

Article

# Use of Nutritional Requirements for *Brettanomyces bruxellensis* to Limit Infections in Wine

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**Abstract:** Specific vitamin requirements of the wine spoilage yeast, *Brettanomyces bruxellensis*, were evaluated. Previous studies had not taken into influences of ethanol or nutrient carry-over between sequential transfers into vitamin-omitted media. Knowing nutritional needs, limiting growth of the yeast in wine by selective removal of important vitamins was investigated. Six strains of *B. bruxellensis* were grown and sequentially transferred into single vitamin-omitted media. None of the strains required *p*-aminobenzoic acid, folic acid, nicotinic acid, *myo*-inositol, pantothenic acid, or riboflavin. While some needed thiamin depending on the absence/presence of ethanol, growth of all strains was greatly affected by biotin. Here, concentrations  $\geq 0.2$   $\mu\text{g/L}$  were required to achieve yeast populations  $>10^6$  cfu/mL for high ( $10^4$  cfu/mL) or low ( $10^2$  cfu/mL) initial inoculums. At concentrations  $<0.2$   $\mu\text{g/L}$ , culturabilities either remained unchanged or increased less than two logs after 40 days. Since the protein avidin binds irreversibly to biotin, egg whites containing avidin or the purified protein were added to a wine to diminish bioavailability of the vitamin. While biotin concentrations were reduced, populations of *B. bruxellensis* achieved were decreased by one to three logs, thereby supporting further development of biotin depletion strategies in winemaking.

**Keywords:** *brettanomyces*; nutrition; vitamins; biotin; avidin; wine

## 1. Introduction

The spoilage yeast *Brettanomyces bruxellensis* is generally regarded as detrimental to wine production [1]. Unfavorable sensory descriptors of “leather”, “clove”, “spice”, “smoke”, “animal”, “stable”, “earth”, “cardboard”, and “medicinal” are among those used to describe the characteristics of wine spoiled by this yeast [2–4].

Although low amounts of sugar ( $<0.275$  g/L) or yeast assimilable nitrogen ( $<6$  mg·N/L) remaining after primary fermentation are sufficient for spoilage during red wine aging [5–7], little is known regarding its requirements for trace nutrients (e.g., vitamins and minerals). Pyridoxine, thiamine, and/or biotin are thought to be required by *B. bruxellensis*, as evidenced by deterred growth in vitamin-omitted media [8–10]. However, previous studies did not account for the effects of vitamin carry-over between media transfers [11], genetic heterogeneity between strains [8,12], or the presence of ethanol in the test media as would be present in wine [13,14].

*B. bruxellensis* can grow over a wide range of pH, temperatures, oxygen availability, sulfites, and ethanol levels [13–17]. As such, winemakers frequently rely upon multiple strategies to limit microbial spoilage [18]. Individual strategies have included the applications of sulfites [15,16,19], filtration [20], extreme temperatures [8,15,17,21], ethanol [13,14], ozone [22], pulsed electric field [23], low electric current [24], chitosan [25], dimethyl dicarbonate [26,27], and others. In fact, combinations of these strategies can help limit infections through synergistic interactions and variable tolerances by strains [28,29].

In addition to these individual strategies, another potential method to reduce spoilage could be removal of required nutrients. For example, if a given vitamin were uniformly required by *B. bruxellensis*, reducing its bioavailability in wines may help limit infections. Thus, the objectives of this study were to (a) determine specific vitamin requirements of *B. bruxellensis* while accounting for vitamin carry-over and presence of ethanol, and (b) evaluate strategies to selectively remove specific vitamin(s) from wine. In general, all strains studied required biotin whose removal from wine resulted in limiting growth over an incubation period of 40 days.

## 2. Materials and Methods

### 2.1. Strains

*B. bruxellensis* strains B1b, E1, I1A and N2 were originally described by Jensen et al. [16] (Washington State University, Pullman, WA, USA). Strains 2049 and 2091, isolated from New Zealand and French wines, respectively, were obtained from the Phaff Yeast Culture Collection (University of California, Davis, CA, USA). All strains were stored in 30% *w/w* glycerol at  $-80^{\circ}\text{C}$ , maintained on WL agar (Fisher Scientific, Waltham, MA, USA), and grown at  $25^{\circ}\text{C}$ .

### 2.2. Nutritional Requirements

Vitamin needs were assessed in a growth medium described by Kurtzman et al. [30] with all reagents and vitamins obtained from Sigma-Aldrich (St. Louis, MO, USA). Briefly, the base medium consisted of glucose (10 g/L),  $(\text{NH}_4)_2\text{SO}_4$  (5 g/L),  $\text{KH}_2\text{PO}_4$  (0.85 g/L),  $\text{MgSO}_4$  (0.5 g/L),  $\text{K}_2\text{HPO}_4$  (0.15 g/L), NaCl (0.1 g/L),  $\text{CaCl}_2$  (0.1 g/L), DL-methionine (20 mg/L), DL-tryptophan (20 mg/L), L-histidine monohydrochloride (10 mg/L),  $\text{H}_3\text{BO}_3$  (500  $\mu\text{g/L}$ ),  $\text{MnSO}_4$  (400  $\mu\text{g/L}$ ),  $\text{ZnSO}_4$  (400  $\mu\text{g/L}$ ),  $\text{FeCl}_3$  (200  $\mu\text{g/L}$ ),  $\text{Na}_2\text{MoO}_4$  (200  $\mu\text{g/L}$ ), KI (100  $\mu\text{g/L}$ ), and  $\text{CuSO}_4$  (40  $\mu\text{g/L}$ ) and was adjusted to pH 3.5 with HCl. The vitamins examined were *p*-aminobenzoic acid (200  $\mu\text{g/L}$ ), biotin (2  $\mu\text{g/L}$ ), folic acid (2  $\mu\text{g/L}$ ), *myo*-inositol (2 mg/L), nicotinic acid (400  $\mu\text{g/L}$ ), calcium pantothenate (400  $\mu\text{g/L}$ ), pyridoxine hydrochloride (400  $\mu\text{g/L}$ ), riboflavin (200  $\mu\text{g/L}$ ), and thiamin (1 mg/L) at concentrations recommended by Kurtzman et al. [30]. Vitamins were individually sterile-filtered through 0.22  $\mu\text{m}$  filters and aseptically added to previously autoclaved media (15 min at  $121^{\circ}\text{C}$ ). All solutions and media were wrapped in aluminum foil to prevent potential light degradation and glassware was rinsed in 1 N HCl after washing to remove any trace residual material.

Media were transferred into  $15 \times 150$  mm Klett tubes (4.2 mL), fitted with Identi-Plug foam inserts (Jaece Industries, North Tonawanda, NY, USA) and plastic dilution blank caps, and autoclaved. Once cooled, vitamin solutions and a mixture of distilled water/ethanol were aseptically added to yield 5 mL total volume containing 0% or 10% *v/v* ethanol. Strains of *B. bruxellensis* were inoculated into triplicate tubes at  $10^4$  cfu/mL. Turbidity was assessed using a Klett-Summerson photoelectric colorimeter equipped with a green filter (Klett Manufacturing Co., New York, NY, USA). Upon reaching a turbidity of 75 Klett units (or as close as possible), cultures were centrifuged at  $3000 \times g$  for 20 min and pellets washed  $3 \times$  with 10 mL 0.2 M phosphate buffer (pH 7.0) made up of 9.36 g/L potassium phosphate monobasic, anhydrous and 32.73 g/L sodium phosphate dibasic heptahydrate (Fisher Scientific). After the final wash, cells were resuspended in 5 mL 0.2 M phosphate buffer and re-inoculated (1% *v/v*) into  $15 \text{ mm} \times 150 \text{ mm}$  Klett tubes containing 5 mL fresh media. Harvesting cells and reinoculation into vitamin-omitted media was repeated to yield three sequential transfers ( $S_1 \rightarrow S_2 \rightarrow S_3$ ).

Using the medium described above, the influence of concentrations of biotin on *B. bruxellensis* growth was evaluated. Culturable populations were determined by Autoplate<sup>®</sup> 4000 spiral plater (Spiral Biotech, Bethesda, MD, USA) using WL agar spread plates. Starter cultures were prepared by harvesting cells and sequential reinoculation (twice) into biotin-omitted media.

### 2.3. Biotin Removal

Residual sulfites present in a commercial red wine (2014 Carlo Rossi Burgundy, Modesto, CA, USA) were removed by addition of 3% *v/v* H<sub>2</sub>O<sub>2</sub> as confirmed by measurement of total SO<sub>2</sub> using the aeration-oxidation method [31]. Glucose (0.5 g/L), fructose (0.5 g/L), yeast extract (0.1 g/L), and *p*-coumaric acid (20 mg/L) were added to the wine prior to filtration through 0.45 μm (Fisher Scientific). Based on the initial concentration of biotin in the wine as determined by the microbiological method described by Angyal [32], additional vitamin was added to yield a total concentration of 2 μg/L. The wine was distributed into 1 L sterilized bottles before addition of either purified avidin (Fisher Scientific) or egg whites, which contain avidin and are used as fining agents in the wine industry. Avidin powder (0.2 g) was added to dialysis tubing (MWCO = 1000 kDa, Fisher Scientific) and continually mixed on a stir plate for 24 h. Egg whites (27 g) were mixed with 40 mL distilled H<sub>2</sub>O and 0.85 g KCl with 8 mL of this suspension was added to the wine which was continually mixed for 14 days. Wine aliquots were removed at various time points and immediately frozen (−80 °C) for biotin quantification. Remaining wines were filtered through 0.45 μm, divided into triplicate 500 mL bottles (330 mL each), and inoculated with biotin-omitted starter cultures of *B. bruxellensis* strains 2091 or I1a at initial populations of 10<sup>4</sup> or 10<sup>2</sup> cfu/mL.

### 2.4. Statistics

Differences in average maximum populations (x<sub>max</sub>) calculated as log values were evaluated using ANOVA and Tukey’s HSD by XLSTAT (Addinsoft, New York, NY, USA). Figure data points were presented as mean ± standard deviation (vertical bars).

## 3. Results

Strains of *B. bruxellensis* were inoculated into synthetic media lacking one of nine vitamins and without/with addition of 10% *v/v* ethanol. Neither strain E1 or I1a required *p*-aminobenzoic acid, folic acid, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, nor thiamin, as evidenced by strong growth after three transfers into vitamin-omitted media (Table 1). With 10% ethanol added, both strains exhibited delayed growth in the absence of *myo*-inositol or thiamin, an effect not observed in media without ethanol. However, growth of strains E1 and I1a was delayed and then limited (<75 Klett) by the third transfer in media without biotin regardless of ethanol content.

**Table 1.** Growth of *Brettanomyces bruxellensis* strains E1 and I1a after sequential transfers in defined synthetic wine medium.

Omitted Vitamin	Strain											
	E1						I1a					
	0% Ethanol			10% Ethanol			0% Ethanol			10% Ethanol		
	S <sub>1</sub> *	S <sub>2</sub>	S <sub>3</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>
None (Control)	++	++	++	++	++	++	++	++	++	++	++	++
<i>p</i> -Aminobenzoic acid	++	++	++	++	++	++	++	++	++	++	++	++
Biotin	++ <sup>d</sup>	+	-	++ <sup>d</sup>	++ <sup>d</sup>	+	++ <sup>d</sup>	+	-	++ <sup>d</sup>	++ <sup>d</sup>	+
Folic acid	++	++	++	++	++	++	++	++	++	++	++	++
<i>myo</i> -Inositol	++	++	++	++ <sup>d</sup>	++ <sup>d</sup>	++ <sup>d</sup>	++	++	++	++	++ <sup>d</sup>	++ <sup>d</sup>
Nicotinic acid	++	++	++	++	++	++	++	++	++	++	++	++
Pantothenic acid	++	++	++	++	++	++	++	++	++	++	++	++
Pyridoxine	++	++	++	++	++	++	++	++	++	++	++	++
Riboflavin	++	++	++	++	++	++	++	++	++	++	++	++
Thiamin	++	++	++	++ <sup>d</sup>	++ <sup>d</sup>	++ <sup>d</sup>	++	++	++	++ <sup>d</sup>	++ <sup>d</sup>	++ <sup>d</sup>

\* Relative turbidity in first (S<sub>1</sub>), second (S<sub>2</sub>), or third (S<sub>3</sub>) culture transfers defined as ++ (>75 Klett); + (10–74 Klett); or – (<10 Klett); <sup>d</sup> Growth reached 75 Klett but was delayed by more than 24 h compared to the control.

Requirements for biotin, *myo*-inositol or thiamin were further studied using additional yeast strains (Table 2). Similar to E1 and I1a, strains B1b, N2, 2049, and 2091 exhibited only limited growth

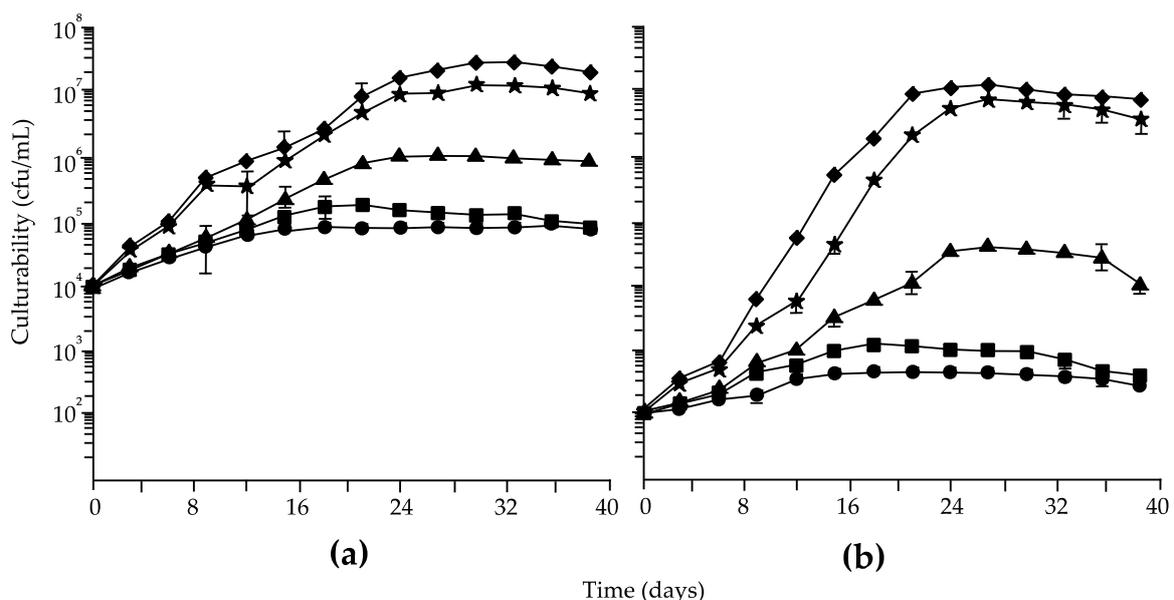
by the third transfer in the absence of biotin regardless of ethanol content. Without *myo*-inositol, none of the strains were affected in media lacking ethanol, while B1b and N2 exhibited delayed growth with 10% ethanol. Exclusion of thiamin produced a range of responses, with no growth by B1b (without ethanol) or 2091 (with ethanol) and delayed growth by N2 or 2049 by the third transfer.

**Table 2.** Growth of *Brettanomyces bruxellensis* strains B1b, N2, 2049, and 2091 after sequential transfers in defined synthetic wine medium.

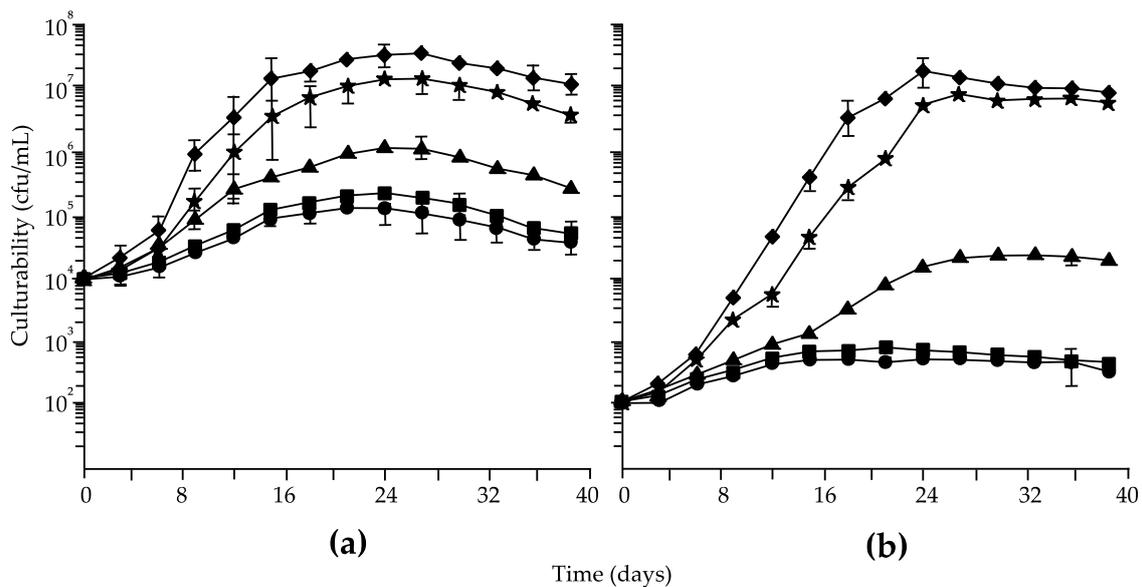
Ethanol (% v/v)	Omitted Vitamin	Strain											
		B1b			N2			2049			2091		
		S <sub>1</sub> *	S <sub>2</sub>	S <sub>3</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>
0%	None (Control)	++	++	++	++	++	++	++	++	++	++	++	++
	Biotin	++	++ <sup>d</sup>	+	++ <sup>d</sup>	++ <sup>d</sup>	+	++ <sup>d</sup>	++ <sup>d</sup>	+	++ <sup>d</sup>	++ <sup>d</sup>	+
	<i>myo</i> -Inositol	++	++	++	++	++	++	++	++	++	++	++	++
	Thiamin	++	+	-	++ <sup>d</sup>	++	++	++ <sup>d</sup>	++	++	++	++	++
10%	None (Control)	++	++	++	++	++	++	++	++	++	++	++	++
	Biotin	++	++ <sup>d</sup>	+	++ <sup>d</sup>	++ <sup>d</sup>	+	++	++ <sup>d</sup>	+	++	++ <sup>d</sup>	-
	<i>myo</i> -Inositol	++ <sup>d</sup>	++ <sup>d</sup>	++ <sup>d</sup>	++	++ <sup>d</sup>	++ <sup>d</sup>	++	++	++	++	++	++
	Thiamin	++ <sup>d</sup>	++ <sup>d</sup>	++ <sup>d</sup>	++ <sup>d</sup>	++ <sup>d</sup>	++ <sup>d</sup>	++	++ <sup>d</sup>	++ <sup>d</sup>	++	+	-

\* Relative turbidity in first (S<sub>1</sub>), second (S<sub>2</sub>), or third (S<sub>3</sub>) culture transfers defined as ++ (>75 Klett); + (10–74 Klett); or – (<10 Klett); <sup>d</sup> Growth reached 75 Klett but was delayed by more than 24 h compared to the control.

Biotin dependence was further investigated by culturing *B. bruxellensis* in the presence of varying concentrations (0 to 2 µg/L). In general, the extent of growth depended on the concentration of biotin present as well as initial populations. Here, strains 2091 (Figure 1) and I1a (Figure 2) could be recovered from biotin-omitted media where populations only increased less than one log 40 days after inoculation. When concentrations increased from 0.002 to 2 µg/L, average maximum populations ( $x_{max}$ ) also increased. With an initial inoculum of 10<sup>4</sup> cfu/mL, strains 2091 (Figure 1a) and I1a (Figure 2a) both reached  $x_{max}$  values of >2.0 × 10<sup>7</sup> cfu/mL in the medium containing 2 µg/L biotin while peak populations were <3.1 × 10<sup>5</sup> cfu/mL with 0.002 µg/L. At a lower initial inoculum (10<sup>2</sup> cfu/mL), a bigger effect was noted. Here, strains 2091 (Figure 1b) and I1a (Figure 2b) reached  $x_{max}$  values of >1.7 × 10<sup>7</sup> cfu/mL with 2 µg/L biotin but <1.4 × 10<sup>3</sup> cfu/mL at the lowest concentration tested.

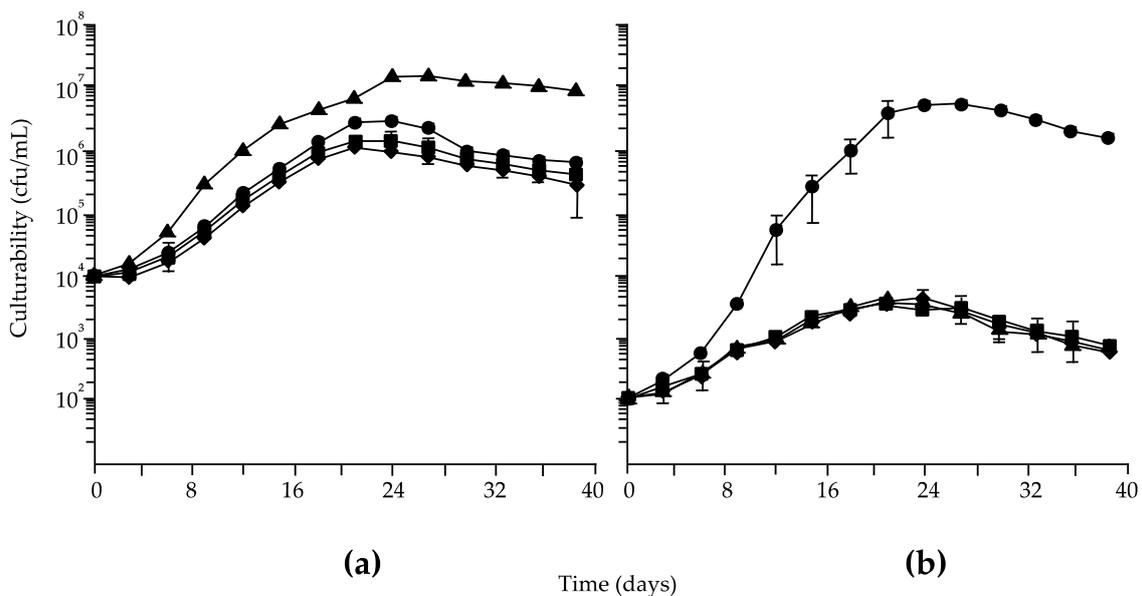


**Figure 1.** Culturabilities of *Brettanomyces bruxellensis* strains 2091 inoculated at (a) 10<sup>4</sup> or (b) 10<sup>2</sup> cfu/mL in media containing 0 (★), 0.002 (●), 0.02 (▲), 0.2 (■), or 2 (◆) µg/L biotin.

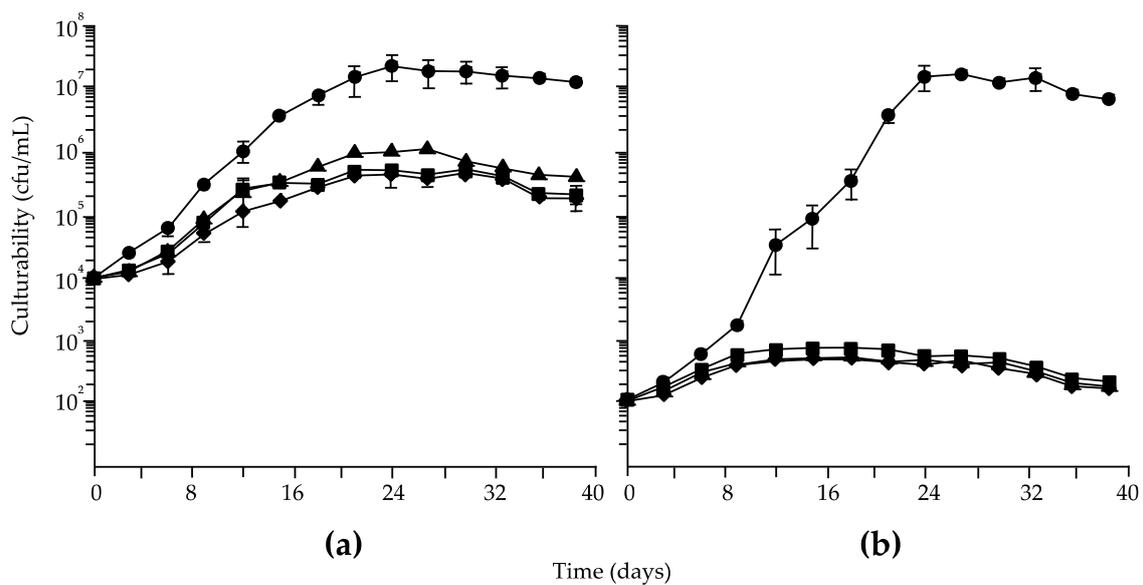


**Figure 2.** Culturabilities of *Brettanomyces bruxellensis* strains I1a inoculated at (a)  $10^4$  or (b)  $10^2$  cfu/mL in media containing 0 (★), 0.002 (●), 0.02 (▲), 0.2 (■), or 2 (◆) µg/L biotin.

Based on the impact of biotin on *B. bruxellensis*, methods were evaluated which could make the vitamin biochemically unavailable to thereby limit growth. Here, addition of egg whites or avidin to a red wine decreased biotin concentrations from an initial concentration of 1.9 µg/L down to <0.15 µg/L (data not shown), which resulted in decreases of  $x_{max}$  for the two strains studied. When inoculated at  $10^4$  cfu/mL, strains 2091 (Figure 3a) and I1a (Figure 4a) both achieved a  $x_{max}$  of  $>1.5 \times 10^7$  cfu/mL in untreated wines while those with egg whites or avidin added reached lower populations. Impacts of these treatments were more pronounced at a lower initial inoculum level ( $10^2$  cfu/mL) where populations of strains 2091 (Figure 3b) and I1a (Figure 4b) were  $<4.5 \times 10^3$  cfu/mL in treated wines. In comparison, untreated wines achieved  $x_{max}$  of  $>4.9 \times 10^6$  cfu/mL.



**Figure 3.** Culturabilities of *Brettanomyces bruxellensis* strains 2091 inoculated at (a)  $10^4$  or (b)  $10^2$  cfu/mL in wines with no additions (◆), with egg whites for 24 (▲) or 336 h (■), or with avidin suspended in dialysis tubing for 24 h (●).



**Figure 4.** Culturabilities of *Brettanomyces bruxellensis* strains I1a inoculated at (a)  $10^4$  or (b)  $10^2$  cfu/mL in wines with no additions (◆), with egg whites for 24 h (▲) or 336 h (■), or with avidin suspended in dialysis tubing for 24 h (●).

#### 4. Discussion

Initially, the vitamin requirements of two *B. bruxellensis* strains isolated from Washington state wines [16] were determined both with and without 10% ethanol, conditions similar to growth in wine. These strains were chosen for differences in region of isolation, antimicrobial tolerance, and genetic cluster [8,16]. Similar to previous work [8,9,11], growth of strains E1 and I1a was unaffected by the absence of *p*-aminobenzoic acid, folic acid, nicotinic acid, *myo*-inositol, pantothenic acid, or riboflavin. Thiamin was also not required by the yeast without ethanol, in agreement with some [9,33] but not others [8,10]. Similarly, pyridoxine was not found to be required, supporting some studies [10,13,33,34] but contradicting others [9]. Finally, biotin was required by both strains, in agreement with previous findings [8,10] but not others [9,33]. While additional strains studied (B1b, N2, 2049, and 2091) all required biotin, results regarding thiamin and *myo*-inositol varied by strain and presence of ethanol. In fact, *myo*-inositol was only required by strains B1b and N2 in 10% ethanol, possibly due to ethanol-induced increases in yeast membrane fluidity [35], which could affect vitamin transport. From a winemaking perspective, these findings imply that vitamin requirements between strains will differ depending on the stage of vinification when infection occurs, i.e., before or after alcoholic fermentation.

Vitamin carry-over and strain (genetic) diversity may have contributed to discrepancies in the literature regarding nutritional requirements of *B. bruxellensis*. As growth was more affected by biotin omission by the third transfer than in the first, this potentially explains why the early work of Burkholder et al. [9] did not report a requirement for this vitamin. However, Madan and Gulati [33] found none of the vitamins tested to be required despite accounting for vitamin carry-over. The present findings suggest that, with the exception of biotin, strain (genetic) diversity and presence of such components as ethanol also affects requirements. Here, strains E1, I1a, B1b and N2 were assigned to genetic cluster b while strains 2049 and 2091 were in clusters a and c, respectively [8]. Biochemically, biotin is involved in pyruvic acid carboxylation and the synthesis of pyrimidine bases, pyridine nucleotides, proteins, polysaccharides, and fatty acids. As such, deficiencies have been linked to damage of the yeast plasma membrane [36], as well as limiting maximum populations achieved in wine.

Because biotin was required by all strains examined and concentrations were correlated to growth in media, the possibility that making this vitamin unavailable to limit spoilage in a commercial wine

was evaluated. Avidin, a protein found in egg white [37], selectively binds biotin [38] and has been applied as a means to remove biotin from chemical solutions [39,40]. With dissociation constants ( $K_d$ ) of  $10^{-15}$  M at neutral pH, this binding complex is one of the strongest bonds known [38]. In fact, dried egg white (1 g) can bind approximately 11.5  $\mu\text{g}$  biotin [37]. Because a single molecule of avidin can bind four of biotin, the concentration of avidin added, either as pure protein or present in egg white, was in 50% excess in order to maximize vitamin removal. On average, grape musts from Washington contain approximately 2  $\mu\text{g}/\text{L}$  biotin [41]. However, avidin can also react with phenolics present in wine [42], thereby rendering the protein inactive against biotin. To compensate, dialysis tubing with small enough porosity to prevent the migration of most phenolics (1000 MWCO) has been used [40].

Compared to the untreated controls, growth in treated wines significantly reduced  $x_{\text{max}}$  at both inoculum levels ( $10^4$  and  $10^2$  cfu/mL) and for both strains (2091 and I1a). The greater contrast was noted in wines inoculated with  $10^2$  cfu/mL, an important observation because low populations of this yeast can lead to spoilage [1]. Because these two strains represented two different genetic clusters and regions of isolation [8,16], reductions in their populations suggests that avidin-based treatments may be effective against other strains. However, as some growth was still found to occur in treated wines, the use of avidin-based treatments should be considered as one of multiple hurdles necessary to limit spoilage. Although avidin has not been approved for use in winemaking, egg whites are readily used for clarifying commercial wines [1]. As biotin may be required by other microbial species beneficial to winemaking (e.g., alcoholic and malolactic fermentation), methods to remove biotin should be employed after completion of all fermentations. Future research should test for synergistic effects between avidin-based treatments and other hurdles known to help control *B. bruxellensis*.

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**Author Contributions:** Nicolas H. von Cosmos and Charles G. Edwards conceived and designed the experiments, analyzed data, and wrote the manuscript; Nicolas H. von Cosmos performed the experiments.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

MWCO	Molecular Weight Cut-Off
cfu	colony forming units

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