

Population Dynamics and Effects of *Brettanomyces bruxellensis* Strains on Pinot noir (*Vitis vinifera* L.) Wines

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Replicated, sterile Pinot noir (*Vitis vinifera* L.) wines were individually inoculated with one of six strains of *Brettanomyces bruxellensis* (Ave, M, 216, Vin 1, Vin 4, or Vin 5) at initial numbers <50 cfu/mL. In two separate studies, population changes were monitored for 23 months, or until cell densities peaked and subsequently declined to ≤30 cfu/mL. Significant variation was noted in both growth rate and stationary phase population densities among strains. The concentration of selected volatile components was monitored using solid-phase microextraction GC/MS. Large increases in the concentration of 4-ethylphenol occurred after titers reached 2.5×10^5 cfu/mL. *Brettanomyces*-inoculated wines were found to have detectable concentrations of ethyldecanoate, isoamyl alcohol, 4-ethylguaiaicol, and 4-ethylphenol, with some significant differences in their concentrations among strains. Duo-trio testing suggested sensory differences between the control and all inoculated wines and among wines inoculated with strains Ave and Vin 5, M and 216, and M and Vin 4. Consensus training analysis suggested that all *Brettanomyces* wines had a greater perception of earthy, smoky, spicy, and cardboard descriptors than uninoculated control wines.

Key words: *Brettanomyces bruxellensis*, 4-ethylphenol, 4-ethylguaiaicol, cumulative cell exposure

Brettanomyces bruxellensis has historically been responsible for wine spoilage and credited with costing the worldwide wine industry millions of dollars annually (Fugelsang 1997). The yeast can cause wines to develop unpleasant odors that have been variously described as Band-Aid, ammonia, mouse droppings, burnt beans, and barnyardlike (Chatonnet et al. 1992, Hock 1990, Licker et al. 1999). Some winemakers suggest that the presence of *Brettanomyces* may contribute to complexity or may accelerate wine aging. *Brettanomyces bruxellensis* (formerly *B. intermedius*) has been the species most frequently identified in *Brettanomyces*, or Brett, wines (Sponholz 1993). To our knowledge, most wines suspected of being contaminated with *B. bruxellensis* have not been adequately characterized in terms of the strain(s) involved.

Brettanomyces bruxellensis is known to ferment low concentrations of sugar and ethanol (Kurtzman and Fell 1998), producing a wide range of metabolites, including volatile phenols, such as 4-ethylphenol and 4-ethylguaiaicol (Chatonnet et al. 1988, 1992, 1995; Dubois and Dekimpe 1982; Heresztyn 1986a, 1986b), and medium-chain fatty acids, including octanoic, dodecanoic (Rozes et al. 1992), isobutyric, isovaleric, and 2-methylbutyric acids (Fugelsang 1997). Other odor-active compounds produced by *Brettanomyces* spp. include 2-phenylethanol, isoamyl alcohol, *cis*-2-nonenal, *trans*-2-nonenal, β -damascenone, and ethyl decanoate (Licker et al. 1999). Individually or collectively, these compounds may influence the sensory profile of a wine.

Ethylphenols have been cited as contributors to the Brett aroma of some wines (Chatonnet et al. 1988, 1990; Dubois et al. 1971, Schreier et al. 1980, Singleton and Noble 1976). The two-step mechanism for 4-ethylphenol formation involves a carboxylase-catalyzed decarboxylation of the phenolic acid directly to vinylphenol, and conversion of vinylphenol into ethylphenol (Chatonnet et al. 1992, 1995). Historically, the presence of ethylphenols in wines was attributed solely to the growth of *Brettanomyces* (Dubois 1983, Williams 1974). Chatonnet et al. (1990, 1995) determined that the concentration of both ethyl and vinyl phenols produced by lactic acid bacteria in red wine was insignificant compared to the odor thresholds. 4-Ethylguaiaicol is responsible for eliciting odors described as bacon and smoky (Aldrich Flavors and Fragrances Catalog, Sigma-Aldrich, Milwaukee, WI) and has a reported sensory threshold of 0.047 mg/L (Chatonnet et al. 1990). 4-Ethylphenol is associated with medicinal, phenolic, and pungent descriptors and has a reported sensory threshold of 0.23 mg/L (Chatonnet et al. 1990). These two volatile phenols, together with guaiaicol, have been reported to be responsible for the Brett character of some wines (Chatonnet et al. 1995). Licker et al. (1999) reported that wines with high, medium, and no Brett character had 4-ethylphenol concentrations of 3.0, 1.74, and 0.68 mg/L, respectively. Chatonnet et al. (1992) proposed that 4-ethylphenol be used as a marker for past and present *Brettanomyces* activity. However, Gerbaux et al. (2002) established that certain enological practices, such as the use of enzymes and thermal processing, influence the level of volatile phenols produced by *Brettanomyces*. The influence of different strains of *B. bruxellensis* on the production of these and other volatiles has not been fully elaborated.

The worldwide winemaking community is divided regarding the potential merits or deficiencies imparted by the growth

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of *Brettanomyces* in wine. The current study suggests that these differences may result from strain variation, length of time for *Brettanomyces*-wine contact, and the ratio of current to cumulative (total) cell exposure. This study evaluated the growth of six strains of *B. bruxellensis* in Pinot noir wines. Selected volatiles and sensory differences post-*Brettanomyces* fermentation were also investigated.

Materials and Methods

Grape and wine processing. Sonoma County, California, Pinot noir fruit (24.4 Brix, 4.3 g/L titratable acidity, pH 3.81) was crushed, without addition of sulfur dioxide and inoculated at a level of 2×10^6 cfu/mL using re-isolated *Saccharomyces cerevisiae* Simi White (Lallemand, Montreal, Canada). The inoculum was prepared by growth on YM agar (Bacto® YM Broth [Difco Laboratories, Detroit, MI] with 2% w/v Agar, type E [Sigma Chemical Co., St. Louis, MO], adjusted to pH 5 with citric acid) for six days, and a single isolated colony was transferred to 50 mL of heat-sterilized grape juice diluted 1:2 with sterile water. This volume was increased incrementally, using sterile-filtered (0.45 µm) juice, to an inoculum volume approximating 3% (v/v) of the fermentation. Progress was followed microscopically using standard methods (Fugelsang 1997).

Prior to starter inoculation, grape must was acidulated at 1 g/L, using tartaric acid. Fermentation was carried out at 20 to 22°C. A single nutrient supplement of diammonium phosphate (250 mg/L) was added at 15 Brix. Cap solids were mixed back into the fermenting juice twice a day. At 0 Brix, the solids phase was pressed (0.5 bar). Free-run and press fractions were combined and collected into 5-gallon demijohns and transferred to cold storage (4.4°C) followed by postfermentation clarification and cross-flow filtration (nominal 0.20 µm). Filtered wine was transferred to heat-sterilized 4-L containers and treated with Velcorin™ (dimethyldicarbonate, DMDC; Bayer Corporation, Pittsburgh, PA) at 600 mg/L. A stir bar was placed in each container to facilitate mixing.

Strain selection, preparation of starter cultures, and inoculation. Studies were designed to follow population changes of *B. bruxellensis* in Pinot noir table wine from low (<50 cfu/mL) inoculum levels through active growth, culminating in death/decline. The growth cycle of each strain was considered completed when titers dropped below 30 cfu/mL.

Twenty-four wine-isolated strains of *B. bruxellensis* were received from Lallemand (Montreal, Canada). Each was screened for growth in wine (R. Thornton 1999, personal communication). From this group, six were selected for further study. Each strain was transferred from growth medium to 250-mL aliquots of previously sterilized (DMDC 600 mg/L) Pinot noir wine and incubated for two weeks, at which time numbers exceeded 10^6 cfu/mL, as determined by visual cell-counting methods (Fugelsang 1997).

Once starters reached 10^6 cfu/mL, appropriate volumes of inoculum were transferred to 4-L containers (four replicates plus controls) of sterilized Pinot noir. Target inoculum was

<50 cfu/mL, which was verified by plating membrane-filtered aliquots of each strain. Inoculated wines and controls were incubated and maintained at 21.0°C.

Two separate studies were conducted. Study one involved six strains. Study two further evaluated observations noted in study one, particularly the apparent extended death and decline phase among several strains.

Microbial sampling. Each wine was aseptically sampled on a weekly basis from time of inoculation through the death and decline phase. Each 4-L container was stirred for 15 min prior to sample removal. During growth and stationary phases, appropriately diluted (0.5- and 1-mL) aliquots were prepared directly as pour plates. During early and late death/decline phases, multiple volumes of samples were membrane-filtered (0.45 µm). Membranes and their collected cells were subsequently transferred to YM agar. At sampling, 25-mL aliquots of each culture were collected and frozen (-20°C) for subsequent chemical analyses. All plates were incubated at 26.7°C for seven to eight days, at which time colony counts were made. Cumulative cell exposure (CCE) was defined as the sum of the colony forming units (cfus) for a particular date and all of the preceding sampling dates.

Chemical analysis. Free volatiles were determined by headspace solid-phase microextraction (HE-SPME) as described by Whiton and Zoecklein (2000), coupled with gas chromatography/mass spectrometry (GC/MS). The HE-SPME sampling was performed by desorbing a divinylbenzene Carbowax® (Supelco, Bellefonte, PA) fiber in the injection port of a Hewlett-Packard (Palo Alto, CA) 5890 GC, coupled to a Hewlett-Packard 5972 MS operating in full scan *m/z* 30 to 300 at 2.8 scans/sec. The GC column was a 30 m x 0.25 mm DB-Wax (J&W Scientific, Folsom, CA) with helium carrier gas at 11 psi and a linear velocity of 36 cm/sec. Injections were made in the splitless mode at 40°C, held for 5 min, then ramped 6°C/min to 230°C. The injector temperature was 250°C and the transfer line temperature was 240°C.

A control wine sample used in study one was spiked with 4-ethylphenol and 4-ethylguaiaicol and analyzed in triplicate using HE-SPME. Recovery for both compounds was 48% at 0.5 mg/L additions (n = 6), compared to 77% for each compound recovered from a spiked model solution of 10% (v/v) ethanol, pH 3.2. These results confirm the work of Whiton and Zoecklein (2000) on the impact of ethanol in the recovery of these volatile phenols. Data reported in the current study have not been adjusted for recovery loss.

Wines were analyzed for alcohol (v/v) by distillation and for pH using an Accumet® model 20 (Fisher Scientific, Pittsburgh, PA) pH/conductivity meter, as described by Zoecklein et al. (1995). HPLC analysis of the organic acid profile of each wine replicate was conducted at three stages during this study to monitor possible malolactic fermentation resulting from contamination. Analysis was conducted using an isocratic system (Hewlett-Packard, model 1100) at 230 nm and a Fast Acid® 110 x 7.8 mm column (Bio-Rad, Hercules, CA). Concentration of sugars (fructose plus glucose, galactose, and

trehalose) was determined by derivatization and GC/MS, as described by Whiton et al. (1985).

Sensory analysis. Following *Brettanomyces* population decline to <30 cfu/mL, wines were racked and bottled. For study one, sensory evaluations of bottled wines were performed using duo-trio difference testing as described by Meilgaard et al. (1991) with 12 trained evaluators. Twenty-five mL of wine was presented under red light and in colored glasses to negate any wine color differences. All samples were presented in standard ISO glassware at 20°C. For the duo-trio test, each *B. bruxellensis*-inoculated wine was tested against the others and the control in a balanced, complete design with aroma and flavor combined using evaluators familiar with wine and the testing technique. Significance of the duo-trio sensory evaluations was determined using one-tailed tests ($p \leq 0.1$) as described by Meilgaard et al. (1991).

For the consensus training analysis, 12 experienced wine evaluators (10 male, two female) from the wine industry and

the academic community served as panelists. In four training sessions of approximately two hours, panelists were provided with aroma standards and selected wines from the experiment and were instructed on the test procedure. The formal evaluations occurred in individual booths in a well-ventilated room under standard conditions over a four-week period, with no more than two evaluations in one day. Six or seven wines were presented per session in a different random order to each panelist for each of the triplicate evaluations. Twenty-five mL of wine was presented in ISO glassware at 20°C. Descriptive analyses were performed using stepwise multiple regression procedures of SAS statistical software (SAS Institute, Cary, NC).

Results and Discussion

Study 1. Variation was noted in the growth rate of *Brettanomyces bruxellensis* strains as monitored by cfu counts (Figure 1). *Brettanomyces* spp. are suspected to have postmortem

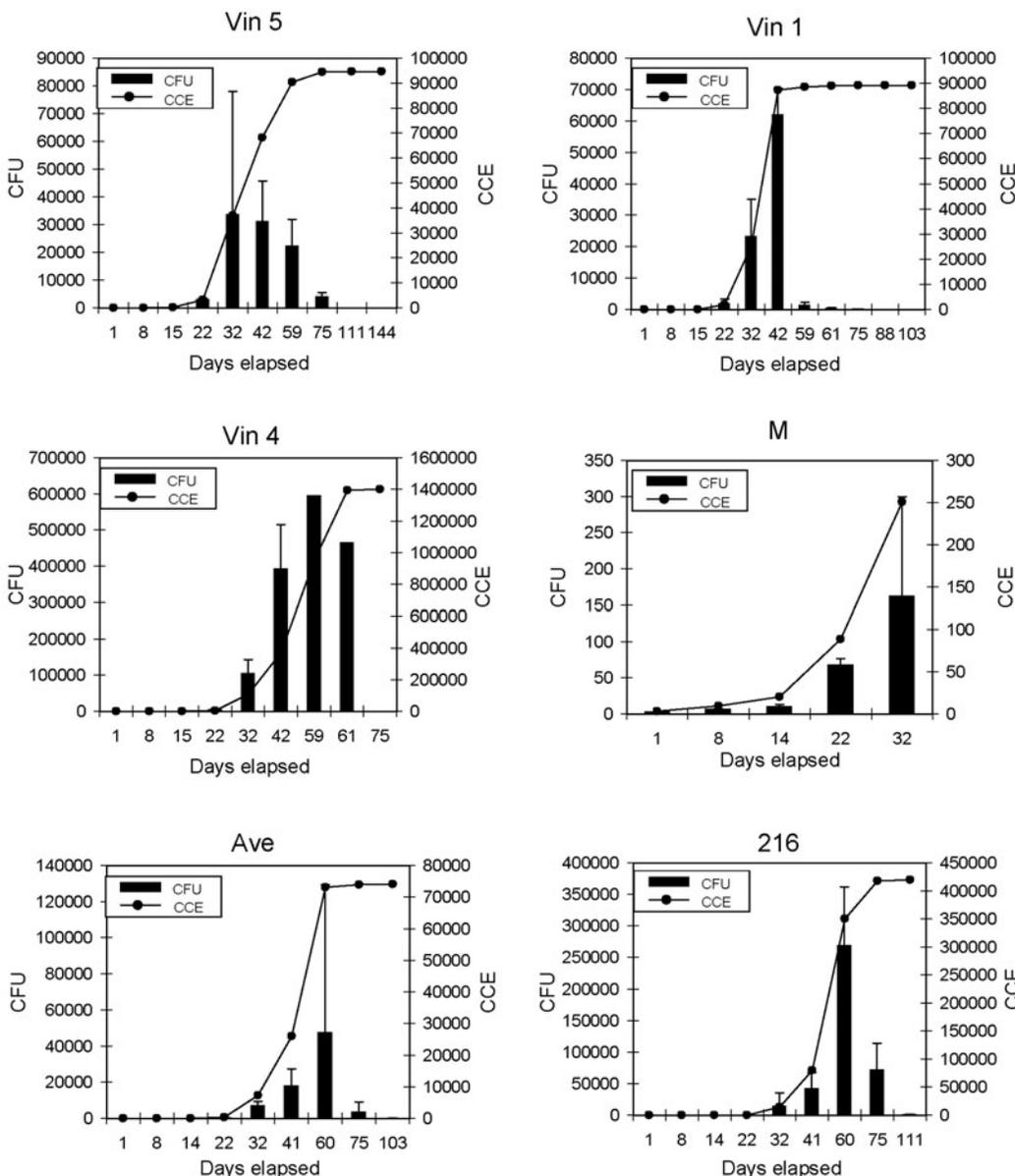


Figure 1 Colony forming units/mL and cumulative cell exposure (CCE) of six strains of *Brettanomyces bruxellensis* in Pinot noir wine: Vin 5, Vin 4, Ave, Vin 1, M, and 216.

sensory implications. This study considered cumulative cell exposure (CCE) during the growth cycle. Thus, CCE was defined as the sum of the cfus for the sample date and all preceding dates.

Highest cfus were observed with the Vin 4 strain, which peaked at 59 days postinoculation. The Vin 4 cfus were approximately four times those of strain 216, and averaged about 14 times higher than strains Ave, 1, and 5, which were roughly similar to one other. Strain M was characterized by poor growth. The highest CCE was observed with Vin 4, which was more than three times that of the next most prolific organism, strain 216. As with cfus, CCEs were similar among strains Ave, Vin 1, and Vin 5.

At the completion of growth, wine pH (mean 4.0) and alcohol percent (v/v) (mean 13.6) did not differ among strains, their replications, or controls. There were slight differences in titratable acidity, which ranged from 4.78 g/L in the control wines to 5.34 g/L in strain Vin 4 wines. Strains 216, Ave, Vin 5, and Vin 1 all had similar titratable acidity, which averaged 5.06 g/L. HPLC analysis of organic acids during and at the completion of the *Brettanomyces* growth phase confirmed the absence of malolactic fermentation among strain replications.

The control wines had no detectable ethyl-2-methylbutyrate, guaiacol, 4-ethylguaiacol, or 4-ethylphenol (Table 1). Among inoculated wines at the completion of the *Brettanomyces* growth phase, the concentration of ethyl-2-methylbutyrate and guaiacol was near or below the detection limit (0.01 mg/L). The levels of isoamyl alcohol, ethyldecanoate, isovaleric acid, and 2-phenylethanol varied somewhat among the strains. Wines inoculated with strain Vin 5 had among the highest concentrations of isoamyl alcohol, guaiacol, and 2-phenylethanol, while wines inoculated with Vin 4 had the highest concentrations of isovaleric acid. All wines had similar concentrations of ethyldecanoate, with the exception of Vin 5.

The concentration of 4-ethylguaiacol and 4-ethylphenol at the completion of the *Brettanomyces* growth phase differed among strains (Table 1). Strains 216 and Vin 5 were distin-

guished from M and had the highest concentrations of both metabolites. Both 4-ethylphenol and 4-ethylguaiacol were positively but weakly correlated to CCEs ($R^2 = 0.48$ and 0.52 , respectively). Strains Vin 1, 216, and Ave demonstrated maximum 4-ethylphenol concentrations after CCEs reached $6-8 \times 10^4$ cfu/mL. Vin 5 did not produce similar 4-ethylphenol concentrations until CCE reached 1.5×10^5 cfu/mL (data not shown). Strain M was characterized by limited cell growth and correspondingly limited 4-ethylphenol production.

Chatonnet et al. (1992, 1995) demonstrated the importance of winemaking practices, including barrel storage, on *Brettanomyces* metabolite production. Cultivar is also believed to influence the concentration of volatile phenols. Concentrations of 4-ethylphenol were relatively low, even when accounting for recovery loss, but above the reported sensory threshold. However, the wines in this study were perceived as possessing a Brett character, possibly suggesting the importance of other metabolites. Licker et al. (1999), using gas chromatography-olfactometry Charm Analysis technique and gas chromatography-mass spectrometry, found that the odor activity of three volatile phenols (4-ethylphenol, 4-ethylguaiacol, and guaiacol) was half or less that of isovaleric acid. They reported that isovaleric acid (3-methylbutanoic acid), described as having a rancid character, was the dominant odorant in high *Brettanomyces* wine. In the current study, the concentration of this acid in the control wines was similar to that found in *Brettanomyces* wines, with the exception of Vin 4, which had the highest cell population.

Duo-trio sensory analysis suggested that there were differences among the *Brettanomyces*-inoculated and control wines and among strains (Table 2). As a general rule, a minimum of 16 test subjects are desirable for a duo-trio test; however, fewer may be employed if the differences are large (Meilgaard et al. 1991). Sensory differences appeared evident between wines inoculated with strains Ave and Vin 5, M and 216, and M and Vin 4. However, the limited number of panels may have increased the beta error in this study. Stepwise multiple regression analysis of the aroma descriptors used in the consensus training analysis indicated that the most important attributes differentiating *Brettanomyces* strains from control wines were earthy, smoky, spicy, and cardboard descriptors, as indicated in Table 3.

Study 2. Study two was initiated to further investigate observations noted in study one, particularly the apparent extended death and decline phase among several strains. Growth profiles for strains 212, 216, and Vin 3 were compared for 712 days post-*Brettanomyces* inoculation (Figure 2). Significant variations in growth characteristics, population densities, and

Table 1 Concentration (mg/L) of selected volatile compounds produced by six strains of *Brettanomyces bruxellensis* in Pinot noir.

Compound	Control	<i>B. bruxellensis</i> strain					
		Ave	M	216	Vin 1	Vin 4	Vin 5
Ethyl-2-methylbutyrate (mg/L)	0.00b ^a	0.00b	0.00b	0.01ab	0.00b	0.02a	0.01ab
Isoamyl alcohol (mg/L)	230ab	224ab	208b	227ab	223ab	221ab	240a
Ethyldecanoate (mg/L)	0.37a	0.37a	0.36a	0.42a	0.39a	0.41a	0.20b
Isovaleric acid (mg/L)	1.5bc	1.3c	1.3c	1.5bc	1.4bc	2.3a	1.8b
Guaiacol (mg/L)	0.00b	0.00b	0.00b	0.01ab	0.00b	0.01ab	0.02a
2-Phenylethanol (mg/L)	21.0ab	20.7abc	18.4c	21.7ab	21.5ab	19.8bc	22.8a
4-Ethylguaiacol (mg/L)	0.00b	0.05ab	0.00b	0.12a	0.08ab	0.06ab	0.09a
4-Ethylphenol (mg/L)	0.00b	0.15ab	0.01b	0.44a	0.24ab	0.16ab	0.37a

^aSignificance of LSD test of treatment means. Means with the same letter are not significantly different at the $p \leq 0.05$ level.

Table 2 Results of duo-trio sensory evaluations of Pinot noir wines inoculated with different strains of *Brettanomyces bruxellensis*.

Comparison	Correct responses vs total
Control vs Ave	9:12*
Control vs M	11:14*
Control vs 216	20:28*
Control vs Vin 1	10:13*
Control vs Vin 4	11:12*
Control vs Vin 5	12:16*
Ave vs M	6:12
Ave vs 216	8:12
Ave vs Vin 1	5:12
Ave vs Vin 4	6:12
Ave vs Vin 5	9:12*
M vs 216	20:24*
M vs Vin 1	6:12
M vs Vin 4	11:15*
M vs Vin 5	9:14
216 vs Vin 1	6:15
216 vs Vin 4	7:12
216 vs Vin 5	5:12
Vin 1 vs Vin 4	7:12
Vin 1 vs Vin 5	7:12
Vin 4 vs Vin 5	7:12

*Indicates significant differences at the $p \leq 0.1$ level when measured by duo-trio significance testing.

Table 3 Stepwise selection summary of aroma attributes differentiating *Brettanomyces*-inoculated from control Pinot noir wine.

Description	$p > F$
Plastic	0.7251
Metallic	0.7129
Woody	0.7045
Ammonia	0.6933
Floral	0.6465
Rancid	0.5350
Fruity	0.2088
Vegetative	0.1279
Cardboard	0.0467
Spicy	0.0161
Smoky	0.0116
Earthy	0.0006

4-ethylphenol production were observed over the 712-day sampling period. Following 712 days, three successive sampling periods covering 1.5 months showed no difference in cell concentration. Strain 213 reached a maximum of 4.3×10^4 cfu/mL between days 137 and 158, followed by a gradual decrease to 543 cfu/mL by day 712. Strain 216 followed a similar trend, reaching a population maximum of 3.8×10^4 cfu/mL between days 137 and 158, followed by a rapid drop-off to 2×10^3 cfu/mL between days 217 and 265. Strain 216 underwent a secondary growth cycle, beginning on sample date 307 and lasting through day 473, reaching a maximum of 1.5×10^4 cfu/mL on day 434. Strain Vin 3 also demonstrated

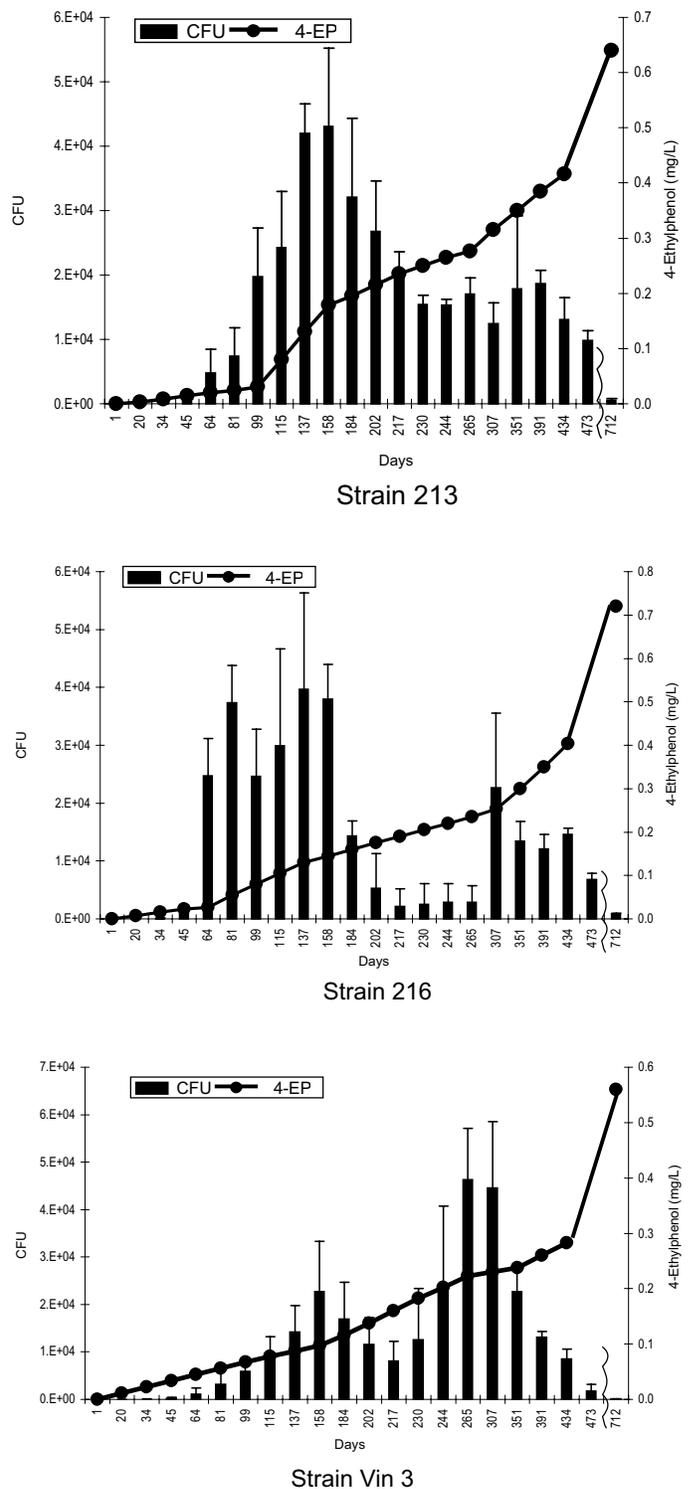


Figure 2 Colony forming units/mL and concentration (mg/L) of 4-ethylphenol produced by three strains of *Brettanomyces bruxellensis* in Pinot noir wine.

two growth phases: initial population peaked at day 158 (2.3×10^4 cfu/mL) and dropped off to 8×10^3 cfu/mL on day 217, followed by a more robust period of activity, reaching a maximum of 4.6×10^4 cfu/mL between days 265 and 307. Numbers dropped to <100 cfu/mL by the conclusion of the study on day 712.

The production of 4-ethylguaiacol followed a trend similar to 4-ethylphenol, but did not differ among strains at day 473 (Table 4). The concentrations of 2-phenylethanol and isovaleric acid did not change as a result of *Brettanomyces* growth. Ethyldecanoate showed a maximum concentration after the count reached 5×10^3 cfu/mL (data not shown) and was similar among strains (Table 4). The concentration of isoamyl alcohol did not differ among strains but was lower than in control wines.

The production of 4-ethylphenol followed similar patterns among strains, with limited increase during the early growth periods, followed by a rapid and relatively uniform increase (Figure 2). The usefulness of 4-ethylphenol as an index of cfu was evaluated over the course of study two. The concentration of 4-ethylphenol continued to increase after cfus declined and, with each strain, 4-ethylphenol trended more strongly with cumulative exposure, rather than cfus. The production of CCE and 4-ethylphenol by each strain over the course of the 712-day study is shown in Figure 3. Vin 3 produced the highest concentration of 4-ethylphenol relative to CCE over the entire growth period, followed by strains 213 and 216. Although the presence of 4-ethylphenol tracked linearly with CCE, significant increases in the analyte concentration were most apparent toward the end of the growth cycle, particularly for strains 213 and 216, possibly suggesting autolytic release. This may suggest the importance of monitoring, using methods other than cfu counts, such as peptide nucleic acid analysis (Connell et al. 2002).

Charpentier and Feuillat (1993) reported that ethanol may react with autolytic products, including various fatty acids, forming volatile ethyl esters, which affect the aroma of wine. McMahon et al. (1999) determined the location of β -glycosidase activity for many of the strains employed in this study. Most strains evaluated demonstrated enzyme activity against a model glycoside in permeabilized or lysed cells, possibly suggesting the ability for enhanced hydrolysis in wines stored sur lie.

Strain 216 demonstrated an increase (80%) in 4-ethylphenol, corresponding to an increase in CCE from 2.3 to 2.8×10^5 cfu/mL. Similarly, strain 213 increased by the same percentage, with a change in CCE from 2.3 to 3.0×10^5 cfu/mL. Levels of 4-ethylphenol in Vin 3 increased 30%, corresponding to a CCE increase from 4.0 to 4.1×10^5 cfu/mL.

The concentration of several sugar substrates at day 0, day 158, and day 434 during study two is shown in Table 5. Fructose plus glucose declined by an average of 42.7% by day 434 for all strains. During this same period, the trehalose concentration for each strain declined from an average of 111.7

Table 4 Concentration (mg/L) of selected volatile compounds produced by three strains of *Brettanomyces bruxellensis* in Pinot noir wines at 473 days postinoculation.

Compound	Control	<i>B. bruxellensis</i> strain		
		216	213	Vin 3
4-Ethylphenol	0.0 c ^a	0.40 a	0.38 a	0.31 b
4-Ethylguaiacol	0.0 b	0.13 a	0.10 a	0.12 a
2-Phenylethanol	19.4 a	18.7 a	19.6 a	19.3 a
Isovaleric acid	1.28 a	1.18 a	1.32 a	1.30 a
Ethyldecanoate	0.22 b	0.56 a	0.51 a	0.48 a
Isoamyl alcohol	225 a	175 b	186 b	180 b

^aLSD analysis of treatment means. Means with the same letters are not significantly different at $p \leq 0.05$ level.

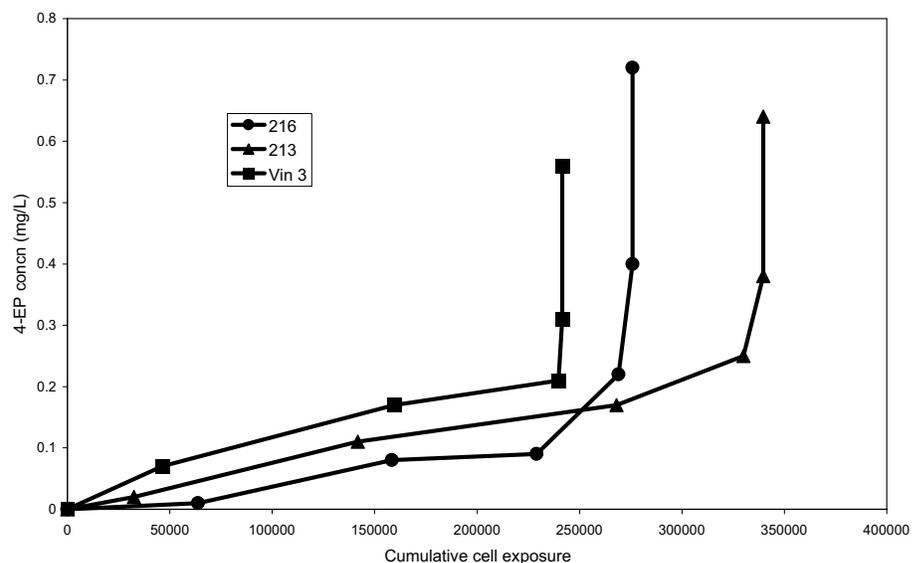


Figure 3 Cumulative cell exposure and concentration (mg/L) of 4-ethylphenol (4-EP) produced by three strains of *Brettanomyces bruxellensis* in Pinot noir wine.

Table 5 Effect of *Brettanomyces bruxellensis* strains on the concentration of residual sugars in Pinot noir wines.

Strain	Day	Sugars (mg/L)		
		Fructose, glucose	Galactose	Trehalose
216	0	1243	86	129
	158	920	82	10
	427	700	43	0
213	0	2000	78	162
	158	1366	73	12
	434	1167	67	0
Vin 3	0	1433	51	120
	158	1058	53	9
	434	773	44	0

mg/L to zero. The vast majority of this substrate was used in the relatively early stages of growth. For example, strain 213 demonstrated a maximum cell concentration at or near day 158, when 88% of the trehalose had been metabolized (Table 5, Figure 2). Chatonnet et al. (1995) investigated the role of sugars (glucose, fructose, galactose, and trehalose) on the production of ethylphenols. They concluded that 275 mg/L of residual sugars was a sufficient amount of carbon to produce threshold levels of ethylphenols.

Conclusion

This investigation followed the growth cycle of several strains of *Brettanomyces bruxellensis* in Pinot noir wine for up to 712 days. To approximate conditions that might occur in commercial winemaking, each strain was initially inoculated at <50 cfu/mL. Growth cycles varied from 61 days postinoculation for Vin 1 to over 712 days for strains 213, 216, and Vin 3. One strain demonstrated poor growth and did not complete its growth phase. Among those strains that completed the growth cycle, the time required to achieve maximum titer varied significantly. Further, the decline phase of the growth cycle varied among strains. Wines inoculated with strains 213, 216, and Vin 3 were found to support relatively dense titers.

The study also found a poor correlation between the marker metabolite 4-ethylphenol and colony forming units at any stage in the growth cycle. Rather, 4-ethylphenol trended with cumulative cell exposure, suggesting that the metabolite may be released upon yeast cell death and autolysis.

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