezer in 15% (v/v) liquid medium with glycerol and YPMG (3 g. L-1 yeast extract, 3 g. L-1 malt extract, 5 g. L-1 peptone and 10 g. L-1 glucose).

Table 1. Yeast strains used in this work.

Strain[a]	Specie	Source
NCYC 2889^{T}	*Saccharomyces kudriavzevii*	National Collection of Yeast Cultures (NCYC™), London, UK
Voss Kveik	*Saccharomyces cerevisiae*	The Yeast Bay™, San Leandro, EUA
Wallonian Farmhouse Ale III	*Saccharomyces cerevisiae*	The Yeast Bay™, San Leandro, EUA
KKH1, KKH2 and KKH3	*S. kudriavzevii x S. cerevisiae (Voss Kveik)*	This study
KWH1, KWH2 and KWH3	*S. kudriavzevii x S. cerevisiae (Wallonian Farmhouse Ale III)*	This study

[a]T = type strain

Design of interspecific hybrids by rare hybridization

Natural auxotrophic mutants (*lys*- and *ura*-) of the parental strains were selected on α-aminoadipic and 5-fluoroorotic acid agar plates, respectively as described by (Boeke, Trueheart, Natsoulis and Fink, 1987). Auxotrophy was confirmed by the inability to grow on minimal selection agar medium (0.67% yeast nitrogen base without amino acids, 3% glycerol, 3% ethanol and 2% agar). A suspension of vegetative cells from *S. kudriavzevii ura-*, *S. cerevisiae* WLNIII and *S. cerevisiae* Voss Kveik lys- was grown overnight at 25 °C by inoculating a single colony into 50 mL of YPM. The culture was centrifuged at 5000×*g* for 5 min, after which the cells were first washed once and then resuspended in sterile H_2O to a concentration of 10 g centrifuged wet yeast mass L^{-1}. One hundred µl of the resulting suspensions from both parental strains, with complementary auxotrophic markers, were transferred together to 1 ml YPM medium in a sterile 2 ml Eppendorf tube. The tubes were vortexed and incubated statically at 25 °C for 7 days. After incubation, the tubes were centrifuged at 5,000×g for 5 min and the supernatant was removed. 500 µl of starvation medium (0.1 % yeast extract and 0.1 % glucose) was added, and tubes were incubated for at least 2 h at room temperature. Tubes were then vortexed and 100 µl aliquots were spread onto minimal selection agar (without uracil or lysine). Plates were incubated at 25 °C and prototrophic colonies appeared after 3–7 days.

Hybrid analysis by PCR and RFLP

Genomic DNA was extracted using the YeaStar™ Genomic DNA kit (Zymo Research Corporation, Irvine, CA, USA) following the manufacturer's recommendations. DNA concentrations were measured on a NanoDrop 2000 spectrophotometer (wavelength 260 nm) (Thermo Scientific, Wilmington, DE, USA). DNA purification was made using Monarch® Genomic DNA Purification Kit (New England Biolabs Inc, Ipswich, Massachusetts, USA) following the manufacturer's recommendations. The extracted genome was used to confirm the hybridization status by amplification of rDNA-PCR (ITS1 and ITS2) using the primers ITS1 (5′- TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′), and digestion of amplicons using the *HaeIII* restriction enzyme (New England BioLabs, USA) as described previously (Pham *et al*., 2011). The six hybrid strains (from *S. kudriavzevii* X WLNIII and from *S. kudriavzevii* x Voss Kveik) that were chosen for further characterization were named KKH1, KKH2 and KKH3 (*S. kudriavzevii* x Voss Kveik hybrids) and KWH1, KWH2 and KWH3 (*S. kudriavzevii* X WLNIII hybrids), showed in Table 1.

ITS sequencing and assembling

The internally transcribed spacer (ITS) regions of the yeast strains were amplified using ITS1 and ITS4 primers (Pham *et al*., 2011). Sequencing of samples was performed by ACTGene Análises Moleculares Ltd. (Center for Biotechnology, UFRGS, Porto Alegre, RS, Brazil) using the automatic sequencer AB 3500 Genetic Analyzer equipped with 50 cm capillaries and POP7 polymer (Applied Biosystems). DNA templates were labeled with 2.5 pmol of the specific primer and 0.5 μL of BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in a final volume of 10 μL. Labeling reactions were performed in a LGC XP Cycler with a initial denaturing step of 96 ºC for 3 min followed by 25 cycles of 96 ºC for 10 sec, 55 ºC for 5 sec and 60 ºC for 4 min. Labeled samples were purified by 75% isopropanol precipitation followed by 60% ethanol rinsing. Precipitated products were suspended in 10 μL Hi-DiTM formamide (Applied Biosystems), denatured at 95 ºC for 5 min, ice-cooled for 5 min and electroinjected in the automatic sequencer. Sequencing data were collected using the software Data Collection 3 (Applied Biosystems) programmed with the following parameters: Dye Set "Z"; Mobility File "KB_3500_POP7_BDTv3.mob"; BioLIMS Project "3500_Project1"; Run Module 1 "FastSeq50_POP7_50cm_cfv_100"; and Analysis Module 1 "BC-3500SR_Seq_FASTA.saz". Resulting Data Collection files (.ab1; electropherograms) were converted into FASTA files (.seq; text) by the Sequence Analysis Software v. 6 (Applied Biosystems) with standard parameters. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 3.25464950 is shown (Figure 3). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1102 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar, Stecher, Li, Knyaz and Tamuka, 2018).

Hybrid selection to further characterization

The hybrid selection criteria was based on a previous fermentation trials in ELISA plates in which 3 mL of 1.044 kg/m^3 malt extract wort with pre-isomerized hop extract Tetrahop (up to 10 IBU) (Barth-Hass) was inoculated with ~10^8 cells/mL of the hybrids KKH1-KKH10 and KWH1-KWH10 (cultivation in 50 mL flasks for 2 days containing YMPG at room temperature of 23ºC). The density was measured daily utilizing a handheld ATC (automatic temperature compensation) portable refractometer. The strains that achieved the higher attenuation level was chosen for further characterization.

Wort preparation and Laboratory Scale Wort Fermentations

The wort used for beer fermentations was obtained from Tupiniquim Brewery, (Porto Alegre, RS, Brazil). The 12 IBU hopped wort was made using Brazilian 2-row malt to an original specific gravity of 1.045 kg/m³. The wort was boiled for 60 minutes prior to use and cooled to the desired temperature utilizing the brewery heat exchanger. Fermentations were performed in 1000 mL fermenters containing 800 mL of wort. Yeast cells were first grown in 50 mL flasks for 2 days containing YMPG at room temperature. These precultures were used to inoculate fermenters equipped with airlocks to maintain anaerobiosis at a density of ~10^8 cells/mL. The temperature was kept constant during the whole length of the fermentation at either 17ºC, 25ºC and 35ºC.

Wort and beer analysis

The specific gravity of samples was determined from the degassed fermentation samples using an Anton Paar Alcohol and Extract Meter Alex 500 (Anton Paar GmbH, Austria). The pH was measured utilizing an AK90 pHmeter (Akso, Sweden). Identification and quantification of volatile yeast-derived flavor compounds were performed on a Perkin Elmer Clarus 680 GC (USA) gas chromatograph equipped with a flame ionization detector (FID) and Clarus GC software. Chromatographic separation was performed using a ZB-WAXplus column (60 m x 0.25 mm x 0.25 µm) from Zebron (USA) and helium gas as a carrier at a flow rate of 1.0 mL.min^{-1}. Temperature programming was performed at an initial oven temperature of 40ºC for 5 minutes, increasing by 2 ºC per minute to 220 ºC. Positive compound identification was performed by comparing the retention time obtained for the sample against the volatile compound standards injected

under the same conditions. Quantification was performed by internal standardization with 3-octanol according to Arcari *et al*. (2017).

Statistical Analysis

Statistical analysis was performed on the fermentation and metabolite data utilizing one-way ANOVA and Tukey's test employing the "stats" package in R (http://www.r-project.org/).

Results and Discussion

Hybrid construction and confirmation

The type strain of *S. kudriavzevii* NCYC2889 (Table 1) was successfully hybridized with Wallonian III and Voss Kveik through the rare mating of *lys*- and *ura*-auxotrophic diploid parental cells, respectively. Hitherto, it was generated 100 possible allotetraploid hybrids in which 50 are *S. kudriavzevii* NCYC2889 x Voss Kveik and 50 S. *kudriavzevii* NCYC2889 x *S. cerevisiae* (Wallonian III) (KKH1-KKH50 and KWH1-KWH50). From the 100 candidates, 20 was already confirmed as hybrids. The confirmation of hybridization was performed utilizing (i) growth in minimal medium, (ii) amplification of the polymorphic ITS1/ITS2 region by PCR with further RFLP in which the amplicon (~880 bp) was digested with *HaeIII* restriction enzyme generating a pattern of ~ 320 bp, ~240, bp, ~180 bp and ~140 bp for *S. cerevisiae*, ~484 bp, ~229 bp, ~123 bp and ~5 bp for *S. kudriavzevii* and for the hybrids, a sum of the parental patterns (figure 1), (iii) sequencing of the ITS1/ITS2 region with further alignment using all the possible hybrids and the parental species. Phylogenetic analysis was conducted in MEGA X (figure 3). *Candida glabrata* (*Nakaseomyces/Candida* clade), *Kluyveromyces lactis* (*Kluyveromyces* clade), and *Cryptococcus neoformans* (*Filobasidiella* clade) were tested as outgroup species. The ITS region was used to confirm species identification due to its status as a barcode gene. The analysis of the sequences showed that the hybrids strains have a higher identity with sequences corresponding to *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii* species rather than the outgroup species. The KKH1-KKH3 and KWH1-KWH3 strains were identified as *S. cerevisiae* x *S. kudriavzevii*-type hybrids based on internally transcribed spacer (ITS) sequencing that differentiates from the original parental ITS sequence.

Laboratory fermentations

For the fermentation tests, it was chosen three interspecific hybrids (KKH1-KKH3) derived from hybridization between *S. kudriavzevii* NCYC2889 and *S. cerevisiae* (Voss Kveik) and three hybrids (KWH1-KWH3) from *S. kudriavzevii* NCYC2889 and *S. cerevisiae* (Wallonian III). All hybrids have displayed good attenuation performance, reaching values between 86 % (KKH1-KKH3) and 92.8 % (KWH1-KWH3) (Figure 2). Taking in consideration that *S. kudriavzevii* NCYC2889 parental strain does not ferment maltotriose and dextrins, the attenuative capacity comparisons of its hybrids was made with the *S. cerevisiae* parental strains (Voss Kveik and Wallonian III) that are able to ferment complex sugars (maltotriose in the case of Voss Kveik and maltotriose/dextrins in the case of Wallonian III). The tests performed in 12 °P in beer 20 IBU hopped wort at 25 °C (pH 5.45 ± 0.2) indicates that KKH1-KKH3 performed significantly better than the Voss Kveik parental species, reaching the final gravity (1.006 kg/m³ ± 0.0003) after 144-168 hours, 24 hours before Voss Kveik ($P < 0.001$, one-way ANOVA with Tukey's post-hoc test). On the other hand, KWH1-KWH3 reached final gravity (1.003 kg/m³ ± 0.001) after 168 hours of fermentation, 24 hours after parental strain Wallonian III being significantly sluggish than parental ($P < 0.001$). Regarding the fermentation test made in 35 °C, a similar pattern, thought, was observed between KKH1-KKH3 and Voss Kveik performances, reaching final gravity (1.006 kg/m³ ± 0.001) in 72-96 hours ($P < 0.001$). Concerning KWH1-KWH3 and Wallonian III in high temperature fermentation, similarly with the behaviour at 25 °C, the parental strain fermented significantly faster ($P < 0.001$) than the hybrids KWH1 and KWH3, although both attenuated to the final gravity of 1.003 kg/m³ ± 0.001 after 120-144 hours. Interestingly, at 17 °C, Voss Kveik, Wallonian III and its hybrids reached low final gravities with similar values than 25 °C and 35 °C (1,006 ± 0.001 for kveik-derived hybrids and 1,023 ± 0.001 for Wallonian III-derived hybrids) but, for it, it was necessary more 24-96 hours of fermentation (KWH1-KWH3 significantly slower than parental, $P < 0.001$). Usually, the wort initial pH has values near 5.00 while in the end of fermentation the pH has dropped to approximately 4 and this is often left without measurement (Boulton and Quain, 2001). For our tests, the initial pH was a standard beer wort value of 5.45 ± 0.2 for all samples. The final pH values are summarized in the table 2. For the fermentation at 25 °C, the final pH value was, in average, 4.20 ± 0,2 for all strains including the hybrids. On the other hand, for fermentations at 35 °C the yeasts displayed a lower pH (KKH3 showed a pH of 3.71) wherein KKH1-KKH3

presented a slightly higher pH than the Voss Kveik parental strain (3.70) with the following values 3.79, 3.86 and 3.76, respectively. By its turn, KWH1-KWH2 demonstrated values of 3.79 and 3.77 compared to 3.92 of the parental Wallonian III strain. The final pH of the test in 17 °C indicates that KKH1 got a pH 3.87 in comparison of 4.01 from Voss Kveik and 4.21 and 4.19 for KKH2 and KKH3, respectively. On the other hand, the pH values for KWH1-HKH3 was 3.88, 3.85 and 3.86 versus 3.78 from Wallonian III. It has been recently shown that there is a search for natural-acidifying yeast in order to produce sour beers without the need for mixed fermentation between brewing yeast and acid-producing bacteria (e.g. *Lactobacillus* and *Pediocccus*) as in the case of *Lachancea thermotolerans* (Domizio, House and Joseph, 2016). A slightly tart/sour beer pH reached at 35 °C range indicates a high release of organic acids, being possibly interesting to the application in the sour beer market).

Table 2. Final pH values in the different temperature conditions (17 °C, 25 °C and 35 °C). Initial pH for all samples were 5.45 ± 0.2.

	NCYC2889	VOSS KVEIK	WLN III	KKH1	KKH2	KKH3	KWH1	KWH2	KWH3
17 °C	4.22	4.01	3.78	3.87	4.21	4.19	3.88	3.85	3.86
25 ºC	4.20	4.31	4.27	4.27	4.39	4.40	4.21	4.13	4.15
35 °C	4.14	3.70	3.92	3.79	3.86	3.76	3.79	3.77	3.71

Flavour-active compounds in beers

In this work, we performed fermentation trials in a common Pilsner wort aiming the analysis of attenuation performance and production of volatile compounds by-products. From the 42 compounds tested, it was observed a production above the threshold of 12 of them (table 3). From these 12 compounds, only 5 was identified above the threshold in the samples fermented with the parental strains (ethyl butanoate, isovaleric acid, ethyl decanoate, diethyl succinate and γ-nonalactone), in which ethyl decanoate was present in the Wallonian III strain, but no in its hybrids. By its turn, ethyl butanoate was detected above the threshold only in NCYC2889 and its hybrids KKH2 and KKH3 in which the hybrids produced significantly more than NCYC2889 ($P < 0.001$). Interestingly, ethyl hexanoate was found only in the hybrids KKH1 and KWH1 and phenylethyl acetate only in KKH3 and KWH1. Furthermore, the following compounds was produced just by

KKH1: ethyl acetate, acetaldehyde and ethyl pentanoate along with 2,3-pentanedione that was not produced by its parental strains. Besides that, ethyl decanoate was produced only for KWH3 and not by any of its parental strains. Intriguingly, diethyl succinate was detected above the threshold by all strains tested but KKH2 (KKH3, KWH1 and KWH3 produced significantly more than NCYC2889 and KWH3 produced significantly more than WLNIII, $P < 0.001$). It is interesting to note, however, that diethyl succinate (fruity flavour) is a sign of beer aging (esterification of ethanol with succinic acid). The following compounds was also found above the threshold: isovaleric acid (5/9 of the strains tested) and γ-nonalactone (6/9 of the strains tested) which are probably hop-derived compounds (Meilgaard, 1982; Leffingwell, 1978; Vanderhaegen *et al.*, 2003; Preiss *et al.*, 2018).

Table 3. Triplicate average values in mg/L (ppm) of the volatile compounds quantified by GC-FID in the temperature condition tested (25 ºC). Light blue indicates flavour compounds identified above the threshold and dark blue, compounds produced above the threshold and significantly higher than one or both parental species ($P < 0.001$, one-way ANOVA). x= undetected.

Compound (Threshold)	NCYC2889	VOSS KVEIK	KKH1	KKH2	KKH3
Ethyl acetate (30 ppm)	0,91777	1,87405	113,06717	0,3231667	22,804147
Acetaldehyde (20 ppm)	3,75435	1,47499333	31,98429	10,5024	3,82546
2,3-butanedione (0,07 ppm)	x	0,05825	0,0754333	0,06419	0,05522
Ethyl butanoate (0,4 ppm)	0,52662333	0,06753	0,1565267	0,9872	1,17785
Ethyl pentanoate (0,9 ppm)	0,02531333	0,13185	1,4781367	0,6059133	X
Ethyl hexanoate/Ethyl caproate (0,21 ppm)	x	x	0,29266	x	x
Isovaleric acid (0,5 ppm)	x	x	2,0776133	2,17875	0,5619233
Ethyl decanoate (0,2 ppm)	0,02484667	x	0,00335	0,1039367	0,3057733
Diethyl succinate (1,2 ppm)	1,58491	5,6216	2,44248	0,4343333	6,83862
Phenethyl acetate (3,8 ppm)	0,05854	0,11247333	x	x	19,662967
γ-nonalactone (0,042 ppm)	0,49202333	x	x	x	0,2807633
Ethyl cinnamate (µg/L) (0,016 ppm)	x	x	0,12451	0,3531633	x
Compound (Threshold)	NCYC2889	WLNIII	KWH1	KWH2	HWH3
Ethyl acetate (30 ppm)	0,91777	1,85869	21,87961	0,20303	0,0376933
Acetaldehyde (10-20 ppm)	3,75435	1,67874333	x	5,02737	x
2,3-butanedione (0,07 ppm)	x	0,06786333	0,0566467	0,0543	x
Ethyl butanoate (0,4 ppm)	0,52662333	0,03925	0,10309	0,03979	0,24708
Ethyl pentanoate (0,9 ppm)	0,02531333	0,14719	x	x	x
Ethyl hexanoate/Ethyl caproate (0,21 ppm)	x	x	0,23175	x	x
Isovaleric acid (0,5 ppm)	x	0,64379	x	x	1,26375
Ethyl decanoate (0,2 ppm)	0,02484667	2,07726333	x	0,0878733	0,0024067
Diethyl succinate (1,2 ppm)	1,58491	5,76641	6,2700733	3,0331	10,166037
Phenethyl acetate (3,8 ppm)	0,05854	0,33985333	21,61878	x	0,0129
γ-nonalactone (0,042 ppm)	0,49202333	0,75805	0,71078	0,2913	0,5599933
Ethyl cinnamate (µg/L) (0,016 ppm)	x	x	x	x	x

Conclusion and perspectives

In the current work, we have generated six unique hybrids between *S. kudriavzevii* and two strains *S. cerevisiae* isolated from farmhouse brewing-related environments. From the tests regarding attenuation levels and biosynthesis of aromatic compounds, the hybrids performed in a fashion that aggregate characteristics from the parental strains. Further molecular and phenotypic studies are necessary to elucidate de role of ester-associated genes *ATF1* and *ATF2* for the biosynthesis of the esters above (Meilgaard, 1982; Pires, Teixeira, Brányik, and Vicente, 2014; Verstrepen *et al*., 2003). Furthermore, it is necessary to investigate *PAD1* and *FDC1* functionality by accessing the development of phenolic compounds such as 4-vinylguaiacol (4-VG) during fermentation through the quantification by GC-FID and subsequent sequencing of these genes in order to evaluate the presence or absence of mutations that allows its enzyme functionality or unfunctionally (McMurrough *et al*., 1996; Mukai, Masaki, Fujii and Iefuji, 2014; Mukai, Masaki, Fujii, Kawamukai and Iefuji, 2010). Besides that, the flocculation capacity, which is related mainly to *FLO1, FLO5, FLO8, FLO9, FLO10* and *FLO11* needs to be explored as well as the consumption of glucose, fructose, sucrose, maltose, maltotriose and dextrins by utilization of HPLC technique and sequencing of dextrin fermentation related gene *STA1* which encodes to a glucoamylase enzyme (Krogerus, Magalhães, Kuivanen and Gibson, 2019; Vidgren, Londesborough and Brew, 2011). On the other hand, the characteristics studied in the present research gathered together indicates a possibility for a broad range of application of KKH1-KKH3 and KWH1-KWH3 in the fermentative industry, including those of bioethanol, wine and, specially, the brewery sector.

Figures

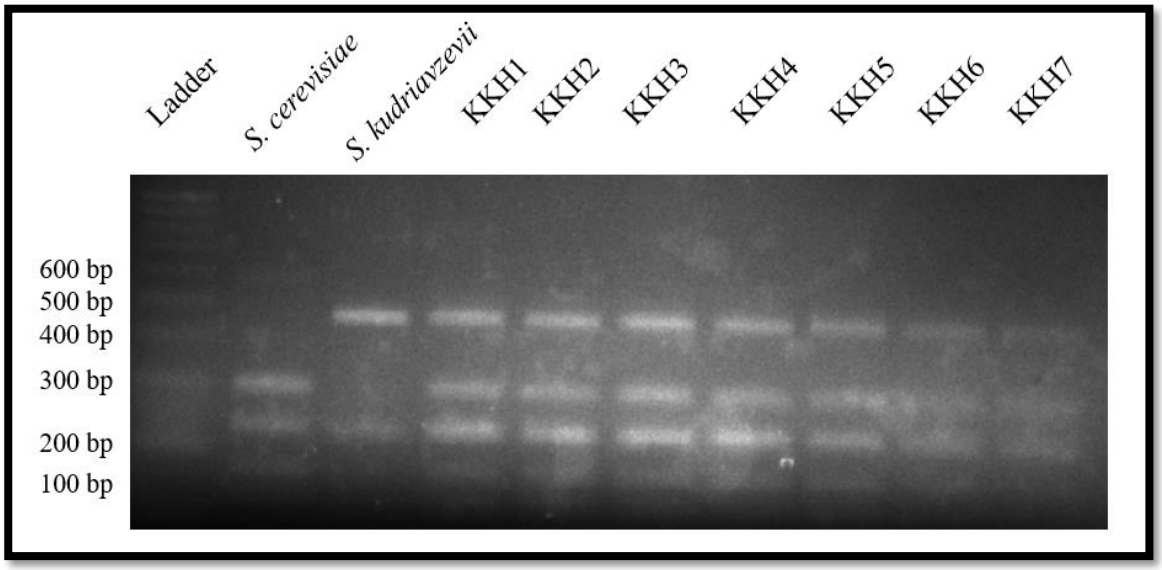
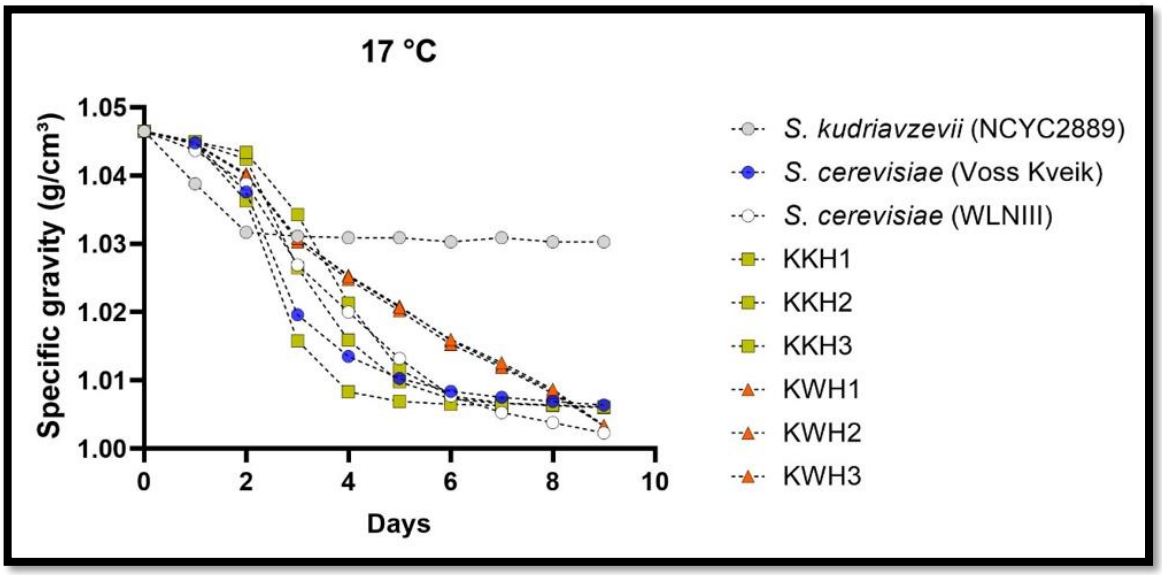
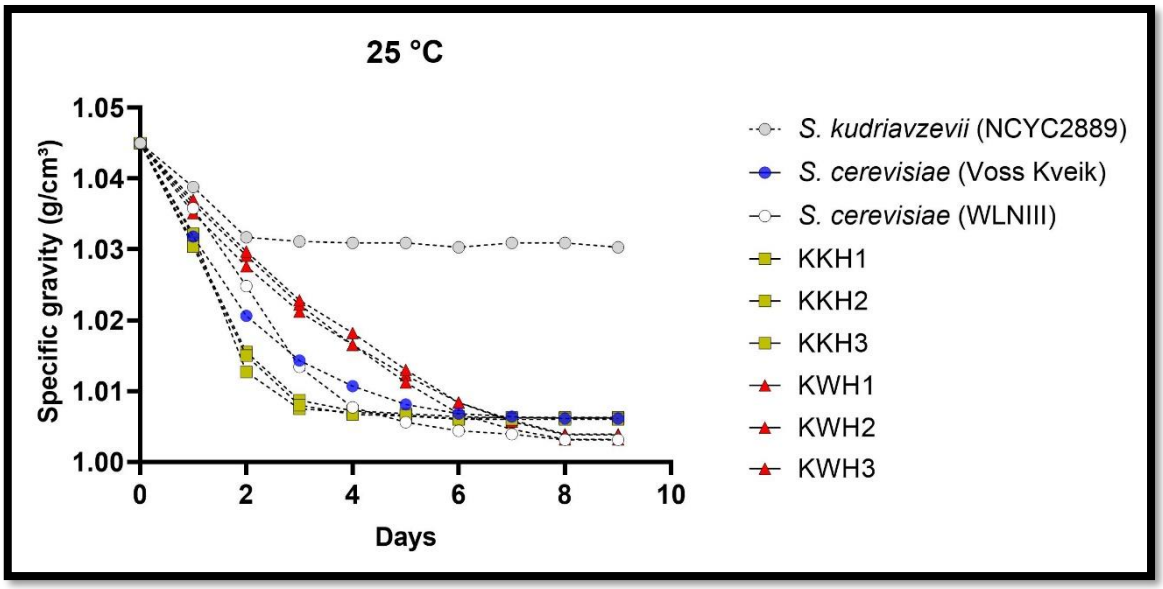


Figure 1. Hybridization confirmation of the the *S. kudriavzevii* and *S. cerevisiae* (Voss Kveik) possible hybrids KKH1-KKH7 via ITS1/ITS4 PCR-RFLP analysis.

A



B.



C.

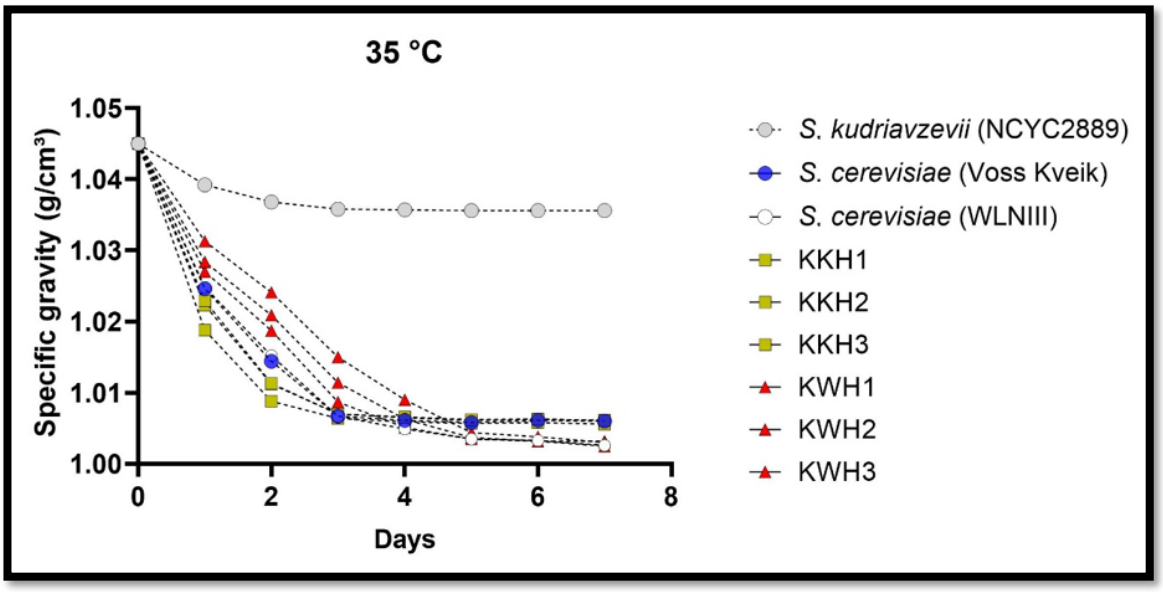


Figure 2. Fermentative performances of KKH1-KKH3 and KWH1-KWH3 in brewing wort. Duplicate average values plotted, SD for all samples are less than SD = ± 0.001. Fermentation tests in A. 17 °C, B. 25 °C and C. 35 °C.

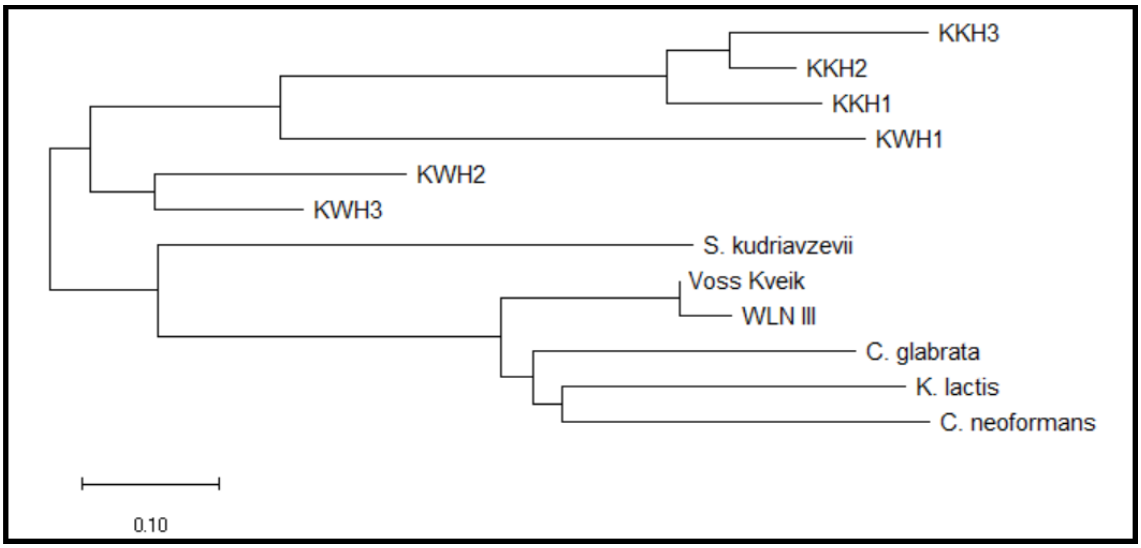


Figure 3. Phylogenetic tree with all ITS1/ITS2 sequences align (KKH1-KKH3, KWH1-KWH3 and the parental strains).

Acknowledgements

Conflict of Interest Statement

The authors declare that they have no competing interests.

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4. Discussão geral

A domesticação de leveduras, ocorrida pelo processo de seleção artificial, gerou uma considerável diversidade genotípica e fenotípica. Nesse sentido, atualmente, existem inúmeras leveduras cervejeiras distintas disponíveis para utilização em cervejarias, das quais se destacam as cepas da espécie *S. cerevisiae* (ales), como, por exemplo, as recém popularizadas Kveiks e *S. pastorianus* (lagers) (GALLONE *et al.*, 2016; PREISS *et. al*, 2018). Ainda que exista um número abundante de cepas de leveduras cervejeiras, há uma crescente demanda por cepas alternativas que representam a possibilidade de (i) gerar novos produtos em decorrência de atributos fermentativos como a formação de compostos aromáticos como ésteres, fenóis e ácidos orgânicos e (ii) economia por meio da otimização das plantas fabris ao utilizar leveduras que apresentam uma velocidade de fermentação mais eficiente (KROGERUS, 2017).

É importante ressaltar, também, que muitas cepas cervejeiras são, na realidade, híbridos interespecíficos (~ 25 %), e que, ao reconstruir esses híbridos em laboratório, há uma melhor compreensão das origens evolutivas dessas cepas, assim como a possibilidade da geração de novos híbridos com características únicas (KROGERUS, 2017; GALLONE *et al.*, 2019 & LANGDON *et al.*, 2019).

A partir da lógica de criação de novos híbridos interespecíficos em laboratório com potencial aplicação na indústria cervejeira, conforme discutido no capítulo 1, foram construídos cinquenta possíveis híbridos entre a cepa de *S. cerevisae* Voss Kveik e a cepa-tipo de *S. kudriavezevii* (NCYC2889) por meio do método de hibridização rara. Dessa totalidade, confirmou-se dez híbridos por ferramentas de biologia molecular como PCR/RFLP e sequenciamento da região ITS. Desses, três híbridos foram escolhidos para posterior caracterização em mosto cervejeiro.

Os híbridos selecionados (KKH1-KKH3) demonstraram uma boa performance fermentativa, alcançando valores de atenuação similares à cepa parental de *S. cerevisiae* Voss Kveik, mas em menos tempo, o que pode representar que ao utilizá-los em cervejarias, os tanques de fermentação podem se otimizados, com maior produção de volume de cerveja (KROGERUS, 2017). Os resultados de atenuação aparente (~86 %) tornam as cepas híbridas estudadas distintas pois esses altos valores ocorreram em uma notável amplitude de temperatura de fermentação (17 °C - 35°C), o que é incomum para a maioria das cepas cervejeiras, uma vez que usualmente existe uma faixa de temperatura

mais restrita para que se alcancem os valores máximos de atenuação. Essa característica é importante pois indica que pode não ser necessário o controle de temperatura de fermentação, o que representa uma notável redução de custos para as cervejarias (WHITE & ZAINASHEFF, 2010; KROGERUS, 2017).

Os híbridos apresentaram produção acima do limiar de percepção de uma gama de ésteres na temperatura testada (25 °C), que não foram produzidos pelas cepas parentais, como pentanoato de etila (que remete à maçã vermelha e melão), hexanoato de etila (maçã e anis-estrelado), cinamato de etila (frutado) e fenetil acetato (doce, mel e floral), além de decanoato de etila (brandy, frutado e uva), gerado, também, pelas cepas parentais. Os ésteres compreendem o grupo de compostos aromáticos mais importantes gerados durante a fermentação de cervejas (PIRES *et al.*, 2014). Assim, as leveduras híbridas geradas nesse trabalho diferenciam-se pela variedade de ésteres produzidos, o que pode representar um importante recurso para a indústria cervejeira. Ainda, os dados obtidos a partir da medição de pH pós-fermentação sugerem que KKH1-KKH3 têm, potencialmente, uma alta produção de ácidos orgânicos, o que sugere que essas cepas podem ser empregadas na produção de cervejas levemente ácidas. Conforme discutido no capítulo 1, o mercado de cervejas ácidas vem crescendo e, portanto, a demanda por leveduras que acidificam o mosto naturalmente, também. Desse modo, nossos dados sugerem que leveduras alternativas que atenuem mais que, por exemplo, *Lachaceae thermotolerans* (produtora de altas concentrações de ácido láctico, porém com baixa atenuação), podem ser geradas a partir da hibridização de cepas de kveiks, como a Voss Kveik e *S. kudriavzevii*.

Ainda é necessário caracterizar uma série de traços genotípicos e fenotípicos das cepas híbridas geradas, como (i) a herança de DNA mitocondrial, importante para o crescimento de leveduras híbridas em baixas temperaturas, (ii) a biossíntese de compostos de aroma e sabor como álcoois superiores e compostos fenólicos, (iii) a capacidade de floculação, fermentação de açúcares complexos como as dextinas e (iv) a tolerância aos diversos estresses presentes na fermentação cervejeira. No entanto, os dados obtidos até o momento de taxas de atenuação, produção de ésteres e pH pós-fermentação indicam que as cepas híbridas KKH1-KKH3 são promissoras para aplicação na indústria cervejeira, assim como a cepa Voss Kveik apresenta potencial para a geração de novos híbridos interespecíficos.

5. Conclusão geral

A hibridização interespecífica em laboratório vem ganhando força e se reafirma cada vez mais como uma ferramenta valiosa no que diz respeito à construção de cepas alternativas de leveduras cervejeiras e com possível aplicação na indústria. Nesse sentido, o presente trabalho visa ampliar o conhecimento acerca de híbridos interespecíficos na indústria cervejeira no que diz respeito à escolha de diferentes cepas, métodos de hibridização, confirmação e caracterização dos híbridos gerados. A partir dos dados obtidos nessa pesquisa, é possível inferir híbridos entre *S. cerevisiae* de diferentes origens e ainda pouco conhecidos, como a cepa Voss Kveik, pertencente a um grupo filogeneticamente distinto de cepas cervejeiras estudadas até o momento (Kveiks) e *S. kudriavzevii* podem ser gerados por meio dos métodos descritos acima. Além disso, é possível inferir que a cepa Voss Kveik da espécie *S. cerevisiae* pode servir como chassi para a formação de híbridos interespecíficos que contenham características fermentativas distintas das cepas atualmente disponíveis e que coincidem com as demandas atuais da indústria cervejeira.

6. Conclusões específicas

- As cepas Voss Kveik (*S. cerevisiae*) e NCYC2889 (*S. kudriavzevii*) podem servir como chassis para a formação de híbridos interespecíficos pelo método de hibridização rara a partir da geração de mutantes auxotróficos para *Lis- e Ura-*.
- Três híbridos selecionados para caracterização (KKH1-KKH3) demonstraram performance que superou as cepas parentais nas temperaturas de 25°C e 35 °C no tempo para alcançar a densidade específica final, enquanto um dos híbridos (KKH1) foi mais rápido que a cepa parental na temperatura de 17 °C.
- KKH1-KKH3 demonstraram produção de mais ésteres acima do limiar de percepção que as cepas parentais.
- Os valores de pH finais após a fermentação indicam que KKH1-KKH3 podem ter potencial para a aplicação na indústria de cervejas ácidas.Devido às suas características fermentativas, os híbridos gerados nesse trabalho apresentam potencial para aplicação nas indústrias vinícola e, especialmente, a cervejeira.

7. Perspectivas

- Determinar qual é a herança parental do DNA mitocondrial, que está diretamente relacionado à criotolerância de leveduras híbridas.
- Avaliar traços fenotípicos como (i) a expressão dos genes *ATF1 e ATF2* de modo a compreender sua contribuição para a formação dos ésteres observados nessa pesquisa, (ii) a expressão e sequenciamento os genes *PAD1 e FDC1* para verificar se há biossíntese de compostos fenólicos como o 4-vinilguiacol, assim como quantificar essa molécula por meio da utilização de GC-FID, (iii) capacidade de floculação dos híbridos gerados, observando, também, a expressão dos genes *FLO1, FLO5, FLO8, FLO9, FLO10* e *FLO11,* além de executar uma análise espectrofotométrica.
- Avaliar precisamente a assimilação e fermentação dos carboidratos glicose, frutose, maltose, maltotriose e dextrinas por meio da técnica de HPLC.
- Verificar se há a presença do gene *STA1,* de modo a averiguar se a capacidade atenuativa dos híbridos está relacionada com a expressão da enzima glicoamilase, que permite a fermentação de dextrinas.
- Avaliar a tolerância a diferentes estresses fermentativos, como térmico, etanólico, oxidativo, de pH, osmótico e depleção de nutrientes.
- Realizar o sequenciamento do genoma das leveduras híbridas e determinar sua ploidia, visando compreender os aspectos genômicos associados às cepas estudadas.

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9. Apêndice

Molecular and biochemical aspects of *Brettanomyces* in brewing

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Molecular and biochemical aspects of *Brettanomyces* in brewing

Marcelo Menoncin and Diego Bonatto*

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 dextrins 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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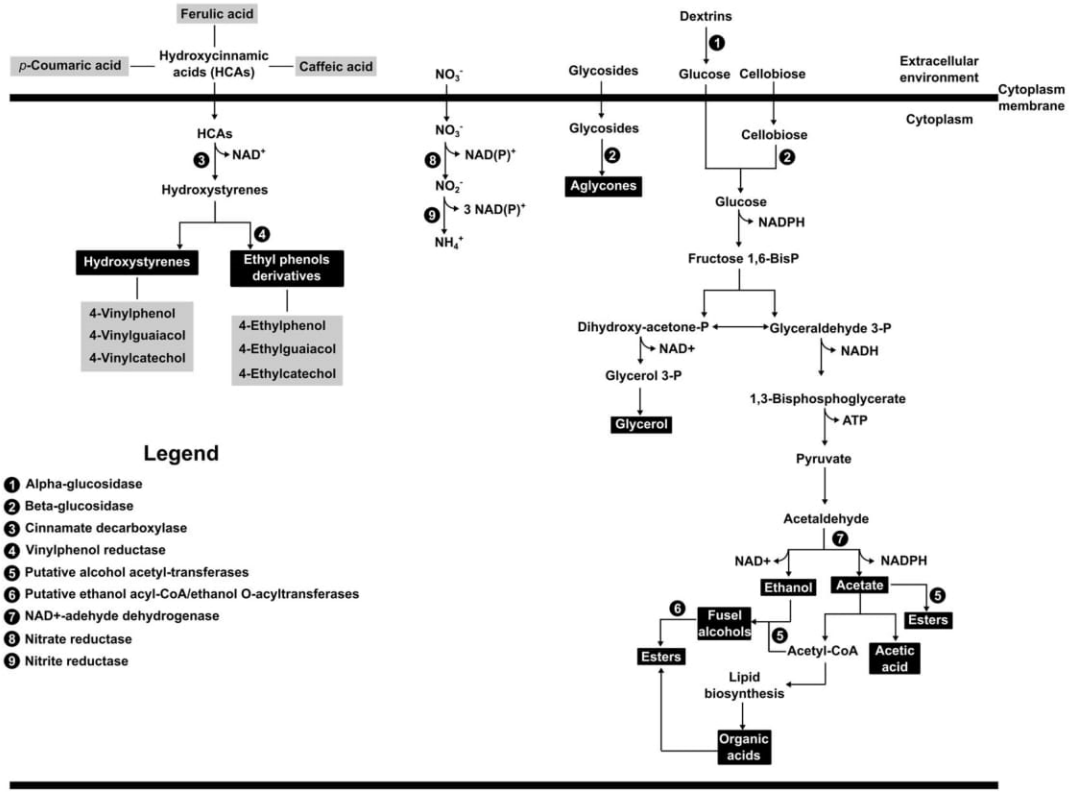

Figure 1. Schematic overview of the main metabolic pathways in *Brettanomyces* species during beer fermentation, focusing on the key enzymes linked to flavour active compound biosynthesis and the regulation of the redox balance (NAD^+/NADH) associated with the Custers effect. The flavour active compounds 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 methylotropic 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 multigene 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

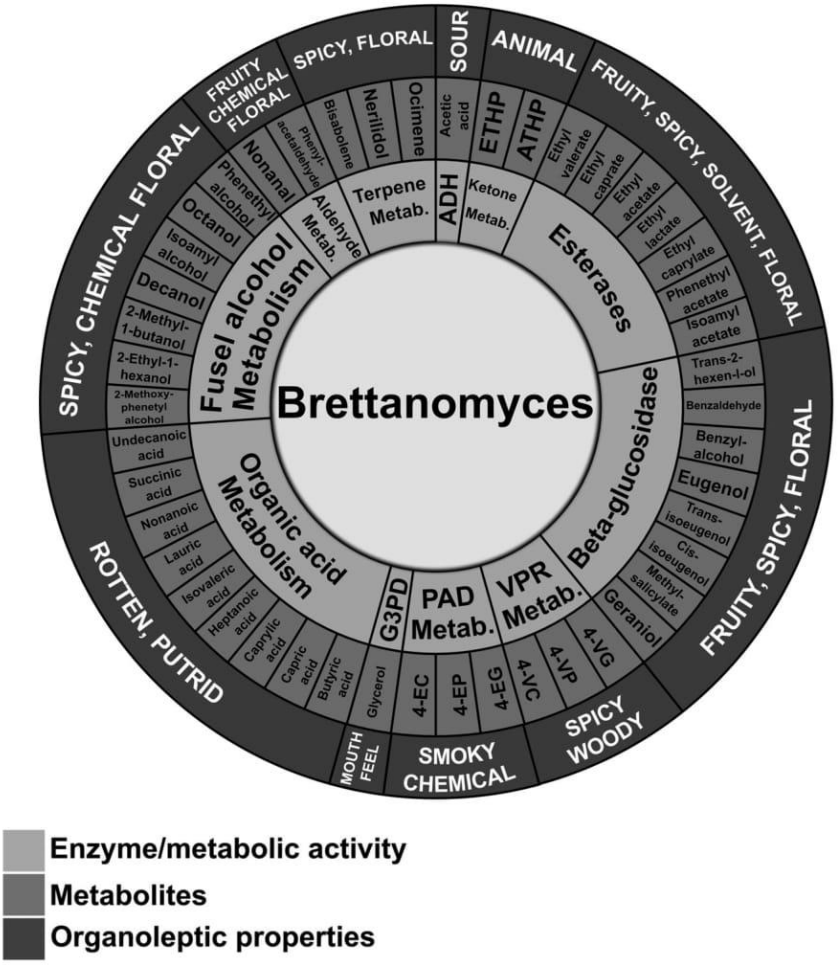

Figure 2. Aroma/flavour wheel containing the major metabolic pathways, enzymes and metabolites produced by different *Brettanomyces* species during beer fermentation. The aroma/flavours, enzymes/metabolic pathways and metabolites are indicated in the wheel by different grey shadows, defined in the legend below the wheel. Abbreviations: ADH, NAD^+-aldehyde dehydrogenase; ETHP, 2-ethyltetrahydropyridine; ATHP, 2-acetyltetrahydropyridine; VPR, vinylphenol reductase; 4-VG, 4-vinylguaiacol; 4VP, 4-vinylphenol; 4-VC, 4-vinylcatechol; PAD, phenylacrylic acid decarboxylase; 4-EG, 4-ethylguaiacol; 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	*Brettanomyces* species	*S. cerevisiae* (ale yeast)	*S. pastorianus* (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

[a]Diastatic *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-l-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.

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7. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS:

MENONCIN, M. & BONATTO, D. 6ta. JORNADAS SUDAMERICANAS DE BIOLOGÍA Y BIOTECNOLOGÍA DE LEVADURAS. Aspectos Moleculares e Bioquímicos da fermentação com *Brettanomyces* aplicada na Indústria Cervejeira. 2018.

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