Influence of *Brettanomyces custersianus* Upon the Activity of *Saccharomyces cerevisiae* Strains During the Tumultuous Phase of Vinification

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ABSTRACT

The aim of this work was to evaluate the influence of Brettanomyces custersianus *on the metabolic activity of* Saccharomyces cerevisiae *during the tumultuous stage of wine production. The Cabernet Sauvignon grape must with the skin was inoculated with individual cultures of* Sacch. cerevisiae *and with mixed cultures of* Sacch. cerevisiae *and* Br. custersianus*. During the 6-day tumultuous phase of fermentation, the highest ethanol production and the highest sugar consumption were obtained with the strains without* B. custersianus*. Fermentations carried out with the addition of* Brettanomyces *metabolites, acetic acid and 4-ethylphenol, showed that only the former inhibited the growth of both* Sacch. cerevisiae *strains used. In some cases,* Br. custersianus *could affect the rate higher alcohols production and their final concentrations during the tumultuous phase of vinification.*

Key words: *Dekkera/Brettanomyces,* wine, vinification, Custer effect

INTRODUCTION

The presence of *Brettanomyces* can devalue the quality of wine due to the production of off-flavours such as horse sweat, Band Aid, barnyard, and burnt plastic. Not all the characteristics produced by *Brettanomyces* are universally disliked. This genus grows and survives in wine with high ethanol levels and low sugar supply. Silva et al. (2004) showed that ethanol can be used by *Br. bruxellensis* and *Dekkera anomala* as the sole carbon and energy source and that their growth was dependent on the concentration of alcohol in the culture media. Conterno et al. (2006) showed that only 26% of 35 *Brettanomyces* strains could grow on ethanol as a sole carbon source. The strains of *Dekkera/Br. bruxellensis* isolated by Conterno et al. (2006) could be distinguished in a (European), b (Americas) and c (United States and New Zealand) groups according to 26S rDNA and physiological aspects. The group b (Americas) is particularly difficult to control in winery environment due to both its high tolerance to SO_2 and high level of 4-ethylphenol and 4-ethylguaiacol production. According to Renouf and Lonvaud-Funel (2007), the grape is

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definitely the primary source of *Brettanomyces* and in being so, the prevention starts in the vineyard.

Microorganisms other than *Dekkera* or *Brettanomyces* also are volatile phenols formers. According to Chatonnet et al. (1997), among the lactic acid bacteria evaluated, *Lactobacillus plantarum* was the only bacterium capable of forming important quantities of 4-ethylphenol, a volatile phenol with a disagreeable odour of "wet horse". The amount produced by these bacteria was lower than that formed by *Br. bruxellensis*. The synthesis of volatile phenols by these bacteria is inhibited by procyanidic tannins. The synthesis of these compounds by *Br. bruxellensis* is not affected by this polyphenolic compound found in red wines. The production of 4-ethylphenol and 4-ethylguaiacol has been shown to be highly variable. Some strains produce large amount of both the compounds while other strains produce low amounts (Conterno et al., 2006). Therefore, the chromatographic analysis of 4-ethylphenol and 4-ethylguaiacol, used as a method to detect the presence of *Brettanomyces* in wine, may not be useful for many strains. Moreover, other compounds, such as tetrahydropyridines (Heresztyn, 1986) and acetic acid which negatively affect the analytical profile of the wine are produced by *Brettanomyces*. These compounds can not be detected by measuring 4-ethylphenol and 4-ethylguaiacol.

It should be stressed that *Br. custersianus* is a negative Custer species (Scheffers and Wiken, 1969) and is also found in wine with the same frequency as *Br. bruxellensis* (Querol et al., 1990). This species does not produce acetic acid from glucose. This organic acid is formed when ethanol is used as the carbon or energy source (Freer et al., 2003). It is well known that high levels of acetic acid can be detrimental not only to wine quality as it imparts a vinegary/acetone-like aroma (Eglinton and Henschke, 1999a) but have also been associated with sluggish/stuck fermentations (Bisson, 1999) and with incomplete fermentation (Eglinton and Henschke, 1999). If this is true, *Br. custersianus* should not affect the *Sacch. cerevisiae* metabolism only at the beginning of the tumultuous phase of the vinification process, period in which the concentration of sugar is high and ethanol is low. The inhibition process should progressively increase as soon as the ethanol is formed.

The main purpose of this study was to evaluate the negative impact of *Br. custersianus* on the metabolic activity of neutral and killer *Sacch. cerevisiae* strains during the tumultuous stage of the wine production and establish the point in which this impact definitely started.

MATERIALS AND METHODS

Microorganism

The neutral strain *Saccharomyces cerevisiae* Embrapa 1vvt/97, the killer strain *Saccharomyces cerevisiae* Embrapa 91B/84 and *Brettanomyces custersianus* NRRL Y-6653 were used. For single treatments the strains used were 1vvt/97 (T1) and 91B/84 (T2). For mixed cultures, the strains employed were 1vvt/97+Brett (*Sacch. cerevisiae* Embrapa 1vvt/97 and *Br. custersianus* NRRL Y-6653) (T3) and 91B/84+Brett (*Sacch. cerevisiae* Embrapa 91B/84 and *Br. custersianus* NRRL Y-6653) (T4). The experimental measurements were analysed as a completely randomised design with four treatments and three repetitions. The yeasts were maintained in G7 medium (da Silva and de Almeida, 2006) and must agar (da Silva, 1996).

Inoculum

The inocula were prepared in a must containing media as described by da Silva (1996). Six Fernbach flasks containing 1 L of culture medium were sterilised at 121°C for 30 min. The medium was inoculated with the neutral strain *Sacch. cerevisiae* Embrapa 1vvt/97, the killer strain *Sacch. cerevisiae* Embrapa 91B/84 and *Br. custersianus*. The liquid cultures were incubated at 25°C and 150 rpm in a rotatory shaker (New Brunswick G-27, Edison, N. J., USA). After 16 h of growth, 2 L of each strain containing media were aseptically transferred to three 20 L glass Carboys containing 10 L of must media. The system was aerated with 2 vvm of filter sterilised air and maintained at 25°C for 24 h. The air was sterilised by filtering through a 0.2 µm Millipore (Millex-FG $_{50}$) filter. After 24h, the aeration system was stopped. Then a glass stopper in the bung-hole that allowed the gas to escape through a water trap was inserted at the top of the Carboys and this anaerobic process was maintained for 16 h. The cultures were centrifuged at 10,000 x g for 15 min and the wet weight of the cells was determined. The initial amount of the cells added

was equivalent to the amount present in 1.7 L of a culture (10% v/v) of *Sacch. cerevisiae* Embrapa 1vvt/97 and *Sacch. cerevisiae* Embrapa 91B/84 for 17 L of grape must. The initial concentration of cells of the mixed cultures corresponded to the amount of cells present in 850 ml of culture *Sacch. cerevisiae* and 850 ml of *Br. custersianus*.

Tumultuous phase

The grapes were passed through a mechanical destemmer-crusher to remove the stems and release the juice. The juice and the skins were transferred to 12 carboys (three carboys for each treatment). After inoculation, the cultures were maintained at 24°C during all stages of the vinification process. The tumultuous phase was performed with the skins and lasted 6 days.

Metabolic response of Sacch. cerevisiae to single and combined inoculation with Br. custersianus in sterile grape must:

Test tubes containing 10 ml of steam-sterilised grape must were inoculated with 1 ml of a single cell suspension containing 10^7 cell/ml of *Sacch. cerevisiae* 1vvt/97, *Sacch. cerevisiae* 91B/84 and *Br. custersianus* NRRL Y-6653. A mixed culture was performed with 0.5 ml of a suspension containing 10^7 cell/ml of *Sacch. cerevisiae* 1vvt/97 and 0.5 ml of *Br. custersianus* NRRL Y-6653. The another mixed culture was prepared with 0.5 ml *Sacch. cerevisiae* 91B/84 and 0.5 ml of *Br. custersianus* NRRL Y-6653. The metabolic activity of the microorganisms was measured by CO_2 evolution according to Giudici and Zambonelli (1992). The experimental measurements were analysed as a completely randomised design with five treatments and five repetitions.

Chemical Analysis

Analysis of anaerobic metabolites

Aliquots of 100 ml of medium were distilled and the original volume of the samples was filled up with distilled water to a volume of 100 ml. The ethanol was then determined by measuring the density of the distillate with an Anton-Paar DMA58 densitometer. The temperature of the sample was controlled with a water bath (Haag-G, West Germany). The concentration of acetaldehyde, ethylacetate and higher alcohols were measured by capillary gas chromatography (Perkin Elmer AutoSystem XL) equipped with a flame-ionisation detector (FID), a capillary column (0.25 mm i.d. by 50 m - CPWAX57B), and a split/splitless injector. Splitless injections were made onto the column at 40°C (5-min hold) with oven programming from 40°C (2°C/min) to 60°C (10 min), then (15°C/min) to 200°C (18 min). The 4-methyl-pentanol-2 was used as internal standard and the separation was performed under a constant flow of 1.8 ml He/min at 30 psi head pressure, 85.7 ml synthetic air/min and 53.1 ml H_2/min.

Analysis of reducing sugar

The reducing sugar and sucrose assays were done by the reduction of Fehling alkaline cupric solutions as described by Ribéreau-Gayon et al. (1982). When sucrose was used, hydrolytic procedure with 1 ml HCl was performed before the analysis.

Volatile Acidity

The volatile acidity was done by distillation as described by Ribéreau-Gayon et al. (1982). In short, the samples were steam distilled with Cazenave-Ferre equipment followed by titration with NaOH (0.1 N). The results were expressed as mEq/L.

Inhibition of Sacch. cerevisiae *by 4-ethyl-phenol and acetic acid*

The assay for the detection of inhibition by 4-ethylphenol was performed by preparing a stock solution containing 1g/L 4-ethylphenol (Merck). The stock solution was sterilised by filtering through a 0.45 µm Millipore filter and added to grape must to give concentrations of 0 (control), 4.46, 9.90, 14.78 and 19.61 µg/ml. The assay for the detection of inhibition by acetic acid was performed adding commercial acetic acid (Merck) to grape must to give concentrations of 0 (control), 9.90, 19.61, 29.13, 38.46 and 47.62 µL/ml. The metabolic activity of both the assays was measured by CO_2 evolution (Giudici and Zambonelli, 1992).

Statistical analysis

The regression analysis was performed by using R Program for Debian GNU/Linux (Venables et al., 2004; Logan, 2005). R version 2.7.1 (2008-06-23) Copyright (C) 2008 The R Foundation for Statistical Computing.

RESULTS AND DISCUSSION

Activity of Sacch. cerevisiae during tumultuous phase of vinification

The activity of *Sacch. cerevisiae* seemed to be repressed by the presence of *Br. custersianus* (Fig. 1). The statistical analysis with regard to 24 h consumption revealed no significant difference between the two single cultures (T1 and T2) and between the single culture *Sacch. cerevisiae* 91B/84 (T2) and the mixed culture *Sacch.*

cerevisiae 1vvt/97+Brett (T3) (P>0.05). The difference between the two mixed cultures (T3 and T4) was significant (P<0.05). The other differences (T1 and T3, T1 and T4, T2 and T4) were highly significant (P<0.01). This suggested that the reduced consumption of sugar in the mixed culture was not only due to the lower concentration of *Sacch. cerevisiae* at the time of inoculation in relation to the single cultures. The ethanol formation in 24 h was not significantly different between the two mixed cultures (P>0.05).
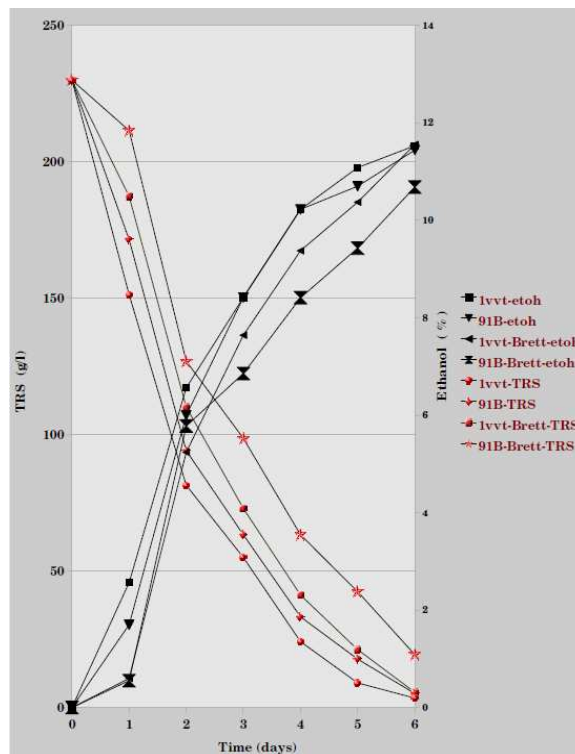

Figure 1 - Total reducing sugar (TRS) and ethanol (etoh) production by single culture of *Sacch. cerevisiae* 1vvt/97 and 91B/84 and mixed culture of *Sacch. cerevisiae* 1vvt/97 with *Br. custersianus* and *Sacch. cerevisiae* 91B/84 with *Br. Custersianus.*

The other differences were highly significant (P<0.01). The strain *Sacch. cerevisiae* 1vvt/97 proved to be metabolically more active than the strain *Sacch. cerevisiae* 91B/84 in producing ethanol only when *Br. custersianus* was not added. Taking the consumption and the ethanol formation into account, it was observed that in 24 h of fermentation, the metabolism of *Sacch. cerevisiae* 1vvt/97 in presence of *Br. custersianus* was deviated to the products other than ethanol. In fact, the only significantly lower ethanol yield ($Y_{p/s}$) was obtained with the mixed culture *Sacch. cerevisiae* 1vvt/97 *Br. custersianus* (P<0.05). At

the end of tumultuous phase, there was no significant differences between the treatments with regard to yield (P>0.05) but the ethanol produced at that time by the mixed culture *Sacch. cerevisiae* 91B/84+Brett was highly significant lower than those of treatment with either combination (P<0.01). This showed that *Br. custersianus* could cause impact on the metabolism of *Sacch. cerevisiae* 91B/84. It should be considered that microorganisms other than *Sacch. cerevisiae* and *Br. custersianus* were present during the process of wine production.

Higher alcohols are not less important compounds in the wine. With regard to these compounds, the two strains of *Sacch. cerevisiae* used presented different production rates. It was interesting to note that the strain 91B/84 had a higher linear rate of 3-methyl-1-butanol production than the strain 1vvt/97 (Table 1). The linear production rate was even higher in the presence of *Br. custersianus*. The strain 1vvt/97 t presented lower production rate and also increased its synthesis in the presence of *Br. custersianus*. It seemed that this microorganism was a 3-methyl-1-butanol producer or an inductor of this higher alcohol. The linear rate of production 2-methyl-1-butanol was also higher for the strain 91B/84 than for 1vvt/97. In the presence of *Br. custersianus*, the strain 1vvt/97 increased its production rate, whereas the strain 91B/84 had its rate decreased (Table 1). As the rates with and without *Br. custersianus* were similar, the presence of this microorganism did not affect the linear rate of the production of 2-methyl-1-propanol (Table 1), albeit the rate of production was higher with the strain 1vvt/97.

Table 1 - Linear regressions for 3-methyl-1-butanol and 2-methyl-1-butanol production between the third and the sixth day of tumultuous fermentation

Sacch. cerevisiae and *Br. custersianus*			
3-methyl-1-butanol	Linear equation	r^2	Pr(>F)
1vvt/97	71.058t+20.164	0.9889	P<0.01
91B/84	75.208t+108.424	0.9976	P<0.01
1vvt/97+Brett	74.629t-22.753	0.9968	P<0.01
91B/84+Brett	93.698t+0.924	0.9812	P<0.01
2-methyl-1-butanol	Linear equation	r^2	Pr(>F)
1vvt/97	23.975t+5.030	0.9953	P<0.01
91B/84	39.7060t+6.2230	0.9998	P<0.01
1vvt/97+Brett	26.1540t-14.2830	0.9991	P<0.01
91B/84+Brett	36.738t-27.421	0.9938	P<0.01
2-methyl-1-propanol	Linear equation	r^2	Pr(>F)
1vvt/97	12.227t+3.506	0.9846	P<0.01
91B/84	10.3250t+20.2000	0.9882	P<0.01
1vvt/97+Brett	12.798t-2.666	0.9843	P<0.01
91B/84+Brett	10.325t+10.020	0.9183	P<0.05

These results showed that the two cultures with the strain 91B/84 did not present significant difference with regard to propanol-1 concentration after six days of tumultuous phase (P>0.05). In all other cases, the difference was highly significant (P<0.01). This meant that not only the concentration of this compound but also the interference of *Br. custersianus* on the production also depended upon the yeast strain. Rankine (1967) showed that the amounts of n-propanol, iso-butanol and iso-amyl plus active amyl alcohol produced during the fermentation of grape juice varied considerably not only according to the yeast but also according to the varieties of grapes (*Vitis vinifera*) used.

Metabolic response of Sacch. cerevisiae to single and to combined inoculation with Br. custersianus in sterile grape must
The activity of *Sacch. cerevisiae* was clearly repressed by the presence of *Br. custersianus* (Fig.

2 and 3). The metabolic inhibition can be better evaluated by the rates of CO_2 evolution. An exponential evolution phase was detected between the fourth and the ninth days for both the mixed and pure cultures. The rates of CO_2 production are depicted in Table 2. The highest rates of CO_2 were obtained with the strain 1vvt/97 regardless of single, or mixed culture. The inhibition of the metabolism seemed to start five days after inoculation for the strain *Sacch. cerevisiae* 1vvt/97 (Fig. 2) and after nine days for the strain *Sacch. cerevisiae* 91B/84 (Fig. 3). Statistical analysis performed after 18 days fermentation showed no significant differences in the CO_2 evolution between the two single cultures of *Sacch. cerevisiae* (T1 and T2) and the two mixed cultures (T3 and T4) (P>0.05). Highly significant differences were found in CO_2 evolution between the single and mixed cultures (T1 and T3, T2 and T3, T1 and T4, T2 and T4) (P<0.01). It is well known that *Brettanomyces* species are ethanol and

acetic acid producers. As a Custer positive species, *Br. bruxellensis* is dependent on the level of aeration. Uscanga et al. (2003) showed that the higher the oxygen supply, the greater the acetic acid formation and the lower the ethanol production. Ciani and Ferraro (1996) observed that the absence of an increase in acetic acid in wines did not exclude the presence of *Brettanomyces* and its high acetic acid production was dependent on the presence of oxygen. The conversion of acetaldehyde to acetic acid is an oxidative process, which results in a drop in $NAD^+/NADH$ ratio. As a consequence, the glycolytic flux stops at the level of glyceraldehyde-3-phosphate. The acetic acid formation is limited by the availability of H-acceptors as oxygen, acetoin, acetone and dihydroxyacetone. In anaerobic conditions, the concentration of these organic H-acceptors can be limited; if available, the anaerobic inhibition of fermentation is abolished. These facts explain the weak activity of *Br. custersianus* when used as pure culture (Fig. 2). *Br. custersianus* did not affect the *Sacch. cerevisiae* metabolism only at the beginning of the tumultuous phase of the vinification process, but also during the period when the concentration of sugar was high and ethanol was low. The inhibition process was progressively increased (Fig. 2 and 3) probably due to the ethanol formation with subsequent production of acetic acid.
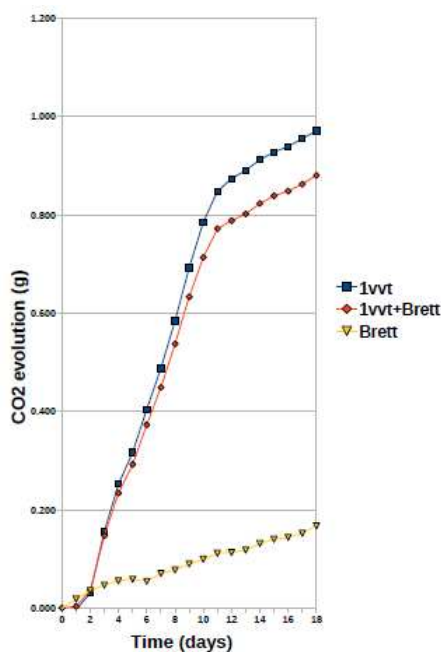

Figure 2 - Yeast activity of *Sacch. cerevisiae* 1vvt/97 and *Br. custersianus* measured by CO_2 evolution. Single and mixed cultures.

Table 2 - Exponential regressions for CO_2 evolution between the fourth and the ninth day of single and mixed fermentations.

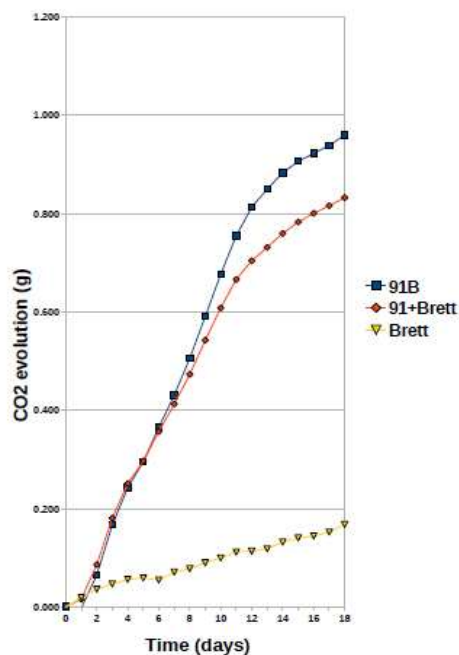| Sacch. cerevisiae and Br. custersianus			
Strains	Exponential equations	r^2	Pr(>F)
	Single		
1vvt/97	$0.1158e^{0.202t}$	0.9944	P<0.01
91B/84	$0.1219e^{0.1778t}$	0.9961	P<0.01
	Mixed		
1vvt/97+Brett	$0.1082e^{0.1998t}$	0.9944	P<0.01
91B/84+Brett	$0.1381e^{0.154t}$	0.996	P<0.01

Figure 3 - Yeast activity of *Sacch. cerevisiae* 91B/84 and *Br. custersianus* measured by CO_2 evolution. Single and mixed cultures.

Effect of acetic acid and 4-ethyl-phenol on yeast metabolism

The addition of acetic acid to the grape must provoked immediate metabolic inhibition of both the *Sacch. cerevisiae* strains (Fig. 4 and 5). The 1vvt/97 strain behaved in a fashion similar to 91B/84. They showed sensitivity to the lowest concentration of acetic acid used (2.5 µl/ml). As seen from Figures 4 and 5, at that concentration, the inhibition was acetic acid limited. These results showed that these strains could also be sensitive to other wine-related microorganisms, especially to those acetic acid producers, which were much more commonly found in any phase of the vinification process than were the *Brettanomyces* species. According to Thomas et al. (2002), it was the total concentration of acetic acid that determined the extent of yeast growth inhibition and not the concentration of undissociated acid alone. Narendranath et al. (2001) showed that the effect of acetic acid depended upon the medium composition and that the length of the lag phase of the growth curve increased exponentially with the increasing concentrations of acetic or lactic acid. It was observed that the length of the lag phase of the CO_2 evolution also tended to increase as the concentrations of acetic acid augmented (Fig. 4 and 5). Cássio et al. (2006) demonstrated that acetic acid and some others monocarboxylic acids were competitive inhibitors of DL-lactate transport, suggesting that these acids used similar process of transport of DL-lactate, proton-lactate symport. This transport was only detectable when the medium was depleted in glucose. The uncharged form, however, entered the yeast cell by simple diffusion even in glucose-grown cells. This could lower the intracellular pH, increasing the extrusion of H^+ (Calahorra et al., 1987) and the ATP expenditure (Eraso and Gancedo, 1987).

These conditions are very stressing to the yeast cells (Dorta et al., 2006). The acetic acid is one of agents that provoke oxidative stress in yeast, inducing apoptosis (Mrozeck and Kufel, 2008).
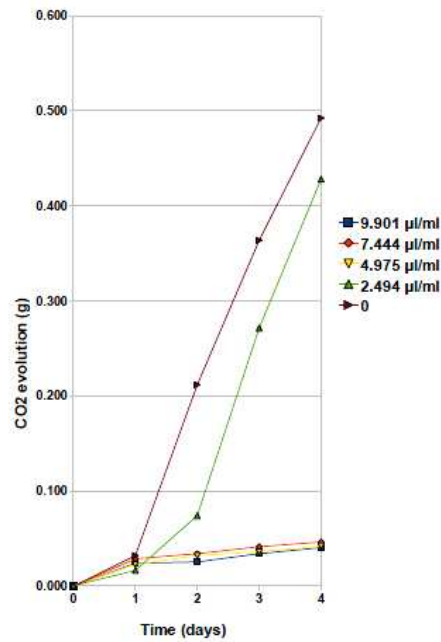
Figure 4 - Inhibition of the *Sacch. cerevisiae* 1vvt/97 activity provoked by the presence of acetic acid.
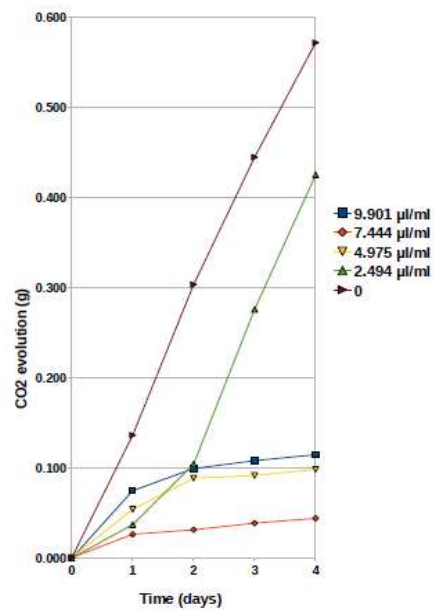

Figure 5 - Inhibition of the *Sacch. cerevisiae* 91B/84 activity provoked by the presence of acetic acid.

Graves et al. (2006) showed that the inhibition of ethanol production by acetic acid increased as the concentration of solids in the medium increased and the pH of the medium declined. There are membrane proteins that protect the yeasts against the potential toxicity of weak organic acids secreted by the competitor organisms. One of these proteins was identified as ATP-binding cassette (ABC) transporter Pdr12 (Piper et al., 1998). Piper et al. (2001) suggested that Pdr12 transporter was used to actively transport acid anions or alcohol molecules to the opposite side of the membrane and released them into the aqueous phase of the periplasm. This lowered the

intracellular level of these compounds. This active transport could explain the lack of total collapse of the two yeast strains activity in must containing 2.5 µl/ml of acetic acid. Maiorella et al. (1983) reported that the acetic acid increased the ATP requirement for maintenance functions, decreased the ratio of specific growth rate/ethanol productivity, disrupted the cell membrane and altered the cell morphology.

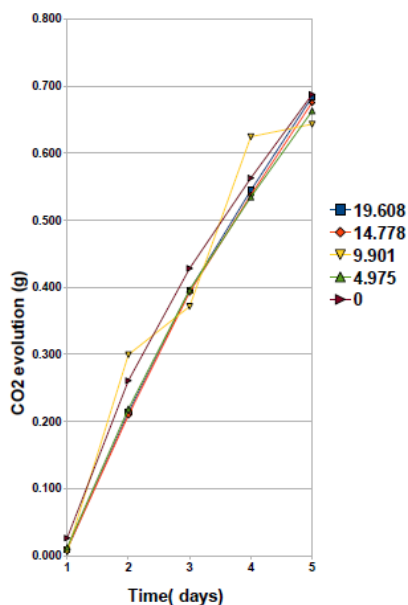The addition of 4-ethyl-phenol to grape must did not affect the metabolism of both the yeasts (Fig. 6 and 7).

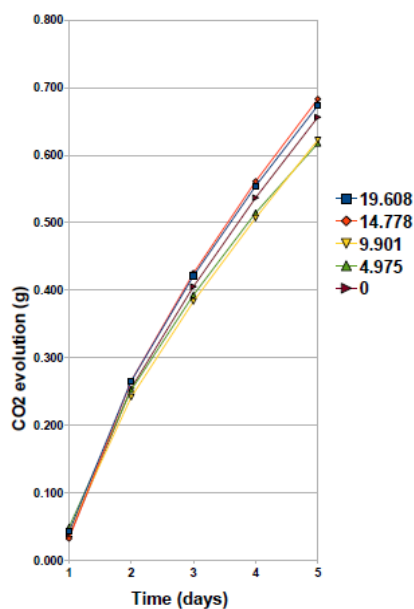**Figure 6 -** Activity of *Sacch. cerevisiae* 1vvt/97 in presence of 4-ethylphenol (µg/ml).

Figure 7 - Activity of *Sacch. cerevisiae* 91B/84 in presence of 4-ethylphenol (µg/ml).

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