MOLECULAR SO2 EFFECT ON *BRETTANOMYCES BRUXELLENSIS* POPULATIONS DURING A ONE-YEAR STORAGE

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Introduction

Brettanomyces bruxellensis is one of the main red wine spoilage microorganism. Few stabilization techniques are available to limit and prevent *B. bruxellensis* growth in wine, SO_2 addition being mostly used. However, there is a lack of clear consensus among the scientific and winemakers communities on the efficient SO_2 concentrations to use. This may be due to *B. bruxellensis* strain variability, as well as the influence of the wine parameters (pH, temperature, ethanol concentration, SO_2 combining molecules) on SO_2 dissociation[1, 2]. Indeed, only molecular SO_2 is effective against microorganisms. This study aims at observing the behavior of three different *B. bruxellensis* strains during red wines storage with different initial molecular SO_2 concentrations (from 0 to 0.8 $mg.L^{-1}$). Cell growth and volatile phenol production were monitored for 30 days and after one year storage.

Materials and methods

B. bruxellensis strains from the IOEB Collection, named L0417, L0463 and L0516 were used to inoculate five different batches of the same sterile red wine in which SO_2 had previously been adjusted to reach 0, 0.2, 0.4, 0.6 and 0.8 $mg.L^{-1}$ molecular SO_2. Inoculations were aimed to reach an initial population of 10^3 $cells.mL^{-1}$. Microbial evolution was monitored 10, 20,30 days and one year after inoculation by direct epifluorescence filter technique (DEFT), culture plating and q PCR (Vineo™ *Brettanomy*test, Bio-Rad). All analyses were performed on two independent samples of each wine and in duplicate. Volatile phenols (VP),(ethyl-4-phenol and ethyl-4-guaiacol) concentrations were estimated on days 10, 30 and one year after by SBSE/GC/MS on two independent samples of each wine.

Results and discussion

SO_2 evolution during the storage of wines

SO_2 was monitored twice, first before *B. bruxellensis* inoculation to ensure that SO_2 adjustments in the different wine batches had been exactly done. A second estimation was performed 15 days after inoculation and showed that total, free and molecular SO_2 had drastically decreased (data not shown). Although a chemical oxidation of the wine cannot be excluded, it could also be conceived that the yeast may have either oxidized SO_2 into sulfate or reduced it into H_2S, and produced SO_2 combining molecules such as acetaldehyde. This could partly explain the greater resistance to SO_2 of *B. bruxellensis* as previously reported in the literature but deserves further investigations[(3)].

Effect of SO_2 addition on *B. bruxellensis* population

In absence of SO_2 all *B. bruxellensis* strains developed, up to to 10^7 UFC. mL^{-1} after 30 days when counted by q PCR, and 10^6 $cells.mL^{-1}$ by DEFT. Results were quite similar for wines initially sulfited with 0.2 to 0.6 $mg.L^{-1}$ molecular SO_2. Differences started to merge for the 0.8 $mg.L^{-1}$ molecular SO_2 for which little or no population evolution could be seen until 30 days. Hence, SO_2 addition tempered *B. bruxellensis* development, but could not, within the concentration range tested, decrease the population (Table 1).

Table 1: B. bruxellensis populations after 30 days storage in sulfited wines.

	Initial molecular SO_2									
L0417	0		0.2		0.4		0.6		0. 8	
q PCR UFC.mL-1 (sd)	1.8E+07	(3.1E+06)	1.4E+07	(2.1E+06)	1.4E+07	(3.1E+06)	8.4E+06	(2.1E+06)	1.3E+05	(6.7E+04)
DEFT cells.mL-1 (sd)	3.1E+05	(4.0E+04)	1.9E+05	(1.3E+04)	1.4E+05	(2.7E+04)	1.6E+05	(4.7E+03)	1.0E+04	(2.0E+03)
Culture UFC.mL-1 (sd)	1.5E+06	(9.2E+04)	1.0E+06	(1.3E+05)	1.0E+06	(8.5E+04)	7.8E+05	(2.1E+05)	5.9E+05	(3.0E+04)
L0463	0		0.2		0.4		0.6		0.8	
q PCR UFC.mL-1 (sd)	1.7E+07	(2.1E+06)	1.7E+07	(4.4E+06)	1.4E+07	(1.2E+07)	7.8E+06	(5.6E+06)	2.1E+04	(4 .7E+03)
DEFT cells.mL-1 (sd)	1.9E+06	(4.6E+05)	1.3E+06	(1.0E+05)	1.1E+06	(9.7E+04)	1.2E+06	(6.4E+04)	4.7E+03	(0.0E+00)
Culture UFC.mL-1 (sd)	7.4E+05	(7.8E+04)	5.0E+05	(3.5E+0)	4.1E+05	(2.9E+05)	3.6E+05	(2.5E+05)	0.0E+00	(0.0E+00)
L0516	0		0.2		0.4		0.6		0.8	
q PCR UFC.mL-1 (sd)	1.6E+07	(3.7E+06)	1.7E+07	(1.5E+06)	1.5E+07	(3.9E+06)	1.5E+07	(6.1E+06)	4.9E+06	(1.2E+06)
DEFT cells.mL-1 (sd)	3.9E+06	(1.9E+05)	2.5E+06	(2.5E+05)	2.5E+06	(8.3E+04)	2.0E+06	(1.5E+05)	9.5E+05	(3.4E+04)
Culture UFC.mL-1 (sd)	6.3E+05	(1.6E+03)	6.2E+05	(1.5E+03)	5.5E+05	(1.4E+03)	5.7E+05	(1.5E+03)	2.3E+05	(2.1E+04)

More precisely, the limiting effect of SO_2 started to be effective for L0417 and L0463 at 0.6 mg.L^{-1} initial molecular SO_2, but a true limitation by reduction of 1 to 3 log was shown for an initial molecular SO_2 of 0.8 mg.L^{-1} after 30 days. For the third strain, SO_2 effect was less active and the population limitation only transitory as shown Figure 1. Hence results clearly show that the effect of SO_2 is strain dependent[3].

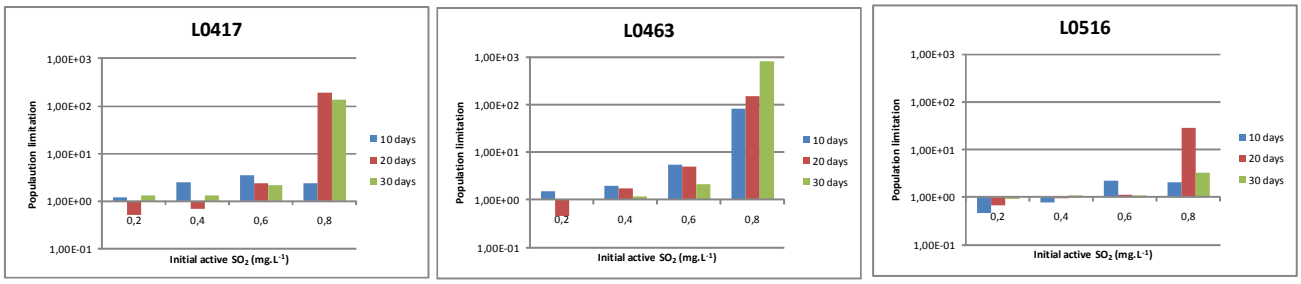

Figure 1 : Population limitation effect of initial addition of SO_2 on three B. bruxellensis strains. The population limitation is represented by the ratio in between a population non exposed to SO_2 and the same strain exposed to increasing amounts of molecular SO_2.

Effect of SO_2 on the cultivability of *B. bruxellensis* strains

SO_2 addition also had an effect on the cultivability of the strains (Table 1). Just after inoculation, populations were cultivable for all strains for 0 to 0.6 mg.L^{-1} molecular SO_2. At 0.8 mg.L^{-1} no cultivable populations were detected for strains L0417 and L0463 (data not shown). Figure 2 shows the total biomass accumulated during 30 days, calculated with q PCR (confirmed by DEFT) and culture plating counts. One strain, L0463, never really regained cultivability (a slight transitory cultivability was noted on day 10), whereas strain L0417 regained complete cultivability and strain L0516 never lost it totally.

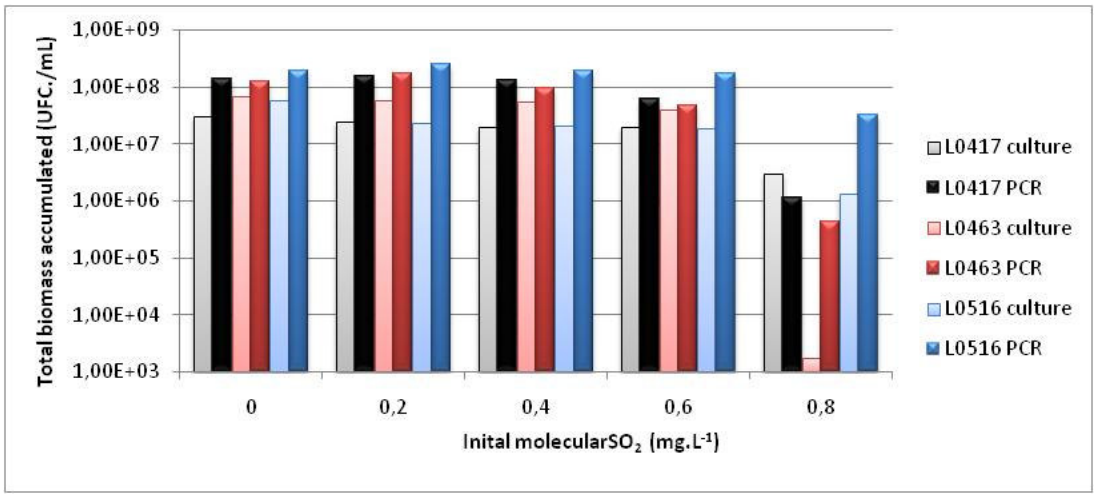

Figure 2 : Effect of initial SO_2 addition on the cultivability of the total biomass accumulated during 30 days storage. Microbial counts were determined by either q PCR (and were confirmed by DEFT, for which no difference of more than 1 log was visualized) or culture plating. The biomass accumulated was calculated as the integral of the population throughout the 30 days storage using the trapezoidal rule.

Volatile phenols production during storage of wines

VP were produced importantly in all wines, up to 1.5-2.5 $mg.L^{-1}$ after 30 days and 2.0-6.0 $mg.L^{-1}$ after one year, depending on the strain and the initial SO_2 content. There was a linear relationship between VP production and cultivable populations, but only when initial molecular SO_2 was less than 0.6 $mg.L^{-1}$ (Figure 3). However in the wines containing 0.8 $mg.L^{-1}$ initial molecular SO_2, only strain L0463 was no more cultivable at day 30. In addition at this high SO_2 concentration, the VP concentrations were not related to the cultivable population (except for strain L0463). The high biomass of strains L0516 and L0417 produced either large amounts of VP for the first one or no VP for the second (Figure 3).

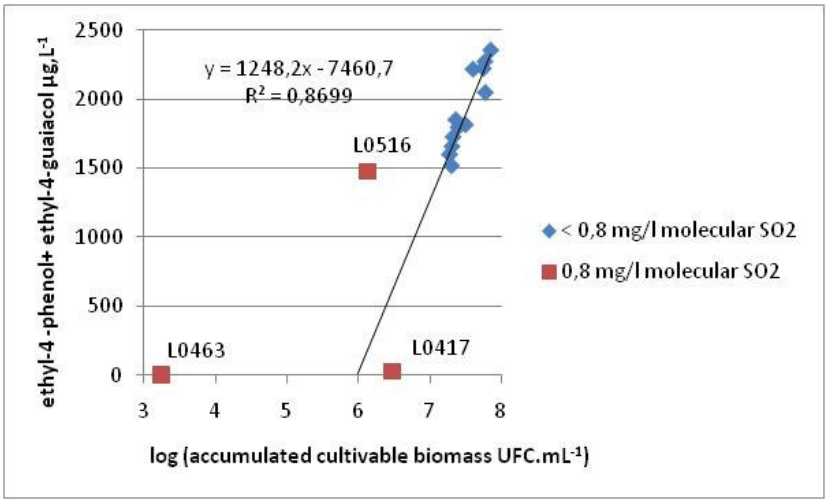

Figure 3: Relationship in between ethyl-4-phenol + ethyl-4-guaiacol production and accumulated cultivable biomass. The biomass accumulated was calculated as the integral of the population throughout the 30 days storage using the trapezoidal rule.

Table 2 shows the microbial counts by q PCR and culture plates at two different times, just after inoculation and after one year storage. As illustrated with strain L0417, the initial absence of cultivable cells at day 0 could not predict the subsequent growth that occurred during the year of storage and which was responsible for the production of about 2 $mg.L^{-1}$ of VP. The q PCR result of 1.3×10^4 eq $UFC.mL^{-1}$ was more appropriate. In consequence, q PCR seems to be a much more reliable tool to estimate the long term volatile phenol production risk. The benefits of the use of molecular tools to monitor the risk related to spoilage microorganisms and food-borne pathogens has been reported elsewhere[4]

Table 2 : Evaluation of the volatile phenols production risk using different B. bruxellensis population estimation tools for a wine sulfited to an initial molecular SO_2 of 0.8 $mg.L^{-1}$.

	L0417		**L0463**		**L0516**	
	q PCR (eq $UFC.mL^{-1}$)	Culture ($UFC.mL^{-1}$)	q PCR (eq $UFC.mL^{-1}$)	Culture ($UFC.mL^{-1}$)	q PCR (eq $UFC.mL^{-1}$)	Culture ($UFC.mL^{-1}$)
one day after inoculation	1.3E+04	0.0E+00	6.1E+03	0.0E+00	2.1E+05	2.4E+03
after one year storage	3.2E+07	1.2E+05	0.0E+00	0.0E+00	5.4E+07	5,9E+06
ethyl-4-phenol + ethyl-4- guaiacol produced ($\mu g.L^{-1}$)	2644		0		2037	

Conclusion

This work confirms that *B. bruxellensis* development can be tempered by SO_2 addition if a sufficient molecular concentration of 0.8 $mg.L^{-1}$ is achieved. However differences do exist among strains in terms of cellular multiplication and cultivability. This in turn has an impact on VP production. Our investigations show that for limited SO_2 concentrations, cultivable populations are directly linked to ethyl-4-phenol and ethyl-4-guaiacol production. This is no longer true for molecular SO_2 concentrations of 0.8 $mg.L^{-1}$. Finally, q PCR is a much finer tool for risk prediction of VP production.

References

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