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Studies on the Wine Spoilage Yeast *Brettanomyces bruxellensis*: 4-Ethylphenol Production and Improved Detection

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Abstract

Brettanomyces bruxellensis is a major threat to wine industry due to its spoilage ability characterized by high production of volatile phenols, mainly 4-ethylphenol. The horse sweat odor, characteristic of this phenol, causes large economic losses to wineries. A better understanding of the behavior of this yeast and better detection methods may lead to a decrease in 4-ethylphenol incidence in red wines worldwide.

In the present work, we studied: (i) the ability of *B. bruxellensis* to enter the viable but nonculturable state by using both vital staining and plate counts to distinguish between viable and culturable cells; (ii) the production of 4-ethylphenol at different growth phases; (iii) the improvement of selective culture media; (iv) the application of a Real-Time PCR protocol for the rapid detection of *B. bruxellensis*.

The existence of a viable but nonculturable state was evidenced during growth in synthetic medium ranging from 2% in strain ISA 2211 to 71% in strain ISA 1791 of the viable cells. The production rate of 4-ethylphenol was maximum when the precursor p-coumaric acid was added during exponential growth and decreased in stationary phase with incubation time. The developed selective medium presented recovery rates higher than the general purpose medium GYP and selectivity similar to DBDM. Response time lasted from 3 to 5 days while DBDM colonies appeared only after 12 days or more of incubation. Real-Time PCR showed to be an easy and faster method for a highly selective detection, taking 3 hours to obtain a positive response. The detection threshold was 700 cells/mL which may be decreased using sample concentration by centrifugation. However, results were 3.7 times higher than the viable counts, probably due to the DNA of dead or lysed cells.

Collectively, this work represented a step forward in understanding the spoiling behavior of this yeast species and enabled the development of better detection methods for *B. bruxellensis*.

Keywords: *Brettanomyces bruxellensis*, wine spoilage, viable but nonculturable state, 4-ethylphenol, selective growth media, Real-Time PCR

Resumo

Brettanomyces bruxellensis é uma grande ameaça na indústria vinícola devido à sua capacidade de alteração do vinho. O cheiro a suor de cavalo, característico deste fenol, cria grandes perdas económicas na indústria vinícola. Um maior conhecimento do comportamento desta levedura e melhores métodos de detecção podem levar a uma diminuição na incidência de 4-etilfenol em vinhos tintos.

Neste trabalho estudámos: (i) a capacidade de *B. bruxellensis* entrar no estado viável mas não cultivável usando coloração vital e contagem de placas, distinguindo células viáveis de cultiváveis; (ii) a produção de 4-etilfenol em diferentes fases de crescimento; (iii) o melhoramento de meios de cultura selectivos; (iv) a aplicação de um protocolo de Real-Time PCR para uma rápida detecção de *B. bruxellensis*.

O estado viável mas não cultivável foi demonstrado durante o crescimento em meio sintético, variando entre 2% da população viável na estirpe ISA 2211 e 71% na estirpe ISA 1791. A taxa de produção do 4-etilfenol foi máxima quando o percursor ácido p-cumárico foi adicionado durante a fase exponencial e menor na fase estacionária. O meio de cultura selectivo desenvolvido apresentou taxas de recuperação superiores às do meio de cultura GYP e selectividade idêntica ao DBDM. O tempo de resposta foi de 3 a 5 dias comparativamente aos 13 dias ou mais no DBDM. O Real-Time PCR mostrou ser um método de detecção específico, fácil e rápido, com resultados em 3 horas. O limite de detecção de 700 células/mL pode ser reduzido concentrando a amostra por centrifugação. No entanto, este método sobrestima a quantidade em 3,7 vezes, provavelmente devido ao DNA de células mortas ou lizadas.

Em suma, este trabalho representa um passo em frente no conhecimento do comportamento de alteração desta espécie e permitiu o desenvolvimento de melhores métodos de detecção de *B. bruxellensis.*

Palavras-chave: *Brettanomyces bruxellensis*, alteração de vinhos, estado viável mas não cultivável, 4-etilfenol, meio de cultura selectivo, Real-Time PCR

1 Introduction

1.1 The wine economy and market

Biotechnology, as we know it, can be defined as the use of biological systems in industrial processes. As so, it's easy to link it to the wine industry since wine is undoubtedly the oldest biotechnological effort since signs of large-scale winemaking activities date to, at least, 5000 BC (Borneman et al., 2013). In the biotechnology world, the economy and market surrounding the industry are of extreme importance. However, it is not easy to study the wine industry economy since it comprehends 3 sections of the agriculture sector: the agriculture activity by itself, the agriculture industry and the market of agriculture products.

In 2011, the agriculture sector in Portugal had 28 thousand companies from which 82% were microenterprises, 17.6% small and medium enterprises and only 0.4% were big enterprises (Banco de Portugal, 2012) with an active population of 500 thousands (Pordata, 2013).

The wine industry is an important part of the agriculture in Portugal representing 13% of the total agriculture generated value in 2007 being the second most valuable product in the vegetal production with 861 millions € (Instituto da Vinha e do Vinho I.P., 2009).

The wine industry itself is analyzed mainly by the Organisation internationale de la Vigne et du Vin (OIV) in an international view and by Instituto da Vinha e do Vinho (IVV) with more emphasis on the national situation. OIV is an intergovernmental organization of a scientific and technical nature, of recognized competence for its works concerning vines, wines and other vine-based products (Organisation internationale de la Vigne et du Vin, 2011). This organization is responsible for the regulation of the viti-viniculture practices and treatments, aiming at a standardization of the sector. All the OIV member countries approved the International Code of Oenological Practices which constitutes a technical and legal reference document for the sector (Organisation internationale de la Vigne et du Vin, 2013a). The mission of the IVV consists in coordinate and control the institutional organization of the vitiviniculture sector, audit the system of quality control, following the European Union policy and prepare the rules for its implementation (Instituto da Vinha e do Vinho I.P., 2013).

Wine production

In 1975 the production was around 313 millions of hl decreasing to 266.8 millions of hl in 2011 (Organisation internationale de la Vigne et du Vin, 2013b, 2013c, 2013d). In 2011, the wine production of Europe represented 66.5% of the total world production, however, in 2001 it represented 73% (Instituto da Vinha e do Vinho I.P., 2012). Comparing 2008 with 2012, we see that the only countries with an increase of growth rate of wine production from were Australia, Chile (with an impressive 45%), China and Portugal (Figure 1.1) (Organisation internationale de la Vigne et du Vin, 2013d). From Figure 1.1, we can also evaluate the 10 major wine producers that represent 80% of the global production and they are,: France, Italy, Spain, USA, Argentina, China, Australia, Chile, South Africa and Germany (Instituto da Vinha e do Vinho I.P., 2012).

Country	mhl	Growth rate 2008/2012
Argentina	11778	-20%
Australia	12660	2%
Brazil	2917	-21%
Bulgaria	1337	-17%
Chile	12554	45%
China	14880	18%
Czech Rep.	470	-44
France	41422	-3%
Germany	9012	-10
Greece	3150	-19%
Hungary	1874	-46%
Italy	40060	-15%
New Zealand	1940	-5%
Portugal	6141	8%
Romania	3311	-36%
Russian Feder.	6400	-10%
Spain	30392	-15%
South Africa	10037	-1%
USA	20510	-1%

Figure 1.1 - Growth rate of wine production from 2008 to 2012 for main wine producers worldwide (Organisation internationale de la Vigne et du Vin, 2013d).

Wine consumption

Because of the financial crisis, the wine consumption started to fall since 2007 (Organisation internationale de la Vigne et du Vin, 2013b). In 2007 the world consumption of wine was around 255 or 251 millions of hl and until the year 2009 where it stabilizes, the world consumption dropped around 11 millions of hl. The European countries continue leading by far the world consumption with 65% in 2009. The major wine consumers in descending order are summarized in Figure 1.2.

Another interesting view is the consumption per capita per year. With this, we can consider the population of a country and estimate the individual human consumption of a country. The countries with a major individual human consumption per year in 2011 are Luxembourg with 49.8 L per capita per year, France with 46.4 and Portugal with 42.6 (Organisation internationale de la Vigne et du Vin, 2013b, 2013c, 2013d).

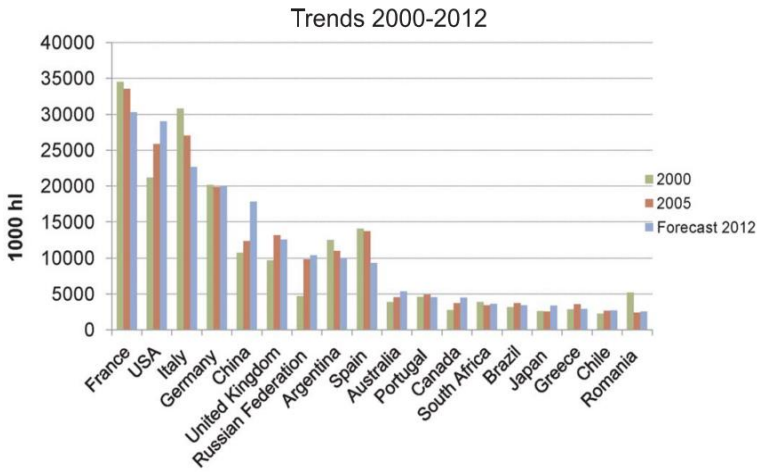

Figure 1.2 - Consumption of wine in leading countries and in 3 different years (Organisation internationale de la Vigne et du Vin, 2013d).

Wine market

The wine market is important in the wine economy and we can see how important it is when we know that from 10 liters of wine consumed, 4 of them have always been exported or imported (Organisation internationale de la Vigne et du Vin, 2013c). Exports of wine worldwide are increasing since 2000 with an exception of a small decrease in 2008 (-3.5% from 2007) where the financial crisis took an important role. In 2000 the exports were around 60 millions of hl but in 2012 the exports ascend to 101.4 millions of hl (+69%). If we talk about the wine trade in value, in 2000 the sum of all exports was around 13700€ but in 20011 the number goes up to 23245€ (+70%) (Instituto da Vinha e do Vinho I.P., 2012; Organisation internationale de la Vigne et du Vin, 2013b, 2013c, 2013d).

The major wine exporters in the year of 2011 were Italy, Spain and France (Organisation internationale de la Vigne et du Vin, 2013c, 2013d). Portugal comes in 10th place with almost 3 millions of hl in 2011 and 3.27 millions hl in 2012 (Instituto da Vinha e do Vinho I.P., 2012; Organisation internationale de la Vigne et du Vin, 2013d). The top importers countries of Portuguese wine are Angola with 0.5 million of hl, France with 0.325 and Germany and United Kingdom with around 0.150 each (Portugal, 2009).

1.2 Wine microbiology

Wine microbiology represents a complex interaction of microorganisms there present. The microorganisms found in wine, are present on grapes before their reception in the vinery. Louis de Pasteur, in 1872, was the first to study wine microbiology and showed that the microorganisms responsible for wine fermentations are present on the surface of grapes in the vineyard environment and that they play an important role in wine quality (Barata et al., 2012; Martins et al., 2012; Renouf et al., 2005).

The diversity present on the grapes surface that can support the harsh conditions of the wine, play an important role on wine production. The high concentration of sugars, the nitrogen, the salts, some trace elements and oxygen in grape must become a selective ecosystem. In fact, at the start of vinification, many microorganisms undergo sequential substitutions due to competitive exclusion from less adapted ones (Figure 1.3A) (Fugelsang and Edwards, 2007; Perrone et al., 2013).

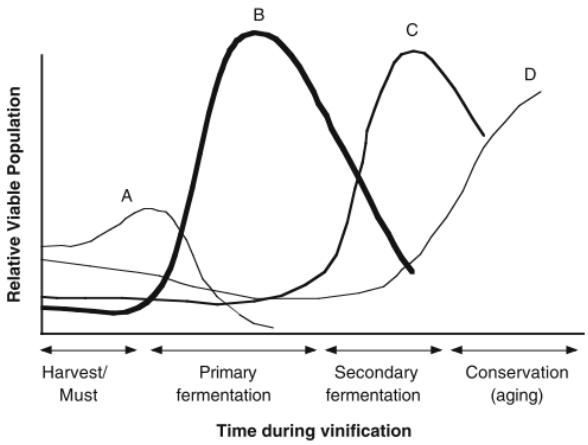

Figure 1.3 - Microbiological population during vinification. (A) non-*Saccharomyces* yeasts, (B) *Saccharomyces* spp., (C) *Oenococcus oeni* and (D) spoilage microorganisms.

Wine microbial consortium

The wine microbial consortium (WMC) consists of yeasts, acetic acid bacteria and lactic acid bacteria that can grow in conditions such as the high alcohol concentration, low pH and low nutrients availability. The consortium can be divided in 3 groups (Barata et al., 2012):

1. Innocent or innocuous species, easily controllable species, meaning they can't spoil wine when good manufacturing practices (GMP's) are used. GMP's consist mainly in washing and sanitizing tanks, lines, and other equipment between each use and the proper use of chemical preservative agents or physical treatments like filtration or pasteurization (Loureiro and Malfeito-Ferreira, 2003);
2. Fermentative species, responsible for sugar and malic acid conversion;
3. Spoilage *sensu stricto* species responsible for wine spoilage when GMP's are used.

In the first group, regarded as innocent, are acetic acid bacteria (AAB) mostly from the genera *Acetobacter* like *Acetobacter pasteurianus.* AABs can spoil wine with an excessive production of acetic acid from ethanol, however, they can easily be controlled using GMP's in wine production (Barata et al., 2012; Bartowsky et al., 2003; Martins et al., 2012).

In the second group, we find one of the most important species of the WMC, *Saccharomyces cerevisiae* (Figure 1.3B). This fermentative yeast is the major responsible for the alcoholic fermentation (AF) of turning grape juice into wine, but others (*S. bayanus* and *S. paradoxus*) can do or cooperate in the fermentation (Perrone et al., 2013). In the exothermic reaction of the AF, the yeast consumes the sugar and produces carbon dioxide, increasing temperature and alcohol, inhibiting more sensitive microorganisms. Even though *S. cerevisiae* is responsible for wine AF and easily found

in wine and wineries, it is rarely found in healthy grapes. First, Martini (1993) concluded that *S. cerevisiae* does not live in nature at all and could only be found in the winery environment. This was proven wrong in 1999, where Mortimer and Polsinelli (1999) demonstrated that even rarely, *S. cerevisiae* was found about 0.05 to 0.1% in healthy berries and 25% in damaged ones with numbers between 10^5-10^6/berry. So, the origin of the yeast responsible for wine AF holds a still open debate regarding if it is maybe the first domesticated microbe (Loureiro and Malfeito-Ferreira, 2003; Martini, 1993).

Another important species in the second group of the WMC is *Oenoccocus oeni,* the best adapted lactic acid bacteria (LAB) to the stressful wine environment. *O. oeni* is the most common representative agent in spontaneous malolactic fermentation (MF) (Figure 1.3C) however, other species of LAB like *Lactobacillus*, *Pediococcus* and *Leuconostoc* may also participate in it. Due to its characteristics, *O. oeni* is the selected bacteria for commercial starter cultures worldwide. In MF, normally after AF, the malic acid is converted into lactic acid with production of CO_2. This bioconversion (and not a real fermentation) helps to increase the pH, increasing the microbial stability of wines. Also, the MF plays an important role to the wine aroma and flavor profile. After MF is completed, other species are still able to metabolize residual sugar, the sugar not used by previous microorganisms, spoiling the wine with off-flavors or ropiness (an increase in viscosity and a slimy mouth feel). *O. oeni*, just like *S. cerevisiae*, is also rarely detected in grapes being the others mentioned LABs much more frequently observed in the vineyard (Barata et al., 2012; Lerm et al., 2010; Martins et al., 2012; Renouf et al., 2005).

As for the third group of WMC, the group of species that are responsible for wine spoilage when GMP's are used, it includes *Lactobacillus* spp. and *Pediococcus* spp. Their activity can continue after the MF, when they are most likely to spoil the wine (Barata et al., 2012; Lerm et al., 2010). However, in this third group, yeasts are the main intervenient since improvement in GMPs like equipment design, sanitation procedures, better use of preservatives and wine technological advances led to the extinction of most traditional bacterial spoilage (Loureiro, 2003). The spoilage yeasts are technologically relevant typically due to their ability to produce diverse secondary metabolites. The most common are: acetaldehyde by film-forming yeasts during bulk storage; hydrogen sulphide by *S. cerevisiae* during fermentation with nitrogen shortage; volatile phenols by *Dekkera/Brettanomyces bruxellensis* between fermentation, during storage or after bottling; sediment and cloudiness formation by *Zygosaccharomyces bailii* (Barata et al., 2012; Malfeito-Ferreira, 2010a).

Even using preventive measures, some hazards are difficult to avoid like the production of volatile phenols by *Dekkera/Brettanomyces bruxellensis* posing a major concern to wine quality and wine economy today (Loureiro and Malfeito-Ferreira, 2006, 2003; Malfeito-Ferreira, 2010a; Oelofse et al., 2008). The most critical points for contamination with the spoilage yeas *B. bruxellensis* is between the two fermentations, in bottling line and at the aging step (Figure 1.3C), especially when this aging is made in wood barrels because of the difficult to sanitize them. Commonly, *B. bruxellensis* isn't present in white wines and one of the reasons is that wood barrels are usually used to age red wines instead of white proving the importance of this critic point of contamination (Guzzon et al., 2011; Loureiro and Malfeito-Ferreira, 2003; Oelofse et al., 2008).

Control measures

Wine spoilage can be a big problem since it causes depreciation or rejection of the product, leading to serious economic losses (Fugelsang and Edwards, 2007; Loureiro and Malfeito-Ferreira, 2003; Malfeito-Ferreira, 2010a; Oelofse et al., 2008; Zuehlke et al., 2013). Most of the hazards mentioned above, can be avoided by current preventive or curative measures. This control normally involves the use of chemical compounds, filtration or even temperature control. The chemical compounds normally used are sulphur dioxide (SO_2) and dimethyl dicarbonate (DMDC). The most used preservative agent in winemaking, SO_2, may inhibit the growth of microorganisms but does not necessarily kill them so the timing of the addition and concentration added is of extreme importance (Fugelsang and Edwards, 2007). For example, after AF and before MF starts, the addition should be minimal or inexistent to let LAB make the fermentation. However, some spoilage microorganisms may intervene in this step. The other preservative commonly used in winemaking is DMDC. DMDC activity is related to metabolic enzymes inhibition. It has shown to be effective too, but is little soluble in water and it needs expensive special equipment to ensure product homogenization. Moreover, this chemical is toxic by ingestion and inhalation before his hydrolysis (Costa et al., 2008; Fugelsang and Edwards, 2007; Organisation internationale de la Vigne et du Vin, 2013e).

1.3 *Dekkera/Brettanomyces*

History and taxonomy

The first description of the *Brettanomyces* genus was made by Claussen in 1904. Claussen isolated the yeast responsible for a slow stock beer secondary fermentation of an old English stock beer producing typical strong flavors. Back then, the flavors produced by this yeast were characteristic of British beers (Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008).

Regarding wine contamination, it was only around 1950s that *Brettanomyces* was isolated in French, South African and Italian wines (Fugelsang and Edwards, 2007; Zuehlke et al., 2013). However, some authors say that the first appearance of *Brettanomyces* genus was in 1933 in a French grape must under the name of *Mycotorula intermedia* (Loureiro and Malfeito-Ferreira, 2006). In fact, this nomenclature was revised in 1940 by Custers in the first systematic study on *Brettanomyces* (Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008).

Originally, the genus *Brettanomyces* included the species *B. bruxellensis*, *B. lambicus*, *B. clausenii* and *B. anomalus*, all of them described by Custers and reproducing asexually by budding. Later, in 1964, Van der Walt and Van der Kerken introduced the genus *Dekkera* to the taxonomy after they observed the production of ascospores by *Brettanomyces* (Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008). Thus, the genus *Brettanomyces* refers to the anamorph state, which means the asexually reproducing stage of the life cycle and the genus *Dekkera* to the teleomorph state, which means the sexually reproducing stage of the cycle even though they are the same microorganism.

Currently, there are 5 species belonging to the genus *Brettanomyces*: *Brettanomyces custersianus*, *Brettanomyces naardenesis*, *Brettanomyces nanus*, *Brettanomyces anomalus* and *Brettanomyces bruxellensis*. In the genus *Dekkera* (perfect or teleomorph state) are now recognized the teleomorphs

D. anomala and *D. bruxellensis* (Fugelsang and Edwards, 2007; Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008; Zuehlke et al., 2013). From the 5 species of the genera, *B. bruxellensis* (*D. bruxellensis* anamorph form) is the primarily associated with wine contamination being the best represented species (Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008; Zuehlke et al., 2013).
Even though technically incorrect, the name *B. bruxellensis*, is commonly seen in a winemaking context since the sporulating form or ascospores were never reported in wine (Oelofse et al., 2008). That is probably why it is not uncommon to see the denomination "*Dekkera/Brettanomyces* spp." used frequently in wine research (Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008).

The cells of the *Dekkera/Brettanomyces* genus are spherical, ogival or cylindrical to elongate with typical dimensions of 2 to 7µm (Figure 1.4). Occasionally, cells can even appear in form of pseudomycelium (Fugelsang and Edwards, 2007; Smith, 2011a, 2011b; Zuehlke et al., 2013). *Brettanomyces* reproduces asexually by multilateral budding and *Dekkera* reproduces sexually by asci forming one to four hat-shaped or spherical ascospores. The ascospores tend to agglutinate when released (Smith, 2011a).

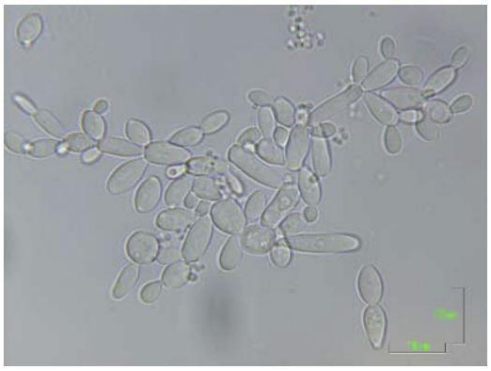

Figure 1.4 – Morphological differences of *Brettanomyces* spp. under optical microscope observation at 1000x amplification (Suárez et al., 2007).

Growth conditions

B. bruxellensis is a slow growth yeast, nutritionally low demanding and dependent on specific carbon sources. *B. bruxellensis* can grow using the monosaccharides glucose, fructose, galactose and the disaccharides sucrose, maltose, cellobiose and trehalose (Conterno et al., 2006; Smith, 2011a).

B. bruxellensis has an optimal growth temperature range between 25°C and 32°C being able to grow from 10°C to 37°C. Some authors however, reported a complete loss of viability if some strains were submitted to 36°C for less than 12 hours (Barata et al., 2008b; Brandam et al., 2008; Zuehlke et al., 2013).

The capacity to resist the wine acidic condition (pH≈3.5) is known since *B. bruxellensis* was detected in wine, however, a recent study showed that *B. bruxellensis* could grow at even more acidic conditions such as pH 1.5 (Bassi et al., 2013; Conterno et al., 2006).

The resistance to ethanol is also a remarkable feature of this yeast. It was shown by Barata et al. (2008a) that, in synthetic media, some strains could grow in conditions of 15.0% (v/v) of ethanol and most of the strain was able to grow under 14.5% (v/v). Although the authors couldn't grow the strains in a 14% (v/v) ethanol adjusted wine, it was observed growth in real wine with 13.8% (v/v). The authors suggest that, under winery conditions, cells are better adapted to overcome environmental stresses. In previous studies, initial ethanol levels of 13% (v/v) or even 11.4% (v/v) in synthetic media were shown to be enough to limit *B. bruxellensis* growth (Loureiro and Malfeito-Ferreira, 2006). These different values can be explained by the combination of various factors, in example, high ethanol and

low pH results in an increased loss of viability of *B. bruxellensis* when compared to the same treatments alone (Bassi et al., 2013).

Another important factor for the growth of *B. bruxellensis* is the concentration of the main preservative in wines, the sulphur dioxide. The resistance to sulphur dioxide is dependent on its presence in molecular sulphur dioxide since this form is not electrically charged. The sulphur dioxide exists either in free or bound forms and within the free form, a pH equilibrium exists between molecular, bisulfite and sulfite forms (du Toit et al., 2005; Fugelsang and Edwards, 2007; Loureiro and Malfeito-Ferreira, 2006).

Different results regarding the resistance to sulphur dioxide have generated controversy in this topic. Some authors refer that *B. bruxellensis* is sensible to free sulphur dioxide higher than 30mg/L and others reported growth under more than 30mg/L (Agnolucci et al., 2010; Barata et al., 2008a). As it was said previously, pH has an important effect on the concentration of molecular sulphur dioxide and this can be the reason for this differences (Barata et al., 2008a; du Toit et al., 2005; Zuehlke et al., 2013). An example of this is a reported loss of viability in white wines due to the efficiency of sulphur dioxide at lower pH environment (Loureiro and Malfeito-Ferreira, 2006).

B. bruxellensis is also affected by DMDC. The concentration of *B. bruxellensis* in wine affect the efficiency of DMDC, it was showed that for high cell concentrations (10^6 cfu/mL) it was needed 300mg/L of DMDC, a higher concentration than the legal limit. For concentrations of 10^4 cfu/mL, the population was killed with 200mg/L of DMDC, being this the legal limit. Its inhibitory effect is transitory because it decreases over time, causing single addition to be insufficient (Costa et al., 2008; Malfeito-Ferreira, 2010a; Renouf et al., 2008).

B. bruxellensis uses oxygen for ATP production but, if not, it can ferment to produce energy being considered a facultative anaerobe yeast (Smith, 2011a). In the presence of oxygen, the glucose metabolism rate increases and the fermentation of glucose is also stimulated, contrarily to what happens with *S. cerevisiae*. This effect is known as Custer's effect, which is attributed to a temporarily repression of the alcoholic fermentation under anaerobic conditions (Aguilar Uscanga et al., 2003; Loureiro and Malfeito-Ferreira, 2006; Smith, 2011a; Zuehlke et al., 2013). Recently, it was demonstrated that under anaerobic conditions, nitrate assimilation abolishes the Custer's effect and, with this and the intrinsic characteristics of *B. bruxellensis*, this species could become a new industrially relevant ethanol-producing organism (Galafassi et al., 2013).

Secondary metabolism

The *Dekkera/Brettanomyces* genus is known to produce high amounts of acetic acid. This was the main reason that sparked interest in wine industry to this yeast (Loureiro and Malfeito-Ferreira, 2006; Malfeito-Ferreira, 2010b; Oelofse et al., 2008). Usually, in the glycolytic pathway, ethanol would be produced from the acetaldehyde; however, in this yeast, the acetaldehyde has a strong tendency to be oxidized to acetic acid. Because of the production of the acetic acid, NADH accumulates in the cell and the need to maintain the NAD+/NADH balance makes the oxygen important as an electrons acceptor in the NADH to NAD+ reaction. In fact, the availability of oxygen greatly affects the amount of

acetic acid formed being higher under aerobic conditions (Aguilar Uscanga et al., 2003; Fugelsang and Edwards, 2007).

The acetic acid constitutes more than 90% the volatile acidity of a wine, and an increase in acetic acid concentration can detriment wine quality since it gives a vinegar taint to the wine (Oelofse et al., 2008). It is also known that high levels of acetic acid like those produced by *B. bruxellensis* can be sufficient to slow or even stuck the wine fermentation showing it is sufficient to inhibit or kill other microorganisms (Fugelsang and Edwards, 2007; Oelofse et al., 2008). The ability to produce acetic acid from *B. bruxellensis* species depends on many factors as it was said previously and it is common to see heavily contaminated wines with *B. bruxellensis* showing normal levels of acetic acid (Loureiro and Malfeito-Ferreira, 2006).

Other secondary metabolites produced by the *Dekkera/Brettanomyces* genus are the tetrahydropyridines. All species of *Dekkera/Brettanomyces* are capable of producing the 2-acetyltetrahydropyridin (ETHP) and the 2-acetylpyrroline (ATHP). These chemical compounds are responsible for the mousy off-flavor in wines, also known as mousiness. The mousy off-flavor has been described as resembling the smell of mice urine. It produces a very disagreeable taste in mouth and is extraordinarily persistent (may exceed 10 minutes). At wine's pH, this compounds are not volatile so only after contact with the saliva this mousiness is perceived (Fugelsang and Edwards, 2007; Malfeito-Ferreira et al., 2009; Snowdon et al., 2006; Suárez et al., 2007).

Another group of secondary metabolites produced by *B. bruxellensis* are the biogenic amines. They are produced mainly by decarboxylation of amino acids and if consumed in excessive amounts can cause headache, nausea, diarrhea and red skin coloration just to name a few health problems. The effect of the biogenic amines is even worse in presence of alcohol since it inhibits the diamine oxidase enzyme, responsible for the conversion of these biogenic amines into harmless products. However, the production by the yeast *B. bruxellensis* has little importance given the small production rate and the lack of some of the most threatening biogenic amines (Caruso et al., 2002; Fugelsang and Edwards, 2007; Oelofse et al., 2008; Vigentini et al., 2008).

Without underestimating the previous referred secondary metabolites produced by *B. bruxellensis*, one of the most serious microbial problems of modern enology worldwide are in fact the volatile phenols. Because of its importance, it will be given an entire chapter on this subject (Chapter 1.4).

Viable but non culturable state

The viable but nonculturable (VBNC) state is characterized by a dormant physiological state where microorganisms fail to grow in culture media when still viable retaining metabolic activity (Fugelsang and Edwards, 2007; Oelofse et al., 2008; Serpaggi et al., 2012; Zuehlke et al., 2013). This state is induced as a response to environmental stress factors like osmotic pressure, temperature, oxygen concentration and others (Agnolucci et al., 2010; Fugelsang and Edwards, 2007; Zuehlke et al., 2013). It is known since late 1900 that bacteria could enter in a VBNC state, however, it was only suggested in the year 2000 that wine yeasts like *B. bruxellensis* could also enter in this state (Millet and Lonvaud-Funel, 2000). The confirmation was reported by du Toit et al. (2005).

VBNC cells of *B. bruxellensis* display a significant reduction of cell size when compared to the viable and culturable ones (Agnolucci et al., 2010; Serpaggi et al., 2012; Umiker et al., 2012). This fact can be a mechanism that allows the reduction in energetic consumption of the cells. For *B. bruxellensis in wine*, it is believed that SO_2 is the main trigger to a VBNC state (Agnolucci et al., 2010; Serpaggi et al., 2012; Umiker et al., 2012; Willenburg and Divol, 2012; Zuehlke et al., 2013). Serpaggi et al. (2012) was the first to demonstrate that eukaryotic cells could exit the nonculturable state when favorable environmental conditions are restored; in this case, *B. bruxellensis* regain the capacity to grow in culture media when the stress factor (SO_2) was removed.

Recent reports demonstrate that the spoilage metabolism of *B. bruxellensis* is still active in VBNC cells. The VBNC cells can produce volatile phenols even though the amounts are roughly half of the produced by normal cells. Therefore, *B. bruxellensis* in VBNC state can also be harmful for wine quality since this quantity is sufficient to be detected by the consumer (Agnolucci et al., 2010; Laforgue and Lonvaud-Funel, 2012; Serpaggi et al., 2012). This topic is of extreme importance for the wine industry perspective. VBNC state could lead to false negatives in plate counting when wine is analyzed and consequently the necessary measures to control *B. bruxellensis* are not done leading to economic losses. Thus, is of extreme importance better detection methods then plate counting.

1.4 Volatile Phenols

The main reason why VPs are so important is due to their spoilage effects: the "horse sweat" off-flavor and off-taste especially in premium red wines matured in oak barrels (Loureiro and Malfeito-Ferreira, 2006; Malfeito-Ferreira et al., 2009).

The formation pathway

The capacity of *B. bruxellensis* to produce volatile phenols was first demonstrated in 1986 (Heresztyn, 1986). The production of VPs is based on a two-step reaction illustrated in Figure 1.5. The precursors hydroxycinnamic acids p-coumaric, ferulic and caffeic are decarboxylated into the hydroxystyrenes 4-vinylphenol (4-VP), 4-vinylguaiacol (4-VG) and 4-vinylcathecol (4-VC) respectively by a cinnamate decarboxylase then, the hydroxystyrenes are reduced by a vinylphenol reductase into the ethylphenols 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG) and the 4-ethylcathecol (4-EC) respectively (Fugelsang and Edwards, 2007; Kheir et al., 2013; Loureiro and Malfeito-Ferreira, 2006; Malfeito-Ferreira et al., 2009; Oelofse et al., 2008; Zuehlke et al., 2013).

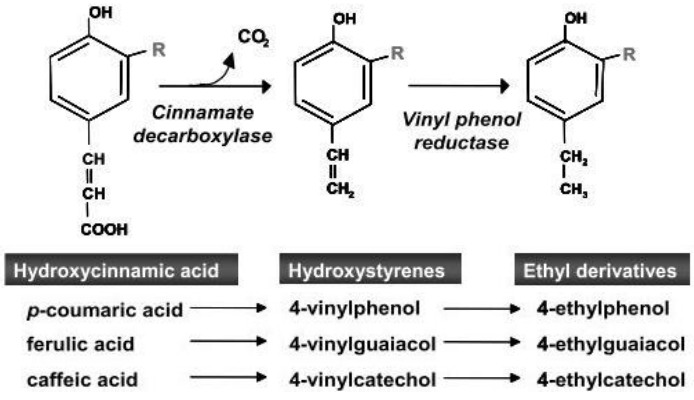

Figure 1.5 – Synthesis of volatile phenols from the hydroxycinnamic acids.(Oelofse et al., 2008)

Hydroxycinnamic acids are present in grapes and are extracted to the juice mainly in the maceration process and some may be converted into vinylphenol and ethylphenol derivatives depending on the microbial population (Kheir et al., 2013; Malfeito-Ferreira et al., 2009).

The decarboxylation reaction can be achieved by a large number of bacteria and yeasts, like *S. cerevisiae* during fermentation. However, the reduction of the vinylphenols derivatives to the ethylphenols are much rarer (Loureiro and Malfeito-Ferreira, 2006; Malfeito-Ferreira et al., 2009; Oelofse et al., 2008). This reaction has been reported to have no inhibition by substrate excess and no inhibition by product. Plus, conversion rates of 90% have been reported for *B. bruxellensis* (Kheir et al., 2013). *B. bruxellensis* can theoretically benefit from species like *S. cerevisiae* that can produce vinyl phenols since *B. bruxellensis* can produce 4-EP from 4-VP in the absence of the hydroxycinnamic acids (Dias et al., 2003; Fugelsang and Edwards, 2007).

The conversion of the others hydroxycinnamic acids besides p-coumaric acid has not been much studied. Knowing that caffeic acid exists in wine in higher concentration, a higher concentrations of 4-EC could be expected, however, some published results show the opposite probably because the utilization of caffeic acid is less preferable then p-coumaric acid (Malfeito-Ferreira et al., 2009).

It is still not known when a higher production rate of 4-EP is observed during the growth of *Brettanomyces*. Some authors say the production is higher in stationary phase, others report a higher production rate in exponential phase and others showed that the production occurred roughly between the two previous phases (Conterno et al., 2013; Dias et al., 2003; Kheir et al., 2013). Recently, it was reported that the production of ethyl phenol was higher in a richer ethanol environment. Ethanol increased lag phase duration and limited the cell growth but not the metabolic efficiency to produce ethyl phenols (Conterno et al., 2013).

Presence in wines and quality depreciation

Wine spoilage by microorganisms is not easy to define. Microbial metabolites, especially in fermented foods and beverages play an important role in the taste and aroma and their pleasantness or displeasure is very subjective. The spoilage is so dependent of cultural reasons, habits and opinion makers that become hard to define it. One great example of this is the presence of volatile phenols in red wines due to the presence of *B. bruxellensis*. Some consumers and opinion makers prefer wine with some level of volatile phenols stating it gives a distinctive aged character to young red wines. Others consider, even at low concentrations, that it makes wine unpleasant due to diminished flavor complexity. Also, the mixture of different aromas are experienced differently than the aromas alone which makes it even harder (Fugelsang and Edwards, 2007; Kheir et al., 2013; Loureiro and Malfeito-Ferreira, 2006; Malfeito-Ferreira, 2010b).

Wines affected by *Dekkera/Brettanomyces* have various sensory descriptors like “spicy”, “smoky”, “leather”, “cedar”, “medicinal”, “animal”, “wet dog”, “barnyard”, or one of the most known “horse sweat” (Fugelsang and Edwards, 2007; Loureiro and Malfeito-Ferreira, 2006). The effect does not end in odors, the volatile phenols in high concentrations also affects the mouthfeel with an increase in astringency and bitterness described as metallic taste (Loureiro and Malfeito-Ferreira, 2006).

The detection/preference threshold can measure the spoilage effect with sensorial activity. This threshold is defined as the minimum concentration under which 50% of the tasters, in a 70-person jury, detect/reject the sample. As said before, the matrix in which the aroma is inserted plays an important role in the detection. An example of this, in Bordeaux red wines, the preference threshold for 4-EP is around 620 µg/L, however, when 4-EG is present in a concentration of one tenth of the 4-EP (normal rate in wines by not always observed), the detection/preference threshold decreases to 426 µg/L. If the concentrations of these compounds are inferior to this threshold, volatile phenols can increase the complexity of wine aroma. Above this threshold, the ethyl phenols dominate the aroma and affect the mouthfeel with metallic notes. Wines, in this case, are normally rejected by the majority of the consumers but still remain pleasant for others due to the reasons previously said (Fugelsang and Edwards, 2007; Kheir et al., 2013; Loureiro and Malfeito-Ferreira, 2006; Malfeito-Ferreira et al., 2009). This thematic is so subject that other authors, Licker et al.(1998), described thresholds that far exceed the above mentioned: a “high Brett” wine contains 3000 µg/L of 4-EP, a medium one 1700 µg/L and a “no Brett” wine 690 µg/L.

It is not easy to estimate the real incidence of volatile phenols contamination in wines, however, Loureiro and Malfeito-Ferreira (2003), estimated that more than 25% of the red wines have concentrations of ethyl phenols higher than the detection/preference threshold of 620 µg/L. More recently, based in data from analytical laboratories, the incidence of volatile phenols in a concentration superior to 690 µg/L show a range from 6% to 74% depending on the year and locations of the wines (Loureiro and Malfeito-Ferreira, 2006). However, these values are overestimated because winemakers normally send to analyzes samples suspected to have problems (Malfeito-Ferreira et al., 2009).

The detection/preference threshold of the other volatile phenol, the 4-EC, is around 50 µg/L. Moreover, the hydroxycinnamic acid precursor, the caffeic acid, is present in relatively high concentrations. Knowing that 4-EC is described as having a phenolic smell similar to that of 4-EP, the differences in sensorial detection and the quantification of 4-EP and 4-EG analyzed by Gas Chromatography (GC) may be explained by the hidden presence of 4-EC (Loureiro and Malfeito-Ferreira, 2006; Malfeito-Ferreira et al., 2009).

Non-*Brettanomyces* species and volatile phenols

Besides *Brettanomyces* many wine related microorganisms like *Oenococcus oeni*, several *Lactobacillus* species, *S. cerevisiae*, *Pichia guilliermondii*, and several *Candida* species can produce vinyl phenols from the hydroxycinnamic acids (Fugelsang and Edwards, 2007; Kheir et al., 2013; Loureiro and Malfeito-Ferreira, 2006). However, like it was said before, few can reduce the vinyl phenols to produce ethyl phenols. Those who can in fact produce ethyl phenols effectively are *B. bruxellensis* and *B. anomala*, *Pichia guilliermondii* and some species from the genus *Candida*. Still, *B. bruxellensis* poses the main threat to wine quality since *Candida species* are more associated with soy sauces. *P. guilliermondii*, apparently, can’t produce 4-EP in wines with normal conditions (12% (v/v) alcohol and 3.5 pH) since they lose viability after 24h and *B. anomala* is less common (Barata et al., 2013; Dias et al., 2003; Fugelsang and Edwards, 2007; Kheir et al., 2013; Loureiro and Malfeito-Ferreira, 2006; Malfeito-Ferreira et al., 2009; Oelofse et al., 2008; Zuehlke et al., 2013).

Detection by gas chromatography

Gas chromatography (GC) has been used by wineries or external laboratories for detection and quantification of spoilage activity of *Brettanomyces* namely, the chemical compound 4-ethylphenol.

GS is a chromatography method for detection of volatile compounds. The method enables a separation of the compounds present in a sample since some compounds elute faster than the other giving different retention times for each compound. The intensity measured by each compound is proportional to its concentration. With this said, the GC method can detect and quantify volatile phenols of a sample after phenols extraction from wine.

As far as we know, *Brettanomyces* is the only microorganism growing in wines that produces high concentrations of 4-EP. Therefore, GC analyses of a wine can act as an indicator to its presence or previous presence. However, many authors reported that ethyl phenols synthesis is strain dependent and population dependent which precludes a direct comparison with cell concentration (Barata et al., 2013; Dias et al., 2003; Fugelsang and Edwards, 2007; Fugelsang and Zoecklein, 2003; Loureiro and Malfeito-Ferreira, 2006; Sangorrín et al., 2013).

The detection of volatile phenols has faster results than plate counts but it is usual to see wine with high levels of volatile phenols and no growth in culture media. These results can be explained by the VBNC state or viability loss of *Brettanomyces* after production of EPs. On the other hand, low levels of EPs with positive results in plate counting indicate a serious threat for wine quality demanding fast action (Laforgue and Lonvaud-Funel, 2012; Loureiro and Malfeito-Ferreira, 2006; Serpaggi et al., 2012).

The major drawback of this method is that it is not a good preventive detection method since when it is detected the presence of ethyl phenols it may be too late to do something about the spoiled wine.

1.5 Detection and Quantification

The detection and quantification of *B. bruxellensis* in wine is extremely important due to the spoilage activity of this yeast and the consequently economic losses for the wine industry. The detection of this yeast should be fast, so the enologists can act fast and prevent high spoilage, accurate with a low detection limit, to know how to treat the contaminated wine and not too expensive so the cost to analyze the samples be cost effective.

The most common and suitable method of detection and quantification of *B. bruxellensis* is the plate counting. However, the burden of the costs of equipment needed for a simple plate counting, the limitation of advanced instruments for other techniques and the requirement of skilled labor in small/medium wine enterprises made wineries ask for external laboratories help (Fugelsang and Edwards, 2007; Loureiro and Malfeito-Ferreira, 2006).

It is known how difficult it is the isolation in plate media of *Brettanomyces* from the environment, especially when heavily contaminated with other yeasts (Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008). However, it is common for the external laboratories to use classical plating techniques associated or not with other molecular methods or a microscopic evaluation of cell morphology. Although a microscopic observation can help, this yeast may have various morphologies depending on different factors.

Culture media

A culture media is a liquid (normally named broth) or solid mixture of components to able microorganisms to multiply. A successful cultivation of microorganisms requires the medium to have all nutritional demands of the desired group of microorganisms. There are also some selective agents that select a growth of a specific group of microorganisms in detriment of others resulting in a selective media. This is the most common technique to identify microorganisms in food industry (Loureiro and Malfeito-Ferreira, 2006).

Commonly, media for yeasts maintenance are based on glucose, peptone and yeast extract with some differences in concentration and pH. Some authors suggest that vitamins such as thiamine and biotine are beneficial to the growth of *Brettanomyces* although some authors disagree (Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008).

Another general culture medium used for yeasts is WL. This media also called WLN (WL-Nutritional) is used for determination of total viable yeast population (Fugelsang and Edwards, 2007). This media contains a pH indicator, bromocresol green, which changes media color when acidification of media is observed.

The culture medias above mentioned are not selective for *Brettanomyces* spp.. For the selective growth of *B. bruxellensis* present in wine, one of the more commonly used selective agent is cycloheximide (Actidione®). This antibiotic inhibits protein biosynthesis in many eukaryotes including *Saccharomyces*, the most common yeast of the alcoholic fermentation in wine. *B. bruxellensis* on the contrary, can resistant to the concentrations commonly used (10-100 mg/L). When *B. bruxellensis* is the predominant yeast in the wine, a non-selective media added of cycloheximide is efficient enough. It is now usual to see in many research articles the use of a WL-Differential medium (WLD) or WL-Cycloheximide (WLC). This is due to the addition of cycloheximide in the WL culture media to select *Brettanomyces* against *Saccharomyces*. Unfortunately, some other wine spoilage yeasts including *Hanseniaspora uvarum*, *Pichia guilliermondii* and *Schizosaccharomyces pombe*, when present, can also grow in 50 mg/L of cycloheximide (Fugelsang and Edwards, 2007; Loureiro and Malfeito-Ferreira, 2006; Morneau et al., 2011; Oelofse et al., 2008; Zuehlke et al., 2013).

Rodrigues et al. (2001) presented the *Dekkera/Brettanomyces* Differential Medium (DBDM). This medium uses 6% (v/v) ethanol as a two way selective agent. First, to reject species that can't support growth with 6% ethanol concentrations and second, to select species that can grow with ethanol as sole carbon source. This media also contains 6.7 g/L of yeast nitrogen base (YNB), the pH indicator bromocresol green, 10 mg/L of cycloheximide for the purpose above mentioned and 100 mg/L of p-coumaric acid. The latter compound, as above mentioned, is the precursor of the 4-EP which leads to a distinct phenolic off-odor if *Brettanomyces* spp. grow in this media. *Pichia guilliermondii* is, theoretical, the single false positive in this medium since it can grow in DBDM, produce acetic acid and produce 4-EP in sufficient rate to identify the off-odor (Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008; Rodrigues et al., 2001; Zuehlke et al., 2013). However, this media has a major limitation since it was demonstrated that not all strains of *B. bruxellensis* can use ethanol as sole carbon source. Some authors reported low frequencies (26-39%) of *B. bruxellensis* strains that could use ethanol as sole carbon source (Conterno et al., 2006; Echeverrigaray et al., 2013). To support

this, Morneau et al. (2011) measured colonies size in WL with increasing concentrations of cycloheximide and DBDM. The results showed that some strains couldn't grow or grew poorly on DBDM. However, when *Brettanomyces* was inoculated in DBDM with a small volume of wine, the strains grew better. The author proposes 2 reasons for these observations: an inability of those strains to quickly metabolize ethanol as a sole carbon source or a lack of an unidentified nutrient. It is also important to refer that, given the stressful conditions of this media and the slow growing ability of *Brettanomyces*, an incubation time of two weeks or more is needed. This result takes too much time which can lead to the spoilage of the wine even before it is known that is contaminated (Fugelsang and Edwards, 2007; Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008).

Renouf et al. (2007) developed an enrichment media named EBB medium. This media contained 200 mL/L of grape juice, 4% ethanol, malt extract, yeast extract, ammonium and magnesium sulfate and tween 80. With this media, after 10 days of enrichment, either no *B. bruxellensis* was detected or it largely outnumbered the other yeasts present in the sample.

Although plating on selective media is the normal and most common technique used in food industry, this method is yet insufficient for detection and quantification of *B. bruxellensis* in the winemaking process where a period of a week is crucial. Given this, the lack of an effective culture media to rapid presume the presence of *Brettanomyces* spp. makes place for a large and needy market for new low cost products due to its recurring need.

Molecular methods

Given the *B. bruxellensis* slow growth in selective culture media, a rapid and reliable identification of this spoilage yeasts demands modern techniques and molecular approaches have shown its potential.

Stender et al. (2001) used fluorescence microscopy for detection and quantification of *Brettanomyces* spp. in wine. This method is based in a known technique called fluorescence in situ hybridization (FISH) known since 1982 (Langer-Safer et al., 1982). This technique uses oligonucleotide probes fluorescently marked to hybridize to a sequence of interest *in situ*. It is then possible to see in whole-cell preparations under a fluorescent microscope the target cells appearing as bright fluorescent colors depending on the fluorescent dyes. After Stender et al., Dias et al. (2003), used the same method but directly in pellets of centrifuged wine. This method is a high sensitivity way to specifically detect and quantify the presence of *B. bruxellensis* with detection limit depending on the volume of centrifuged samples. If not centrifuged, the microscopic-based technique is only suitable when contamination is high (around 10^4 cells/mL).

Other molecular techniques that are fast, sensitive and accurate normally involves polymerase chain reaction (PCR). This technique, developed in 1983 by Kary Mullis (Mullis, 1990), is now one of the most used methods in molecular biology laboratories. As it is commonly known, PCR involves 3 steps: denaturation of DNA helix, annealing of the primers and elongation of the complementary sequence. The product of these thermal cycles is an immense amplification of the target sequence. Based on PCR, several methods for detection and/or quantification of *Brettanomyces* spp. have been reported.

The first PCR-based method used to detect *Brettanomyces* spp. was reported by Ibeas et al. (1996). This method consisted in a nested-PCR with detection results in 10 hours and no need of isolation or

culture being used directly in wine. The nested-PCR is used normally to prevent unspecific binding of the primers using in this case a second set of primers to amplify a region within the first one (Ibeas et al., 1996; Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008).

After the year 1996, a lot of studies used PCR-based methods for the identification of *Brettanomyces* spp. In 1999, a random amplification of polymorphic DNA (RAPD-PCR) (Mitrakul et al., 1999) and a restriction fragment length polymorphisms (RFLP-PCR) (Esteve-Zarzoso et al., 1999) were reported. After this date, many PCR-based methods were reported to identify *Brettanomyces* spp. using the specific 5.8S ribosomal genes and their flanking internal transcribed spacer (ITS1 and 2) (Egli and Henick-Kling, 2001; Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008; Suárez et al., 2007; Zuehlke et al., 2013). However, these studies relied on fresh colonies and so the isolation problem of culture media above mentioned remained. Cocolin et al. (2004), compared the PCR-based techniques like restriction endonuclease PCR (RE-PCR), denaturing gradient gel electrophoresis (DGGE) and a dot blot RNA against the classical method of plate counting. The main fault reported by the authors and also commonly attributed to other direct methods is the high detection limit of 10^4 cfu/mL. At this concentration of *Brettanomyces* spp. wine can already be spoiled. However, some of these techniques can be rendered more sensitive if wine is centrifuged first (Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008).

In the Ibeas et al. (1996) report, it was also used a dot blot hybridization for simple present or not present *Brettanomyces* spp. analyzes. In Cocolin et al. (2004) work above mentioned, the dot blot RNA test was also a present or not present analyzes in wine samples, however, a detection limit of around 10^4 cfu/mL was shown from serial dilutions. This year, Cecchini et al. (2013) reported a dot blot method which allows the detection of *B. bruxellensis* in wine samples without amplification steps or enrichments with a 10 cells/mL detection limit. These results show a promising tool for winemakers hence it gives quick results with a great sensitivity.

In 2007, the use of a recent method called loop-mediated isothermal amplification (LAMP) to detect and quantify *Brettanomyces* spp. was reported by Hayashi et al. (2007). This method, developed by Notomi et al. (2000), relies on an enzyme with strand displacement activity under isothermal conditions. It uses four to six primers recognizing six to eight regions of the target DNA sequence. The auto-cycling reaction lead to accumulation of a large amount of the target DNA and other reaction by-products, such as magnesium pyrophosphate (a white precipitate), allowing a rapid detection by spectrophotometry (Hayashi et al., 2007; Njiru, 2012; Notomi et al., 2000). Hayashi et al. (2007) reported a detection limit of 10 cfu/mL in a 60 minutes method showing the usefulness of this method for quality control in wineries.

Real-Time PCR

Real-Time PCR (RT-PCR) also known as quantitative PCR (Q-PCR) is a PCR-based method where the DNA amplification during a normal PCR is measured after each cycle (hence the term real-time). The amount of DNA/RNA can be measured by detection of fluorescent reporters that give increased fluorescence signal when the number of amplicons (product PCR molecules) is greater. There are different fluorescent dyes used for detection in RT-PCR: double-stranded DNA binding dyes like

SYBR green, dye molecules attached to the reaction primers or probes that hybridize with PCR product during amplification. Since the PCR is based on an exponential increase of amplicons, the exponential phase of the reaction can yield information on the initial amount of the target: if the target sequence is abundant, the amplification starts in earlier cycles; if the sequence is in low concentration, amplification takes place in later cycles.

This method was first used to quantify *Brettanomyces* spp. by Phister and Mills (2003). The authors reported a 3 hours test with a detection limit of 10 cells/mL of wine. After this, in the following year, Delaherche et al. (2004) reported another RT-PCR method but the detection limit was 10^4 cells/mL instead. The reason of this difference is because the latter author used a different protocol, probably because the previous work was not known yet since there was no reference to it in this article. In 2007, a personal communication by the same authors referred that a change of the DNA extraction method improved the detection limit to the same 10 cfu/mL of Phister and Mills work (Loureiro and Malfeito-Ferreira, 2006; Oelofse et al., 2008; Zuehlke et al., 2013).

Tessonnière et al. (2009) tested six different protocols of DNA extraction and isolation from wine and three PCR reaction compositions to select the best method. The selected method allowed a detection limit of 31 cfu/mL.

Willenburg and Divol (2012) compared the efficiencies of DNA and mRNA as template and concluded that mRNA was a better template. One of the reasons was that DNA can remain intact for more time than mRNA and this cause an overestimation of cell numbers due to dead cells. The authors also showed that cells in VBNC state induced by SO_2 addition were detected by RT-PCR and not by plate counting which is of great importance due to reason previously mentioned. The detection limit was around 10 cells/mL of wine. The other article published in the past year was from Tofalo et al. (2012). In this work, the authors have also compared plate counting analyzes with RT-PCR and they also found some wine samples with negative results in plate counting but positive in RT-PCR probably due to the VBNC state of *B. bruxellensis*. The authors also tested different commercial kits for DNA extraction since it is one of the most critical steps in no-culture PCR-based applications and found out that DNAPowerSoil® Isolation Kit (MO BIO Laboratories) gave the best results and was fast and simples to use. A detection limit of 10 cells/mL was also achieved.

Portugal and Ruiz-Larrea (2013) also reported a comparison of RT-PCR with conventional plate counting method. From 324 wine samples, there were 31.8% that gave positive results in RT-PCR and negative in plate count and only 7.7% that gave positive in plate count and negative in RT-PCR. The false negative results were probably due to low yeast populations of <100 cfu/mL. These low concentrations could be lost in DNA extraction or washing stages. The group of 31.8% false negative in plate count was explained by the authors as nonviable *Brettanomyces* cells and DNA that still persist in wine. The authors however did not give importance to a probable VBNC state in these samples. Thus, they report that the choice is basically based on price and time. If winemakers want rapid and efficient detection they should choose RT-PCR but if winemakers want an efficient and low-cost method they should choose the plate counting.

1.6 Objectives

Despite the threat *B. bruxellensis* poses to the wine industry, there are much to learn about this yeast and much to do in order to help wineries decreasing the incidence of the phenolic odor in their wines with faster and efficient detection. The production of 4-ethylphenol is not yet fully understood and disagreements about in which growth phase, the production rate is higher and if cells without active growth are able to produce this compound, still exists. DBDM shows to be highly selective but has the disadvantage of large incubation times and using ethanol as sole carbon source. Real-time PCR seem promising for specific and rapid detection but it is expensive for the most wineries. To better understand the behavior of this yeast and find new solutions to wineries, we established the following objectives:

- To study the difference in viability and culturability as well as evaluate the 4-ethylphenol production in the absence of actively growing populations.
- To develop a new selective medium with a rapid, labor free and efficient detection.
- To make a Real-Time PCR protocol with lower costs for the detection of *B. bruxellensis* in our laboratory.

2 Materials and Methods

2.1 Species and strains

All strains were kept in slants of GYP medium with addition of 5 g/L of calcium carbonate at 4°C. When needed, a loop full was inoculated in GYP agar plate and incubated at 25°C until isolated colonies appeared.

The strains used as well as their origin are summarized in Table 2.1.

Table 2.1 - Source and code of used strains.

Species	ISA nº	Source[a]
Brettanomyces bruxellensis	1146	UCD 605
	1717	White wine (Estremadura, Portugal)
	1791	Red wines (Dão, Portugal)
	2101	Red wines (Alentejo, Portugal)
	2114	Red wines (Ribatejo, Portugal)
	2150	Red wine (Portugal)
	2172	Red wine (Spain)
	2173	Red wine (California, USA)
	2202	Red wines (ISVEA, Italy)
	2206	Red wine (Estremadura, Portugal)
	2211	Red wines (Douro, Portugal)
	2297	Wood barrique after red wine storage
	2298	Insect in winery
Dekkera anomala	1654	IGC-5161, bees wine culture
Candida tropicalis	1359	Beer
C. halophila	1982	MUCL 29967^{T}
Kloeckera apiculata	1189	CECT-1120
Pichia anomala	1478	IGC-4121^{T}
P. guilliermondii	2105	Grapes
	2131	Red wine
Saccharomyces bayanus	1730	No reference
S. cerevisiae	1000	IGC 4072, Commercial starter (Fermivin®)
Zygosaccharomyces bailli	1307	Sparkling wine production line
	2295	No reference
Z. hellenicus	2284	Sour rotten grapes

[a] UCD (University of California, Davis, USA); IGC (Gulbenkian Institute of Science, Oeiras, Portugal); MUCL (Micothéque de la Université Catholique de Louvain, Belgium); CECT (Coleccion Española de Cultivos Tipo, Valencia, Spain).

2.2 Chemical compounds

The chemical compounds used were of the highest quality available. They were: WL Differential Agar (Conda Laboratories, Madrid, Spain), Glucose (COPAM, Loures, Portugal), Yeast extract (Biokar Diagnostics, Beauvais, France) Peptic digest of meat USP (Biokar Diagnostics), Agar (Iberagar, Coina, Portugal), Yeast nitrogen base (Difco Laboratories, Detroit, USA), Alcohol (AGA, Prior Velho, Portugal), p-coumaric acid (Sigma-Aldrich, Saint Louis, USA), Ringer's Solution (Biokar Diagnostics), Methylene blue (Merck, Darmstadt, Germany), Bromocresol green (Sigma-Aldrich), Bromophenol blue (Merck), Cycloheximide (Calbiochem, Darmstadt Germany), Chloramphenicol (Sigma-Aldrich), Delvocid® (pimaricine) (DSM, Heerlen, Netherlands), Dimethyl Sulfoxide (Sigma-Aldrich), Diethyl ether (VWR International, Radnor, Pennsylvania), n-Hexane (Merck), 3,4-dimethylphenol (Thermo Fisher Scientific, Waltham, USA)**,** DNA PowerSoil® Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA), SsoFast™ Evagreen Supermix (Bio-Rad Laboratories, Hercules, USA) and SYBR® Select Master Mix (Life Technologies, Carlsbad, USA).

DNA primers

The primers used by Phister and Mills (2003) DBRUXR (5'-GAAGGGCCACATTCACGAACCCCG-3') and DBRUXF (5'-GGATGGGTGCACCTGGTTTACAC-3') were used for the Real-Time PCR analysis in this thesis and were made in STAB VIDA (Caparica, Portugal). These primers were designed to target the D1/D2 domain of the 26S rRNA gene.

2.3 Equipment

All the spectrophotometric measurements were taken with a Boeco S-20 spectrophotometer. All media and solutions were prepared using a weighing-machine Radwag PS450lx except measurements with less than 1 g in which was used the weighing-machine Mettler AE160. pH adjustments was made using a Schott instruments blue-line 14pH electrode. All microscopic quantification needed was done using a hemocytometer in a Leitz-Dialux 20 microscope. Incubation of plates was made in a Sanyo MIR-162 incubator. Liquid growth media was incubated in a Panasonic MIR-154-PE incubator with orbital shaker. When needed, an Eppendorf centrifuge 5415D and a vortex was used.

GC analysis

All chromatographic measurements were taken with a Varian CP-3800 Gas Chromatographer with a Stabilwax 30m, 0.25mm, 0.25 µL column.

Real-Time PCR

Normal RT-PCR material was used like disposable transparent adhesive opti-seal and a 96 wells plaque. For DNA extraction, all material needed was provided in DNA PowerSoil® Isolation Kit (MO BIO Laboratories). For RT-PCR method, an iQ5 and iCycler (Bio-Rad Laboratories) were used. Milli-Q water autoclaved was used in every RT-PCR reaction.

2.4 Software

The software used was Bio-rad iQ5 V2.0 and Galaxie chromatography workstation V1.9.3.2.

2.5 General methods

Growth media preparation

All media were prepared using a weighting-machine and distilled water. When needed, the pH was adjusted using NaOH or HCl solutions before agar addition. After media preparation, all media were autoclaved at 121°C for 20 minutes.

GYP media consists in 20 g/L of glucose, 5 g/L of yeast extract, 5 g/L peptone and 20 g/L agar (if solidified); WL media is prepared according to the manufacturer's instructions with an addition of 4 g/L of agar for a better solidification; Medium A consists in 20 g/L of glucose, 5 g/L of yeast extract, 5 g/L peptone, 22 mg/L of bromocresol green, 10 mg/L of cycloheximide, 100 mg/L chloramphenicol, 50 mg/L of Delvocid® previously dissolved in 5 mL of DMSO and 20 g/L agar (if solidified) adjusted to pH 5.4; Medium B consists in 20 g/L of glucose, 5 g/L of yeast extract, 5 g/L peptone, 22 mg/L of bromophenol blue, 10 mg/L of cycloheximide, 100 mg/L chloramphenicol, 50 mg/L of Delvocid® previously dissolved in 5 mL of DMSO and 25 g/L agar (if solidified) adjusted to pH 4.5.

DBDM media was prepared as follows: A main solution is prepared by 22 mg of bromocresol green dissolved in 800 mL of distilled water. pH is adjusted to 5.4 and 20 g of agar are added before autoclave sterilization. A solution with 6.7 g of yeast nitrogen and 140 mL of distilled water is prepared. Another solution with 100 mg/L of p-coumaric acid and 60 mL of total ethanol is prepared. Next, the 2 solutions are mixed and pH is adjusted to 5.4. Sterilization of the solution is made by vacuum filtration with 0.22µm membrane and added along with 10 mg/L of cycloheximide, 100 mg/L chloramphenicol, 50 mg/L of Delvocid® previously dissolved in 5 mL of DMSO to the main solution.

Determination of 4-ethylphenol

Determination of 4-ethylphenol in synthetic media was measured according to the following protocol:

Adjust the pH of a sample to 8. Add 0.5 mL of 3,4-dimethylphenol in a 10mg/L solution (internal standard) to a 10 mL volumetric flask and fill it with the sample. Transfer all volume to a 25 mL volumetric flask and add 4 mL of 50% ether-hexane. Place a magnet inside and mix in a magnetic stirrer for 5 minutes. Decant, recovering the aqueous phase to the same flask and the organic phase for a vial with a stopper. The aqueous phase is submitted to 2 more extraction with an addition of 2 mL of 50% ether-hexane instead of 4. The organic phase is always recovered to the same vial. The transparent phase is transferred with a Pasteur pipette to a GC vial for posterior GC analyses.

The program used for GC analysis was as follows: Initial temperature 50°C, increase in temperature 10°C/min, second temperature 215°C, second increase in temperature 20°C/min and final temperature 250°C for 10min. Injector temperature was at 230°C and detector temperatures were 250°C. Hydrogen was used as a carrier gas at 2.0 mL/min. The sample volume injected was 2 µL.

The data analysis of the GC results was made using the Galaxie chromatography workstation software.

DNA extraction and Real-Time PCR

In every procedure of the DNA extraction and the Real-Time PCR preparation was done wearing gloves. DNA extraction was according the manufacturer's instructions. Real-Time PCR reaction mixture was prepared for a final volume of 20 µL in each well. Each reaction well contained the following: 10 µL of Master Mix from Life technologies or Bio-Rad, 8.76 µL of Milli-Q autoclaved water, 0.12 µL of DBRUX-F primer, 0.12 µL of DBRUX-R and 1 µL of DNA (excluding the no template control where water was added instead). All samples were analyzed in triplicate. The RT-PCR program was as described by Tofalo et al. (2012) with a minor modification: the denaturation temperature was of 95°C instead of 94°C in every cycle.

The data analysis of the RT-PCR results was made using the Bio-Rad iQ5 v2.0 software.

2.6 Impact of growth phase in the nonculturable *Brettanomyces bruxellensis* population and 4-ethylphenol production

For the nonculturable population trial:

Single fresh colonies of two *Brettanomyces bruxellensis* strains, ISA 1791 and ISA 2211, were inoculated in a 100 mL Erlenmeyer flask with 50 mL of GYP broth and incubated in orbital shaker incubator at 25°C and 130 rpm for 2-3 days. After this incubation, Optical Density at 620nm (OD_{620nm}) was measured and it was transferred volume to a new erlenmeyer flask with 50 mL of GYP broth until near 0.05 of OD_{620nm} was reached. For each strain, 2 inoculations were made with several hours of separation to limit blank spaces on growth curve. Incubation in orbital shaker incubator at 25°C and 130 rpm was made. Every 3 hours, cell concentration (viable and non-viable cells) was measured by hemocytometer count with vital staining, the absorbance at 620nm was measured and serial dilutions were inoculated in GYP plates. This procedure was done until stationary phase was observed (approximately 4 days). Trials were done in duplicate for each strain.

Specific growth rate was calculated as the slope of the trend line obtained by the graphical representation of ln(absorbance) vs. time in hours at exponential growth phase. Doubling time was calculated as the ln(2) divided by the specific growth rate.

For the 4-ethylphenol production trial:

A single fresh colony of *B. bruxellensis* ISA 1791 was inoculated in a 100 mL Erlenmeyer flask with 50 mL of GYP broth and incubated in orbital shaker incubator at 25°C and 130 rpm for 2-3 days. After this incubation, the absorbance at 620nm was measured and it was transferred volume to 4 different 250 mL Erlenmeyer flasks with 100 mL of GYP broth until near 0.05 of absorbance at 620nm was reached. In each flask, 1 mL of a 10 mg/mL p-coumaric ethanol solution was added in different times of incubation. In one flask the addition was made immediately after inoculation, the second flask after 1 day, the third after 2 days and the fourth 3 days. The incubation was made in orbital shaker incubator at 25°C and 130 rpm. Cell concentration (viable and non-viable cells) was measured by hemocytometer count with methylene blue coloration, the absorbance at 620nm was measured, serial

dilutions were inoculated in GYP plates and 13 mL of the growth solution was stored in a sterile falcon at -4°C for posterior phenols extraction and GC analysis.

This full procedure was repeated once more with the difference of p-coumaric addition times. In the second trial, only 3 flasks were used and the addition of 1 mL of a 10 mg/mL p-coumaric ethanol solution was made after 1 days of incubation, the second it was added after 4 days and in the third flask after 7 days.

Production rates were obtained by the slope of the 4-ethylphenol calculated by GC analysis vs. time in days.

2.7 Growth features of *Brettanomyces bruxellensis* on existent culture media

A single fresh colony of 13 *B. bruxellensis* strains (ISA 1146, ISA 1717, ISA 1791, ISA 2101, ISA 2114, ISA 2150, ISA 2172, ISA 2173, ISA 2202, ISA 2206, ISA 2211, ISA 2297, ISA 2298) was inoculated in 50 mL Erlenmeyer flasks with 50 mL of GYP broth and rubber stoppers with a syringe inserted to create a micro oxygenation environment. Flasks were incubated at 25°C and homogenized every day with absorbance at 620nm measured. When in exponential phase (0.5 – 1 Abs), 1 mL of each strain was transferred to a 1.5 mL tube and centrifuged at 13.2 G's for 10 minutes. The supernatant was discarded and 1 mL of Ringer Solution was added. Then serial dilutions were made and inoculated in GYP plates, DBDM plates and WL with added 100mg/L p-coumaric, 10 mg/L of cycloheximide and 100 mg/L chloramphenicol plates. Every day colonies size was measured using a common scale for 20 days. Records from the observed morphology were also made.

2.8 Development of a new selective medium

A single fresh colony of every species present in Table 3.5 was streaked into medium A and medium B. Plates were observed after 3 to 5 day to check for microbiological growth, growth medium color change and phenolic smell (only if growth was observed).

A single fresh colony of *B. bruxellensis* strains (ISA 1791 and ISA 2211) was inoculated in two 50 mL Erlenmeyer flask with 50 mL of GYP broth and rubber stoppers with a syringe inserted to create a micro oxygenation environment. Flasks were incubated at 25°C and homogenized every day and OD_{620nm} was measured. Just after inoculation and when in exponential phase (0.5 – 1 Abs), 1 mL of each strain was transferred to a 1.5 mL tube and centrifuged at 13.2 G's for 10 minutes. The supernatant was discarded and 1 mL of Ringer Solution was added. Then serial dilutions were made and inoculated in GYP, WL, medium A and medium B plates. Cell concentration (viable and non-viable cells) was also measured by hemocytometer count with vital staining. Colony size was measured using a common scale over 20 days.

2.9 Real-Time PCR implementation

To optimize the method, a single fresh colony of *B. bruxellensis* ISA 1791 and ISA 2211 were subject to DNA extractions. DNA was stored at -4°C until RT-PCR analysis. RT-PCR was made with 5 serial dilutions of the DNA extracted using different reaction mixtures (data not shown) until a reaction mixture was selected. The selected reaction mixture was the one with lower costs and better efficiency in the calibration curve. Posterior quantification analysis used the reaction mixture selected. To compare classic microbiological methods with RT-PCR, a single fresh colony of *B. bruxellensis* ISA 1791 was inoculated in a 100 mL Erlenmeyer flask with 50 mL of GYP broth and incubated in orbital shaker incubator at 25°C and 130 rpm for 3-4 days (stationary phase). Cell concentration (viable and non-viable cells) was measured by hemocytometer count with vital staining, the absorbance at 620nm was measured, and serial dilutions were inoculated in GYP, medium A and medium B plates. DNA extraction was made for every dilution followed by Real-Time PCR analysis.

The same procedure was done for the ISA 2211 strain using only 2 dilutions for DNA extraction and Real-Time PCR analysis. This 2 known cell concentrations of ISA 2211 strain were used as unknown concentrations in RT-PCR detection method.

3 Results and Discussion

3.1 Impact of growth phase in the nonculturable *B. bruxellensis* population and 4-ethylphenol production

The growth of *B. bruxellensis* ISA 2211 measured by hemocytometer, plate counting and optical density is illustrated in Figure 3.1. Regarding absorbance readings, the yeast showed a lag phase of 20 hours and an exponential phase of 20 hours. The specific growth rate was 0.096 h^{-1} and the maximum concentration of viable cells achieved was 10^8 cells/mL. The values of cell viability (measured by hemocytometer count with vital staining) followed the absorbance evolution after the end of lag phase. Culturable cell concentration overlapped the cell viability curve indicating that most of the viable cells were also culturable. Only an average of 2.09% of the viable population was in a viable but nonculturable state. The concentration of nonviable cells increased with time being about 3% in average of the total cells during all experiment.

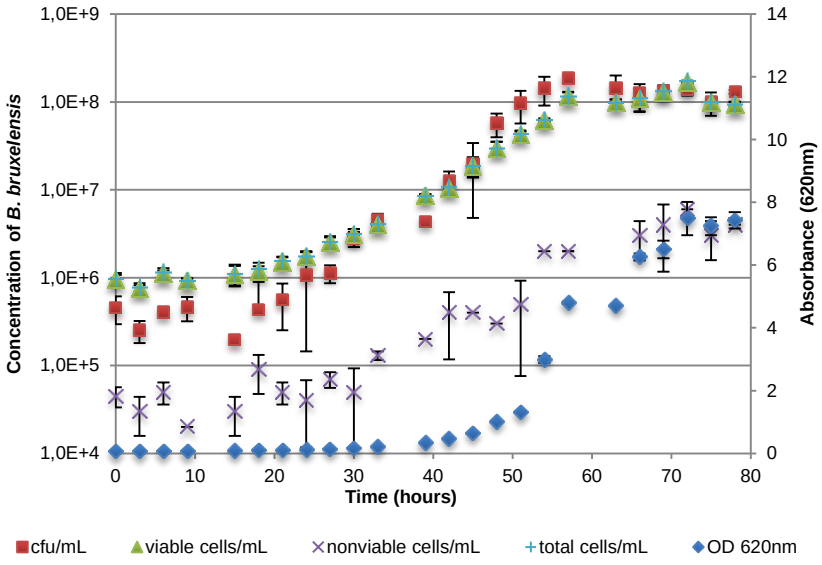

Figure 3.1 - Growth of *B. bruxellensis* ISA 2211 strain in GYP medium. Concentration of *B. bruxellensis* was measured in GYP plate (cfu/mL), hemocytometer count with vital staining (viable cells/mL, nonviable cells/mL and total cells/mL) and absorbance at 620nm.

The growth of *B. bruxellensis* ISA 1791 measured by hemocytometer and plate counting and optical density is illustrated in Figure 3.2. This strain showed a lag phase of around 30 hours and an exponential phase of near 20 hours. The specific growth rate was 0.104 h^{-1} and the maximum concentration of viable cells of 10^8 cells/mL was also achieved by this strain indicating that, under these conditions, this is the maximum concentration achievable by *B. bruxellensis*. Cell viability followed the absorbance evolution at 620nm after the end of lag phase. These parameters are consistent with those from *B. bruxellensis* ISA 2211 strain. However, the concentration of the nonviable population of strain 1791 was about 8.4% average of the total cells.

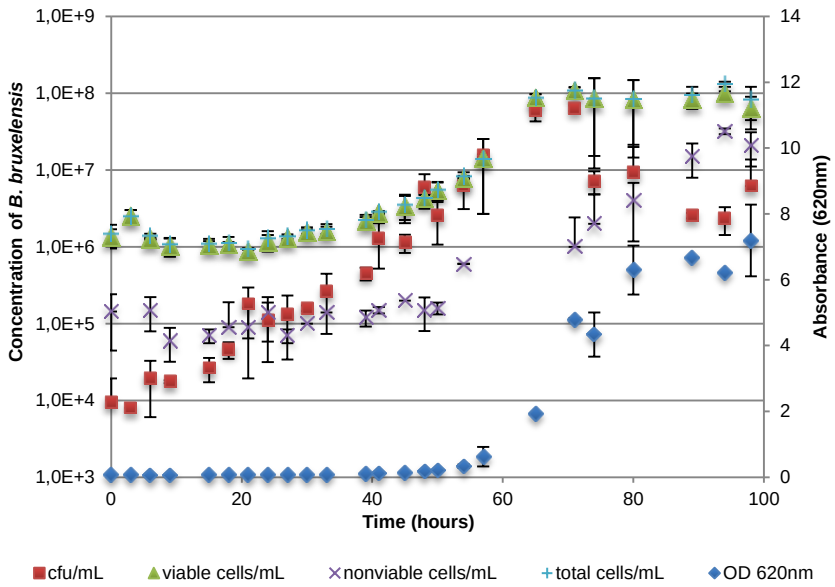

Figure 3.2 - Growth of *B. bruxellensis* ISA 1791 strain in GYP medium. Concentration of *B. bruxellensis* was measured in GYP plate (cfu/mL), hemocytometer count with vital staining (viable cells/mL, nonviable cells/mL and total cells/mL) and absorbance at 620nm.

Regarding *B. bruxellensis* ISA 1791 (Figure 3.2), in lag phase, it seems that the nonculturable population started to decrease being insignificant only when growth reached the exponential phase. Soon after the exponential phase, part of the viable population entered again into the VBNC state. The culturable population of this strain appeared to be 10 to 100 fold less than the viable cells with an exception in the exponential phase. More so, the viable but nonculturable population was, in average, 71.44% of the viable population, a significantly higher value than the one obtained from strain ISA 2211.

These results regarding the ability of *B. bruxellensis* to enter in a VBNC state are in agreement with previous reports (Agnolucci et al., 2010; Laforgue and Lonvaud-Funel, 2012; Serpaggi et al., 2012); however, no stress factor was added. We hypothesized that the stress implicated in this growth was the secondary metabolite acetic acid, however, no determinations of acetic acid concentration was done. Acetic acid is known to be inhibitory to several microorganisms as well as to *Brettanomyces* spp. (Aguilar Uscanga et al., 2003; Fugelsang and Edwards, 2007; Oelofse et al., 2008). The reason why strain ISA 2211 did not present significant viable but nonculturable population when compared to ISA 1791 can be explained by a higher resistance to acetic acid concentrations.

Some important growth parameters were calculated and are summarized in Table 3.1. The results obtained are in agreement with previous works from Brandam et al. (2008) and Barata et al. (2008b). Specific growth rate of both strains were around 0.1 h^{-1} like previously said. The time needed for *B. bruxellensis* to double its concentration, meaning the doubling time, was near 7 hours for both strains reason why it is considered a slow growing yeast (Smith, 2011b). The other parameter calculated was the concentration of viable cells present in a solution with 1 unit of absorbance at 620nm.

Table 3.1 – Parameters calculated from the growth curves of *B. bruxellensis* ISA 2211 and ISA 1791 strains.

Strain	Specific growth rate (h^{-1})	Doubling time (h)	Viable cells in 1 OD unit
ISA 2211	0.096	7.26	$2.80x10^7$
ISA 1791	0.104	6.68	$2.04x10^7$

The strategy to study the effect of growth phase in the production of 4EP was to follow the growth of *B. bruxellensis* ISA 1791 by optical density, hemocytometer and plate counting and add the precursor p-coumaric acid at different incubation times. The results are illustrated in Figure 3.3.

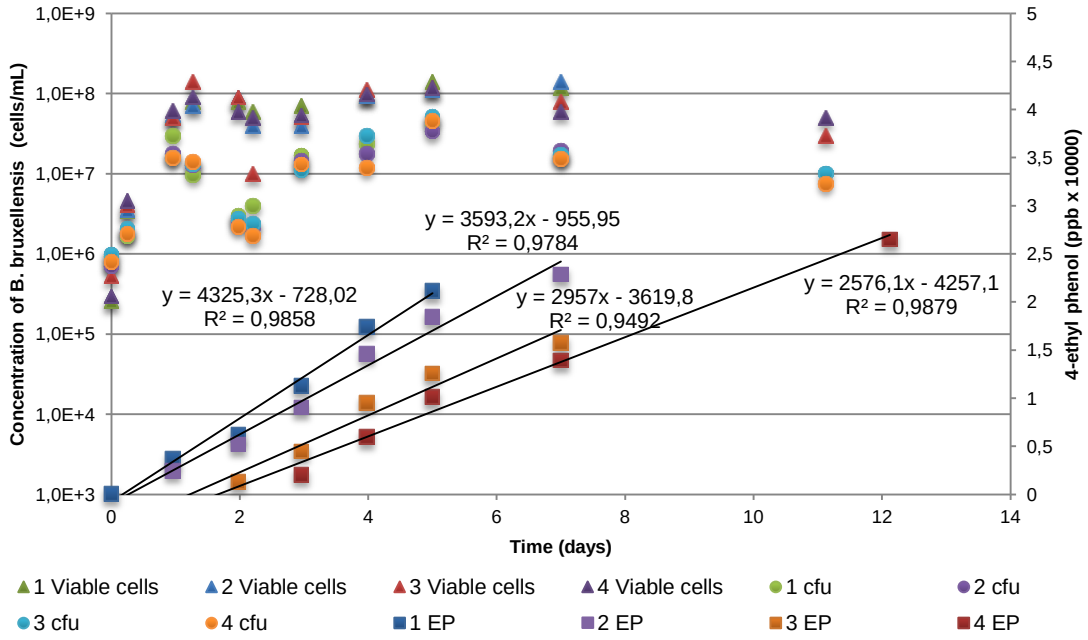

Figure 3.3 - Growth of *B. bruxellensis* ISA 1791 in synthetic culture medium GYP. (1) Addition of p-coumaric acid at day 0, (2) addition of p-coumaric acid at day 1, (3) addition of p-coumaric acid at day 2 and (4) addition of p-coumaric acid at day 3.

As it can be observed, the number of viable cells was always superior to the number of culturable cells by 74.6% average, demonstrating the existence of the viable but nonculturable state. The production rate of 4EP decreased with time of cultivation as it is summarized in Table 3.2. Even though the production rate decreased, 6 days after p-coumaric addition every flask had concentrations of around 25 mg/L of 4EP.

The results indicate that, in fact, the production rate decreases with incubation time and we hypothesize that this is due to loss of enzymatic activity with time. It was also demonstrated that cells without active growth, here represented as stationary cells can produce 4EP with significant rates.

Table 3.2 - Production rate determined for each 4 flasks with the addition of p-coumaric at different times. Production rate was determined by the slope of the trend line of 4EP concentration vs. time.

Time until p-coumaric addition	Production rate (mg/L/day)
0 days	4.3
1 day	3.6
2 days	3.0
3 days	2.6

In a second trial, the addition of p-coumaric was made after 7 days of inoculation to better evaluate the production of 4EP by stationary phase cells. The results are illustrated in Figure 3.4.

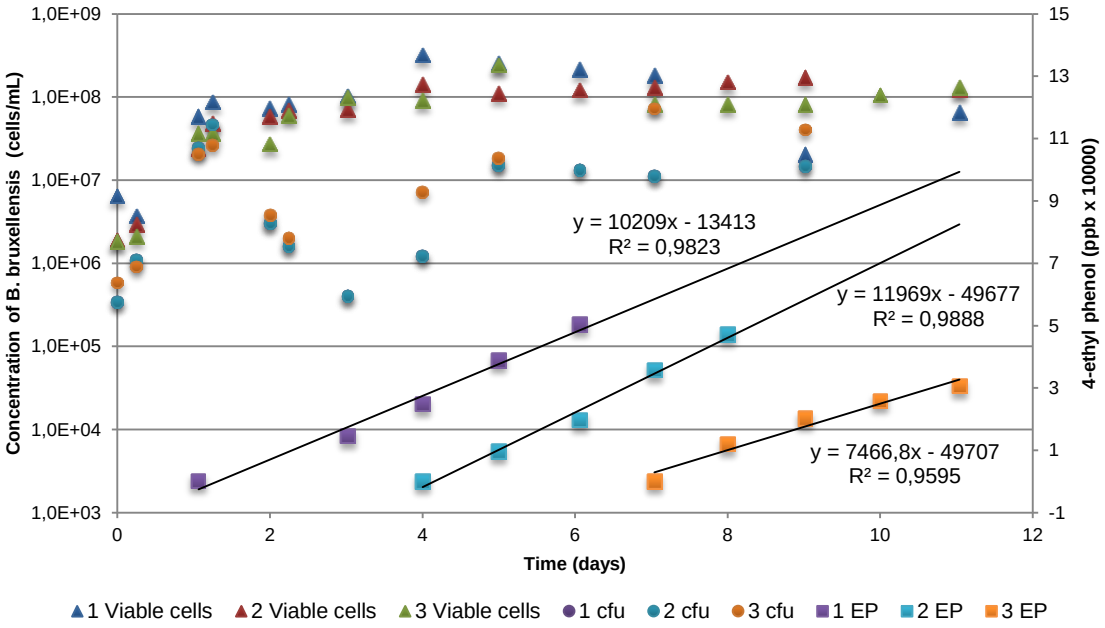

Figure 3.4 - Growth of *B. bruxellensis* ISA 1791 in synthetic culture medium GYP. (1) Addition of p-coumaric acid at day 1, (2) addition of p-coumaric acid at day 4 and (3) addition of p-coumaric acid at day 7.

In this trial, the number of viable cells was again superior to the number of culturable cells by 70.1% average, demonstrating once again the existence of a viable but nonculturable state.

The production rate of 4EP decreased with cultivation time as summarized in Table 3.3. 5 days after p-coumaric addition, concentrations of around 50 mg/L of 4EP were determined in the two cultures where p-coumaric addition was made earlier (1 day after and 4 days after incubation). In the one with the later addition of p-coumaric acid (7 days after incubation) a significant lower value around 30.5 mg/L of 4EP was determined (-39%).

The addition of p-coumaric after 1 day or 4 days of incubation time showed no significant difference. However, when the addition of the precursor was made after 7 days of incubation we conclude the same as before: that the production rate decreases with incubation time. We can again observe that stationary cells can in fact produce 4EP in significant rates since 30.5 mg/L of 4EP was achieved in only 5 days.

Table 3.3 - Production rate determined for each 3 flasks with the addition of p-coumaric at different times. Production rate was determined by the slope of the trend line of 4EP concentration vs. time.

Time until p-coumaric addition	Production rate (mg/L/day)
1 day	10.2
4 days	12
7 days	7.5

Contrarily to Conterno et al.(2013) we found a higher production rate of 4EP at lower incubation times than at stationary phase. Dias et al. (2003) showed however that 4EP occurred between mid-exponential and stationary phase. We found that 4EP production starts just after exposure of *B. bruxellensis* cells to p-coumaric acid. Some discrepancies exist in which phase the production is higher (Kheir et al., 2013) and we demonstrate here that the production starts soon after exposure to the precursor is added and the higher incubation time the less able cells are to produce 4EP.

Given the results demonstrated, it is relevant to say that stationary phase cells still produce 4-ethylphenol at high production rates which is of great interest for understanding wine spoilage under realistic wine conditions.

3.2 Growth features of *Brettanomyces bruxellensis* on existent culture media

To evaluate the efficiency of several culture media to grow *B. bruxellensis*, we plated 13 strains on GYP, modified WL and DBDM. During incubation, colonies were observed and measured during incubation time and the evolution of the maximum diameter of each strain in each medium is illustrated in Figure 3.5.

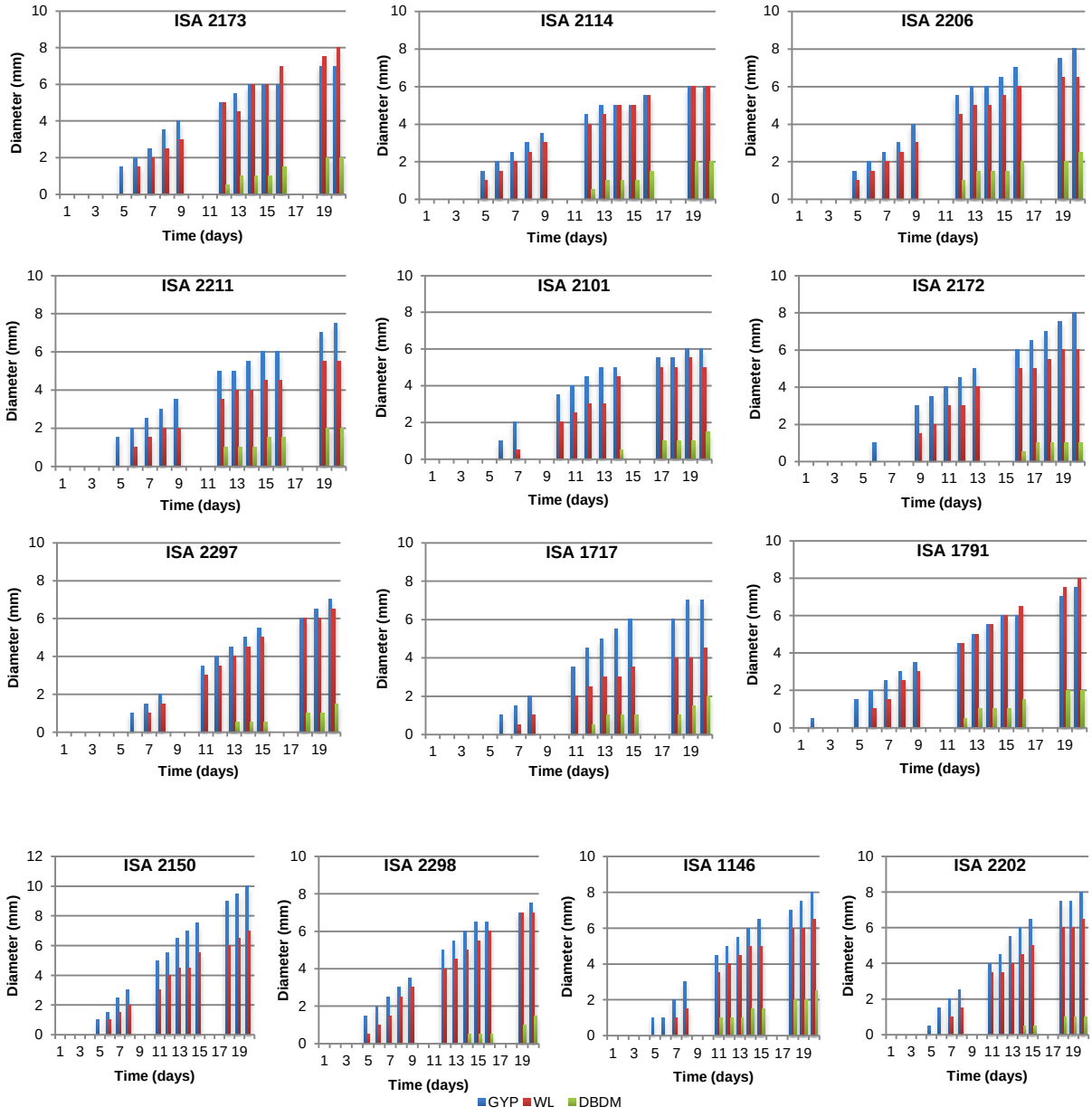

Figure 3.5 - Evolution of the maximum diameter for every 13 strains of *B. bruxellensis* growth in solid GYP, modified WL and DBDM.

In Table 3.4 are summarized the results obtained from all strains and media. GYP colonies appear first as pin points after an average of 3 days. In WL medium, colonies appear normally after 4 days and are normally 1 mm shorter than the ones in GYP. As for colonies in DBDM, it was shown that it is needed 13 days in average to be able to detect them. After 20 days, the strains with larger colonies on DBDM had a maximum diameter of 2.5 mm while in GYP or WL the maximum diameter observed was 10 mm and 8mm respectively.

Table 3.4 - Important data from the incubation of 13 strains of *B. bruxellensis* on solid GYP, WL and DBDM media.

Culture media	Average time to detect (days)	Colony size after 7 days (mm)	Strains detected before 14 days (%)
GYP	3	2.2	100
WL	4	1.2	100
DBDM	12	0	69

From the data recovered from this experiment, we concluded that DBDM medium is in fact an inefficient method for the type of detection required in wineries where a short response time is crucial. Only 69% of the *B. bruxellensis* strains studied were detected before 14 days of incubation and none was observed after 7 days. This is partially in agreement to Morneau et al. (2011) results where they reported that some strains grew poorly on DBDM. It is also stated by several other authors that the majority of *B. bruxellensis* strains cannot use ethanol as sole carbon source (Conterno et al., 2006; Echeverrigaray et al., 2013; Morneau et al., 2011). However, this was not observed in our experiment since only 1 from the 13 strains tested did not grow in DBDM after 20 days.

DBDM is slowly loosing market in the wine industry leaving a large market to explore due to the obtainment of poor results with this medium (Conterno et al., 2006; Fugelsang and Edwards, 2007; Morneau et al., 2011; Zuehlke et al., 2013). The results demonstrated and the reasons previously said enhanced our willingness to create a new culture medium to rapid detect and quantify *Brettanomyces* spp..

3.3 Development of a new selective medium

To evaluate the selectivity of the new growth media, different yeasts were streaked in medium A and Media B. The results were obtained after 3-5 days of incubation and are listed in table 3.5. A positive test result is characterized by positive growth, a modification in color of the growth medium and a phenolic smell. Only when these 3 factors appear together a result can be labeled as positive.

Table 3.5 - Evaluation of medium selectivity by growth, acid production and phenolic smell inoculated with several yeast species.

		Medium							
	ISA	Growth		Acid production		Phenolic smell		Test result	
Species	strain	A	B	A	B	A	B	A	B
Brettanomyces	2211	+	+	+	+	+	+	+	+
bruxellensis	1791	+	+	+	+	+	+	+	+
	2202	+	+	+	+	+	+	+	+
Dekkera anomala	1654	+	+	+	+	+	+	+	+
Candida tropicalis	1359	+	+	+	+	-	-	-	-
C. halophila	1982	+/-	+/-	-	-	-	-	-	-
Kloeckera apiculata	1189	+	+	+	+	-	-	-	-
Pichia anomala	1478	+/-	+/-	-	-	-	-	-	-
P. guilliermondii	2131	+	+/-	+	-	+	-	+	-
	2105	+	-	+	-	+	-	+	-
Saccharomyces bayanus	1730	-	-	-	-	-	-	-	-
S. cerevisiae	1000	-	-	-	-	-	-	-	-
Zygosaccharomyces bailli	1307	-	-	-	-	-	-	-	-
	2295	+	+	-	-	-	-	-	-
Z. hellenicus	2284	+	+/-	+	-	-	-	-	-

Medium A and medium B have only two differences: the pH and the pH indicator. According to the results obtained, besides the expected *Brettanomyces*/Dekkera spp., only *Pichia guilliermondii* had a positive result in medium A while in medium B this species did not grow in 3 to 5 days. Based only in the final test result, this medium showed a selectivity similar to DBDM (Loureiro and Malfeito-Ferreira, 2003; Morneau et al., 2011; Rodrigues et al., 2001; Stender and Kurtzman, 2001) with results in only 3-5 days in contrast to 14 days of DBDM incubation which could lead to wine spoilage before the contamination is known (Fugelsang and Edwards, 2007; Loureiro and Malfeito-Ferreira, 2006; Oelofse

et al., 2008). The limitation of DBDM regarding using ethanol as sole carbon source (Conterno et al., 2006; Echeverrigaray et al., 2013; Morneau et al., 2011) was also surpassed by the use of glucose as carbon source.

To evaluate the growth features on these media, we inoculated 2 *B. bruxellensis* strains (ISA 2211 and ISA 1791) in GYP, WL, medium A and medium B at different growth phases, lag and exponential, with concentrations measured by hemocytometer with vital staining. The evolution of the maximum diameter was measured in all media and results are shown in Figure 3.6 for strain ISA 2211 and Figure 3.7 for strain ISA 1791.

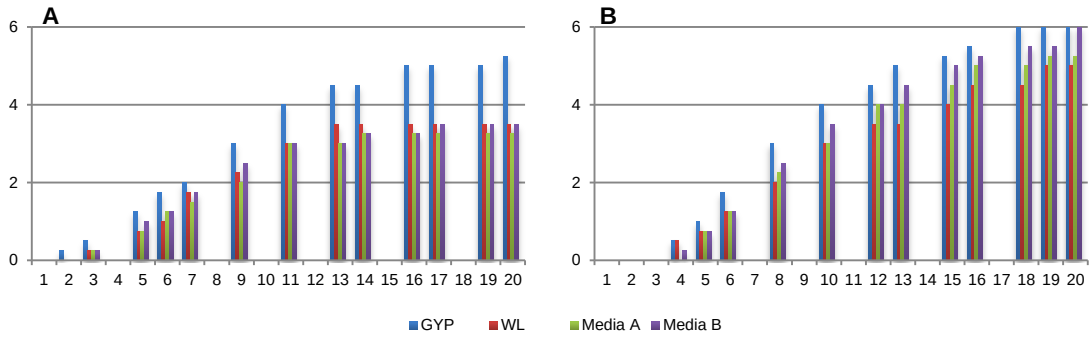

Figure 3.6 - Evolution of the maximum diameter for *B. bruxellensis* strain ISA 2211 in lag phase (A) and exponential phase (B) growth in solid GYP, WL, medium A and medium B.

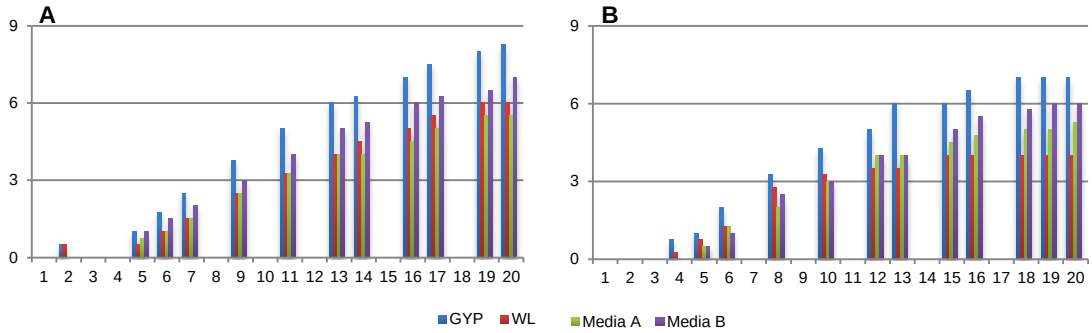

Figure 3.7 - Evolution of the maximum diameter for *B. bruxellensis* strain ISA 1791 in lag phase (A) and exponential phase (B) growth in solid GYP, WL, medium A and medium B.

Analyzing the results from figure 3.6 and 3.7, we concluded that there are no significant differences if cells are inoculated in lag phase or in exponential phase. The reason why strain 2211 in lag phase (Figure 3.6A) and strain 1791 in exponential phase (Figure 3.7B) stopped increasing the diameter at the same rate after day 13 was because of a higher colony concentration on those plates, which didn't let colonies get larger due to both lack of space and lack of nutrients.

Colonies inoculated on GYP medium were around 1 mm bigger than the others media over the 20 days. Colonies in WL, medium A and medium B had roughly the same diameter during incubation time. Comparing our previous trial using DBDM, we concluded that medium A and medium B are more effective for *B. bruxellensis* growth since we observed that colonies appeared in plates between 3 and 5 days on medium A and medium B which is of great advantage for wineries that need a faster result.

The evaluation of cell recovery on culture media is presented in Table 3.6 using viable determination by hemocytometer with vital staining and plate counting.

Table 3.6 – Results regarding two strain in different growth phases. Viable cell concentration was determined by hemocytometer count with vital staining. Culturable cells were determined by plate counting in different media. Percentage of recovery from viable cells on each media is labeled as %.

Strain phase	Viable	GYP	%	WL	%	Medium A	%	Medium B	%
2211 Lag	$1{,}50x10^6$	$5{,}80x10^5$	38.7	$4{,}65x10^5$	31.0	$7{,}45x10^5$	49.7	$6{,}00x10^5$	40.0
2211 Exp.	$1{,}90x10^7$	$2{,}30x10^6$	12.1	$1{,}90x10^6$	10.0	$1{,}85x10^6$	9.7	$2{,}15x10^6$	11.3
1791 Lag	$1{,}70x10^6$	$1{,}60x10^4$	0.9	$1{,}80x10^4$	1.1	$1{,}75x10^4$	1.0	$1{,}30x10^4$	0.8
1791 Exp.	$8{,}00x10^6$	$4{,}40x10^5$	5.5	$7{,}80x10^5$	9.8	$4{,}55x10^5$	5.7	$2{,}90x10^5$	3.6

The results showed that the number of culturable cells (determined by plate counting) were always inferior to the concentration measured using hemocytometer since the recovery rates from viable cells to plate counting were always inferior to 50%. This is mainly due to the presence of a viable but nonculturable state in *B. bruxellensis* cells of this trial. The recovery rate seemed to be dependent on strain. Strain 2211 at lag phase showed higher recovery rates in every media when compared to strain 1791 at the same growth phase. These results are in agreement to our previous results regarding these two strains. Also, the recovery rate seemed to be dependent on growth phase. In strain 2211, lag phase showed to have higher recovery rates than exponential phase, however, in strain 1791 it was observed the opposite.

Medium A and medium B had similar or higher recovery rates than the common media GYP and WL. This result is of great interest since the general purpose media GYP is normally the one used for total quantification of yeasts.

3.4 Real-Time PCR implementation

To study the efficiency of a Real-Time PCR reaction for detection of *B. bruxellensis*, serial dilutions of the DNA extracted from ISA 2211 strain were submitted to a RT PCR. Some reaction mixtures were tested and the one with better efficiency values with lower costs was selected for posterior analysis. Figure 3.8A illustrates the amplification curves obtained by RT PCR analysis of the DNA extracted using the selected reaction mixture.

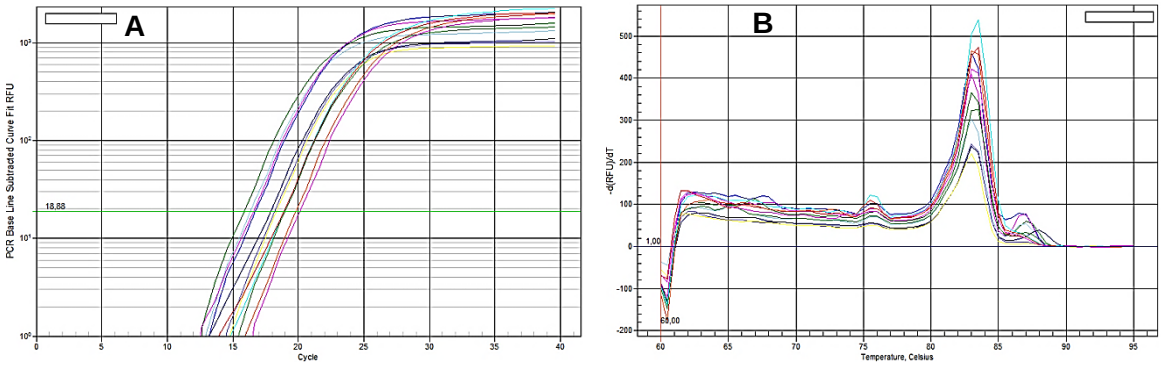

Figure 3.8 - Amplification curves (A) of RT PCR analysis of serial dilution of DNA extracted from cells of *B. bruxellensis* ISA 2211 and melting temperature curves (B) of the generated amplicons.

The specificity of the primers used in this study was previously tested in Phister and Mills (2003) work. In their work, database searches revealed no significant homology to any other microorganism then *B. bruxellensis* except one *D. anomala* entry. The authors also tested the specificity of the primers in 36 other wine-related yeasts and bacteria with no amplification result.

The melting curve, illustrated on Figure 3.8B, was analyzed in order to evaluate the specificity of the RT PCR reaction, meaning the formation of primer-dimer artifacts or amplification of a nonspecific region of the DNA. As observed, no primer-dimer artifacts were shown and neither nonspecific amplifications. The specific melting temperature for this reaction was near 83°C which is similar to the Portugal and Ruiz-Larrea (2013) result of 85°C using the same primers but different reaction.

The threshold cycle (C_t) obtained from the amplification curves allowed the construction of a standard curve (Figure 3.9). The standard curve obtained showed a correlation coefficient of 0.976 and an efficiency of 100.5% indicating a good linearity of the standard curve and that the amount of product doubles perfectly during each cycle respectively. Similar results were obtained for *B. bruxellensis* ISA 1791 with a correlation coefficient of 0.993 and an efficiency of 95.1%. Based on these results we assumed that the extraction method, reaction mixture and RT PCR amplification program were optimized for the detection of *B. bruxellensis*.

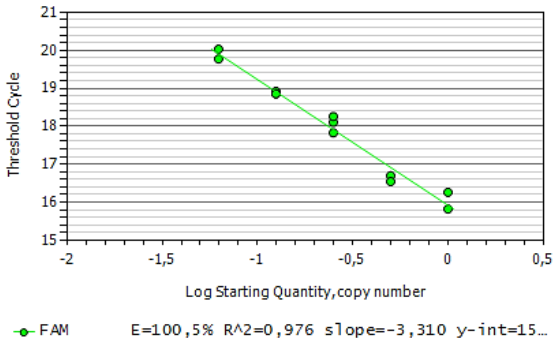

Figure 3.9 - Standard curve obtained from the DNA extracted of *B. bruxellensis* ISA 2211 serially diluted.

The next step was to optimize and compare with classic methods, the quantification of *B. bruxellensis* using RT PCR as a quantification method. For these purposes, DNA was extracted from 5 different cell concentrations, of *B. bruxellensis* ISA 1791 strain inoculated in GYP being the concentrations determined by hemocytometer with vital staining.

No primer-dimer artifacts or amplification of a nonspecific region of the DNA were observed. DNA extracted was used for RT PCR reaction and a standard curve of viable cell concentrations against the threshold cycle was established (Figure 3.10).

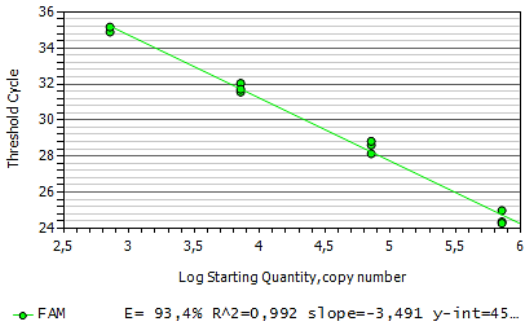

Figure 3.10 - Standard curve obtained from DNA extracted from serially diluted concentrations of *B. bruxellensis* ISA 1791.

The standard curve obtained from RT PCR analysis showed a correlation coefficient of 0.992 and an efficiency of 93.4% indicating similar results from previous analysis of serially DNA dilutions. This standard curve has in consideration the different yield of DNA extraction from a more or less concentrated suspension. Amplification of the DNA extracted from the less concentrated sample was not achieved. Since the less concentrated sample had a concentration of near 70 cells/mL, we concluded that 700 cells/mL should be considered the detection limit with the current method. However recent results regarding RT PCR technique for quantification of *B. bruxellensis* report detection limits of 10 cells/mL, obtained after a previous centrifugation of 10 mL of the sample (Portugal and Ruiz-Larrea, 2013; Tofalo et al., 2012). As so, in future analysis from our laboratory, 10 mL of the samples will be centrifuged to render a lower detection limit.

To simulate unknown samples, 2 samples of GYP broth with serial diluted concentrations of *B. bruxellensis* ISA 2211 were submitted to extraction. The DNA extracted from the 2 samples was submitted to three independent RT PCR reaction and the results were plotted against the standard curve established in the same reaction (Figure 3.11). Colony numbers were quantified by cultivation on GYP and medium B and viable cells were determined by vital staining determination.

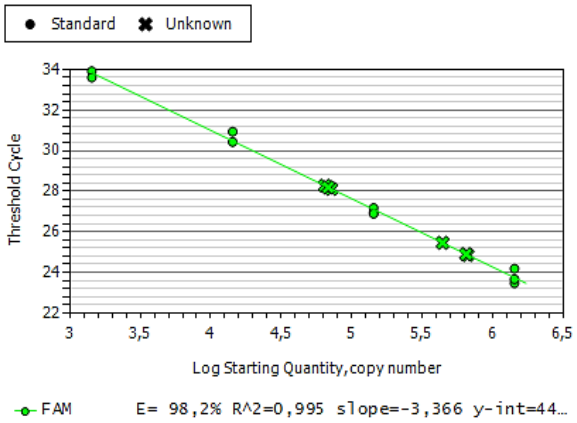

Figure 3.11 - One of the three quantifications tests done using a standard curve obtained from DNA extracted from serially diluted concentrations of *B. bruxellensis* ISA 1791 (●) and DNA extracted from 2 different concentrations of *B. bruxellensis* ISA 2211 (✖).

The standard curves obtained showed similar correlation coefficients and efficiency values, close to the previous ones as expected. Determined concentrations, correlation coefficients and efficiencies from all three reactions are summarized in Table 3.7. Real-Rime PCR quantification results showed to be very similar between independent analysis demonstrating that the assay is reproducible and robust. Correlation coefficients are all at expected values.

Table 3.7 - *B. bruxellensis* ISA 2211 quantification by three Real-Time PCR analysis and their respective correlation coefficients and efficiencies.

	Real-Time PCR quantification		
Viable (cells/mL)	Average 1	Average 2	Average 3
$1.70x10^5$	$5.85x10^5$	$6.04x10^5$	$5.28x10^5$
$1.70x10^4$	$6.81x10^4$	$7.68x10^4$	$6.30x10^4$
Efficiency	98.2 %	87.6 %	98.3 %
R^2	0.995	0.985	0.989

The population quantified by RT PCR method, although within the same order of magnitude as the quantification by hemocytometer, an over estimation was seen for the two concentrations in every analysis. This over estimation was expected since, like Willenburg et al. (2012) concluded, the use of DNA for RT PCR quantification assay amplifies bot viable and DNA from dead or lysed cells that can still be amplified 72 hours after death or cell lyse. Quantification results using RT-PCR, hemocytometer count with vital staining and plate count using GYP medium and medium B are summarized in Table 3.8 and illustrated in Figure 3.12.

Table 3.8 - *B. bruxellensis* ISA 2211 quantification by plate counting on GYP and medium B, by hemocytometer count with vital staining and by RT PCR.

Detection Method	Detected concentration (cells/mL)	Recovery (%)
GYP	5,45E+04	32,06%
Medium B	8,85E+04	52,06%
Hemocytometer	1,70E+05	100,00%
RT PCR	6,33E+05	372,16%

RT-PCR quantification yielded higher counts than vital staining while plate count using either media showed the lowest cell numbers. We believe that the reason why this under estimation exists using culture media is the ability to enter in the VBNC state by *B. bruxellensis*. RT PCR showed an over estimation of 372% compared to hemocytometer quantification. The over estimation of the RT PCR as a quantification method has been reported in several research articles with the reason above mentioned (Portugal and Ruiz-Larrea, 2013; Tofalo et al., 2012; Willenburg and Divol, 2012).

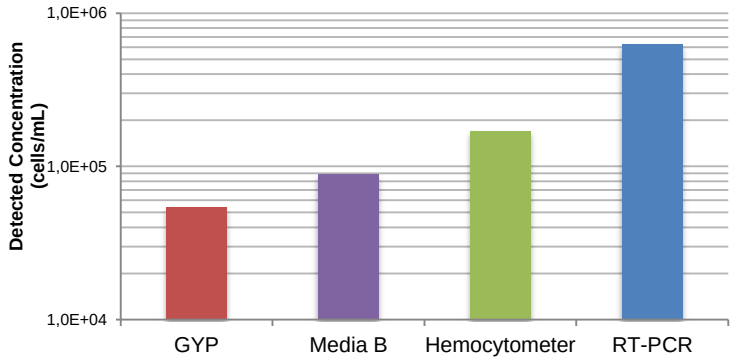

Figure 3.12 - Graphical representation of the quantification difference of *B. bruxellensis* ISA 2211 by plate counting on GYP and Medium B, by hemocytometer count with vital staining and by RT PCR.

4 Conclusions

One of the most serious microbial problems of modern enology worldwide is the volatile phenols produced by the spoilage yeast *Brettanomyces bruxellensis.* The “horse sweat” off-flavor and off-taste that characterize the spoilage effect induces big economic losses to the wine industry. The results presented in this thesis increased our knowledge of the spoilage yeast *B. bruxellensis* and produced a new selective media and a Real-Time PCR protocol for the detection and quantification of this threat to wine quality.

A viable but nonculturable state was observed to be in higher concentration at lag and stationary phases and was easier to induce in some strains than in others. This induction was not made by addition of any chemical compound, we hypothesized that it was induced due to the acetic acid produced and that one strain had higher resistance than the other to this secondary metabolite.

The production of 4-ethylphenol still rises disagreements in scientific community about if cells without active growth can, or cannot, produce this spoilage compound. We showed that this compound is produced during stationary growth phase although with lower rates than in active growing populations. We also demonstrated that the production of 4-ethylphenol starts just after the precursor addition and not in a specific phase of growth. It remains to be determined if this production is done by culturable, viable or both type of cells.

Current culture media were tested and we concluded that the selective media DBDM is inefficient for the rapid detection required by wineries since only after 12 days average colonies were observed. Even after 14 days of incubation, only 69% of the 13 strains tested were detected. As so, the new medium developed in this work showed to be a better alternative for the detection of *B. bruxellensis* since a high selectivity was observed and an incubation time of only 3 to 5 days was needed. *Pichia guilliermondii* was the only positive result from the several yeasts tested, however, this yeast can survive but does not grow under wine’s harsh conditions. When compared to general purpose media like GYP and WL, the new medium showed higher or equal recovery rates from viable population. The recovery rates seemed to be strain dependent and growth phase dependent. The validation of this medium with real wine samples should be done in the future.

The developed Real-Time PCR protocol showed similar efficiency and detection limit and we managed to reduce analysis costs in reaction mixture. The quantification by this method showed results with a detection limit of about 700 cells/mL. However, this detection limit can be reduced by a 10 mL centrifugation of the sample. When this molecular method was compared to classical microbiological methods including the new selective medium, we concluded that an over estimation of the population was observed using RT PCR probably due to dead and lysed cell’s DNA amplification. RT PCR quantification was 3.72 times higher than viable cell population. Further research should be carried out to elucidate this discrepancy.

The better knowledge and detection methods produced by this work will hopefully contribute to a decreased occurrence of wine spoiled by *B. bruxellensis* worldwide.

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