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Biotransformation of hop derived compounds by *Brettanomyces* yeast strains

Marc Serra Colomer,[1,2] Birgitte Funch,[1] Natalia Solodovnikova,[1] Timothy John Hobley[2] and Jochen Förster[1]*

Several hop derived compounds in wort are known to be converted by yeast during fermentation, influencing the overall perception of the beer. A deeper understanding of such metabolic processes during fermentation is needed to achieve better control of the outcome. Here, the interaction between hop derived compounds and the yeast genera *Brettanomyces* was studied. Several *Brettanomyces* strains with different genomic backgrounds were selected, focusing on two traits: beta-glucosidase activity and nitrate assimilation. The role of three beta-glucosidases present in *Brettanomyces bruxellensis* and *Brettanomyces anomalus* and their impact on the final monoterpene alcohol profile was analysed. The beta-glucosidase activity was highly strain dependent, with *B. anomalus* CRL-49 exhibiting the highest conversion. Such activity could not be related to the release of aglycones from hops during fermentation, as a substantial part of such activity was intracellular. Nevertheless, the reduction of geraniol to β-citronellol was remarkably efficient for all *Brettanomyces* strains during fermentation, and it is suggested that two oxidoreductases BbHye2 and BbHye3 may have an influence. Moreover, the transfer of nitrate from hops to wort and its further assimilation by *Brettanomyces* species was analysed. The amount of nitrate in wort proved to be linearly proportional to the contact time of the hops with the wort. The level of nitrate assimilation by yeast was shown to be dependent on the nitrate assimilation cluster (*YNR, YNI, YNT*). Hence, the desired yeast strains may be selected according to the genetic make-up. **© 2020 The Authors. Journal of the Institute of Brewing published by John Wiley & Sons Ltd on behalf of The Institute of Brewing & Distilling**

Keywords: *Brettanomyces bruxellensis*; beta-glucosidase; monoterpene alcohols; nitrate assimilation; beer

Introduction

The craft brewing boom is driving innovation in the brewing industry, and it places demands on new and alternative practices. Recently, the use of non-conventional yeast species has been explored more rigorously, as the application of the right yeast can strongly influence the flavour profile of a beer. *Brettanomyces (*anamorph of *Dekkera)* species have been highlighted for beer flavouring, as their use results in features unachievable with conventional *Saccharomyces* brewing yeast (1,2). One of these is a positive impact on ester production, leading to fruity flavours. Recently it has been reported that there can be synergistic effects on flavour formation between yeast and hops (3,4). The authors found that monoterpene alcohols from hops can be metabolised by brewer's yeast, imparting tropical and citrus flavours, however the exact mechanisms are not fully understood (3,5,6). It is not clear whether similar effects are catalysed by *Brettanomyces* species and an evaluation of this would aid in understanding the mechanisms behind the positive attributes of brewing with this yeast.

Bioconversion of hop compounds by conventional and non-conventional yeasts has been described previously (4). During fermentation, the hop derived monoterpene alcohol geraniol - which gives a characteristic rose flavour - is reduced by yeast to β-citronellol (lime flavour), especially in the early stages of fermentation (Fig. 1) (3,7,8). Such a conversion has been correlated with expression of 'old yellow enzyme' (*Oye2*) by yeast, which is an oxidoreductase responsible for the transformation step (9,10). The citrusy flavour perceived in beers is caused by the synergistic effect of geraniol and citronellol (11). Therefore, an understanding of the interaction of yeast with hop derived compounds is required. *De novo* synthesis of monoterpene alcohols in conventional brewer's yeast has also been reported, in both natural and genetically engineered strains (6,12).

In addition, beer contains aglycones, sugar molecules bound to monoterpene alcohols that are not volatile and are odourless (13). When the glycosidic bond is broken the aromatic monoterpene alcohols are released and can be perceived by the consumer. Beta-glucosidase activity has been linked to this phenomenon (Fig. 1) (5,14). Several studies have focused on screening beta-glucosidase activity in yeasts, and despite the activity being strongly straindependent, *Brettanomyces* species display remarkably high activity (15–17). In one of these studies, a *B. custersianus* strain was found with beta-glucosidase activitiy up to ten fold higher than average (15). It has been reported that *Brettanomyces* can contain two open reading frames (ORFs), which putatively encode for two beta-glucosidases, however the impact of the presence of these genes during brewing has not been explored (18).

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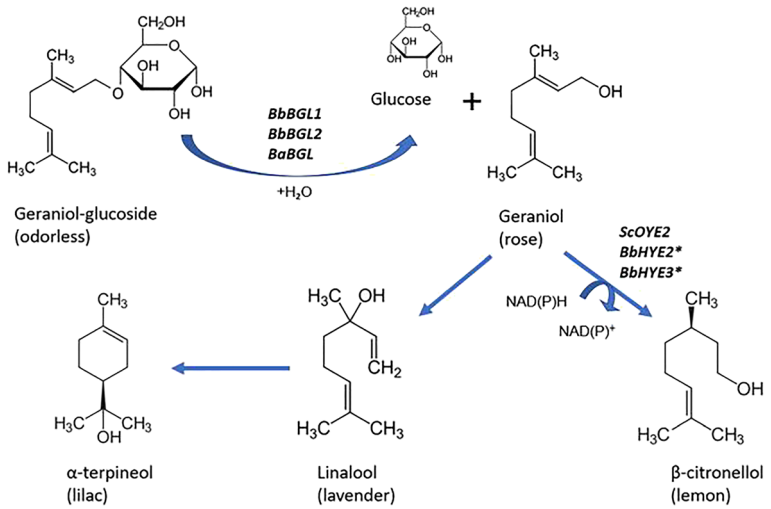

Figure 1. Beta-glucosidase activity on glucosides and bioconversion of monterpene alcohols by yeast. Flavour descriptors adapted from the literature. Genes responsible for the particular reactions are marked in bold. Enzymes marked with (*) are suggested here but not confirmed. Bb = *Brettanomyces bruxellensis*, Ba = *Brettanomyces anomalus*, Sc = *Saccharomyces cerevisiae*. [Colour figure can be viewed at wileyonlinelibrary.com]

Besides the potential for releasing aromatic monoterpene alcohols from hops, *Brettanomyces* can also affect other hop derived compounds. One compound of particular interest is nitrate. There are restrictions on the maximum concentration of nitrate in foods and water. The allowable maximum residual level (MRL) of nitrate in water and beer in the European Union is 50 mg/L. Typically, the concentration of nitrate in beer is much lower with nitrate transferring from hops during boiling and dry hopping (19). Despite assimilating ammonium, conventional brewing yeast cannot assimilate nitrate and the compound has a negative impact on its fermentation performance (20). In a recent publication, nitrate assimilation by a diversity of yeast species was reported, establishing a phylogenetic association with other metabolic traits, such as the biosynthesis of the molybdenum cofactor (21). In certain occasions, *Brettanomyces* yeast species have been reported to assimilate nitrate from several substrates (22–24). This requires a nitrate transporter, nitrate and nitrite reductases, encoded by the genes *YNT, YNR* and *YNI* respectively (23–25). Nitrate reduction to ammonia is dependent on NAD(P)H (26) (Fig. 2). Furthermore, the metabolism of *B. bruxellensis* has been reported to be influenced by the presence of nitrate in media, leading to expression of genes in the pentose phosphate pathway and TCA cycle (27,28). Of particular relevance to brewing, is that under anaerobic conditions the fermentative capacity of *B. bruxellensis* is improved when nitrate is assimilated, leading to higher ethanol concentration and growth rates (29).

In this study for the first time, we investigated the role of the two beta-glucosidase genes present in *Brettanomyces* on monoterpene alcohol release, and sought to confirm that this yeast can reduce nitrate levels in beer. A variety of strains were sequenced, the presence or absence of key genes was determined, and relevant strains were then selected. The activity of beta-glucosidase was assayed in selected *Brettanomyces* strains and the impact on the release of hop derived compounds was examined, during both primary fermentation and secondary fermentation after dry hopping. Finally, worts with different nitrate concentrations were produced and fermented with *Brettanomyces* strains with selected genetic backgrounds. This allowed the impact of hop dosage on wort nitrate concentration and nitrate assimilation by *Brettanomyces* strains to be studied.

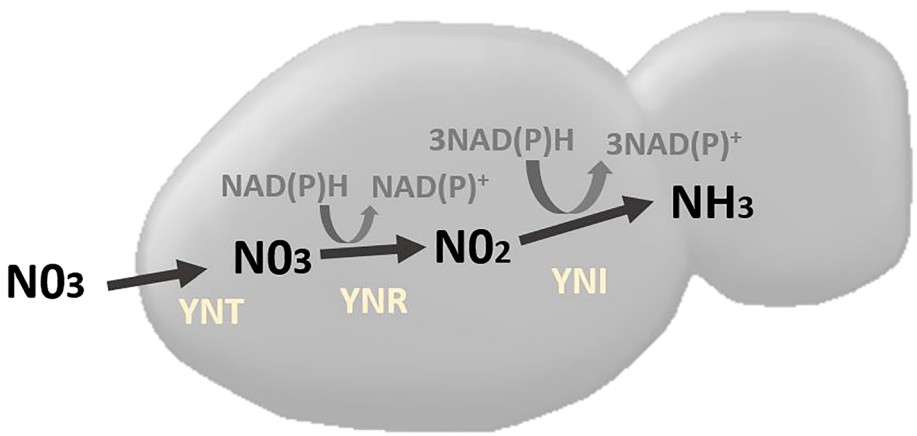

Figure 2. Nitrate assimilation pathway by *Brettanomyces* species. The enzymes responsible for transportation and conversion to NH_3 are indicated in white. Cofactors involved in the reaction are indicated in light grey. [Colour figure can be viewed at wileyonlinelibrary.com]

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Materials and methods

Microbial strains and media

Strains of *Brettanomyces* were obtained from the Carlsberg Research Laboratory's (CRL) collection (CRL-1 to CRL-50 in Table 1). The yeasts were grown on yeast extract (1%, w/v) peptone (2%, w/v) glucose (2%, w/v) (YPD) media, with agar (2%, w/v) added where required. The strains were grown in 100 mL Erlenmeyer flasks containing 50 mL of YPD, under aerobic conditions at 25°C with agitation (100 rpm).

DNA sequencing and bioinformatics

Yeast strains were grown for one week in YPD . Cells were recovered by centrifugation at 4000 g for 5 minutes at 4°C, washed in sterile Milli-Q water and recentrifuged. Samples were sent for DNA extraction and whole-genome sequencing, short insert PE150 library, on Illumina HiSeq4000 (BGI-Tech Solutions, Hong Kong). CLC Genomics Workbench software (www.qiagenbioinformatics.com) was used as a tool for bioinformatics analysis. Genome assembly of strains was performed in CLC software using D*e novo* assembly feature. Genes of interest were found in GenBank, accession number (AKS48905.1, EIF45415.1, AKS48904.1) for BbBGL1, BbBGL2 and BaBGL and (EIF45251.1, EIF45249.1, EIF45250.1) for *BbYNT, BbYNR* and *BbYNI*, respectively. Nucleotide BLAST tool on CLC software was used to identify the presence or absence of each gene.

Beta-glucosidase assay

To test beta-glucosidase activity in *B. bruxellensis* and *B. anomalus*, the yeast strains were grown for one week in yeast extract (1%, w/v) peptone (2%, w/v) cellobiose (2%, w/v) (YPC) media. Extracellular, cell associated and intracellular cell fractions were prepared using the modified method of Daenen et al (15). For the extracellular fraction, 1 mL of culture was transferred to a 1.5 mL Eppendorf tube (ThermoFisher), centrifuged (4,000 g, 5 min, 4°C) and the supernatant collected. The cultures were adjusted to give an optical density at 600 nm of 1. The cells were washed with sterile water and resuspended in phosphate buffered saline (PBS) buffer for the cell associated enzyme fraction. To obtain the intracellular fraction, 0.5 mg/mL zymolyase (ThermoFisher) was added with PBS and incubated for 1 hour at 37°C. Glass beads (425-600 μm, Sigma) were added to the cell fractions and vortexed twice for 20 seconds and kept on ice. The homogenate was centrifuged (14,000 g, 10 min) and the supernatant was collected as the intracellular fraction. The beta-glucosidase activity of each fraction was determined with the MAK129 β-glucosidase assay kit (Sigma Aldrich). The assay was performed in 96 well plates with *p*-nitrophenyl-β-D-glucopyranoside (β-NPG) as the substrate. The reaction was measured at 405 nm after a 20 minutes incubation at 37°C. The results are reported in units/L, where one unit is the amount of enzyme that catalyses the hydrolysis of 1.0 μmole of substrate per minute at pH 7 and 37°C.

Fermentation

To investigate the ability of the *Brettanomyces* strains to release hop aromas, two experimental approaches were performed: i) An all-malt pale wort, 16°P was provided by Jacobsen Breweries for primary fermentation; ii) Jacobsen Indian Pale Ale (IPA) dry hopped beer (Jacobsen Breweries) was used for secondary fermentation, with 1.2% (w/v) glucose added to support yeast growth. Fermentations were performed using strains CRL-1, CRL-2, CRL-19, CRL-49 and CRL-50.

To examine the effect of nitrate, standard CRL pilsner wort (Viking malt) with an original gravity of 16°P was used. Saaz hops were added at 4 g/L. Wort (1.5 L) was transferred to 2 litre glass bottles (Witeg)with the hop pellets contained in a tea bag and the wort was brought to 100°C in a water bath. Three different worts were produced, (i) without an additional boil (W1), (ii) with 30 minutes additional boiling (W2) and (iii) with 1 hour additional boiling (W3). Fermentations were performed using strains CRL-1 and CRL-27.

Strains were propagated in the CRL pilsner wort in 50 mL Erlenmeyer flasks. All fermentations were performed in duplicate in 250 mL Duran bottles containing 200 mL of media. The fermentation was allowed to become anaerobic and the ANKOM RF Gas Production System (ANKOM) used to monitor fermentation performance and CO_2 release. A pitching rate of $1x10^5$ viable cells/mL was used (Cellometer X2, Nexelom Bioscience). No samples were taken during fermentation so as to ensure anaerobiosis. Fermentation was complete when no further CO_2 release could be measured and the beer was stored at -20°C before analysis.

Table 1. Genomic landscape of *Brettanomyces* strains used in the study.

		Beta-glucosidase ORFs			Nitrate assimilation genes		
Strain	Species	BbBGL1	BbBGL2	BaBGL	YNR1	YNI1	YNT
CRL-1	*B. bruxellensis*	X	-	-	X	X	X
CRL-2	*B. bruxellensis*	-	X	-	X	X	X
CRL-19	*B. bruxellensis*	X	X	-	X	X	X
CRL-27	*B. bruxellensis*	X	-	-	-	-	-
CRL-49	*B. anomalus*	-	-	X	X*	X*	X*
CRL-50	*B. bruxellensis*	-	-	-	X	X	X

From sequencing of the strains, (X) indicates the gene or ORF present. (–) indicates the gene or ORF is missing. (*) indicates homologous genes in *B. anomalus*.
*

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Quantification of fermentable sugars, ethanol, monoterpene alcohols and nitrate

Quantitative analyses of carbohydrates in wort was performed by high-performance anion-exchange chromatography (HPLAEC) using a Dionex ICS-3000 system (Thermo Scientific) with a Dionex PA200 IC column (Thermo Scientific) with a flowrate of 0.5 mL/min. Eluent gradients were performed with (A) 50mM NaOH and (B) 50mM NaOH/100mM acetate pressurised with Helium. The column temperature was at 30°C. Injection volume was kept at 10 μL full loop. Samples were filtered through a SEP-PACK C18 (Waters Corporation) and diluted 10x before injection. Reference sugar samples were obtained from Sigma Aldrich and calibration curves were prepared for each measurement.

Ethanol content was quantified with Alcolyser BeerME Analyzing System (www.anton-paar.com). Monoterpene alcohols were quantified using Headspace Gas Chromatography Solid Phase Micro Extraction (HS-GC SPME) following the method described by (30). Nitrate content in wort was determined with HPLC anion exchange column and detected by conductimetry, based on (31). Beer samples were degassed by agitation, filtered through a 0.45 μm filter and diluted 10 times with pure water before injection.

Results and discussion

Genetic characterisation and selection of strains

Six *Brettanomyces* strains with different genomic maps were selected to examine the effect of the presence or absence of genes for nitrate assimilation and beta-glucosidase (Table 1). Five of the strains were *Brettanomyces bruxellensis* and one (CRL-49) *Brettanomyces anomalus*. For studies of the effect of beta-glucosidase on hop flavour release, CRL-1 and CRL-2 were chosen as they contain just one ORF, *BbBGL1* and *BbBGL2* respectively. CRL-19 was selected for having both ORFs and CRL-50 for having neither ORF. Furthermore, CRL-1 was chosen as a representative strain possessing all three nitrate assimilation genes, in comparison with strain CRL-27 lacking the nitrate assimilation loci (Table 1).

Genetic background and beta-glucosidase

Five *Brettanomyces* strains with different arrangements of the beta-glucosidase genes were assayed for enzyme activity. Intracellular, cell related and extracellular activities were measured (Fig. 3). The highest activity in the *B. bruxellensis* strains (up to 74 units/L) was detected in the intracellular fraction of CRL-19, which contains both beta-glucosidase ORFs. In contrast, little activity was detected in the strains with only one or no beta-glucosidase encoding genes. The results indicate that BbBgl2 is more efficient than BbBgl1 and suggests that there could be an additive effect between the two proteins. Interestingly, Kuo and co-workers purified a heterodimeric structure with beta-glucosidase activity from *Dekkera bruxellensis*, consisting of two subunits of 50 and 30 kDa (32). Nevertheless, the amino acid sequences of those two subunits do not correlate to the two ORFs found here for the *Brettanomyces* strains. In contrast to the proteins tested by Kuo (32), our results suggest that such enzymes are primarily located intracellularly, with little secretion (Fig. 3), similar to the findings of Blondin et al (33).

The intracellular fraction of *B. anomalus* CRL-49 showed the highest activity among all the *Brettanomyces* strains tested (144 units/L). The potential of *B. anomalus* beta-glucosidase activity over *B. bruxellensis* has been reported before (16). Interestingly, beta-glucosidase activity was also detected in the cellular fraction of CRL-49, suggesting that the enzyme could be associated with the cell wall in *Brettanomyces anomalus*.

Hop aroma conversion by *Brettanomyces* strains during primary fermentation

Brettanomyces strains producing different beta-glucosidase enzymes were selected to examine whether they could facilitate the release of hop flavour compounds. The effects on primary fermentation (before dry hopping) were examined first, as the *Brettanomyces* strains would be anticipated to have high viability with little release of the intracellular beta-glucosidase. Fermentations proceeded for 10 days, by which time CO_2 release had stopped. The fermentations for all strains were similar, as shown by CO_2 production (Fig. 4A) and ethanol formation (7.5 ± 0.2 %; Table 2). However, *B. bruxellensis* CRL-2 was an exception as it

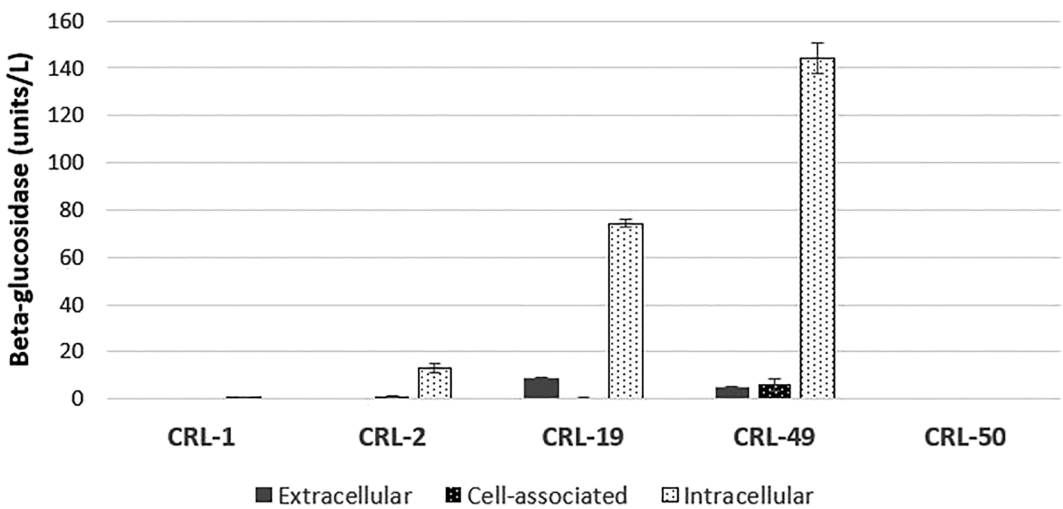

Figure 3. Activity of beta-glucosidase in the different cell fractions of *Brettanomyces* strains. Values are the average of two replicates and error bars show the standard deviation. The results are given in units/L, where one unit is the amount of enzyme that catalyses the hydrolysis of 1.0 μmole of substrate per minute at pH 7 and 37°C.

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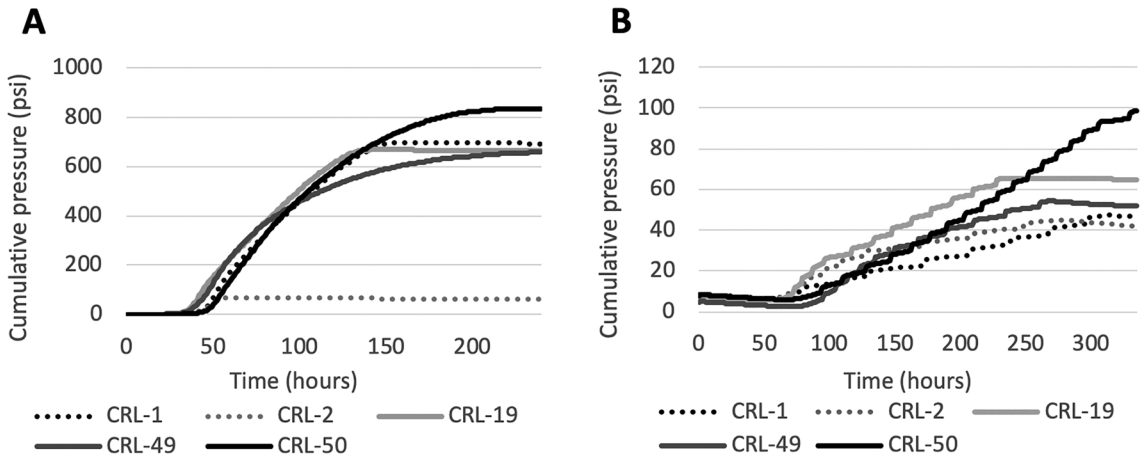

Figure 4. Cumulative pressure measured in primary (A) and secondary (B) fermentation. Strains were fermented in duplicate, and the curve represents the average values.

Table 2. Ethanol and fermentable sugars after primary fermentation and secondary fermentation (shaded). Standard deviation is reported (n=2).

	Ethanol (%, v/v)	Glucose (mg/L)	Maltose (mg/L)	Maltotriose (mg/L)	Maltotetraose (mg/L)
CRL-1	7.49 ± 0.13	4 ± 2	0 ± 0	179 ± 19	3225 ± 150
CRL-2	1.71 ± 0.02	91 ± 16	60215 ± 3559	16674 ± 839	3325 ± 157
CRL-19	7.39 ± 0.08	10 ± 14	22 ± 30	67 ± 46	2686 ± 1072
CRL-49	7.3 ± 0.05	0 ± 0	15 ± 21	3403 ± 388	3064 ± 148
CRL-50	7.75 ± 0.32	37 ± 4	1570 ± 549	19 ± 7	81 ± 1
CRL-1	6.97 ± 0.0	9 ± 1	553 ± 100	9012 ± 721	1414 ± 48
CRL-2	6.82 ± 0.17	12 ± 4	827 ± 332	9711 ± 1883	1396 ± 70
CRL-19	6.91 ± 0.04	10 ± 8	842 ± 264	12396 ± 4254	1377 ± 0
CRL-49	6.96 ± 0.06	9 ± 3	407 ± 137	8518 ± 373	1260 ± 14
CRL-50	7.32 ± 0.39	16 ± 5	785 ± 17	3202 ± 1874	916 ± 140

was not able to metabolise maltose. Ethanol production by CRL-50 was the highest due to its ability to ferment dextrins, as shown by utilisation of maltotetraose (Table 2). At the end of fermentation samples were analysed for monoterpene alcohols and compared to the wort. The results in Fig. 5A show that yeast strains CRL-1 (one ORF) and CRL-50 (no ORFs), which had the lowest beta-glucosidase activities led to the greatest concentrations of β-citronellol, reaching levels up to 31.5 μg/L after fermentation for CRL-50. Furthermore, CRL-2 (lacking one ORF and maltose utilisation) had the lowest conversion of geraniol to β-citronellol. The same pattern was seen in all the strains; the content of geraniol decreased in favour of the production of β-citronellol. Linalool was converted to α-terpineol but at lower rate. Following conventional pathways, myrcene was completely depleted in all cases and isoamyl isobutyrate was slightly increased (34). No correlation between beta-glucosidase activity (Fig. 4) and aroma released from aglycones (Fig. 5) was seen. To our knowledge, such large amounts of β-citronellol are rarely reported without the use of dry-hopping. In *Saccharomyces* yeast, β-citronellol formation has been strongly correlated with Oye2 activity (9). In the *Brettanomyces* genome sequences reported here, two proteins were identified as potential homologs of Oye2 and Oye3, showing domains of flavin mononucleotide (FMN) oxidoreductase activity. A BLAST search revealed a 58% similarity with 'hansenula yellow enzyme' 2 and 3 of *Hansenula polymorpha*. Accordingly, the proteins are referred to as BbHye2 and BbHye3 (35). Accordingly, the results reported here suggest that oxidoreductases BbHye2 and BbHye3 cause stronger monoterpene alcohol conversion than the conventional brewer's yeast ScOye2. This could contribute a more stable redox metabolism under anaerobiosis in *Brettanomyces* by the regeneration of NAD^+ (Fig. 1).

Hop aroma conversion by *Brettanomyces* strains during secondary fermentation

A dry hopped commercial beer supplemented with glucose (1.2%, w/v) was inoculated with the respective strain of *Brettanomyces*, re-sealed in the ANKOM system and refermented for 14 days. At this point, the glucose had been consumed as shown by the CO_2 accumulation curves (Fig. 4B) and between 6.8 and 7.3% (v/v) alcohol had been produced (Table 3). The total amount of monoterpene alcohols were higher in the secondary fermentations compared to the primary fermentations due to dry hopping (Fig. 5B). However, bioconversion of monoterpene alcohols occurred to a similar extent as in the primary fermentationwith, in both cases, the conversion of ca. 25 μg/L geraniol. As in the primary fermentations, *Brettanomyces* strains increased α-terpineol and β-citronellol content while reducing linalool and geraniol. There was no correlation between strain dependent beta-glucosidase activity (Fig. 4) and hop aromas, suggesting that the intracellular beta-glucosidase was

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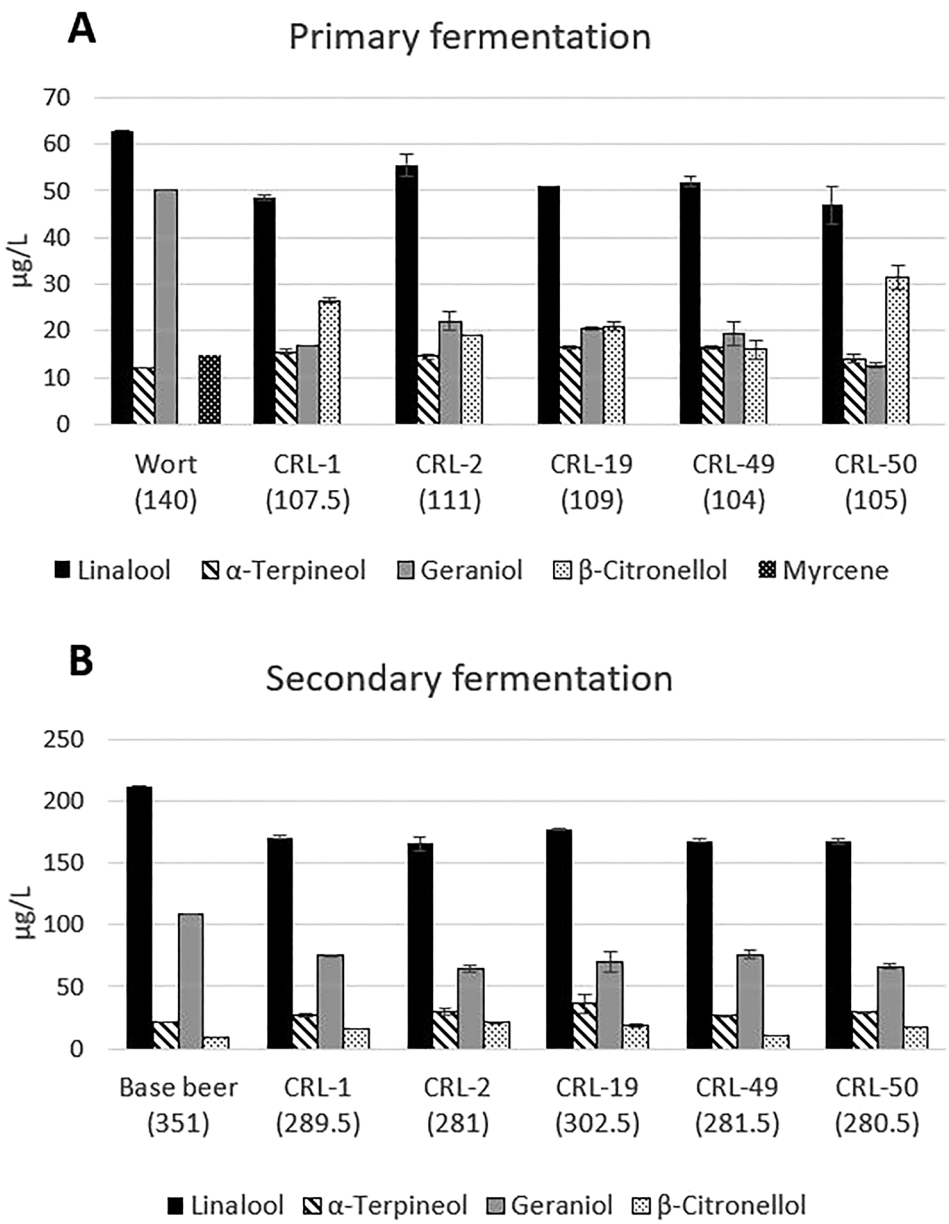

Figure 5. Monoterpene alcohols in beers after fermentation with *Brettanomyces* as primary (A) or secondary (B) yeasts. The sum of monoterpene alcohols by strain is indicated in ug/L. Standard deviation is shown by error bars (n=2).

Table 3. Ethanol production after fermentation of Pilsner wort with varying nitrate content.

Yeast	Media	Hop contact time (min)	Initial nitrate content (mg/L)	Alcohol (%, v/v)
CRL-1	Pilsner Wort 1	0	1.8	7.04 ± 0.12
CRL-1	Pilsner Wort 2	30	11.7	7.02 ± 0.06
CRL-1	Pilsner Wort 3	60	22	6.89 ± 0.21
CRL-27	Pilsner Wort 1	0	1.8	7.2 ± 0.0
CRL-27	Pilsner Wort 2	30	11.7	7.1 ± 0.04
CRL-27	Pilsner Wort 3	60	22	6.96 ± 0.08

not available to release of aglycones from hops. In support of this, Takoi et al 2010 (3) reported the release of glycosidically bound precursors using added Sumizyme BGA, a commercial beta-glucosidase derived from *Aspergillus niger* (3). Nevertheless, a treatment at 50°C for 60 min was included, which is unsuitable for beer. Other studies have reported optimal activity at even higher temperatures (16). Other enzymes such as an extracellular exoglucanase protein encoded by *EXG1* gene have been related

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to non-specific activity of sugar bound molecules (17,32,36,37). Further research is needed to evaluate the specific role of proteins in the release of hop aglycones and the bioflavouring of beerand whether other compounds such as nerol or ergosterol derivatives contribute to the bioconversion.

Effect of hop boiling time on the nitrate content in wort

The transfer of nitrate from hops to wort was investigated by varying wort boiling time with 0, 0.5 and 1 hour of additional boiling. The concentration of nitrate in the worts was respectively 1.8, 11.7 and 22 mg/L This linear (R^2 = 0.9999) increase in nitrate over time is in agreement with previous studies (19). The nitrate levels were below the maximum residual level in the EU (50 mg/L). However, this will be dependent on the hop variety and treatment during cultivation (38). These results confirm that hops are a significant source of nitrate in wort and confirm the risk that long contact times or large hop additions can result in the release of high concentrations of nitrate.

Effect of fermentation by *Brettanomyces* strains on nitrate concentration

Fermentations were conducted in wort containing different concentrations of nitrate with two strains of *Brettanomyces*. Strain CRL-1 contained all the nitrate assimilation genes whereas CRL-27 had none of these genes. Nitrate and ethanol concentrations were measured in final beers, harvested after 9 days of fermentation when CO_2 production had stopped (Fig. 6). The results in Fig. 7 show that the nitrate concentration was unaffected in the fermentation with CRL-27 (without the nitrate assimilation cluster). In contrast, nitrate was almost fully consumed in

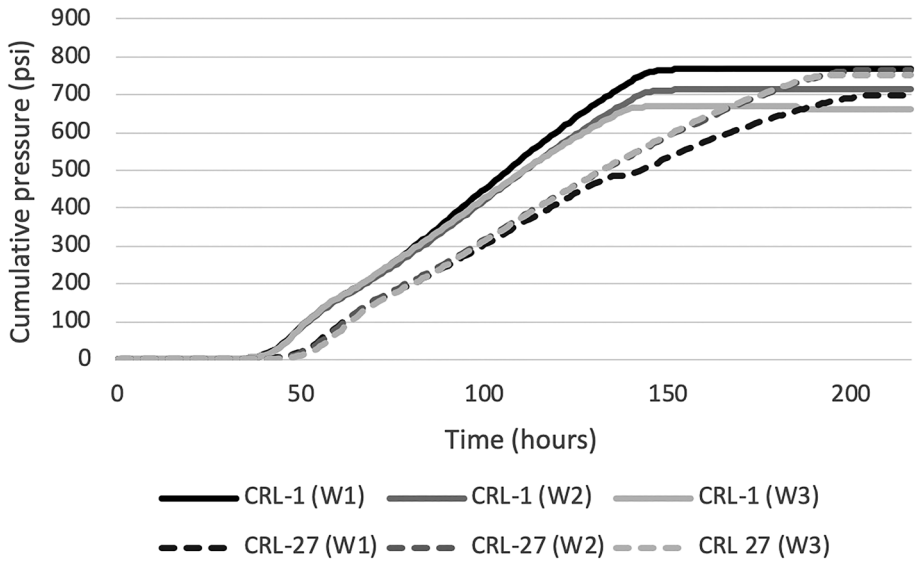

Figure 6. Fermentation of worts with varying nitrate content with *Brettanomyces* CRL-1 (+ nitrate assimilation) and CRL-27 (- nitrate assimilation). Curves represent the average values of duplicate fermentations. Wort 1 (W1) had no additional boiling, Wort 2 (W2) had additional hop boiling for 30 minutes, Wort 3 (W3) was boiled for 1 hour

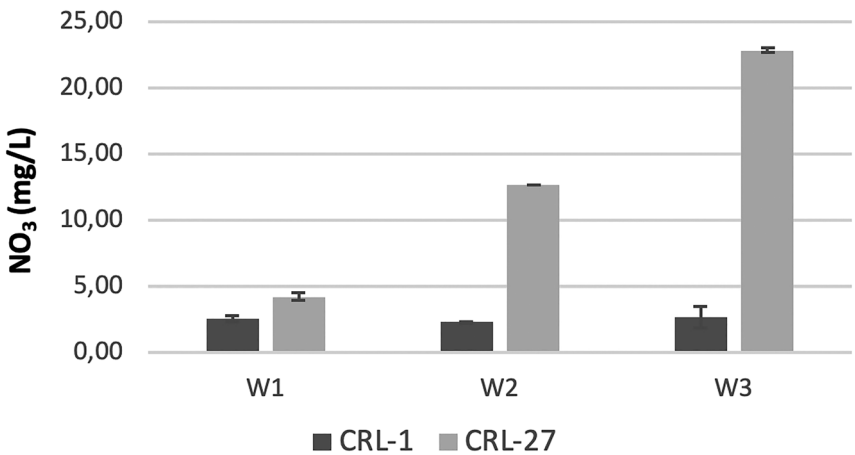

Figure 7. Nitrate content after fermentation with different *B. bruxellensis* strains in worts W1, W2, W3 containing 1.8, 11.7 and 22 mg/ml NO_3 respectively. Wort 1 (W1) had no additional boiling, Wort 2 (W2) had additional hop boiling for 30 minutes, Wort 3 (W3) was boiled for 1 hour . CRL-1 contains the nitrate assimilation loci, CRL-27 does not.

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fermentations with CRL-1, which contained all of the nitrate assimilation genes. Interestingly, ca. 2.5 mg/mL nitrate remained in the CRL-1 fermentations, independent of the starting concentration, suggesting that the capacity of the *Brettanomyces* strain for nitrate reduction is much higher. Furthermore, despite nitrite (NO_2) being the an intermediate in the pathway, it was not detected in any sample. This suggests that the enzyme nitrite reductase is efficient in reducing nitrite to ammonia (NH_3).

Despite wort being rich in amino acids and the preferred nitrogen source in *Brettanomyces* (39), nitrate was efficiently assimilated during fermentation. This has been linked to the need of yeast to reoxidise NAD(P)H, to maintain the redox balance and enable cell growth (28). Examination of the fermentation data suggests that the fermentative metabolism was not affected by the assimilation of nitrate, in contrast to previous studies (28,29,40). This is presumably due to the content of nitrate in wort which was much lower than previously reported (>240 mg/L) (40), suggesting that the nitrate level transferred by hops was not significant enough to make an impact on yeast metabolism.

Conclusions

In this study, the interaction between hop derived compounds and yeast of the genus *Brettanomyces* was studied. In particular, beta-glucosidase activity was found to be highly strain dependent, with *Brettanomyces anomalus* the most efficient in converting β-substrates. The beta-glucosidase activity of *Brettanomyces* strains is almost exclusively intracellular and therefore has little impact on the release of hop derived monoterpene alcohols during fermentation. In addition, the conversion of geraniol into β-citronellol was substantially higher, most likely from a direct contribution of the oxidoreductase proteins BbHye2 and BbHye3. Furthermore, it was demonstrated that nitrate levels in wort increased linearly with time during wort boiling, but the level was reduced during subsequent *Brettanomyces* fermentation. No effect on nitrate levels was seen in a strain lacking the nitrate assimilation genes, confirming that *YNR1*, *YNI1* and *YNT* are required for nitrate metabolism.

Author contributions

MSC designed, performed the experiments and wrote the manuscript, BF provided technical support, NS supported the experimental design, TJH supported writing the manuscript, JF supported the experimental design and overall supervision.

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Conflict of interest

A patent application protecting some of this work has been submitted by Carlsberg Breweries.

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