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CARATTERIZZAZIONE MOLECOLARE, RISPOSTE A STRESS E ATTIVITÀ ENZIMATICHE SPECIFICHE DI CEPPI VINARI DI *DEKKERA/BRETTANOMYCES BRUXELLENSIS*

(AGR16)

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XIV CYCLE

MOLECULAR CHARACTERIZATION, STRESS RESPONSES AND SPECIFIC ENZYMATIC ACTIVITIES OF *DEKKERA*/*BRETTANOMYCES BRUXELLENSIS* WINE STRAINS: STRATEGIES OF ANALYSIS AND CONTROL IN THE OENOLOGICAL FIELD

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0. PREFACE

The yeast species *Brettanomyces bruxellensis* (*Dekkera bruxellensis*, perfect form) was isolated in spontaneous fermented beer at the Carlsberg brewery in 1904. Nowadays, *Dekkera*/*Brettanomyces* spp. have been the subjects of numerous studies focused on enhancing the knowledge of the wine industry since this yeasts is the primary yeast species involved in spoilage of finished wine. In fact, although advances in wine technology have led to effective disappearance of defects due to the action of bacteria, yeasts are now the most feared contaminants leading common effects such as films or sediment formations, cloudiness or haziness, gas production and off-flavours at all stages of winemaking.
Moreover, it is known that *D./B. bruxellensis* is able to grow under a high ethanol concentration as well as at low pH, the typical condition of wine. In a work carried out by Conterno et al. (2006) all the isolates grew at pH 2.5 and 94% of them grew at pH 2.0. A study carried out by Brandam et al (2008) shows the effect of the temperature on *D./B. bruxellensis* growth. Temperature between 15 and 32°C did not affect the final biomass concentration, but the exposure at 35°C caused viable biomass to be reduced up to 75%.
Spoilage caused by *B./D. bruxellensis* was first described by Chatonnet et al. (1995). It is mainly due to the fact that *D. bruxellensis* has several adaptations that let it survive under stressing environments such as fermenting wines:

- ✓ this species remains viable and active in beverages preserved by extreme abiotic stress (anaerobic condition, up to 12-13% ethanol (v/v), minimal amounts of fermentable sugars);
- ✓ the adopted treatments (sulphiting, membrane filtration, transfer of wine to sanitized barrels) are not always effective;
- ✓ the off-flavours produced by *Brettanomyces* include volatile phenols characterised by disagreeable odours;

Despite this species causes large economical loses within the wineries, molecular, genetics, population and evolutionary studies of this yeast are still scarce. Moreover, the genomic structure of *D./B. bruxellensis* is poorly understood. The ploidy status is unclear and also the existence of a sexual life style.

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Conterno L, Joseph LCM, Arvik TJ, Henick-Kling T, Bisson LF, 2006, Genetic and physiological characterization of *Brettanomyces bruxellensis* strains isolated from wines. Am J Enol Vitic 57; 2 139-147.

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1. STATE OF THE ART

The genus *Brettanomyces* was first classified as a species missing of the sexual cycle (anamorph or imperfect form) that reproduce itself throughout multipolar budding (Custers, 1940). The introduction in the modern taxonomy of the genus *Dekkera* was due to the detection of cells able to produce ascospores which reproduces sexually (teleomorph or perfect form) (Van der Walt, 1984). Five species within the genera of *Dekkera*/*Brettanomyces* are currently enclosed to taxonomy: the anamorphs *B. bruxellensis*, *B. anomalus*, *B. custersianus*, *B. naardenensis*, and *B. nanus*, and the teleomorphs *D. bruxellensis* and *D. anomala* (Kurtzman & Fell, 2000; Cocolin et al., 2004; Oelofse et al., 2008). The species mainly associated to the wine environment are considered to be *D./B. bruxellensis* and *B. anomalus*, with the majority of the strains being *D./B. bruxellensis*. The distinction between *Dekkera* and *Brettanomyces* is uncertain with Loureiro &Malfeito-Ferreira (2006) and Oelofse *et al*. (2008) which affirmed that no variation between the anamorph and teleomorph states are detectable by the use of the current molecular DNA techniques. However, a description of the life style of *D. bruxellensis* is still lacking.
The first infections of *Brettanomyces* was found in 1950 by van der Walt & van Kerken (1958), and it has since been reported in wine in wine-producing countries including, but not limited to, France, Germany, Russia, Italy, Spain, Australia, New Zealand, South Africa and the USA. How the yeast spreads is not known in detail, but it has been found on fruit flies of the *Drosophila* species, and one theory that these insects carry it (van der Walt & van Kerken, 1961). Due to their slow growth rate and low final biomass, these yeasts are difficult to be detected and identified throughout the winemaking and ageing process. The presence of this yeast on the surface of the berries of four different grape varieties, i.e. Merlot, Cabernet–Franc, Cabernet–Sauvignon, Petit–Verdot, and Sangiovese, support the hypothesis that *D./B. bruxellensis* belongs to the native grape microflora besides being a possible winery spoilage yeast (Agnolucci et al., 2007; Renouf & Lonvaud-Funel, 2007).

1.1 Genetic features of *Dekkera/Brettanomyces bruxellensis* species

Among yeasts responsible for wine spoilage, *B./D. bruxellensis* is the species on which the scientific community has the highest interest. This fact is documented by an increasing in the international publications in the last ten years (www.isiknowledge.com), and by the beginning of the genome sequencing (Woolfit et al., 2007). The estimated genome size is 19.4 Mb and, throughout the genome survey sequencing, 3,000 genes were identified approximately; 2,606 are protein-coding genes with orthologs in *Saccharomyces cerevisiae,* while 277 genes are orthologs from other *Saccharomycetales* species.
Maximum likelihood phylogenetic analyses suggest that the relationship between *D. bruxellensis*, *S. cerevisiae*, and *Candida albicans* is close to a trichotomy. This result was obtained studying the the conservation of gene order in *D. bruxellensis;* this yeast has a somewhat lower rate of small-scale rearrangements than *C. albicans*.
Approximately 2% of the *D. bruxellensis* genes identified contain introns, and the mean length of the introns is 195 nucleotides. These values are consistent with those found in other hemiascomycetes: the proportion of genes that contain introns varies from 1% in *D. hansenii* to 4% in *S. cerevisiae*, with a concomitant increase in average intron length from around 100 to over 250 nucleotides (Bon et al., 2003).
The genome contains two types of repeated: microsatellites and transposable elements. On the basis of results from other hemiascomycete species (Lasker et al., 2006), it is likely that at least some of these are polymorphic and may thus be of use in future analyses of population diversity. The great majority of transposable elements so far described in hemiascomycete yeasts are LTR retrotransposons, although non-LTR retrotransposons and DNA transposons have been found in *C.*

albicans and *Y. lipolytica* (Goodwin et al., 2001; Casaregola et al., 2002; Neuveglise et al., 2005). The *D. bruxellensis* data contain at least 42 transposable elements, all of which are LTR retrotransposons. In particular, the presence of three putative Ty1 elements may indicate that elements of this class arose at an earlier stage in hemiascomycete evolution and have subsequently been lost from other species in the *C. albicans*/*D. hansenii* clade (as they have been from *Saccharomyces bayanus*, for example). The Ty1-like elements present in *D. bruxellensis* are substantially diverged from one another and from their closest homologs in other species (the most similar has 26% amino acid identity to a Tkm1 element from *Kluyveromyces marxianus*), indicating that they are unlikely to be the result of a recent horizontal gene transfer event.
The proteome of *D. bruxellensis* is enriched for transporters and genes involved in nitrogen and lipid metabolism, among other functions, which may reflect adaptations to its low-nutrient, high-ethanol niche. An adenyl deaminase gene was also identified; it has high similarity to a gene in bacteria of the *Burkholderia cepacia* species complex and appears to be the result of horizontal gene transfer. These data provide a resource for further analyses of the population genetics and evolution of *D. bruxellensis* and of the genetic bases of its physiological capabilities (Woolfit et al. 2007).
In *D./B. bruxellensis* the numbers of chromosomes varied drastically, from 4 to at least 9. The chromosomal patterns show an elevated variation in the number and sizes of their chromosomes and genomes among the different isolates. The sizes of chromosomes differ in the range from approximately 0.75 to 6.5 Mb, and nuclear genomes approximately from 15.5 to 39.05 Mb. When single gene probes were used in Southern analysis, the corresponding genes usually mapped to at least two chromosomal bands, excluding a simple haploid organization of the genome. When different loci were sequenced, in most cases, several different haplotypes were obtained for each single isolate, and they belonged to two subtypes. Phylogenetic reconstruction using haplotypes revealed that the sequences from different isolates belonging to one subtype were more similar to each other than to the sequences belonging to the other subtype within the isolate. Reanalysis of the genome sequence also confirmed that partially sequenced strain (Y879) is not a simple haploid and that its genome contains approximately 1% polymorphic sites (Hellborg & Piškur, 2009).
D. bruxellensis mitochondrial DNA (mtDNA) shares several properties with *S. cerevisiae*, such as the large genome size (76,453 bp), and the organization of the intergenic sequences consisting of spacious AT-rich regions containing a number of hairpin GC-rich cluster-like elements. In addition to a basic set of the mitochondrial genes coding for the components of cytochrome oxidase, cytochrome b, subunits of ATPase, two rRNA subunits and 25 tRNAs, *D. bruxellensis* also carries genes for the NADH dehydrogenase complex (Procházka et al, 2010).
Saccharomyces yeasts degrade sugars to two-carbon components, in particular ethanol, even in the presence of excess oxygen. This characteristic is called the Crabtree effect and is the background for the 'make-accumulate-consume' life strategy, which in natural habitats helps Saccharomyces yeasts to out-compete other microorganisms. A global promoter rewiring in the Saccharomyces cerevisiae lineage, which occurred around 100 mya, was one of the main molecular events providing the background for evolution of this strategy. Many genetic and physiological traits that are similar between *Saccharomyces* and *Brettanomyces/Dekkera* are likely to be a product of parallel evolution. A recent work (Rozpędowska et al., 2011) shows that by the analysis of this promoter, the *D. bruxellensis* lineage separated from the *Saccharomyces* yeasts more than 200 mya. They demonstrate that this yeast uses the same “make–accumulate–consume” life strategy of *S. cerevisiae* to out-compete other microorganisms in natural environments.

1.2 *Dekkera/Brettanomyces bruxellensis* metabolism: Custers effect

D. bruxellensis produced substantial amounts of ethanol under aerobic conditions and could also grow anaerobically like *S. cerevisiae* and its close relatives. In other words, *D. bruxellensis* can be classified as a Crabtree-positive and facultative anaerobic yeast. The main metabolic trait that characterized this species is the "Custrers effect" which is defined as the inhibition of the glucose fermentation to ethanol under strictly anaerobic conditions and its stimulation in the presence of oxygen (Custers, 1940; Henrici, 1941; Skinner, 1947; Barnet et al., 2005). Acetic acid is produced in a high amount during aerobic growth, while no appreciable amounts are formed under anaerobic fermentation. Normally, in yeasts the acetaldehyde oxidation leads to acetate formation troughout acetaldehyde dehydrogenase activity. This enzyme is NAD^+ or $NADP^+$ dependent and its gene is constitutively expressed. This last characteristic a temporary lag phase and subsequent Custers effect is created through the continued drainage of NAD^+ by way of the irreversible conversion of acetaldehyde to acetic acid bringing glycolysis to a stop due to the lack of NAD^+ (Wijsman et al., 1984). Wijsman (1984) further concluded that Custers effect observed in *Brettanomyces* spp. was caused by the inability of cells to restore the redox balance via production of reduced metabolites, specifically glycerol, possibly attributed to the absence of glycerol 3-phosphate phosphatase activity. The production of glycerol has been shown to be important in metabolizing NADH and restoring the redox balance during anaerobic fermentation in other yeasts (Oura, 1977). A study by Aguilar-Uscanga et al. (2003) observed slight glycerol production occurring under anaerobic conditions within a single strain of *Brettanomyces bruxellensis*. Glycerol production might then be variable between strains indicating the ability of the redox to be restored more rapidly, in which case strains would likely be capable of full fermentation with a decrease in the time required. Moreover, Sanfaçon et al. (1976) showed that part of the reason for these metabolic characteristics was a low level of succinic dehydrogenase, which gives high intracellular levels of succinate and an effective block in the tricarboxylic acid cycle, which stops the oxidation of ethanol by this metabolic pathway. However, Scheffers (1961) found anaerobic fermentation could be stimulated by minute amounts of O_2 or the addition of H^+-acceptors such as acetaldehyde, acetone, pyruvic acid and other carbonyl compounds. In particular, the role of oxygen on growth and acetic acid production by *Dekkera*/*Brettanomyces* spp. has been carefully studied in wine and industrial alcoholic fermentations (Ciani & Ferraro, 1997; Abbott et al., 2005a,b). Moderate to low amounts of oxygen has been shown to encourage cell biomass production, with semi-aerobic conditions yielding the greatest cell growth (Aguilar-Uscanga et al., 2003). The levels of ethanol and acetic acid produced during aerobic batch culture are dependent on the levels of aeration with increased concentrations of oxygen lowering growth rates (Aguilar-Uscanga et al., 2003). *Brettanomyces* spp. have been observed to utilize both glucose and ethanol in producing acetic acid under increased levels of oxygen, although Freer (2002) showed not all strains could use both as carbon sources, with high variability seen in the levels of acetic acid produced by different strains.

1.3 "Brett" character in oenology

In oenology, "Brett" character refers to a sensory defect linked to the release of unpleasant aromas by *B. bruxellensis* as it grows in the wine. *B. bruxellensis* has been isolated from several aged wines worldwide in spite of the antimicrobial conditions occurring in the media. Vineyards and grapes may be accounted as important source of such yeast for winery contamination (Agnolucci et al, 2007; Renouf & Lonvaud-Funel, 2007). The sensory effect has been described with various attributes such as "leathery", "pharmaceutical", "burned plastic", "Band Aid®", "smoky", "barnyard" (Licker et al., 1999). The firsts and mostly identified chemicals with "Brett" off-odours are the 4-ethylguaiacol (4EG) and the 4-ethylphenol (4EF) (Chatonnet et al., 1992), but its capability to produce detrimental amounts of acetic acid In certain styles of wine a slight "Brett"

character may be considered to improve complexity (Fugelsang, 1997). Red wines were found to contain up to 6047 µg/l (perception threshold: 440 µg/l) and 1561 µg/l (perception threshold: 40 µg/L) of 4EP and 4EG, respectively. Ethyl phenols production is a two-step metabolic pathway whereby ferulic acid or p-coumaric acid are first decarboxylated to vinylphenols by hydroxycinamate decarboxylases, (HCD), and then further reduced by a vinylphenol reductase (VPR). VPR is the crucial enzyme involved in the production of ethylphenols in wines due to *B. bruxellensis*. Godoy et al. (2008) purified a p-coumarate decarboxylase from *B. bruxellensis* and identified a "putative gene" of a VPR from the same microorganism, although no conclusive evidence of the correlation between the putative gene and the expression of a functional VPR was alleged. A complete characterization of VPR has been hindered till now because of the fast enzyme inactivation following the extraction from the cytoplasm (Harris et al., 2009). However, information either on the biological basis regulating the phenolic metabolism or on the metabolic reason leading the yeast to produce 4EP and 4EG are not available. The possible relation with a detoxification process was evaluated but data at this regard showed to be controversial (Brandam et al., 2008).

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2. AIMS OF THE STUDY

A multilateral approach is required to solve the potential spoilage by *Dekkera/Brettanomyces*: the hygienic level of the environment should be necessarily maintained high by means of adequate procedures and the monitoring of the contamination along the phases of wine production (end of alcoholic fermentation, malolactic transformation, wine aging) is fundamental for an early diagnosis.

New and reliable analytical protocols are needed to a careful detection of *Dekkera/Brettanomyces* in wine. At the moment, cultural methods on selected media and microscopic observation are employed but they are inaccurate and time consuming also because the problem to recover viable cells.

The aim of this thesis was to develop strategies in order to analyse and control the wine spoilage linked to *Dekkera/Brettanomyces bruxellensis* species.

As concern the first issue, spoilage microbial analysis, the present PhD thesis describes:

- ✓ the development of new methods for *Dekkera/Brettanomyces bruxellensis* molecular typing;
- ✓ the phenotypic biodiversity of *Dekkera/Brettanomyces bruxellensis* species.

The topics on the microbial spoilage control were:

- ✓ *Dekkera/Brettanomyces bruxellensis* response to stress conditions.

3. RESULTS AND DISCUSSION

3.1 Development of new methods for *Dekkera/Brettanomyces bruxellensis* molecular typing (1)

Investigation on genome variability among individual represents an important tool to assess their biodiversity and to study their environmental diffusion. The safeguard of microbial strains with technological interest is becoming a strategic activity in food and wine industries. Nevertheless, many species marginally implicated in productions are still poorly studied, and rapid and reliable protocols for their recognition and typing are not well described. This is the case of the yeast *D./B. bruxellensis* that can cause spoilage and increasing economic losses in wine production.

3.1.1 Discrimination of *Dekkera bruxellensis* strains troughout Intron Splice Site PCR analysis (1a)

In oenology, “Brett” character refers to the wine spoilage caused by the yeast *D./B. bruxellensis* and its production of volatile phenolic off-flavours. Although the spoilage potential of this yeast is strain-dependent (Vigentini et al., 2008) only a few works have reported methods to distinguish *D./B. bruxellensis* at strain level (Martorell et al., 2006; Agnolucci et al., 2009; Oelofse et al., 2009); typing protocols appear useful but are time consuming or require sophisticated instrumentations. The setting up of molecular probes designed on genes that are responsible of the off-flavour production could be a partial solution for a rapid detection of dangerous strains since an ethylphenol production around 2 mg/l is considered a benefit for wine (Etiévant et al., 1989). About 40% of the *D. bruxellensis* CBS2499 genome has been sequenced (Woolfit et al., 2007). When the entire genome becomes available, more consistent possible correlations of genetic and physiological aspects to single strain protocols will be arranged. Thus, the availability of comparable results among laboratories throughout databases could represent an important goal to face the increasing problem represented by *D./B. bruxellensis* in the oenological field.
Introns are DNA regions that are not linked to any genetic function, and can vary with low control; changes such as nucleotide substitutions, deletions, or insertions can occur in an intron structure. However, short conserved sequences for the spliceosome assembling are required during the synthesis of mRNA: in *Saccharomyces cerevisiae*, the lariat branch point TACTAAC and the 5′ motif GTATGT consensus sequences are strictly conserved (de Barros Lopes et al., 1996). A recent work identified introns in 40 *D. bruxellensis* genes that are *S. cerevisiae* orthologs; moreover, eight introns were specific to *D. bruxellensis* (Woolfit et al., 2007). In this work, intron splice site PCR amplification (ISS-PCR) was applied to a collection of 17 *D. bruxellensis* strains using primers similar to those employed for *S. cerevisiae*. The main goal was to test and improve the method on *D. bruxellensis* species, since it is a technique that is simple, rapid, and potentially accessible to industrial laboratories with limited molecular expertise and resources.

3.1.1.1 Materials and Methods (1a)

Yeast strains and cultural media

Seventeen *D. bruxellensis* strains from the Centraalbureau voor Schimmel Culture Collection (CBS) were analysed in this study: CBS73, CBS1940, CBS1941, CBS1942, CBS1943, CBS2336, CBS2499, CBS2547, CBS2796, CBS2797, CBS4459, CBS4480, CBS4481, CBS4482, CBS4601, CBS4602 and CBS5006. All strains were cultivated on YPD medium [1% (w/v) Yeast Extract, 2% (w/v) Peptone and 2% (w/v) Glucose] at 25°C for 3-5 days. Cells were maintained at −80°C in YPD medium added with 20% (v/v) glycerol.

DNA amplification

Yeasts were grown in liquid YPD medium with shaking overnight. DNA was extracted from a 5-ml culture as described by Querol et al. (1992) using 500 µg/ml of Zymolyase 100T (USBiological, Massachusetts, USA) as lytic enzyme. The primers employed in the amplification reactions were EI1, EI2, LA1, LA2 (de Barros Lopes et al., 1996) and DbEI1 (5'-CTGGCTTGGTGTAAGT-3′); primer DbEI1 derives from EI1, where in position 14, the T was substituted with an A. One microliter (80-100 ng) of genomic DNA was added to a 24 µl reaction mix consisting of 0.75 µM each primers, 200 µM dNTPs, 1x reaction buffer $MgCl_2$ free, 2.5 mM $MgCl_2$, 5% (v/v) dimethyl sulfoxide (DMSO), 1U *Taq* polymerase (Fermentas, Vilnius, Lithuania). Amplifications were carried on in a Mastercycler ep gradient S (Eppendorf, Hamburg, Germany) following the protocol described for *S. cerevisiae*, but modified by increasing the number of cycles to 40 (de Barros Lopes et al., 1996) and setting the annealing temperatures at 46 and 47°C when EI1/LA2 or DbEI1/LA2 and EI2/LA1 pairs of primers were used, respectively. Multiplex amplifications were carried out as described above using EI1, DbEI1 and LA2 oligonucleotides. Electrophoresis was run in 1.5% (w/v) plus 0.4 µg/ml ethidium bromide agarose gels. PCR products were photographed under GelDoc UV transilluminator (Bio-rad, Hercules, CA, USA).

ISS profiles elaboration

The digitalized gel images were analysed using Quantity One version 4.6.2 (Bio-Rad). The software was used to detect bands, using a match tolerance of 2% (Thompson et al., 2008). A similarity matrix was constructed using Dice's similarity coefficient. Dendrograms were constructed by the unweighted pair group method using arithmetic averages (UPGMA) and the horizontal axis indicated the coefficient of genetic similarity. The repeatability was carried out using four *D. bruxellensis* strains: CBS1940, CBS1941, CBS1942 and CBS1943. PCR reactions were prepared in triplicate for each one of the four strains. Indexes of discrimination among ISS genetic profiles were calculated as described by Hunter and Gaston (1988) according to the repeatability percentage.

3.1.1.2 Results and discussion (1a)

The use of primers that can anneal to specific conserved motifs, such as those designed for ISS-PCR, has been already well described for the inter- and intraspecific characterisation of wine yeasts (ITS, inter-delta elements, SSR, microsatellite markers, etc.). Although the ISS-PCR technique proved to be useful for the typing of *S. cerevisiae* species since it is able to detect the polymorphisms of highly mutable sequences as introns, no reliable results have been shown for the discrimination of *D. bruxellensis* species at strain level (de Barros Lopes et al., 1996, 1998). This has also been proved by the fact that unstable ISS profiles were obtained when yeasts are analysed with the primer EI1 alone (Oelofse et al., 2009). For this reason, primers EI1/LA2 and EI2/LA1 were preliminary employed in pairs to type the *D. bruxellensis* collection. Repeatability tests were performed with primers used singly and in pairs. Results confirmed what has already been reported for the employment of single primers (Oelofse et al., 2009), that stable profiles with a 100% of genetic similarity among identical strains were obtained only if primers were used in pairs (data not shown). Figure 1a shows the genetic profiles obtained through the amplification of the genomic DNAs of *D. bruxellensis* CBS strains with the EI1+LA2 primers pair. Different fragment lengths (bp units) were generated from EI1+LA2 and EI2+LA1 amplifications: the longest amplicons that were separated onto gels measured about 1,200 bps for the former pair and approximately 2,500 bps for the latter one. The cluster analysis generated by the application of UPGMA revealed that 15 and 10 different genotypes could be distinguished by EI1+LA2 and EI2+LA1, respectively. The genetic similarity between the strains ranged from 25 to 100% for EI1+LA2 (Figure 1b) and from

28 to 100% for EI2+LA1 (data not shown). In both analyses, only *D. bruxellensis* CBS2797 showed a genotype very different from the other strains, which was separated from the rest of the collection by an individual branch. Similar indexes of discrimination were calculated for the two couple of primers even if EI1+LA2 generated a percentage slightly higher than EI2+LA1 (98.5 vs. 93.0%).

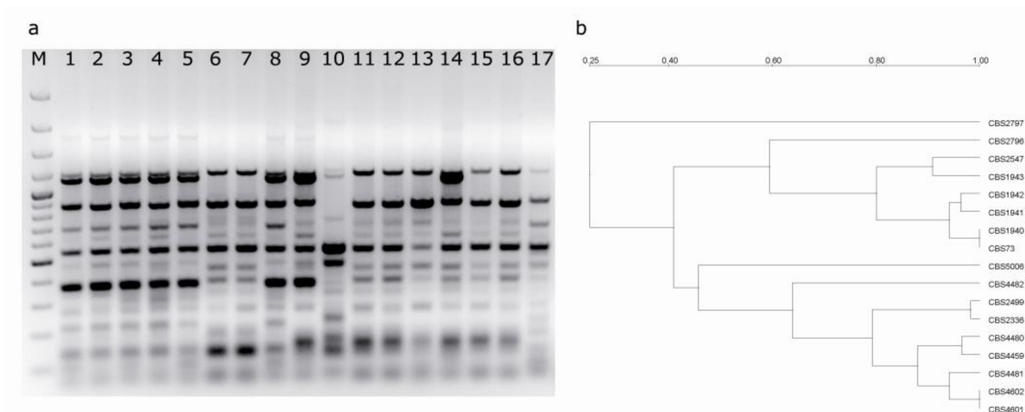

Figure 1
ISS profiles of *D. bruxellensis* strains using a) EI1/LA2 pairs of primers. 1: CBS73; 2: CBS1940; 3: CBS1941; 4: CBS1942; 5: CBS1943; 6: CBS2336; 7: CBS2499; 8: CBS2547; 9: CBS2796; 10: CBS2797; 11: CBS4459; 12: CBS4480; 13: CBS4481; 14: CBS4482; 15: CBS4601; 16: CBS4602; 17: CBS5006; M: 100-bp DNA ladder (Fermentas, Vilnius, Lithuania). b) Dendrogram built by UPGMA analysis comparing ISS profiles obtained after EI1/LA2 amplifications.

Considering that *D. bruxellensis* has an intron content similar to *S. cerevisiae* and that among *D. bruxellensis* genes the most represented 5'motif is GTAAGT instead of GTATGT as reported for *S. cerevisiae* (Woolfit et al., 2007), a new primer was tested targeting this sequence. PCRs were carried out using DbEI1+LA2 pair since, as above described, EI1+LA2 resulted in a higher discriminating ability. Although the substitution of a single nucleotide in the EI1primer sequence led to a change in the molecular weight of fragments (Figure 2a) and increased the range of genetic similarity between the strains (from 19 to 100%), the results indicated that DbEI1+LA2 showed a lower discriminatory index (92.0%) than EI1+LA2 (98.5%). Even in this case, *D. bruxellensis* CBS2797 generated an out group (Figure 2b). Comparing the results obtained from the amplifications with these two oligonucleotides pairs on the same strain, it was evident that no bands with the same molecular weight were detected. This could suggest a really different genetic origin of the amplified fragments.
Therefore, a multiplex PCR was applied on the whole collection using primers DbEI1, EI1 and LA2 (Figure 3a and 3b), with an increase of the discriminatory power up to 99.3%. In conclusion, this work proved to be a simple and reliable method for strain typing of *D. bruxellensis* species. Moreover, further investigation of the fragment sequences could lead to interesting implications on genome structure, gene regulation and sequence evolution as already reported for *S. cerevisiae* (Mattick, 1994).

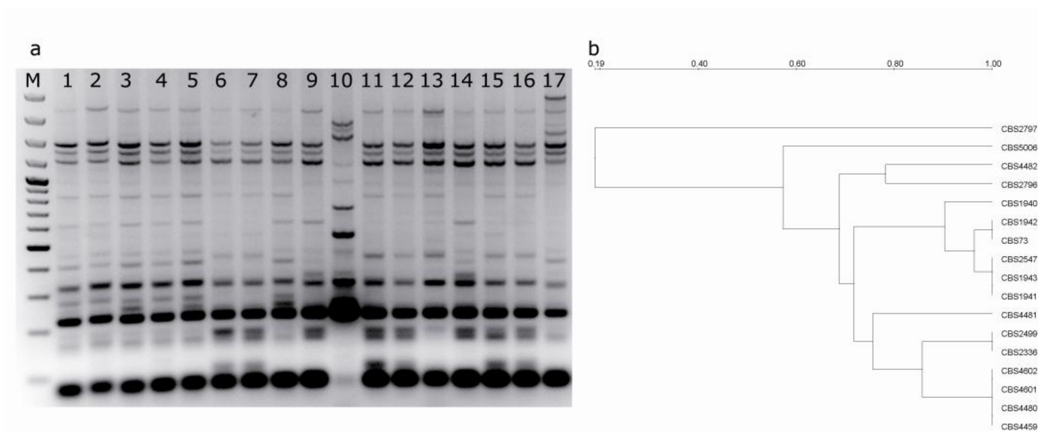

Figure 2
ISS profiles of *D. bruxellensis* strains using a) DbEI1/LA2 pairs of primers. 1: CBS73; 2: CBS1940; 3: CBS1941; 4: CBS1942; 5: CBS1943; 6: CBS2336; 7: CBS2499; 8: CBS2547; 9: CBS2796; 10: CBS2797; 11: CBS4459; 12: CBS4480; 13: CBS4481; 14: CBS4482; 15: CBS4601; 16: CBS4602; 17: CBS5006; M: 100-bp DNA ladder (Fermentas, Vilnius, Lithuania); b) Dendrogram built by UPGMA analysis comparing ISS profiles obtained after DbEI1/LA2 amplifications.

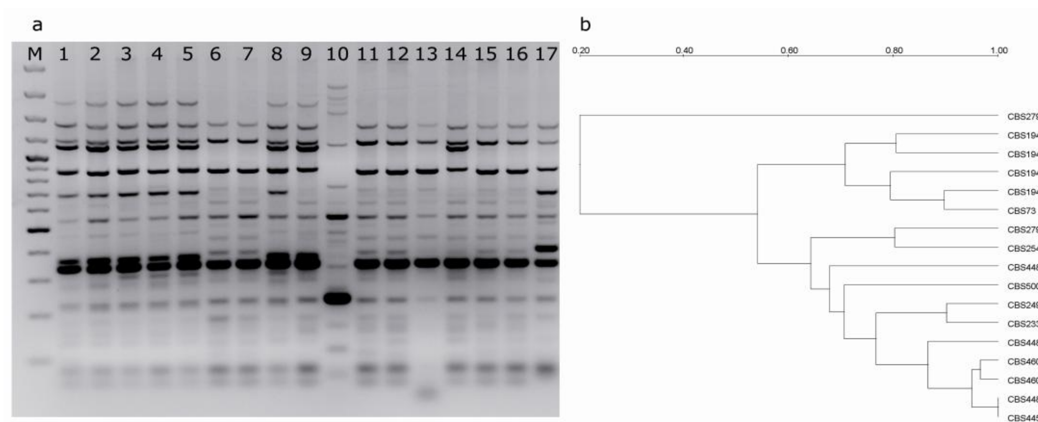

Figure 3
ISS profiles of *D. bruxellensis* strains using a) Multiplex PCR with DbEI1, EI1 and LA2 primers. 1: CBS73; 2: CBS1940; 3: CBS1941; 4: CBS1942; 5: CBS1943; 6: CBS2336; 7: CBS2499; 8: CBS2547; 9: CBS2796; 10: CBS2797; 11: CBS4459; 12: CBS4480; 13: CBS4481; 14: CBS4482; 15: CBS4601; 16: CBS4602; 17: CBS5006; M: 100-bp DNA ladder (Fermentas, Vilnius, Lithuania). b) Dendrogram built by UPGMA analysis comparing ISS profiles obtained after DbEI1, EI1 and LA2 amplifications.

3.1.1.3 References (1a)

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3.1.2 Intraspecific variations of the Intron Splice Site in *Dekkera/Brettanomyces bruxellensis* genome studied by capillary electrophoresis separation (1b)

Studies on the environmental diffusion and the incidence of *D./B. bruxellensis* species have been reported in literature, focusing on the isolation, identification and genotyping of reference and indigenous strains (Martorell et al., 2006; Conterno et al., 2006; Curtin et al., 2007; Agnolucci et al., 2008). These works show an existing high intraspecies polymorphism degree. The recent availability of the partial genome sequence allows the origin of ascomycetous yeasts to be traced (Woolfit et al., 2007) and molecular tools to be set up for the correlation of genetic and physiological data. Recently Hellborg and Piškur (2009) stated that genome polymorphism is due to a fusion event among several genomes or to an increased mutation rate resulting in asexual life style. Moreover, it has to be considered that *D./B. bruxellensis* has mainly been associated with fermented beverages (wine, beer, cider, traditional drinks) where the environmental conditions (high acidity, oxygen limitation, SO_2 and ethanol concentration, starvation, etc.) could be considered as extreme for survival. In particular, a high alcohol level, could play an important role in determination of the frequent genome rearrangements in *D./B. bruxellensis*.
Genetic variations are usually found at a higher frequency in DNA regions that are not linked to any gene function. In this context, frequent nucleotide substitutions, deletions, or insertions may occur in introns modifying their structure and dispersion. Thus, they are considered as good indicators in evolution studies. In *Saccharomyces cerevisiae*, the lariat branch point TACTAAC and the 5’ splice site GTATGT (5’ss) are conserved and were used to build primers for the Intron Splice Site amplification analysis (ISS-PCR) (de Barros Lopes et al., 1996). Since our previous report showed that ISS-PCR analysis on *D./B. bruxellensis* yeasts is reliable but still skill-depending (Vigentini et al., 2011), the main goal of this work is to provide a new optimized set of primers for a simple capillary electrophoresis protocol that can accurately separate the amplified fragments. A further development of the results could include a database collecting both ISS profiles and physiological data as has already been the case for microsatellite patterns in *S. cerevisiae* (Richards et al., 2009) or grape cultivars (This et al., 2004).

3.1.2.1 Materials and Methods (1b)

Strain collection and culture conditions

A collection of 98 yeasts belonging to *Dekkera/Brettanomyces* was analysed in this study. Among them 66 were obtained from the CBS collection: 5 strains of *Brettanomyces custersianus*, 10 strains of *Brettanomyces naardenensis*, 3 strains of *Brettanomyces nanus*, 19 strains of *Dekkera anomala* and 29 strains of *Dekkera bruxellensis* (Table 1). The remaining part of the yeasts collection was composed of 31 *D./B.bruxellensis* wild isolates (Table 2) collected from bottled wines showing the “Brett” character. After appropriate dilutions of wine samples in water plus 10 g/l peptone and plating on WL medium (Merck), isolates with different colour or colony morphology were transferred on YPD medium [1% (w/v) Yeast Extract, 2% (w/v) Peptone and 2% (w/v) Glucose] at 25°C for 5 days. Cells were maintained at -80°C in YPD medium added with 20% (v/v) glycerol. Volatile phenols production was detected as described in the paragraph “*Determination of volatile phenols and biogenic amines*” (3.2.1.1) (Vigentini et al., 2008).

Species	CBS designation	Accession numbers: 26SrDNA/15S rDNA	Species	CBS designation	Accession numbers: 26SrDNA/15S rDNA
Brettanomyces custersianus	4805^T	U76199/AY988564	*Brettanomyces nanus*	1945^T	U76197/AY988544
	4806	AY969094/AY988576		1955	AY969064/AY988546
	5207	AY969098/AY988580		1956	AY969065/AY988547
	5208	AY969099/AY988581	*Dekkera bruxellensis*	73	AY969048/AY988530
	8347	AY969115/AY988597		74^T	DBU45738/AY988531
Brettanomyces naardenensis	6042^T	U76200/AY988565		75	AY969050/AY988532
	6040	AY969102/AY988584		78	AY969053/AY988535
	6041	AY969103/AY988585		96	AY969054/AY988536
	6043	AY969104/AY988586		97	AY969055/AY988537
	6107	AY969107/AY988587		98	AY969056/AY988538
	6108	AY969106/AY988588		1940	AY969058/AY988540
	6115	AY969107/AY988589		1941	AY969059/AY988541
	6117	AY969109/AY988591		1942	AY969060/AY988542
	6118	AY969110/AY988592		1943	AY969061/AY988543
	7540	AY969111/AY988593		2499	AY969068/AY988550
Dekkera anomala	76	AY969051/AY988533		2336	AY969067/AY988549
	77	AY969052/AY988534		2547	AY969069/AY988551
	1938	AY969057/AY988539		2796	AY969070/AY988552
	1947	AY969063/AY988545		2797	AY969071/AY988553
	3026	AY969073/AY988555		3025	AY969072/AY988554
	4210	AY969074/AY988556		4459	AY969077/AY988559
	4211	AY969075/AY988557		4480	AY969080/AY988570
	4212	AY969076/AY988558		4481	AY969088/AY988571
	4460	AY969078/AY988560		4482	AY969089/AY988572
	4461	AY969079/AY988561		4601	AY969090/AY988573
	4608	AY969081/AY988563		4602	AY969091/AY988562
	7250	AY969085/AY988567		4914	AY969095/AY988577
	4462	AY969087/AY988569		5206	AY969097/AY988579
	4711	AY969092/AY988574		6055	AY969084/AY988566
	4712	AY969093/AY988575		8027	AY969086/AY988568
	5111	AY969096/AY988578		5512	AY969100/AY988582
	7654	AY969112/AY988594		5513	AY969101/AY988583
	8138	AY969113/AY988595			
	8139	AY969114/AY988596			

Table 1
Dekkera/Brettanomyces strains from the international CBS collection.

Laboratory designation	Isolation year/ Country
VALT	2008/Sondrio, Lombardy (Italy)
VOLO	2008/Brescia, Lombardy (Italy)
CMR62/1	2004/Alessandria, Piedmont (Italy)
NdA2	2007/Sicily (Italy)
SABA1109	2009/Sondrio, Lombardy (Italy)
SABA1309	2009/Sondrio, Lombardy (Italy)
ML11	2006/Lucca, Tuscany (Italy)
ML12	2006/Lucca, Tuscany (Italy)
ML14	2006/Lucca, Tuscany (Italy)
ML23	2006/Lucca, Tuscany (Italy)
ML24	2006/Lucca, Tuscany (Italy)
ML26	2006/Lucca, Tuscany (Italy)
ML27	2006/Lucca, Tuscany (Italy)
ML28	2006/Lucca, Tuscany (Italy)
VL4	2006/Lucca, Tuscany (Italy)
VL5	2006/Lucca, Tuscany (Italy)
VL11	2006/Lucca, Tuscany (Italy)
VL17	2006/Lucca, Tuscany (Italy)
VL19	2006/Lucca, Tuscany (Italy)
VL22	2006/Lucca, Tuscany (Italy)
VL27	2006/Lucca, Tuscany (Italy)
VL38	2006/Lucca, Tuscany (Italy)
VL39	2006/Lucca, Tuscany (Italy)
VL58	2007/Lucca, Tuscany (Italy)
VL66	2007/Lucca, Tuscany (Italy)
VL67	2007/Lucca, Tuscany (Italy)
VL68	2007/Lucca, Tuscany (Italy)
VL69	2007/Lucca, Tuscany (Italy)
VL70	2007/Lucca, Tuscany (Italy)
VRANAC A	2004/Montenegro
VRANAC B	2004/Montenegro

Table 2
Wild *D./B. bruxellensis* strains isolated from wine samples with “Brett” character.

Strain identification

All DNAs were extracted as described in the paragraph "*DNA amplification*" (3.1.1.1). After RNAs digestion at 30°C for 1h with 0.5 mg/ml RNase (Fermentas, Vilnius, Lithuania), DNA concentrations were determined by measuring the A_{260nm}. Species identification of the wild isolates were carried out using the RFLP analysis of the ribosomal internal transcribed spacers and the 5.8S rRNA gene region as described by Esteve-Zarzoso et al. (1999). The amplified DNAs (10 µl or 0.5-10 µg) were digested with *Hin6*I restriction endonuclease (Fermentas, Vilnius, Lithuania) according to the supplier's instructions. PCR products or their restriction fragments were separated and visualised on 0.8% or 1.5% agarose gels, respectively, with 1x TAE buffer. Isolates showing the same profile were grouped together and one sample per cluster was submitted to the partial amplification and sequencing of the 26S rDNA D1/D2 domain (Primm, Milan, Italy) using primers NL1 and NL4 (Spirek et al., 2003) (Appendix A). Eighty to one hundred ng of genomic DNA were added to a reaction mix consisting of 0.1 µM of each primers, 200 µM dNTPs, 1x reaction buffer $MgCl_2$ free, 2.5 mM $MgCl_2$ and 1U *Taq* polymerase (Fermentas, Vilnius, Lithuania). The amplifications were performed in a Mastercycler ep gradient S (Eppendorf, Hamburg, Germany) according to Spirek et al. (2003). Amplification fragments for yeast identification (26S rDNA) were resolved in 1% (w/v) agarose gels (Agarose electrophoresis grade, Invitrogen) at 100V for 1 h using 1X TAE as running buffer (0.4 M Tris-acetate, 0.01 M Na_2EDTA, pH 8) and detected by EtBr staining (0.5 µg/µl). Bands were visualised under UV exposition (GelDOC, Bio-Rad). PCR products were submitted to sequencing (Primm, Milan, Italy).

Species clustering

Sequences of 26S and 15S rDNA genes of the strains listed in Table 1 were submitted to clustering analysis. Partial amplifications of the mitochondrial 15S ribosomal DNA locus were performed with the primer pairs SSU1/SSU2 and SSU3/SSU5 (Sulo et al., 2009). PCR reactions were carried out on a Robocycler Gradient 40 (AH Diagnostics, Stratagene) in a total reaction volume of 50 µl containing 2U *Taq* DNA polymerase (Amersham, Uppsala, Sweden), 0.2 mM each dNTP, 0.2 µM each primer and 80-100 ng of the genomic DNA. The amplifications were performed according to the following conditions: 94°C initial denaturation for 5 minutes; 30 cycles of 2 minutes at 94°C, 1 minute at 50°C and 2 minutes at 72°C; then a final extension at 72°C for 7 minutes. Twenty microlitres of each 15S rDNA PCR reaction were run on a 1% (w/v) agarose gel (Agarose electrophoresis grade, Invitrogen) and the amplicons were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Hvidovre, Denmark). The purified PCR products were sent to a service company (Eurofins MWG Operon, Ebersberg, Germany) to be sequenced on both the DNA strands.

ISS-PCR fingerprinting of D./B. bruxellensis *species*

Primers used for the ISS-PCR fingerprinting of *D./B. bruxellensis* isolates were LA1, LA2, Db1EI1, and DB1EI2. Oligonucleotides derived from primers for *S. cerevisiae* (de Barros Lopes et al., 1996) whereas Db1EI1 and Db1EI2 were designed on *D. bruxellensis* genomic survey sequences (gss) (GenBank accession numbers from EI011584 to EI026443) (Woolfit et al., 2007) and they were 5'-dye-labelled with 6-Carboxyfluorescein (6-FAM, Primm, Milan, Italy). Db1EI1 (5'-CTGGCTTGGaataGTAAGT-3') and Db1EI2 (5'-CTGGCTTGCTtacttACTTAC-3') are longer than EI1 and EI2 (de Barros Lopes et al., 1996), and contain the "GTAAGT" 5' splice site (5'ss) instead of "GTATGT". The added nucleotides (lower-case typed) are positioned at 10-13 and 11-15 of Db1EI1 and Db1EI2, respectively. The amplifications were carried out in a 25 µl solution containing 150 ng of genomic DNA, 0.75 µM each primers, 200 µM dNTPs, 1x reaction buffer $MgCl_2$ free, 2.5 mM $MgCl_2$, 5% (v/v) dimethyl sulfoxide (DMSO), 1U *Taq* polymerase

(Fermentas, Vilnius, Lithuania). The reactions were performed (for 40 cycles) following the protocol described for *S. cerevisiae* (de Barros Lopes et al., 1996) with few modifications: the annealing temperatures were set at 46°C and 47°C when Db1EI1/LA2 or Db1EI2/LA1 primer pairs were used respectively and, as suggested by Tristezza et al. (2009), the final elongation step was carried out at 72°C for 30 min.
ISS-PCR profiles were separated and visualised on 1.5% (w/v) agarose gels at 100 V for 2 h with 1X TAE and detected by EtBr staining (0.5 µg/µl). Ten microlitres of amplification products added to 2 µl of running marker (6X bromophenol blue) were placed in gel slits. Amplification products range was evaluated by 100bp DNA Ladder (Fermentas, Vilnius, Lithuania). For fragment size determination, PCR products were run in an ABI Prism 310 Genetic Analyzer (Applied Biosystems – Life Technologies), using POP-4 polymer, 310 Genetic Analyzer Buffer with EDTA, and a 47 cm x 50 µm capillary (Applied Biosystems – Life Technologies). Samples were injected for 20 s at 1.5 kV and separated at 8 kV for 80 min with a run temperature of 60°C. DNA sizing was performed with GeneScan-1200 LIZ (Applied Biosystems, Life Technologies, Carlsbad, USA). To optimize the capillary electrophoresis assay, different concentrations of PCR products (1:10, 1:50 and 1:100 dilutions) and different loading volumes (0.9 and 1.75 µl) were tested for each examined concentration. For each sample, 20 µl of formamide (Applied Biosystems – Life Technologies) were added to 0.75 µl of 1200 LIZ and 0.9 µl of PCR products. The samples were denatured at 95 °C for 3 min and then snap cooled on ice prior to loading them into the autosampler tray. Digital profiles were visualised using ABI PRISM GeneMapper 3.7 (Applied Biosystems – Life Technologies) software. Fragments between 50 and 1200bp were scored. Peaks having a relative fluorescent intensity value less than 100 were not scored. Strain CBS74 was used as an internal standard in the different capillary electrophoresis runs.

Assessment of protocol repeatability

To estimate the repeatability error (RE) of ISS-PCRs, the genomic DNA, extracted from five distinct *D. bruxellensis* strains (CBS74 - CBS1942 - CBS1943 - CBS2797 - CBS5206) was used as template in three independent PCR reactions with Db1EI1/LA2 and Db1EI2/LA1 primer pairs. The resulting PCR profiles were displayed by agarose gel and capillary electrophoresis. Percentage of genetic similarity among replicates of each isolates (Dice's similarity index) (Dice, 1945) was calculated and the RE threshold value for strain discrimination was assigned on the basis of replicates having the lowest percentage of genetic similarity.

Data scoring and cluster analysis

Multiple sequence alignment of 26S and 15S rDNA partial sequences (ClustalX algorithm) was analysed with MEGA5 software (Tamura et al., 2011). Evolutionary distances were calculated using the Maximum Likelihood method based on the Jukes-Cantor model and setting the complete deletion from the data set of the gaps and missing data. Bootstrap method consisted of 500 replicates. Electrophoretic patterns generated by agarose gel and GeneMapper 3.7 software were employed to create presence/absence (1/0) matrices. Markers that were present in all samples (monomorphic) were not considered informative and were removed from the data set. Binary matrices were imported into NTSYS-pc 2.1 package (Rohlf, 1998) for cluster analysis. Genetic similarity matrices among genotypes were calculated for dominant multilocus markers according to Dice's similarity index (Dice, 1945), using the SIMQUAL routine. Distance matrices were analysed using UPGMA (unweighted pair group method with arithmetic means) clustering algorithms, through the SAHN-clustering program, and dendrograms were displayed by TREE PLOT program. Bootstrap analysis was assumed to evaluate the reliability of dendrograms. The bootstrap values were calculated by WinBoot package (Yap & Nelson, 1996).

3.1.2.2 Results (1b)

Interspecific diversity among genus Dekkera/Brettanomyces

Two loci, one located in the nuclear and the other one in the mitochondrial DNA, were used to investigate the genetic relationships in *Dekkera/Brettanomyces* yeast genera (Table 1). Figure 4 display that these genetic targets can separate the analysed strains into clusters that contained only individuals belonging to the same species. The results showed that the two most closely related species were *D. bruxellensis* and *D. anomala*; this observation was confirmed by both the phylogenetic trees where a subgroup enclosing only these species was formed (data not shown). As concerns the ability of the investigated loci to discriminate at strain level, the best separation among *D. bruxellensis* and *D. anomala* yeasts was obtained using 26S rDNA gene; nevertheless, only a low level of intraspecific diversity was observed.

Primers design for ISS PCR finger printing for D. bruxellensis *species*

The possibility to discriminate inside the *D. bruxellensis* species was investigated choosing the 5' splice site of introns as a molecular target. To avoid the production of unspecific PCR fragments, since similar 5' ss motifs are shared in hemiascomycetous genomes, the primer design for *D./B. bruxellensis* intron sequences has involved the analysis of the nucleotides flanking this locus. Conserved exonic sequences at the begin and at the end of 5'ss were checked among the gss deposited on GenBank database. In this case, the analysis had an intrinsic limitation since until now only 40% of the total genome of *D. bruxellensis* has been published in gss and some sequences could be repeated. As regards the nucleotides that were detected at the 5' exon/intron boundary, the motif -AATA- was the most frequent; in particular, among the deposited sequences with the 5'ss, 36.5% contained these four nucleotides. On the other hand the downstream region of the 5'ss was characterised by a sequence of five nucleotides -AAGTA- with a frequency of 76.3%. This observation allowed us to describe an hypothetical extension of the 5'ss core of *D. bruxellensis*.

Optimisation and repeatability assessment of the capillary electrophoresis separation

Fluorescence signal intensity of capillary electrophoresis was optimized by testing two different loading volumes (0.9 and 1.75µl) for three different dilutions (1:10, 1:50 and 1:100 dilutions) of PCR products of five different isolates (CBS74, CBS1941, CBS1942, CBS1943, CBS2797, and CBS5206). This subset of concentrations allowed the determination of the best capillary electrophoresis conditions producing an unambiguous chromatogram. The highest number of identified fragments was obtained at a 1:10 dilution for each replicate of the five isolates. The percentage of scored fragments obtained by 1:50 and 1:100 dilutions was on average about, respectively, 47 and 72% lower than 1:10 dilution for 1.75 µl of loading volume and about 53 and 76% lower for 0.9 µl of loading volume. The best chromatograms (absence of off-scale peaks and low percentage of undefined peaks with a florescent intensity value lower than the threshold value) were obtained adopting 1:10 dilution and 0.9 µl of loading volume (Figure 5a). Electrophoresis patterns performed in these conditions were transformed in binary matrices (1 presence, 0 absence of fragment) for the five different isolates and genetic similarity between replicates of each isolate was calculated in order to establish the repeatability of the protocol. The lowest value of similarity among replicates of the same sample evidenced in a UPGMA-based dendrogram was 94% (Figure 6). Thus we assumed this rate as the threshold for the strain discrimination; this result is consistent with the repeatability value obtained by PCR-based molecular markers (Tristezza et al., 2009). In capillary separation, an average of 58 fragments for sample was obtained compared to an average of 15 fragments for samples detected after resolving PCR products on agarose electrophoresis gel. Analysis of electrophoresis patterns of each isolate in different replicates performed by capillary electrophoresis allowed the definition of a size range of 3bp where fragments of different

electrophoretic profiles can be considered the same allele. Robustness of capillary electrophoresis conditions and fragments assignment was evaluated performing bootstrap analysis on the UPGMA-based dendrogram. Bootstrap values were calculated for each cluster corresponding to triplicates of the five isolates. The distinctiveness of the five subgroups is strongly supported by bootstrapping. Bootstrap values, ranging from 73 to 100%, were statistically significant, showing very robust clusters with no misclassifications.

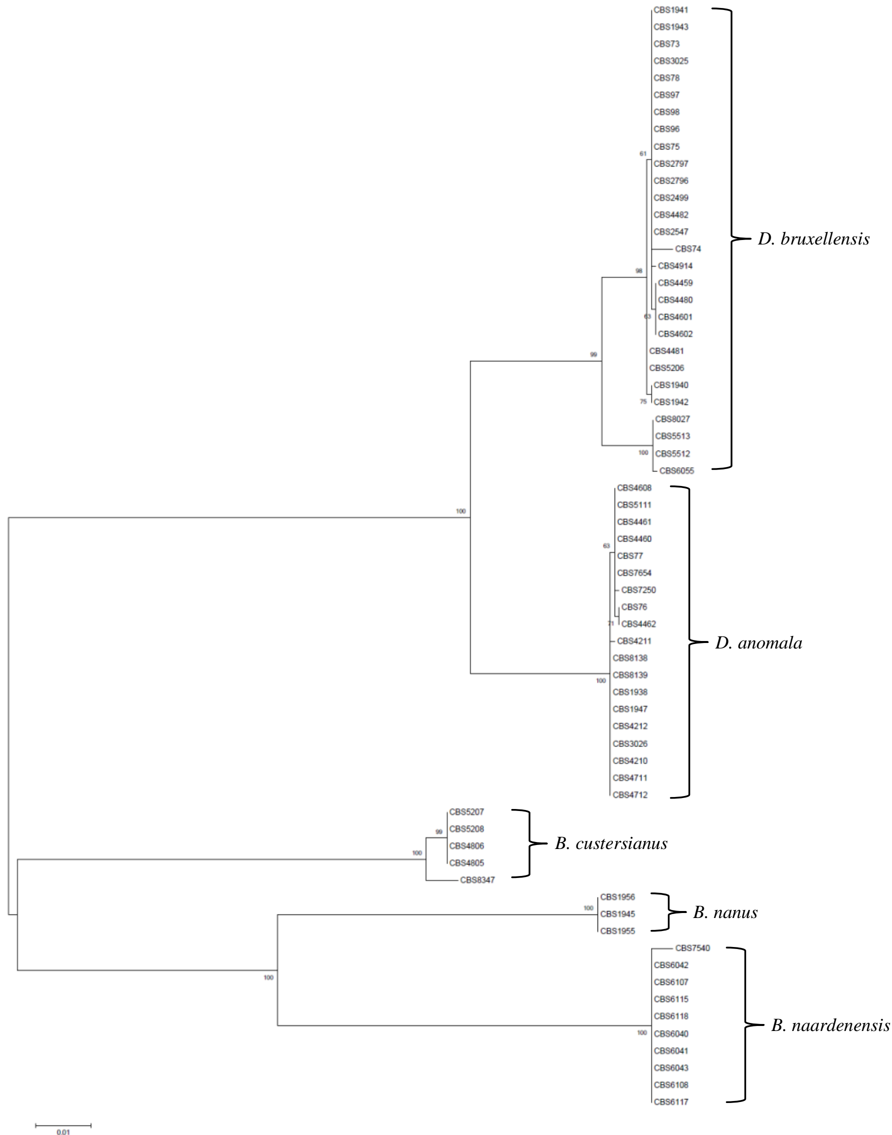


Figure 4

Phylogenetic relationships among *Dekkera/Brettanomyces* CBS strains. Phylogenetic relationships are based on nuclear 26S (D1/D2 fragment) and 15S mtDNA partial sequences. Evolutionary distances were calculated using the Maximum Likelihood method. Bootstrap values (500 replicates) are shown as percentage next to the branches. The bar indicates the number of base substitution per site.

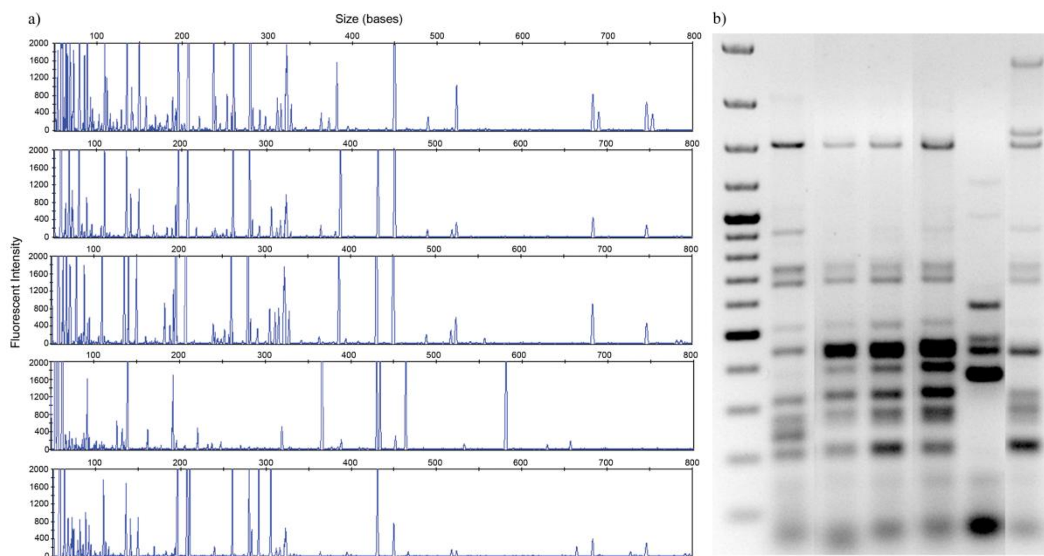


Figure 5
Example of ISS-PCR profiles from five CBS strains obtained using Db1EI1/LA2 primers pair: a) Analysed by capillary electrophoresis. The molecular sizes (expressed in base pairs) and fluorescent intensity are respectively displayed across the horizontal and the vertical axes. From the top: CBS74, CBS1942, CBS1943, CBS2797 and CBS5206 strains. b) Analysed by agarose gel. From the left: DNA marker (100bp DNA Ladder, Fermentas, Vilnius, Lithuania) and CBS strains (in the above mentioned order).

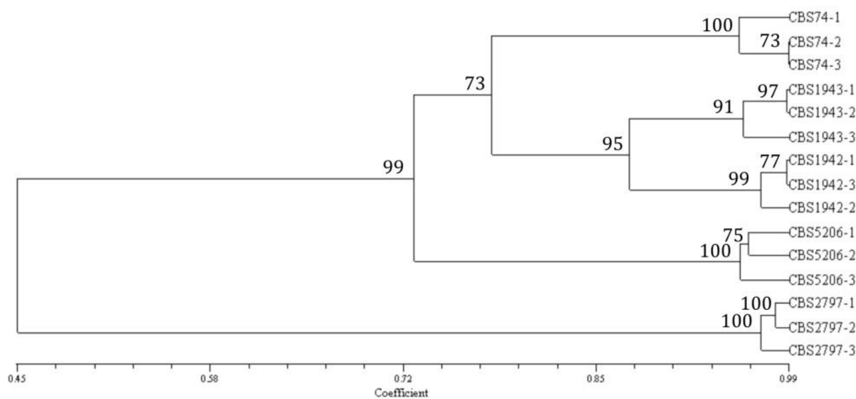


Figure 6
Dendrogram generated from the repeatability test using Db1EI1/LA2 primers pair. The coefficient of similarity among the ISS-PCR profiles is indicated along the horizontal axis. Bootstrap values (500 replicates) are given at each node.

Intraspecific diversity among D./B. bruxellensis *CBS strains*

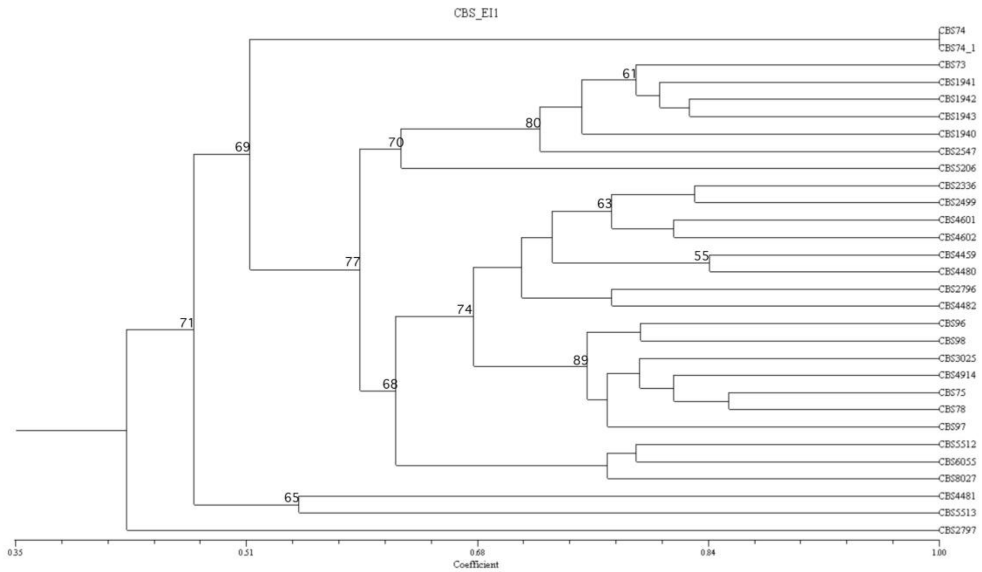
The ISS profiles of CBS strains were firstly analysed to understand if the new molecular target was able to differentiate at strain level. Agarose gels were carried out to set up the PCR conditions and to verify the presence of stable genetic profiles (Figure 5b). Different DNA fragment profiles were obtained from amplifications with Db1EI1/LA2 or Db1EI2/LA1 primers pairs: the longest amplicons that were separated onto gels measured approximately 1500 bp for the former pair and 2000bp for the latter one. For both primer pairs, an average of about 10 bands for each sample was clearly detected, most of them being positioned between 100 and 550bp (Figure 5b). Nevertheless, a smeared and sometimes weak signal could be visualized under the 100 bp reference band.
In our conditions, the separation of ISS-PCR amplicons by capillary electrophoresis showed that the intronic profiles enclosed DNA fragments up to 940bp in length for Db1EI1/LA2 and about 1470bp for Db1EI2/LA1. In any case, most of the detected sequences (visualized as peaks with relative intensity intensity >100 in the electropherograms) were distributed between 50 - 350bp. The total number of peaks was different in relation to both the primer sets and the strain; the average number of peaks was 53 (sd=14) when the amplifications were performed with Db1EI1/LA2 primers pair and 64 (sd=12) when Db1EI2/LA1 primers pair was used. Considering that maximum intron length in *D. bruxellensis* CBS2499 strain is 472bp for the RPL36A gene (Woolfit et al., 2007), the Mean Length of Introns (MLI) for each isolate was calculated as follows: all the lengths (bp) of the DNA fragments in a range between 50-470 bp were added and the result was divided for the number of the considered fragments. The calculation of the mean length of introns values (MLI) in the investigated *D./B. bruxellensis* genomes for both primer pairs was about 198bp, corroborating the value of 195bp reported by Woolfit et al. (2007).
The elaboration of ISS profiles obtained with Db1EI1/LA2 and Db1EI2/LA1 primer pairs generated two dendrograms that were able to separate the CBS strains within a limited range of similarity (from about 35% to 75-85%) (Figures 7a, 7b). This result highlighted the robustness of the protocol. Since the repeatability thresholds of the ISS-PCR profiles resolved in capillary electrophoresis was calculated at 94%, the trees showed that both the primer pairs were able to discriminate all the yeasts at strain level. The bootstrap analysis of these two trees was also comparable, where 43% and 41% of statistically significant nodes were calculated for Db1EI1/LA2 and Db1EI2/LA1 primer pairs, respectively. A further elaboration, consisting in the combination of results obtained from both the primer pairs, improved the bootstrap analysis increasing the percentage of significant nodes at 46% (Figure 8). In terms of biodiversity the most relevant result was that CBS strains were separated at about 55% of genetic similarity in two main clusters containing only isolates collected from wine or other substrates. No relation was annotated regarding the geographic origin of the strains (genetic similarity > 70%).

Typing of D./B. bruxellensis *yeast collection*

Yeasts biodiversity among the 60 isolates of the *D./B. bruxellensis* collection was assessed throughout the analysis of the ISS-PCR profiles that resulted from the amplification with Db1EI1/LA2 and Db1EI2/LA1 primer sets. Fragments of different size were generated from the two primer pairs, and the genetic similarities from clusters analysis among the isolates ranged between 41-98% and 35-83% with Db1EI1/LA2 and Db1EI2/LA1, respectively (Figure 9a and 9b). Actually, two isolates (SABA 1109 and SABA1309) were not separated with Db1EI1/LA2 primer pairs, while they were discriminated with Db1EI2/LA1. As showed by the bootstrap analysis, the best molecular characterisation was achieved drawing together all the ISS-PCR profiles; in fact, although the combination of all the ISS profiles led to a percentage of significant nodes similar to the one obtained using only the Db1EI1/LA2 primer pairs (about 35%), the former elaboration allowed the complete separation between the isolates SABA 1109 and SABA1309 (Figure 10).

Most of the strains grouped into two main clusters at a similarity level of approximately 45% (A) and 47% (B), respectively. Cluster A included almost all the CBS strains without showing any relationship between the ISS-PCR patterns and the geographical area of isolation. However, the groups containing the strains CBS73, CBS1941, CBS1942, and CBS1943 (French wine isolates), CBS2336 and CBS2499 (French wine strains), and CBS4601 and CBS4602 (South Africa wine isolates) shared a similarity higher than 70%. Cluster B grouped most of the wild isolates collected in this work, with the exception of CBS74, the type strain of *D. bruxellensis* isolated from Lambic beer. No evident correlation between genetic profiles and the origin of the samples was found. Nevertheless, the groups containing the strains ML11, VL4, and VL27 (Italian isolates), VL69 and VL70 (Italian isolates), which were collected from wines produced in the same geographic area (Lucca) in 2006, showed a level of similarity higher than 70%. Again, a strong relation was detected for strains SABA1109 and SABA1309 both isolated from the same wine sample. Two strains were positioned outside the main clusters (A and B); one of them derived (ML23) from Tuscan wine samples whereas the other (CBS2797) belonged to CBS collection. It should be noted that CBS2797 showed the lowest similarity within all the investigated yeasts.

a)



b)

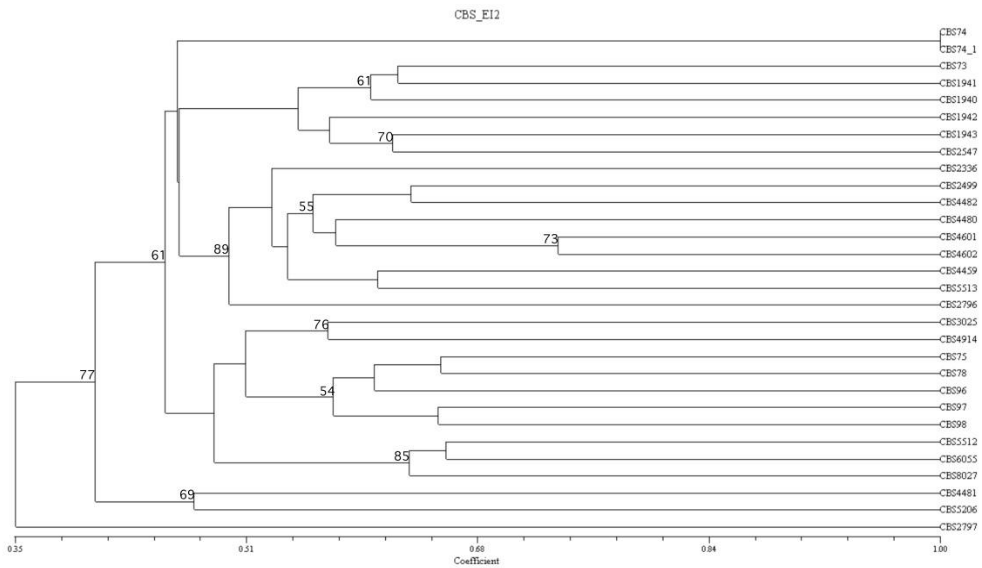


Figure 7

Dendrograms generated by cluster analysis of ISS-PCR patterns obtained from the *D. bruxellensis* CBS strains: a) using Db1EI1/LA2 primer pair; b) using Db1EI2/LA1 primer pair. The coefficient of similarity among the genetic profiles is indicated along the horizontal axis. Only bootstrap values ≥50 (500 replicates) are given.

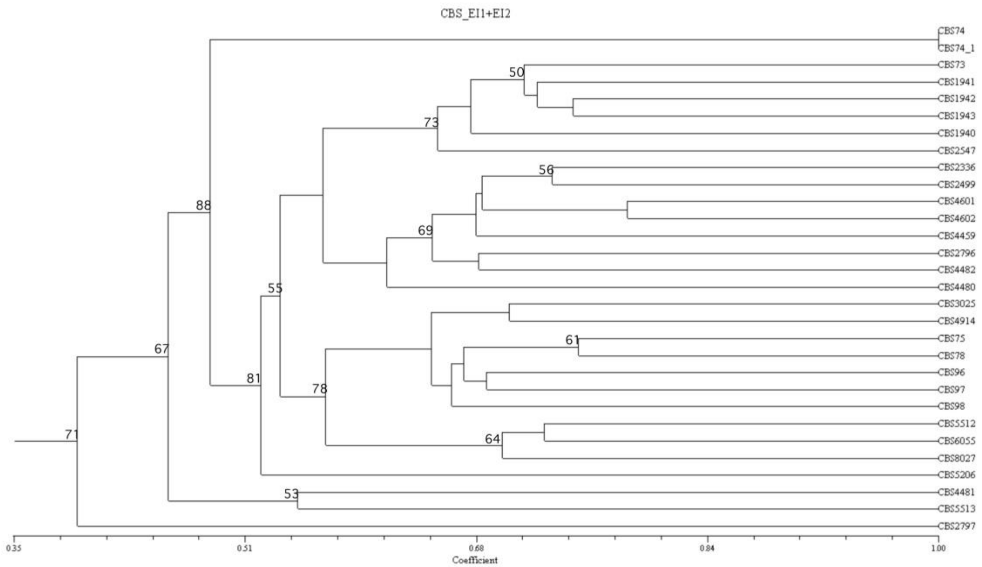


Figure 8

Dendrograms generated by the combination of ISS-PCR patterns of *D. bruxellensis* CBS strains using Db1EI1/LA2 and Db1EI2/LA1 primer pairs. The coefficient of similarity among the genetic profiles is indicated along the horizontal axis. Only bootstrap values ≥50 (500 replicates) are given.

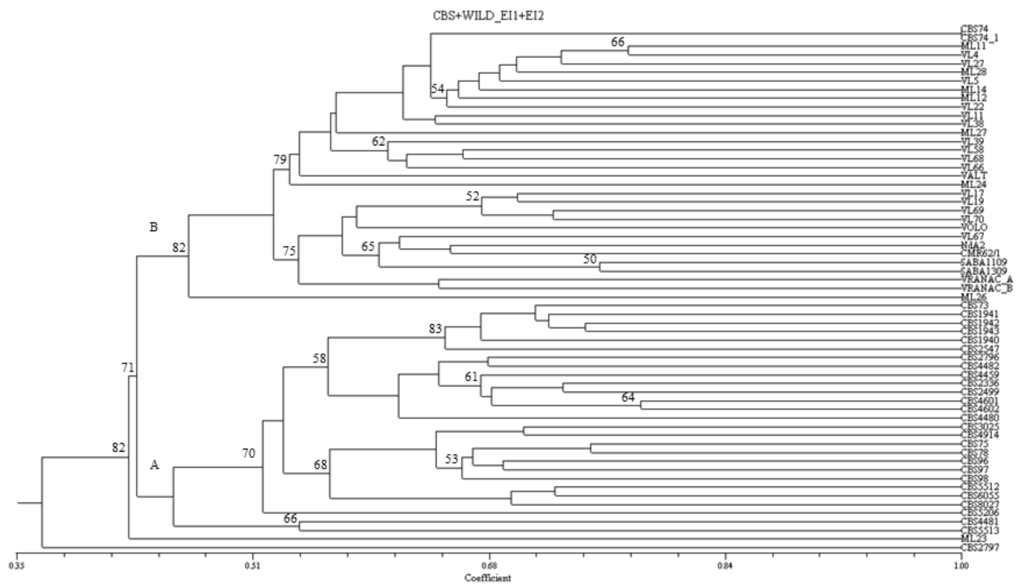


Figure 10

Dendrograms generated by the combination of ISS-PCR patterns of the whole *D. brettanomyces* yeast collection using Db1EI1/LA2 and Db1EI2/LA1 primer pairs. The coefficient of similarity among the genetic profiles is indicated along the horizontal axis. Only bootstrap values ≥50 (500 replicates) are given.

a)

b)

Figure 9

Dendrograms generated by cluster analysis of ISS-PCR patterns of the whole *D. brettanomyces* yeast collection: a) using Db1EI1/LA2 primer pair; b) using Db1EI2/LA1 primer pair. The coefficient of similarity among the genetic profiles is indicated along the horizontal axis. Only bootstrap values ≥50 (500 replicates) are given.

Strain	Volatile phenols production	Reference
CBS73	Low	Conterno et al., 2006/Vigentini et al., 2008
CBS74^T	"	"
CBS1940	"	Vigentini et al., 2008
CBS1941	"	"
CBS1942	"	"
CBS4482	"	"
CBS4601	"	"
CBS4602	"	"
CBS5206	"	"
CBS1943	Medium	"
CBS2499	"	"
CBS2336	"	Conterno et al., 2006/Vigentini et al., 2008
CBS2547	"	"
CBS2796	"	Conterno et al., 2006/Vigentini et al., 2008
CBS2797	"	Conterno et al., 2006/Vigentini et al., 2008
CBS4459	"	Vigentini et al., 2008
CBS4480	"	"
CBS4481	High	"
VL4	Low	Laboratory data
VL5	"	"
VL27	Medium	"
VALT	"	"
NdA2	"	"
CMR62/1	"	"
VL17	High	"
VL19	"	"
VL22	"	"

Table 3

Production of volatile phenols under different growth conditions by some strains of the yeast collection. Low = Volatile phenols between 0-0.5 mg/l; Medium = Volatile phenols between 0.5-2.0 mg/l; High = Volatile phenols > 2.0 mg/l.

3.1.2.3 Discussion (1b)

Saccharomyces and *Dekkera/Brettanomyces* yeasts are both associated with wine fermentation, and are both good ethanol producers (Rozpędowska et al., 2011). Conterno et al. (2006) have reported that the production of volatile phenols represents the most discriminant feature of *D./B. bruxellensis*. On the contrary, Agnolucci et al. (2009) showed that different genetic haplotypes can share only a few metabolic and spoilage profiles when grown in a synthetic medium. Although both works have exploited the combination of several classic genetic tools for yeast typing (SNPs analysis of the 26S rDNA and *ACT1* partial sequences, RAPD, and mtRFLP) the discrimination at strain level has not always provided full intraspecific differentiation. Oelofse et al. (2009) have described a different ability in the production of volatile phenols comparing laboratory media and wine by different strains typed throughout a REA-PFGE protocol. Anyway, strains that differed at genetic level produced similar amounts of ethyl derivates under both conditions confirming the observation of Agnolucci et al. (2009). Recently, other authors have applied on *D./B. bruxellensis* advanced analytical tools to prove their high level of genetic polymorphism (Curtin et al., 2007; Hellborg & Piškur, 2009) but they have not correlated this feature with the spoilage potential. It is very likely that the spoilage activity is a strain dependent character and although many efforts have been made to develop not cultured-dependent systems for the early detection of *D./B. bruxellensis* in wine, the setting up of new molecular methods that can lead to rapid and reliable recognition of spoilage strains is still desirable.

Most hemiascomycetous yeast genomes show a common architectural organisation of the exon/intron sequences (Génolevures, 2000). The model yeast *S. cerevisiae* can be classified as an intron-poor eukaryote where only a small amount of its genes contain introns (4%) (Goffeau et al., 1996; Spingola et al., 1999). The average length of *S. cerevisiae* introns is estimated to be around 265 nt and, as occur in the other eukaryotic organisms, they exhibit adjacent (5' and 3' splice sites) and internal (branch site) conserved motifs used by the spiceosome machinery to process pre-mRNAs. A comparative genome analysis among hemiascomycetous yeast species has revealed a very dynamic nature of the introns size, location and conservation and also in the organisation of the intron splice sites (Bon et al., 2003). In the past, the use of specific primers that recognise the 5'ss -GTATGT- has allowed the identification and discrimination at strain level of a large number of yeast species (de Barros Lopes et al., 1996, 1998). Recently, the setting up of a multiplex PCR protocol throughout the use of modified oligonucleotides that targeted 5'ss -GTAAGT- has confirmed a high polymorphism in the *D./B. bruxellensis* genome (Vigentini et al., 2011).

To build more specific primers for the investigated species, adjacent sequences to 5'ss -GTAAGT- were analysed. In this work, to understand if non-random sequence patterns are conserved in *D./B. bruxellensis* genome. In fact, a non-random vocabulary around the 5'ss could be maintained during evolution to preserve the spliceosome activity as happens for the splice sites themselves. Since the analysis was carried out on the gss deposited sequences (representing approx. 40% of the total genome), our results show that a hypothetical conserved nucleotides usage exists in *D./B. bruxellensis*. In particular, this species exhibits a conserved -AATA- pattern in the region that precedes the 5'ss, instead of the -AAG- observed in other hemiascomycetous yeasts such as S. *cerevisiae* and closely related species. An analogous situation has been reported for *Debaryomyces hansenii* (Bon et al., 2003). However, the high similarity of the above mentioned two patterns supports the hypothesis that, also in *D./B. bruxellensis*, they can both favour the interaction with the T-rich loop inside the spliceosome and allow a recognition mechanism between exons and spliceosome (Ares et al., 1995; Teigelkamp et al., 1995). As concerns the downstream 5'ss, we found more frequently an adenine, as already observed in *D. hansenii*, *Yarrowia lipolytica* and *Pichia angusta*, instead of thymine or guanine detected in other hemiascomycetous species (Bon et al., 2003). On the contrary, an analysis on *D./B. bruxellensis* mtDNA has suggested that this

species could be more closely related to *Saccharomyces*/*Kluyveromyces* genera than to *D. hansenii* and *Candida* spp. lineages (Prochàzka et al., 2010). Further investigation will be required to confirm the 5'ss boundary of *D./B. bruxellensis*.

As regards the genomic diversity of *D./B. bruxellensis*, this study confirms the high level of intraspecific heterogeneity. The most significant result obtained by the new typing protocol application is that the whole yeast collection was clearly discriminated at strain level already at about 80% genetic similarity, a level widely below of the 94% repeatability value where no more clusters among isolates were found (Figure 10). From an ecological point of view the main conclusion is, in comparison to other works carried out in relatively large geographic areas (Curtin et al., 2007; Agnolucci et al., 2009), that no predominant genotypes were found. This could be due to the high resolution of the technique that comprises only two steps (amplification and separation) rather than other more complex analyses where multiple approaches are needed. Since our collection was made up of CBS strains and wild yeasts isolated both inside and outside Italy, some consideration on yeast's geographic (inter- and intra-area) and time dispersion should be considered. In disagreement to what was observed in *S. cerevisiae* (Goddard et al., 2010), we observed that a specific territory such as Italy does not harbour a distinct population of *D./B. bruxellensis* and also that different areas in the country (north, middle and south) do not harbour distinctive communities. The cluster analysis describes an almost complete separation between strains isolated before (from CBS collection) and after (wild strains) the year 2000. This observation could support the assumption that *D./B. bruxellensis* evolves very fast (Hellborg & Piškur, 2009) or to reinforce the idea that the laboratory cultural conditions change the evolutional rates (Ronald et al., 2006).

Based on the dendrogram obtained by the ISS genetic profiles elaboration (Figure 10) and the partial literature and laboratory data on the volatile phenols production (Table 3) we observed that the CBS strains growing in a model wine and showing a genetic clusters at approximately 70% genetic similarity enclosed wine strains able to produce similar off-flavour amounts. This could indicate a possible correlation between the genetic profile and the spoilage ability. We could speculate that 70% is a critical value of similarity since below this level it seems that there is no more correlation between the genetic distance and the ability to produce off-flavours. However, the relationship is loose in the wild isolates that grew directly in wines with different characteristics supporting what was already observed by Oelofse et al. (2009).

3.1.2.4 References (1b)

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3.1.3 Conclusions (1)

The development of new methods that exploit introns as molecular targets for the discrimination of *D./B. brettanomyces* yeasts have proved to be ideal to highlight genetic differences at strain level being reliable and robust. The proposed typing protocols (ISS-Multiplex PCR and ISS-PCR using specific primers for *D./B. bruxellensis*) are not as laborious as karyotyping and REA-PFGE methods are, they do not employ endonuclease enzymes such as AFLP, avoiding artefacts after the electrophoresis separation of the bands. Moreover, capillary electrophoresis does not use image analysis software, which is usually high skill-dependent, for the elaboration of the ISS profiles. A future optimisation could involve a direct colony ISS-PCR.
Considering that a positive correlation between the extend of non-protein-coding DNA and the eukaryotic complexity degree has been observed (Taft et al., 2007), the ISS fingerprinting can represent a useful tool to analyse the evolution rate of a yeast species. It should be underlined that at the technological level the genetic and physiological variations are often not correlated; the compilation of a database collecting both genetic and phenotypic traits of different *D./B. bruxellensis* strains such as ISS-PCR profiles, vinylphenol reductase activity and metabolic features (production of volatile phenols, acetic acid production, ethanol and SO_2 resistance) will offer an efficient way to counteract this spoilage yeast.

3.2 Phenotypic biodiversity of *Dekkera/Brettanomyces bruxellensis* species (2)

D./B. bruxellensis yeasts have developed numerous options in order to adapt and survive to the changing status of the environment. On the other hand, molecular profiling, carried out with several techniques (Martorell et al., 2006; Conterno et al., 2006; Curtin et al., 2007; Agnolucci et al., 2008; Vigentini et al., 2011), has shown a high genetic variability among strains. Therefore, different genetic groups of *D./B.bruxellensis* isolates could be related to distinct physiological characteristics.

3.2.1 Physiological and oenological traits of *Dekkera/Brettanomyces bruxellensis* strains isolated from Tuscan Sangiovese wines (2a)

D./B. bruxellensis is able to produce 4-vinylphenol and 4-ethylphenol from p-coumaric acid, whereas 4-vinylguaiacol and 4-ethylguaiacol from ferulic acid. Recent studies (Silva et al., 2005; Farina et al., 2007) revealed that *D./B. bruxellensis* strains varied in their production of phenolic substances in wine. In samples spoiled by *D./B. bruxellensis* high amounts of acetic acid can be found as well (Vigentini et al., 2008). However, the behaviour of this yeast species in terms of off-flavour production is not yet fully understood, both because only few systematic studies on *D./B. bruxellensis* strains of different origin have been performed and because the cultural media have been poorly standardized or they had little oenological significance. Moreover, some studies have proved that *D./B. bruxellensis* is able to produce neuroactive and vasoactive amines, mainly phenylethylamine from phenylalanine at an average quantity of 10 mg/l and lower amounts of other biogenic amines (BA) (Caruso et al., 2002). Anyway, the ability of *Dekkera/Brettanomyces* yeasts to generate BA seems to be strain dependent (Vigentini et al., 2008). The occurrence of these compounds (i.e. histamine, tyramine, putrescine, cadaverine, phenylethylamine, spermine and spermidine) in wine has been linked to the amino acid decarboxylase activity of lactic acid bacteria, such as *Oenococcus oeni*, but genetic determinants of some decarboxylases have been also identified in *Saccharomyces cerevisiae* (White Tabor & Tabor, 1985), whereas they are unknown for *D./B. bruxellensis*.
In the present study physiological diversity within *D./B. bruxellensis* strains, isolated during winemaking with Sangiovese grapes in some Tuscan wineries of the Chianti area (Italy), was investigated. The strains were characterized for their ability both to grow under a wine model condition by cultural tests and to produce volatile phenols and biogenic amines.

3.2.1.1 Materials and Methods (2a)

Yeast strains

A total of 7 haplotypes belonging to *D./B. bruxellensis* species were analysed in this study (Table 4). They were discriminated at strain level among 84 *D./B. bruxellensis* isolates using RAPD-PCR assay and mtDNA restriction analyses (Agnolucci et al., 2009). *D. bruxellensis* CBS4481 was used as reference strain.

Isolate designation	Molecular pattern*		Haplotype
	MtDNA/ *Hinf*I	RAPD/ OPA-02	
1-20L, 24-27L, 37L, 1-20N, 1T, 2T, 5T-7T, 9T, 10T, 13T-16T, 18T, 19T, 22T-24T, BD1, BD4, BD5, BD8-BD12, BF1-BF3	1	1	1
11T, 17T, 20T, 21T	2	2	2
BD2	1	3	3
BD7	1	4	4
BF4, BF5	2	5	5
3T, 4T, 8T	1	2	6
12T	2	1	7

Table 4

D./B. bruxellensis haplotypes analysed in this work. Isolate designation and origin: BD = Radda in Chianti (SI); BF = Castellina in Chianti (SI); T = Certaldo (FI); N = Greve in Chianti (FI); L = Vino rosso Toscano. * = Agnolucci et al., 2009.

Cultural media and physiological tests

The experiments were carried out in duplicate using a synthetic wine medium (SWM) as previously described by Vigentini et al. (2008). Colonies from YPD agar plates were transferred in YPD liquid medium supplemented with ethanol at 10% (v/v). After growth for 72 h, cells were harvested, washed with sterile water and used to inoculate SWM at 10^5 cells/ml. The culture was then divided into 10 ml aliquots and cultivated at 18 °C in hermetically closed and static tubes with no headspace volume. Each aliquot sample was used once for analyses and experiments lasted 110 days. The dissolved oxygen concentration was measured with a Mettler–Toledo polarographic oxygen probe twice during the experiment: there was about 98% of air saturation at the beginning whereas there was less than 30% of air saturation, corresponding to a value of oxygen concentration <1.8 mg/l, after 110 days. To minimize oxygen diffusion during measurements, the determination was conducted by flushing on the culture surface a certified pure nitrogen gas (SAPIO Srl, Italy) containing less than 5 mg/l of oxygen at a flow rate of 0.5 l/min.

Determination of biomass and culturability

Cell biomass was evaluated by optical density measurement at 640 nm (Beckman DCU640), diluting the sample when necessary. The specific growth rate (μ, h^{-1}) was calculated by linear regression of the ln (OD/ODi) vs. time for the exponential growth phase. Cell culturability (Millet & Lonvaud-Funel, 2000), i.e. the ability to form colonies, was carried out in duplicate on YPD agar plates maintained at 25 °C for 5 days. Results from preliminary tests (56 samples) were submitted to analysis of variance by Statgraphics Plus V.4 (Statistical Graphics corp., 1994); no significant differences were observed between two replicates ($p<0.05$) in both determinations.

Determination of volatile phenols and biogenic amines

Reagents and standards were at least of analytical grade. Coumaric acid, ferulic acid, vinylphenol, vinylguaiacol and ethylphenol were from Aldrich (Steinheim, Germany), ethylguaiacol was from SAFC (Steinheim, Germany). All of the biogenic amines standards were purchased from Fluka (Buchs, Germany) but synephrine (Sigma, Steinheim, Germany) and tryptamine (Merk, Hohenbrunn, Germany). All of the chromatographic solvents were of HPLC grade. HPLC determinations were performed by a Waters 2695 Alliance HPLC module equipped with a Waters 2487 double-wavelength UV detector and a Novapak C18 (4 µm, 3.9×150 mm) column (Waters, Milford, USA). Each sample was submitted to duplicate determination. Vinyl- and ethyl-phenols were evaluated by injecting 50 µl of culture media previously filtered through 0.22 µm PVDF membrane (Millipore, Bedford, USA) as described by Vigentini et al. (2008). Column temperature was set to 30 °C and UV detection was performed at 260 and 280 nm for vinylphenols and ethylphenols respectively. HPLC solvents were water/formic acid 0.2% (v/v) (eluent A) and acetonitrile/ formic acid 0.2% (v/v) (eluent B). Elution was achieved at 1 ml/min flow increasing eluent B from 10% to 35% in 20 min and from 35% to 75% in 6 min. The separation column was finally rinsed with 100% eluent B for 2 min. Run time was 40 min. Biogenic amine analysis was performed according to Krause et al. (1995) and based on pre-column derivatisation with dabsyl chloride and UV detection at 436 nm. This method allows the determination of cadaverine, ethanolamine, heptylamine, hexylamine, histamine, isoamylamine, octopamine, phenylethylamine, putrescine, spermine, spermidine, synephrine, tryptamine and tyramine. Chromatograms were recorded and processed using Millennium software v.4 (Waters, Milford, MA). The per cent standard error for both methods was calculated as 5%.

3.2.1.2 Results (2a)

Growth curves

In order to characterize the seven *D./B. bruxellensis* haplotypes from a physiological point of view, their ability to grow was investigated using a synthetic medium similar in composition to a wine (SWM). Preliminary tests performed with this medium pointed out that *Brettanomyces* grew in aerobic condition showing that SWM did not represent a limiting condition for the cellular growth (Vigentini et al., 2008). All the haplotypes slowly increased their biomass throughout the experiment (110 days) reaching an optical density (OD_{640nm}) ranging between 0.745 (CBS4481 strain) - 1.595 (haplotype 2) (Figure 11). According to their growth rate during the first 10 days of incubation the haplotypes could be grouped in two clusters: cluster "A" comprises the haplotypes 1, 2, 6 and 7, showing a 7.5 folds higher growth rate than the one observed for cluster B, which includes haplotypes 3, 4 and 5 (average specific growth rate 0.0110 h^{-1} and 0.0013 h^{-1}, respectively).

In order to investigate the possibility that isolates belonging to the same haplotype could exhibit physiological differences, the isolates 3T, 4T and 8T, belonging to the haplotype 6, were also cultivated and studied under the conditions as above over 60 days. The three cultures behaved identically: they grew at the same specific rate revealed for cluster "A," retaining their culturability around 4.2×10^6 cfu/ml until the end of experiments (data not shown).

Each haplotypes maintained the ability to form colonies, throughout the 110 days experiment, ranging 10^6-10^7 cfu/ml. Only the reference strain (CBS4481) showed a reduction in the number of colonies forming units, decreasing below 10^6 cfu/ml.

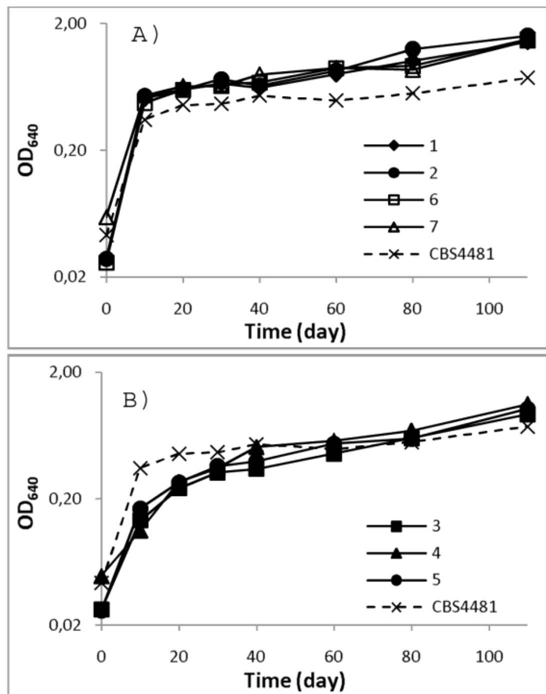

Figure 11

Growth curves of the *D./B. bruxellensis* haplotypes: a) cluster A includes haplotypes 1, 2, 6 and 7; b) cluster B includes haplotypes 3, 4 and 5.

All the haplotypes changed their cell morphology from an elliptic into a branched shape, around 60-80 days after the start of incubation (Figure 12). Pseudohyphal differentiation as well as invasive growth is hypothesised to be adaptation forms which allow yeast cell to look for nutrient rich conditions (Aguilar-Uscanga et al. 2000). In relation to cell wall structure and composition in *D./B. bruxellensis* yeasts no data are available so far. The identification of cell wall structure and composition could allow some interesting mechanisms linked to the colonization in wineries (biofilms) to be elucidated.

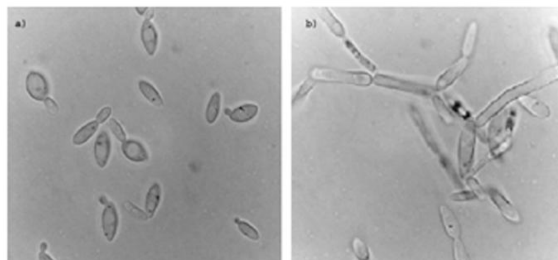

Figure 12

Cell morphology of the *D./B. bruxellensis* haplotype 4, obtained by optical microscopy at: a) the inoculum time and b) 80 days.

Production of volatile phenols and biogenic amines

Volatile phenols (4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol) were analysed by a RP-HPLC method. All the haplotypes metabolised p-coumaric and ferulic acids (Figure 13). No quantifiable amounts of both 4-vinyl derivatives were produced by haplotypes 4, 6, 7 and by the reference strain CBS4481. On the contrary, haplotypes 1, 2, 3 and 5 accumulated 4-vinylphenol, which reached its highest concentration (up to 2 mg/l) after 20 days and then disappeared (data not shown). The production of 4-ethylphenol by haplotypes 2, 6 and 7 occurred mainly during the first 20 days of culture in SWM and this fast production in our growth conditions allowed to define such haplotypes “4-ethylphenol fast producers” (Figure 13a). When the highest concentration of 4-ethylphenol was accumulated by the haplotype 2 (6.84 mg/l after 80 days), an average production of about 0.0013 pg per cell was calculated. On the contrary, haplotypes 1, 3, 4 and 5 could be defined “4-ethylphenol slow producers” since they slowly released 4-ethylphenol all along the experiment (Figure 13c), but their production per cell was higher, (around 0.0019 pg per cell) on an average. The same haplotypes clustering could be done according to the production rate of 4-ethylguaiacol: “4-ethylguaiacol early producers” (haplotypes 2, 6, and 7) accumulated more than 0.5 mg/l within 20 days and “4-ethylguaiacol late producers” (haplotypes 1, 3, 4 and 5) began to release it after 30-40 days (Figure 13b and 13d). Taking into account that the maximum amounts of 4-ethylguaiacol could be detected within 60 days from the inoculum, mostly released by the haplotype 7 (4.67 mg/l), the average production of 4 ethylguaiacol was estimated equal to 0.0022 pg per cell for the early producers and 0.0007 pg per cell for the late producers. The production of volatile phenols by the isolates 3T, 4T and 8T confirmed the results previously reported (Figure 13a and 13b). As shown for haplotype 6, in fact, the maximum amounts of 4-ethylphenol and 4-ethylguaiacol accumulated by each isolates were, after 60 days of growth, about 7 mg/l and 2 mg/l, respectively.

Biogenic amines (Table 5) were evaluated after 60 days and hexylamine was found as the main amine produced (1-4 mg/l). Levels lower than 1 mg/l were detected for the other aliphatic and aromatic amines. Phenylethylamine, which was reported to be produced at a concentration of up to 10 mg/l (Caruso et al., 2002), did not exceed 0.3 mg/l in our experimental conditions. However, strains 1, 3 and 4 were able to synthesize the methylated derivative of phenylethylamine, i.e. octopamine, at a concentration of 0.36 mg/l, as an average. All the isolates belonging to haplotype 6 synthesized levels of biogenic amines as reported in Table 5.

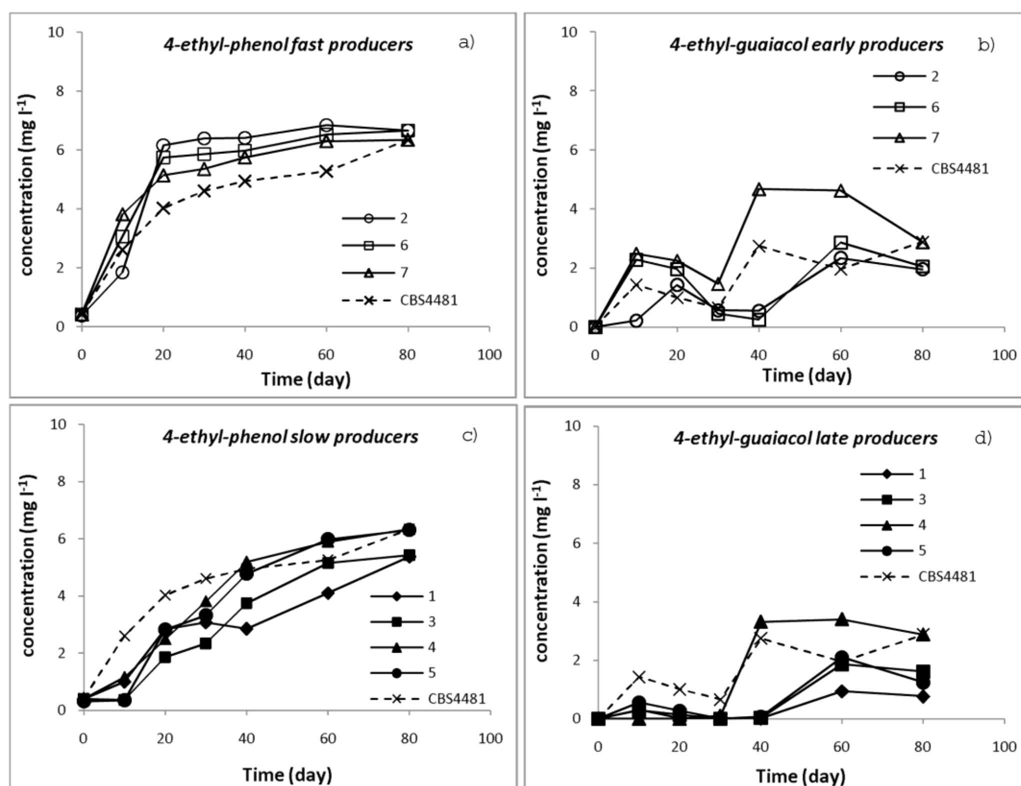

Figure 13

Start time and rate of production of 4-ethylphenol and 4-ethylguaiacol by the *D./B. bruxellensis* haplotypes.

Haplotype	TPM	PEM	IAM	PUT	HXM	CAD	HPM	OCM	SPD
1	nd	0.20	nd	0.30	3.35	nd	0.18	0.51	nd
2	nd	0.15	nd	0.27	3.38	nd	0.07	nd	nd
3	nd	0.21	nd	0.17	3.34	nd	0.14	0.38	nd
4	0.13	0.18	0.15	0.19	1.02	0.16	0.26	0.19	0.14
5	nd	0.20	0.12	0.19	3.49	0.08	nd	nd	nd
6	0.13	0.19	0.11	0.27	0.95	nd	nd	nd	0.11
7	nd	0.23	0.19	0.34	3.92	nd	nd	nd	nd

Table 5

Amounts of biogenic amines in samples collected at 60 days from the incubation. Concentrations are expressed in mg/l. Histamine, synephrine, tyramine and spermine were not detected. TPM = tryptamine, PEM = phenylethylamine, IAM = isoamylamine, PUT = putrescine, HXM = hexylamine, CAD = cadaverine, HPM = heptylamine, OCM = octopamine, SPD = spermidine. nd = not detectable.

3.2.1.3 Discussion (2a)

For the tested haplotypes a strong decrease in the specific growth rate in the semi-aerobic and stressful conditions of growth (because of the presence of 10% (v/v) ethanol), when compared to the 10–100 fold higher values obtained in aerobic tests, was observed (data not shown). This is in agreement with results from literature, although referred to different growth media (Ciani et al., 2003; Silva et al., 2004; Abbott et al., 2005). Moreover, all the tested haplotypes maintained the ability to be cultivated up until 110 days. The relation between *D./B. bruxellensis* genomic diversity and the ability to produce volatile phenols, which represents its potential risk as wine spoilage yeast, is still unclear. A recent study on 32 haplotypes of *Pichia guilliermondii* has indicated a correlation between 4-ethylphenol production and the genetic polymorphism of the strains (Martorell et al., 2006). On the other hand Conterno et al. (2006) demonstrated that different strains from CBS collection belonging to the same 26Sr DNA group can be very similar in off-flavour production but variations in other physiological parameters were also found (i.e. SO_2 tolerance, ethanol resistance). These results of this work showed that among the 7 haplotypes two main metabolic profiles can be identified, which allowed to discriminate “fast and early producers” and “slow and late producers” of 4-ethylphenol/guaiacol. A relationship between growth rate and production kinetic of volatile phenols was detected, as described by Vigentini et al. (2008). In fact, the “fast and early producers” strains were also the “fast growing” haplotypes (cluster A, μ=0.0110 h^{-1}), in contrast to the “slow growing” haplotypes (cluster B, μ=0.0013 h^{-1}) which corresponded to the “slow and late producers.” Haplotype 1, the most widespread, showed an intermediate behaviour: although it clustered in the cluster A, it accumulated the volatile phenols as a slow (4-ethylphenol) and late (4-ethylguaiacol) producer (cluster B). Interestingly, the “fast and early producers” released lower amounts of ethylphenol and almost a three times higher amount of ethylguaiacol (pg/cell) in comparison to the “slow and late producers”. Volatile phenols are reported to be produced during exponential growth phase (Dias et al., 2003). These results confirm and extend the literature data: 4-ethyl derivates were detected already at the beginning of exponential phase of growth. This could mean that the production of ethylphenols is connected with the primary metabolism in reducing NADH.

All *D./B. bruxellensis* strains tested in this study produced high amounts of 4-ethylphenols (about 6 mg/l), ten times with respect to the perception threshold, fixed at 0.62 mg/l (Chatonnet et al., 1992; Loureiro & Malfeito-Ferreira, 2003). Production of BA in wine from *Dekkera/Brettanomyces* was first described in five strains by Caruso et al. (2002) which detected 10 mg/l phenylethylamine as average. Low amounts of other amines were also produced. Production of BA was reported by Vigentini et al. (2008) under similar cultural conditions as described in this paper, but only 1-2 mg/l of polyamines were found. The haplotypes tested in this work produced hardly quantifiable amounts of both aromatic amines and polyamines, whereas hexylamine levels up to 3.9 mg/l could was detected. Slight differences could be found for the haplotypes tested: haplotypes 4 and 6 produced 1 mg/l hexylamine whereas about 3.5 mg/l were found for all the other haplotypes. Surprisingly, octopamine was detected for haplotypes 1, 3 and 4; production of octopamine by yeasts has been overlooked in the past, are here reported for the first time. However, it is not known whether higher levels could be produced by *Dekkera/Brettanomyces* growing in different media like wine. Increased blood levels of such amine in humans were described in patients affected by migraine (D'Andrea et al., 2004, 2007) and it can have important physiological effects after oral consumption even though the concentrations detected in these experimental conditions have little or no significance for human health (Haaz et al., 2006).

3.2.1.4 References (2a)

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3.2.2 Exploration of *Dekkera bruxellensis* biodiversity by studing carbon compounds assimilation, H_2S production, and vinylphenol reductase (VPR) activity (2b)

D./B. bruxellensis has shown to adapt under stressful environments where nutrient limitation is usually severe. Studies on yeast ability to metabolise alternative carbon sources to glucose and fructose have revealed that this species developed the capability to use wood sugars, such as cellobiose, that are extracted from toasted barrel by ethanol during wine aging. Thus, *D./B. bruxelensis* is not considered a natural competitor of *Saccharaomyces cerevisiae* and it is detected in wine after the alcoholic fermentation. Due to the harsh conditions (pH, oxygen limitation, starvation, high ethanol and SO_2 concentrations), the yeast drastically reduces its specific growth rate and, during the exponential phase of growth, it can produce off-flavours (Dias et al., 2003). Although this yeast is classified as spoilage wine yeast on the basis of ethylphenols production, no remarkable annotations have been reported concerning H_2S releasing in wine. This aspect has been deeply investigated in *Saccharomyces cerevisiae* where H_2S is produced in response to nitrogen availability (Mendes-Ferreira et al., 2010); it produces sulphide as an intermediate product of both the sulfate reduction sequence (SRS) pathway and sulphur amino acid biosynthesis.
Since previous works have described the correlation between growth and off-flavours production (Vigentini et al., 2008; Agnolucci et al. 2009), the main goal of this topic was to expand the knowledge regarding the carbon sources that allow the *D./B. brettanomyces* growth and to study the VPR specific activity that characterise strains with different genetic profiles. Moreover, the H_2S production was here tested for the first time on the same yeast collection.

3.2.2.1 Materials and Methods (2b)

Yeast strains

Nineteen *D. bruxellensis* CBS strains were analysed in this study (Table 6). Cultural media and inoculation protocols are described in the following sections.

D. bruxellensis isolate	Substrate of isolation	Identified by/Isolation year/Country
CBS73	Grape must	M.T. Smith/1998/Issoudum (France)
CBS74^T	Lambic beer	M.T. Smith/1990/Munte (Belgium)
CBS1940	Sour wine	M.T. Smith/1990/Besancon (France)
CBS1941	Sour wine	M.T. Smith/1990/Besancon (France)
CBS1942	Sour wine	M.T. Smith/1990/Besancon (France)
CBS1943	Sour wine	M.T. Smith/1998/Besancon (France)
CBS2336	Wine	M.T. Smith/1990/Gironde (France)
CBS2499	Wine	M.T. Smith/1990/Bordeaux-Merignac (France)
CBS2547	Sour wine	M.T. Smith/1990/Dijon (France)
CBS2796	Sparkling Mosselle wine	M.T. Smith/1998/Leinefelde (Germany)
CBS2797	Bordeaux wine	M.T. Smith/1990/Issoudum (France)
CBS4459	Dry white wine	M.T. Smith/1990/Smithfield-Mun (South Africa)
CBS4480	Dry white wine	M.T. Smith/1990/Smithfield-Mun (South Africa)
CBS4481	Champagne	M.T. Smith/1990/Smithfield-Mun (South Africa)
CBS4482	Sherry	M.T. Smith/1990/Smithfield-Mun (South Africa)
CBS4601	Wine	M.T. Smith/1990/Smithfield-Mun (South Africa)
CBS4602	Wine	M.T. Smith/1990/Groot Drakenstein (South Africa)
CBS5206	Grape must	M.T. Smith/1990/Smithfield-Mun (South Africa)
CBS5513	Bantu-beer	M.T. Smith/1990/Smithfield-Mun (South Africa)

Table 6

D. bruxellensis strains from the international CBS collection.

Carbon compounds assimilation

The use of different carbohydrate by *D. bruxellensis* strains was tested using the API/ID32C kit (bioMérieux SA, Marcy-L'Etoile, France) in accordance to the manufacturer's instructions with few modifications (Figure 14).
Before testing, cultures were activated by means of a culture steps in YPD liquid medium [Yeast extract 1% (w/v), Peptone 2% (w/v), Glucose 2% (w/v)] under aerobic conditions at 25°C for 48-72 h. In a final volume of 0.18 ml API C Medium (bioMérieux SA, Marcy-L'Etoile, France) (Table 8), about 10^3 cells were inoculated in each well. Test strips were incubated at 25˚C. Cell growth was verified at 24, 48, 72 and 144 hours. Positive growth results were attributed by the increasing in turbidity in comparison to the negative control.
Assimilation data generated by API/ID32C kit were employed to create a presence/absence (1/0) matrice (1=grown; 0= not grown). Markers that were present in all samples (monomorphic) were not considered informative and were removed from the data set. Binary matrice was imported into DendroUPGMA software for cluster analysis (Garcia-Vallve et al. 1999). A similarity matrice among phenotypes was calculated according to Dice's similarity index (Dice, 1945). Distance matrices were analysed using UPGMA (unweighted pair group method with arithmetic means) clustering algorithms.

Figure 14

API/ID32C strip. Each well, except the negative control, contains a different carbon source.

Compound	Concentration
Ammonium sulphate	5 g
Monopotassium phosphate	0.31 g
Dipotassium phosphate	0.45 g
Disodium phosphate	0.92 g
Sodium chloride	0.1 g
Calcium chloride	0.005 g
Magnesium phosphate	0.2 g
L- Histidine	0.005 g
L- Tryptophane	0.02 g
L- Methionine	0.02 g
Gel powder	0.5 g
Vitamin solution	1 ml
Trace solution	10 ml
pH at 20-25 °C	6.4 – 6.8

Table 8

API C medium composition

Hydrogen sulphide production

H_2S production was assessed by spotting 10µl at 10^6 cell/ml of fresh YPD culture on BiGGY medium (BD, Le Pont de Claix, France). Plates were incubated in aerobic condition at 25˚C for 72 hours. Strains were classified for sulphide production on the basis of color of the colonies: more hydrogen sulphide was produced, darker the colonies were appeared due to the precipitation of bismuth sulphide.

Vinyl Phenol Reductase (VPR) specific activity

Cells in exponential phase of growth were centrifuged at 10.000 *g* for 15 min, washed in phosphate buffer 0.05 M pH 7 and then harvested at 10.000 *g* for 15 min. Pellets were resuspended in the same buffer, added with dithiothreitol 0.1 mM and phenylmethanesulfonyl fluoride (PMSF) 2 mM. Cells were disrupted by subsequently 6 steps of icing and vortexing (1 min ON/1 min OFF). Extracts were prepared in duplicate. Total proteins content was measured by Bradford assay (Bradford, 1976). The determination of the specific activity of VPR was calculated by means of a spectrophotometer with lecture at 340 nm. Enzyme trials were performed as described in Table 7. This assay measures the conversion of 4-vinylguaiacol into 4-ethylguaiacol throughout the VPR activity. The reaction was triggered with 4-vinylguaiacol 0.3M as substrate. Absorbance at 340 nm was registered every minute until no variation was detected. The activity of VPR (U/ml) was calculated according to the following equation:

$$U/ml = (V \times A/min)/(\varepsilon \times d \times v)$$

- ✓ A = Absorbance at 340 nm
- ✓ V = final volume (ml);
- ✓ d = light path (cm);
- ✓ v = sample volume (ml);
  - ✓ ε = extinction coefficient of NADH at 340 nm is 6.22 ($l \times mmol^{-1} x\ cm^{-1}$)
- ✓

The VPR specific activity (U/mg) was calculated as follow:

$$U/mg = (U/ml)/(mg/ml)$$

- ✓ mg/ml = total amount of proteins measured by Bradford assay

Pipette into cuvette	**Blank**	**Sample**
Phosphate buffer 0.05 M pH 7	0.927 ml	0.927 ml
NADH 10mM	0.020 ml	0.020 ml
4-vinylguaiacol 0.3 M	0.003 ml	-
Cell extract	-	0.050* ml
Mix and read absorbance of the solution for 5 minutes. Then, start the specific reaction adding into the cuvette:		
4-vinylguaiacol 0.3 M	-	0.003 ml
Mix and read absorbance of the solution for 5 minutes.		

Table 7

Dosage of VPR activity. * = approx. 250 mg of total proteins

3.2.2.2 Results and discussion (2b)

Table 10 shows the results concerning the carbon sources assimilation using API/ID32C kit. Different growth rates were observed; about 30% of the strains grew slower in comparison to the rest of the yeast collection (144h vs. 72h). Ten different assimilation profiles were obtained by clustering analysis (Figure 16). All isolates were capable of glucose and esculin assimilation, whereas between 85-75% of the isolates assimilated saccharose, metil-αD-glucopyraniside, and palatinose, 74-65% melezitose, maltose, trehalose and cycloheximide, 64-55% galactose and cellobiose, about 37% N-acetyl-glucosamine, between 30-15% glycerol, sorbose, and raffinose, about 5% gluconic acid, levulinic acid, mannitol, and glucosamine. Other sugars (mono- and disaccharides), organic acids or sugar alcohols such as lactic acetic, arabinose, potassium 2-ketogluconate, xylose, ribose, erythritol, melibiose, glucuronic acid, lactose, inositol, and sorbose were not assimilated by any of the isolates confirming what was observed by Conterno et al.

(2006). These authors also reported that cellobiose, and trehalose are in general metabolised. In literature, it has been reported that *B. claussenii* and *B. anomalus* strains are capable of fermenting maltose, lactose and cellobiose (Spindler et al., 1992). In particular, β-glucosidase is the enzyme responsible for the hydrolysis of cellobiose in *Brettanomyces* spp. (Daenen et al., 2008); a symbiotic relationship has been assumed given the longevity of *B. bruxellensis* existence in the oak barrels (Vanderhaegen, 2003).
Results from the experiments on carbon assimilation revealed that about 30% of the analysed yeasts has a own pattern in the utilization of carbonious sources suggesting that the chromosomal rearrangement occurred in this species has direct implications on the physiological state of a strain (Figure 16) (Hellborg & Piškur, 2009). Further investigations should be focused on the understanding of the real status of *D./B. bruxellensis* taxonomy.

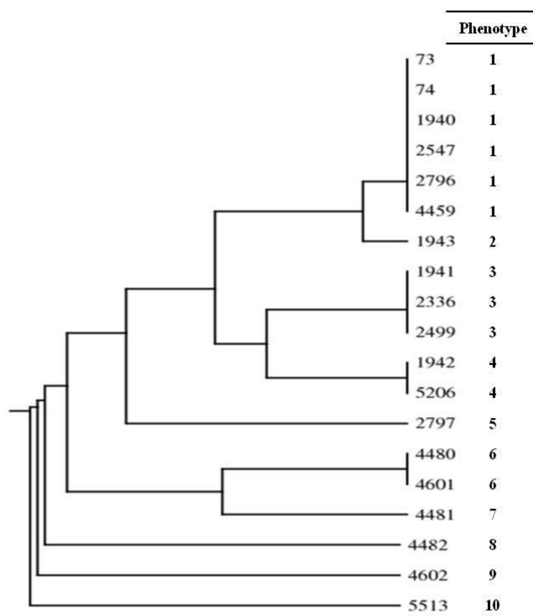

Figure 16

Similarity relationships for carbon sources assimilation among the 19 *D. bruxellensis* strains investigated. Terminal nodes indicate identical assimilation profiles.

CBS strain/ Incubation time	1	2	3	4	5	6	7	8	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
73 - 72h	+	+	+	-	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-	+	-	+	-	+
74 - 144h	-	-	+	+	-	-	-	-	+	+	-	+	+	-	-	+	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
1940 - 144h	-	+	+	+	-	-	+	-	+	+	-	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
1941 - 72h	+	+	+	-	-	-	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
1942 - 72h	+	+	+	+	-	-	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
1943 - 72h	+	+	+	-	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
2336 - 72h	+	+	+	+	-	-	+	+	+	+	-	+	-	-	-	+	-	+	-	-	-	+	-	-	+	-	-	+	-	-	-	+
2499 - 72h	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
2547 - 72h	+	+	+	-	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
2796 - 72h	+	+	+	+	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
2797 - 72h	+	+	+	+	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
4459 - 144h	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
4480 - 144h	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
4481 - 144h	+	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+
4482 - 72h	+	+	+	+	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
4601 - 72h	-	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
4602 - 72h	-	-	+	-	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
5206 - 72h	-	+	+	-	-	-	-	-	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+
5513 - 144h	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+

Table 10

Assimilation of different carbon sources by *D. bruxellensis* strains. 1 = D-galactose; 2 = cycloheximide; 3 = D-saccharose; 4 = N-acetyl-glucosamine; 5 = Lactic acid; 6 = L-arabinose; 7 = D-cellobiose; 8 = D-raffinose; 9 = D-maltose; 10 = D- trehalose; 11 = potassium 2-ketogluconate; 12 = metil-αD-glucopyraniside; 13 = mannitol; 14 = D-lactose; 15 = inositol; 16 = None substrate; 17 = D-sorbitol; 18 = D-xylose, 19 = D-ribose; 20 = glycerol; 21= L-ramnose; 22 = palatinose; 23 = Erythritol; 24 = D-melibiose; 25 = glucuronic acid; 26 = melezitose; 27 = potassium gluconate; 28 = levulinic acid; 29 = D-glucose; 30 = sorbose; 31= glucosamine. Incubation time is expressed in hours.

In order to assess the H_2S production, the strains were plated on BiGGY agar, and the colour of the colonies was evaluated. BiGGY agar exploits bismuth as an indicator for the production of sulphide (Figure 17). The production of sulphide in this medium is thought to be correlated with the basal level of activity of sulphite reductase. Low producers strains were the ones that showed a colony colour ranging from white to light tan, medium producers cells had colonies from tan to light brown, and high H_2S producer yeasts formed colonies from brown to black (Table 11). Most of the yeast collection was composed by high producers of H_2S (63%). This is the first time H_2S is used to discriminate among *D./B. bruxellensis* strains.

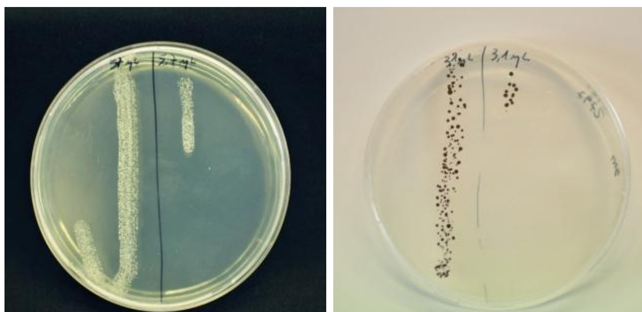

Figure 17

Hydrogen sulphide production on BiGGY medium by *D. bruxellensis*: left) example of a low producer strain; right) example of a high producer strain.

H_2S production (colony colour)	**CBS designation**
Low producers (white to light tan)	4459, 4480, 4601, 4602, 5513
Medium producers (tan to light brown)	4481, 5206
High producers (brown to black)	73, 74, 1940, 1941, 1942, 1943, 2336, 2499, 2547, 2796, 2797, 4482

Table 11

D. bruxellensis strains clustering on the basis of the H_2S production on BiGGY medium.

Absorbance tests, performed in triplicate, showed a good repeatability of the spectrophotometer assay (standard error of 3%).The specific activity of the VPR enzyme was preliminary measured in presence of NADPH as coenzyme. The slow decreasing in the kinetics of reaction obtained for all the strains (A_{340}/min< 0.1), confirmed the low affinity of VPR to NADPH (Tchobanov et al., 2008). On the contrary, the use of NADH led to quantifiable values of VPR activity, especially for CBS4601 strain (Figure 15).

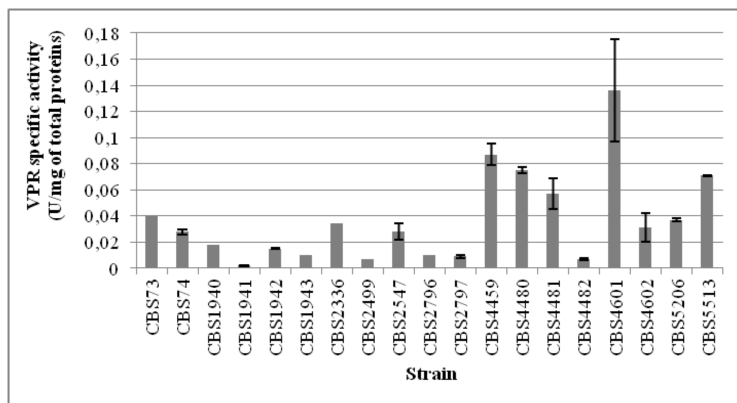

Figure 14

VPR specific activity by *D./B. bruxellensis* strains of the yeast collection. VPR specific activities are expressed as average values and error bars correspond to minimum and maximum detected values.

Three different ranges of VPR specific activity were used to assign the physiological profiles (Table 9). About 74% of the yeasts showed a low activity of VPR (0-0.040 U/mg of total proteins, profile 1 in Table 9), 21% were strains with a medium activity (0.041-0.080 U/mg of total proteins, profile 2 in Table 9), whereas only one strain (5.3%) was classified as a yeast with a high VPR activity (>0.081 U/mg of total proteins, profile 3 in Table 9). These data confirmed that different strains could be discriminated for their physiological aptitude to produce off-flavours (Conterno et al., 2006; Vigentini et al., 2008; Harris et al., 2009).

VPR specific activity (U/mg of total proteins)	**CBS designation**
Low activity (0-0.040)	73, 74, 1940, 1941, 1942, 1943, 2336, 2499, 2547, 2796, 2797, 4482, 4602, 5206
Medium activity (0.041-0.080)	4480, 4481, 5513
High activity (>0.081)	4459, 4601

Table 9

D. bruxellensis strains clustering on the basis of the VPR specific activity.

A negative correlation between VPR specific activity and H_2S production was observed (Table 12). Take into account that volatile phenol production could be used by *D./B. bruxellensis* yeasts to restore the redox balance in anaerobic condition and that in *S. cerevisiae* the liberation of H_2S arises from a reduction of inorganic sulphur troughout the activity of sulphite reductase enzyme (EC 1.8.1.2) (Stratford & Rose, 1985), this result could indicate that strains characterized by a low VPR specific activity have evolved other mechanisms to re oxidise equivalents, among these the capability to exploit the sulphite reduction. For example, the production of glycerol has been shown to be important in metabolizing NADH and restoring the $NADH/NAD^+$ equilibrium during anaerobic fermentation (Oura, 1977). Anyway, Aguilar-Uscanga et al. (2003) observed a slight glycerol production occurring under anaerobic conditions within a single strain of *Brettanomyces bruxellensis*. Further experiments are required to support this correlation.

CBS designation	Phenotypic profile	
	VPR activity	H_2S production
73	1	3
74	1	3
1940	1	3
1941	1	3
1942	1	3
1943	1	3
2336	1	3
2499	1	3
2547	1	3
2796	1	3
2797	1	3
4459	3	1
4480	2	1
4481	2	2
4482	1	3
4601	3	1
4602	1	1
5206	1	2
5513	2	1

Table 12

Phenotypic profile comparison. VPR specific activity: 1 = low activity; 2 = medium activity; 3 = high activity. H_2S production: 1 = low producers; 2 = medium producers; 3 = high producers.

3.2.2.3 References (2b)

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3.2.3 Conclusion (2)

The potential hazard of spoilage when *D./B. bruxellensis* grows in oenological conditions is confirmed since most of the analysed strains (7 different haplotypes and 19 CBS strains) were able to produce volatile phenols or showed a detectable VPR specific activity. Actually metabolic traits, as growth rate and off-flavour production, proved to be related and strain-dependent. In particular, this means that an early detection and identification of “fast-growing yeasts” and “fast volatile phenols producers” could be essential to introduce adequate corrective measures. However, a generalization of these results in relation to wine-making must be considered with caution, since the “fast” and “early” phenotype discovered in this work might not be observed in wine, due to different environmental conditions and nutrient composition. Further investigations on the BA production are required to carry out a reliable risk assessment of this species in wine-making. Formation of octopamine by *D./B. bruxellensis* is here reported for the first time.

The present study extends our knowledge on phenotypic diversity of *D./B. bruxellensis*, it indicates a rather high level of polymorphism regarding the growth rate and the kinetic of the off-flavour production formation. As concern the carbon sources assimilation it confirmed the capability of this yeast to use unconventional substrates for biomass production. Moreover, a negative correlation between VPR activity and H_2S production was observed suggesting an alternative mechanism to reoxydise NADH.

3.3 *D./B. bruxellensis* response to stress conditions (3)

The severity of *B. bruxellensis* related spoilage in wine arises from its ability to survive in spite of the unfavourable environment and the sanitation practices applied to the wood barrels. Such characteristics make the cellar contaminations invasive and difficult to treat. Its capability to grow in barrel aging wine can be partially ascribed to its high resistance to SO_2, to the lack of microbial competitors in the post-fermentation winemaking stage and to its capability to produce both α and β-glucosidase which allows cellobiose, a disaccharide found in toasted wood, to be metabolised (Mansfield, 2002). Very few is known about the mechanism of stress response in *B. bruxellensis* to oenological factors such as SO_2 concentration, starvation or thermal treatment.

3.3.1 *Dekkera bruxellensis* inactivation using low electric current treatment (LEC) (3a)

The control of *Dekkera/Brettanomyces bruxellensis* in must, wine and wine contact surfaces is relevant for wine producers in order to reduce their economic losses; wine industries are constantly seeking to optimize current methods or to find new approaches without modifying the sensorial properties of the final product (Couto et al., 2005). Moreover, alternative systems that avoid the addition of SO_2 and which are able to selectively inhibit spoilage yeasts are considered a good practice. (Barbosa-Canovas et al., 2001; Devlieghere et al., 2004). In recent decades, the use of electric field strength treatment in food processing has progressively increased: in fact more and more research studies are directed towards achieving the inactivation of microrganisms in the food system (Vega-Mercado et al., 1997; Knorr & Heinz, 2001; Raso & Barbosa-Canova, 2003; Abram et al., 2003). Nowadays, the inactivation of bacteria and yeast cells by means of pulsed electric field (PEF) is well documented; PEF technology has been used to preserve fruit juices, such as orange juice, and to delay spoilage by microrganisms (Heinz et al., 2003). Recently, this technology has been implemented in the winemaking process. In recent studies involving winemaking, to reduce the risk of wine spoilage caused by indigenous yeast growth troughout a low electric current technology (LEC) has been shown (Ranalli et al., 2000, 2002; Lustrato et al., 2003, 2006). However, the optimization of the process and evidence of the effective role of each parameter of the innovative treatment with respect to the final goals are required. The aim of this work was to investigate the effectiveness of LEC treatment in controlling spoilage yeast (only *D. bruxellensis*) in winemaking processes and to compare the evolution of volatile compounds in red wine when made without the addition of sulphur dioxide.

3.3.1.1 Materials and Methods (3a)

Yeast strain and growth conditions

Pure culture of selected yeast *D. bruxellensis* strain 4481 (Centraalbureau voor Shimmelcultures (CBS), Utrecht, the Netherlands) was used in this investigation. *D. bruxellensis* CBS 4481 strain was maintained at -80 °C in YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) with 20% v/v glycerol and revitalized on YPD plus 2% w/v agar plates adjusted at pH 5.6. Two parallel experiments called Experiment Without Adaptation (EWOA) and Experiment With Adaptation (EWA), respectively, were performed. Cells from YPD agar plates were inoculated and grown for 72 h in YPD liquid medium containing (EWA) or not containing (EWOA) ethanol at 10% (v/v) (Vigentini et al., 2008). The inocula were prepared as follows: a fresh cell culture grown on YPD broth at 30 °C, and the cell biomass was evaluated by optical density measurement at 640 nm (Beckman DCU640, Milan, Italy) after 3–5 days. According to Dias et al. (2003), adequate volumes of both cultures (EWOA and EWA) in the exponential phase of growth were harvested by centrifugation at 3500 rev/min for 15 min at 4 °C and washed once in a solution of 10 g/l peptone in order to obtain 10^6 CFU/ml in wines. The initial cell concentration in each wine was then confirmed by plate counts.

Monitoring of culturability and viability

Cellular culturability was assessed by plate counting on YPD agar medium in Petri dishes. Yeast growth was determined after incubating the plates at 30 °C for 2 days. Cellular viability was evaluated by ATP determination. A portable bioluminometer Biocounter model P 1.500 (Lumac B.V., Landgraaf, the Netherlands) equipped with a photomultiplier tube set at 7200 Relative Luminose Unit (RLU) with 200 pg ATP in 100 µl was adopted. For the evaluation of ATP content, we used a Microbial Biomass Test kit and Standard ATP assay (Celsis-Lumac B.V., Landgraaf, the Netherlands). Triplicate bioluminescent assays were performed in a Tris–HCl buffer solution (0.025 M; pH 7.75) by adding an adequate dilution of standard ATP to the sample as internal standard (Ranalli et al., 2002). The precision of the technique corresponded to minimum cell concentration of 10^3–10^4 and 30–50 CFU/ml for bacteria and yeast, respectively, as confirmed by previous data (Ranalli et al., 2003).

LEC equipment

The trials employed an electric power unit (De Ponti Application Electronics, Treviglio, Italy), divided into two parallel, separately working sections. The unit had the following technical characteristics: dimensions, 47x12x20 cm; weight, 2.8 kg; working voltage, 220 V; direct current, 0 to 22 V; intensity current, 0 to 200 mA; power, 0 to 4.4 W; frequency, 50 Hz and inversion polarity, 0 to 999 s (Zanardini et al., 2002; Ranalli et al., 2002; Lustrato et al., 2003). Tests were performed for 60 days in cylindrical polyethylene tanks diameter, 20 cm and high, 15 cm. Each test was carried out using a pair of Metal Mixed Oxide (MMO; Metakem GmbH, Usingen, Germany) current, 200 mA and inversion polarity every 60 s. (Figure 18). The energy applied to the samples in each treatment was 1.44 J/kg, corresponding to 0.3439 cal/h. When required, the values of actual amperes and volts applied in the chamber were measured by specific probe Testo 175-S1⁄S2 current⁄voltage data logger (Testo, Milano, Italy) immersed in the wine samples.

Figure 18

View of low electric current equipment and the tanks used under laboratory conditions, under EWA conditions

Samples and experimental procedures

Fifty liters of Montepulciano d'Abruzzo red wine filtered throughout a 0.45µm membrane filter (Millipore, Milan, Italy) was used for the experiments. It was produced in a local winery (Cooperativa San Zenone, Montenero di Bisaccia, Italy) and had the following mean chemical constitution: alcohol 13.50 % v ⁄v, total acidity 5.40 g/l, pH 3.48, total SO_2 120 mg/l and free SO_2 30 mg/l. Two experimental conditions called EWOA and EWA were independently performed.

- ✓ In EWOA (yeasts without adaptation), 25 l of wine were directly inoculated with a yeast viable cell suspension grown in YPD without ethanol (10^6 CFU/ml). The inoculated wine (wine + cells) was then divided into five flasks with a working volume of 2.0 l. Wine sample treatment was performed as follows: (i) control test (wine + cells), (ii) wine + cells, with added SO_2 (80 mg/l), (iii) wine + cells, subjected to 200 mA applied current; (iv) wine + cells, with added SO_2 (30 mg/l) + LEC treatment at 200 mA. Tests were performed under non sterile conditions, on laboratory desk.
- ✓ In EWA (yeasts with adaptation), 25 l of wine were inoculated with a yeast viable cell suspension grown in YPD plus ethanol 10% (initial cell density 10^6 CFU/ml). The inoculated wine (wine + cells) was divided into four flasks with a working volume (2.0 l). Wine sample treatment was performed as follows: (i) control test, (wine + cells), (ii) wine + cells with added SO_2 (80 mg/l), (iii) wine + cells subjected to 200 mA applied current. Tests were performed under sterile conditions, on a constantly functioning sterile cabinet lab desk.

In both experimental conditions, a flask was used as a 'witness' sample, providing a second control, with no microrganisms, no LEC and no SO_2 addition. The experiments took place at room temperature (18–22 °C), and the temperature, and pH values were monitored (Mettler-Toledo AG, Schwerzenbach, Switzerland). Seventy millilitres of liquid paraffin was added to each vessel both to minimize water evaporation and to avoid the contact of wine with oxygen. There was no stirring during the trials.

Chemical and physical analyses

Throughout the fermentative process, beginning at the start-up time, an assessment was made of the pH, the titratable acidity and the ethanol and sugar concentrations in the red wine; standard methods were used (Anonymous, 1990). Wine samples were examined for changes in sensorial characteristics such as colour, aroma and other parameters before and after LEC treatment to evaluate the potential impact of the treatment. According to previous work (Lustrato et al., 2006), in brief, the wines were evaluated by a panel of seven experts. All the panellists were Distaam University staff members (University of Molise, Italy), aged from 20 to 40 and experienced in sensorial wine analysis.

Microscope observations

Microbial growth and the dynamics of cell survival during the electrochemical treatment of the samples as well as for the relative controls (no current) were determined using scanning electron microscopy (SEM) observation scanning (Ranalli et al., 2002). The samples for SEM observation were left to sit overnight in a solution of 2% glutaraldehyde (0.01 M phosphate buffer), and then immersed in 1% osmium tetroxide. A microscope operating at 10 kV was used (Zeiss DSM 940A; LEO Elektronenmikroskopie GmbH, Oberkochen, Germany).

Determination of hydroxycinnamic acids, 4-vinyl and 4-ethyl derivates and biogenic amines in wines

All the HPLC determinations were performed by a Waters 2695 Alliance HPLC module equipped with a Waters 2487 double-wavelength UV detector and a Novapak C18 (4 lm, 3.9x150 mm) column (Waters, Milford, MA, USA). Volatile phenols (vinyl- and ethyl-phenols) and biogenic amines (phenylethylamine, isoamylamine, putrescine, hexylamine, cadaverine, histamine, heptylamine, tyramine, spermidine, octopamine and synephrine) were evaluated according to the method mentioned by Krause et al. (1995). The detection limit for volatile phenol analysis was estimated 0.05 mg/l.

Statistical analysis

All data are expressed by means of tree replications and standard deviation (±SD), submitted for statistical analyses (ANOVA), significance was defined as $P < 0.01$. The sas statistical software package (1997) and CoStat-Statistics Software ver. 6.3 program (http://www.cohort.com/costat.html) were adopted.

3.3.1.2 Results (3a)

Analysis of D. bruxellensis *growth and inactivation under EWOA condition*

The EWOA experimental condition was performed using cells that had not been adapted to ethanol. Figures 19–21 and Table 13 show the results of a multiple series of trials performed on laboratory scale. Figure 19 shows the variation over time of the *D. bruxellensis* yeast survival (inactivation) in the wine for the different treatments: control wine, (witness); wine + cells; wine + cells + SO_2 (80 mg/l); wine + cells + LEC at 200 mA; wine + cells + LEC at 200 mA + SO_2 (30 mg/l).

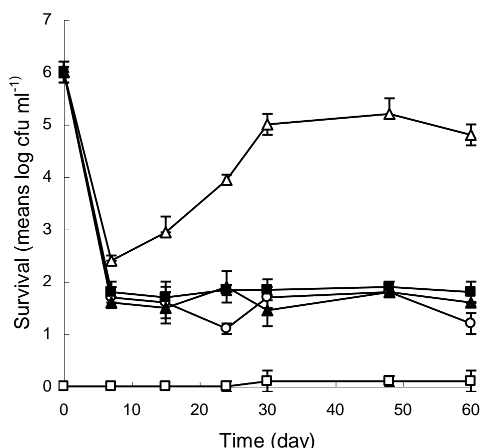

Figure 19

Survival dynamics of *D. bruxellensis* cells (CBS 4481) in Montepulciano d'Abruzzo red wine at different times and for different treatments, under the EWOA conditions. (■) witness, (only wine); (▲) control test, (wine + cells); (○) wine + cells + SO_2, 80 mg/l;(■) wine + cells + LEC at 200 mA;(▲) wine + cells + LEC (200 mA) + SO_2 (30 mg/l).

In all trials, there is a significant decrease ($p < 0.01$) of about 4 log CFU/ml yeast cell viability after 1 day compared with that at time zero in the control test (wine + cells); then, significant effects (decrease 4.5 log CFU/ml) are recorded when the control test is compared with the other treatments (LEC or SO_2 tests). As a consequence, 24 h after starting the experiment, a slight but significant decrease ($p < 0.05$) 0.5 log CFU/ml in the yeast cell viability for LEC or SO_2 treatment compared with that of the control was recorded. The viable cell count in the wine inoculated with cells showed an increase over time with greatest values of log 5.2 UFC/ml at the 30th day. Further, in all the experimental trials lasting 60 days performed with both LEC (200 mA) and SO_2 addition (80 mg/l, 30 mg/l data confirmed the inhibition of the yeast growth. No significant differences were observed among these results ($p < 0.05$). The results shown at 24 h from inoculation in wine indicates that yeast are sub-lethally injured resulting in the reduction of the number of cells; this effect could be explained by the high level of alcohol of wine adopted (13.5%). These injured cells would recover over time if there were no other treatment (control test) or would maintain a constant level of viable yeast if SO_2 were present or LEC were applied. (Dias et al., 2003). Under our experimental conditions, *D. bruxellensis* cells were never present in the wine sample of the control test until the 14th day. Figure 20 shows the mean ATP content in the yeast culture of the *D. bruxellensis* 4481 strain in red wine, for different times and different treatments. The application of 200 mA LEC leads to a reduction in the total ATP content; this reduction was already evident on the seventh day after the start of LEC treatment when compared with all other tests (significant decrease, $p < 0.01$) and it remained evident until the 60th day. Differently, the data in Figure 20 shows from the second to seventh days and after, increments of total ATP (ranging 4-5 ng/ml) are not related to the yeast viable counts as reported in Figure 19, both in test with SO_2 addition to red wine and in the treatment where LEC was applied. The explanation for the phenomenon could be related to the environmental laboratory conditions, without sterile conditions adopted. In fact, a weak microbial viable count was recorded (average log 0.5 CFU/ml). The temperature profile during and at the end of all the experiments was monitored. No variations were recorded in the time interval between the control set and electric treatments; the temperature of the wine during the LEC treatment was 20 °C.

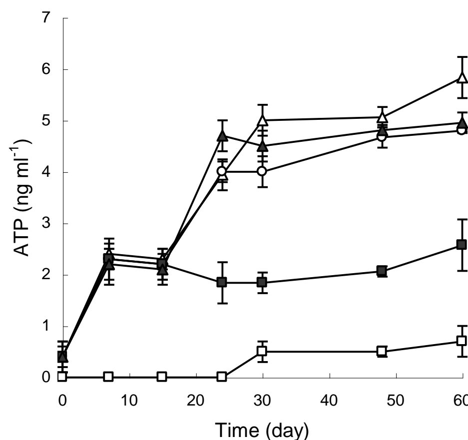

Figure 20

Mean ATP content in Montepulciano d'Abruzzo red wine at different times and for different treatments, under the EWOA conditions. (■) witness, (only wine); (▲) control test, (wine + cells); (○) wine + cells + SO_2, 80 mg/l;(■) wine + cells + LEC at 200 mA; (▲) wine + cells + LEC (200 mA) + SO_2 (30 mg/l).

Hydroxycinnamic acids (*p*-coumaric acid and ferulic acid) and volatile phenols (4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol) were determined throughout HPLC analyses. At the beginning of the experiment, the wine contained 1.96 ± 0.10 mg/l of *p*-coumaric acid and 0.41 ± 0.02 mg/l of ferulic acid. In our conditions, 4-vinyl derivates were not detected, while quantifiable amounts of 4-ethylphenol and 4-ethylguaiacol were accumulated in wine over a period of 60 days. Both the hydroxycinnamic acids were partially depleted in tests (i) and (ii), whereas in tests (iii) and (iv), in which LEC treatment was applied, no decreases were observed (Table 13). As a consequences, 4-ethyl derivates were moderately produced throughout tests (i) and (ii), whereas they were not found in (iii) and (iv). Therefore, LEC treatment was useful to avoid the off-flavours production.

Test	**Concentration (mg/l)**			
	***p*-coumaric acid**	**ferulic acid**	**4-ethylphenol**	**4-ethylguaiacol**
i)	0.35 ± 0.018	0.12 ± 0.006	0.18 ± 0.009	0.15 ± 0.008
ii)	0.57 ± 0.029	0.18 ± 0.009	0.16 ± 0.008	0.11 ± 0.006
iii)	1.90 ± 0.095	0.40 ± 0.020	0.05	0.05
iv)	1.85 ± 0.093	0.35 ± 0.018	0.05	0.05
v)	1.89 ± 0.098	0.42 ± 0.021	0.05	0.05

Table 13

Volatile phenols accumulated in wine after 60 days under the EWA conditions: (i) control test (wine + cells); (ii) wine + cells + SO_2 80 mg/l; (iii) wine + cells + LEC at 200 mA; (iv) wine + cells + SO_2 30 mg/l + LEC at 200 mA; (v) witness, (only wine). A detection limit of 0.05 mg/l has to be considered for every determination.

Analysis of D. bruxellensis *growth and inactivation under EWA conditions*

The experimental condition EWA was applied using cells that had been adapted to ethanol (10% v/v). Figures 22-25 and Table 14 show the results of the present trial performed on laboratory scale. Figure 22 shows that there are no significant variations ($p < 0.05$) between the two treatments (LEC treatment and addition of SO_2), in the viable cell count of *D. bruxellensis* strain 4481 over time. Both the LEC treatment and the SO_2 addition to the red wine resulted in a constant decrease in viable cells, although there were significant decrease effects at 24 h and after 30 days ($p < 0.01$). The mean ATP content on the *D. bruxellensis* 4481 strain in the red wine, for different times and different treatments, is shown in Figure 23. It can be seen that the data of the total ATP content were related to the variations in the yeast growth until day 30, supporting the similarity between LEC treatment and SO_2 addition in the control spoilage process. Table 14 shows the main chemical characteristics of the wines submitted under EWA conditions.

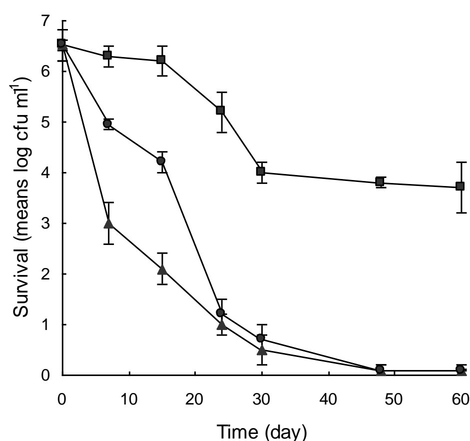

Figure 22

Survival dynamics of *D. bruxellensis* cells (CBS 4481) in Montepulciano d'Abruzzo red wine at different times and for different treatments, under the EWA conditions. (■) control test, (wine + cells); (▲) wine + cells + SO_2 80 mg/l; (●) wine + cells + LEC at 200 mA.

	i)	ii)	iii)	iv)	
Alcohol level	13.0±0.2	13.0±0.2	13.0±0.2	13.0±0.2	% (v/v)
pH	3.2±0.04	3.2±0.4	3.2±0.4	3.2±0.4	-
Total acidity[a]	6.2±0.03	5.4±0.3	5.4±0.3	5.6±0.3	g/l
Volatile acidity[b]	1.2±0.01	0.1±0.2	0.2±0.2	0.6±0.1	g/l
Reducing sugar	1.55±0.1	1.55±0.1	1.55±0.1	1.55±0.1	g/l

Table 14

Oenological parameters of Montepulciano d'Abruzzo wine after 60 days under the EWA conditions. i) control test, (wine + cells); ii) wine + cells + SO_2 80 mg/l; iii) wine + cells + LEC at 200 mA, iv) witness, (only wine). All parameters are given with their standard deviation (n=3). [a] = Expressed as tartaric acid; [b] = Expressed as acetic acid.

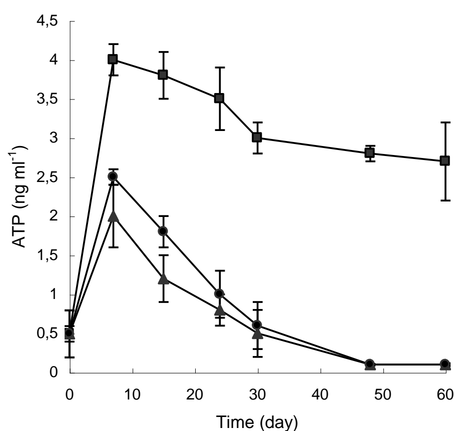

Figure 23

Mean ATP content in Montepulciano d'Abruzzo red wine at different times and for different treatments, under the EWA conditions: (■) control test, (wine + cells); (▲) wine + cells + SO_2 80 mg/l; (●) wine + cells + LEC at 200 mA.

For both the addition of SO_2 and LEC treatment trials, the final pH of the wine was 3.2. In the experiments involving LEC, the quantity of volatile acids, expressed as acetic acid, did not exceed 0.2 g/l, data similar to the results of the trials with SO_2 (0.1 g/l). The control (witness, only wine) value was 0.6 g/l, instead 1.2 g/l (control test, wine + cells). Thus, it can be seen that yeast performance was inhibited by the electric current, confirming the results of the microbiological monitoring.

Kinetics of off-flavours production, under various conditions, until 60 days are shown in Figure 24. In the control test (i) higher amounts of off-flavour compounds were produced when compared to tests (ii) and (iii); in fact, if LEC or SO_2 treatment took place the volatile phenols accumulation was very low and the levels of 4-ethylphenol and 4-ethylguaiacol increased up to 0.10 ± 0.05 and 0.05 ± 0.05 mg/l, respectively.

No formation of biogenic amines was observed in the wine; however, hexylamine, cadaverine, histamine and heptylamine were found at the beginning of the experiment. Thus, these amines were degraded in the tested conditions over time, as reported in Figure 25. Notably, LEC seems to promote this phenomenon; in particular, the concentration of cadaverine, histamine and heptylamine fell to 0 mg/l after 7 days of treatment. Similar behaviour was observed by Vigentini et al. (2008) in *D. bruxellensis* strains, which were able to produce and then to consume polyamines. Nevertheless, no physiological data are available to explain such metabolic activity, and the involvement of LEC should be better investigated.

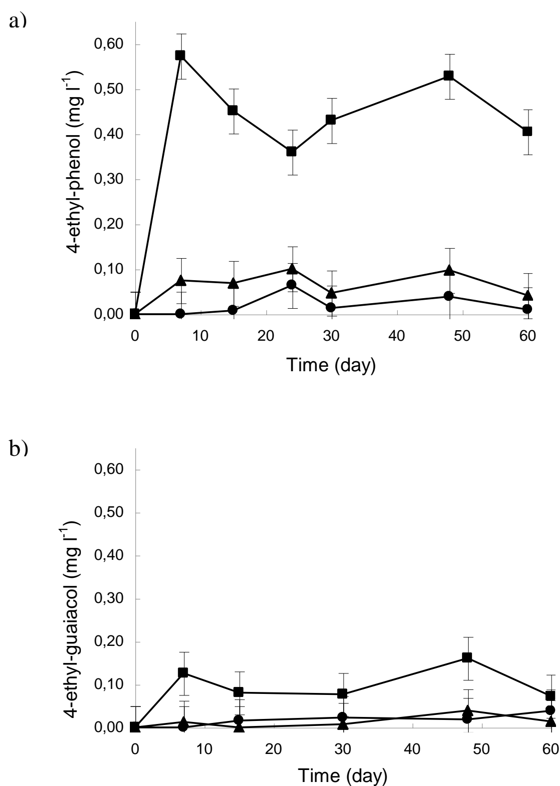

Figure 24

Kinetic of volatile phenols accumulation under the EWA conditions: a) 4-ethylphenol production; b) 4-ethylguaiacol production. (■) control test (wine + cells); (▲) wine + cells + SO_2 80 mg/l; (●) wine + cells + 200 mA. A detection limit of 0.05 mg/l has been applied.

SEM observations of *D. bruxellensis* cells after LEC treatment (200 mA at 72th h) using a pair of MMO electrodes are shown in Figure 26. The LEC treatment applied to yeast cultures caused alterations in the morphology and integrity of the cells. The rupturing of the membrane system with the loss of cell organization was evident when compared with the control [(a) untreated; (b) LEC]. Moreover, no evident changes in colour, odour or other criteria (floral, reduced, acetic) were observed at the 60th day in the wine before and after LEC treatments at 200 mA intensities.

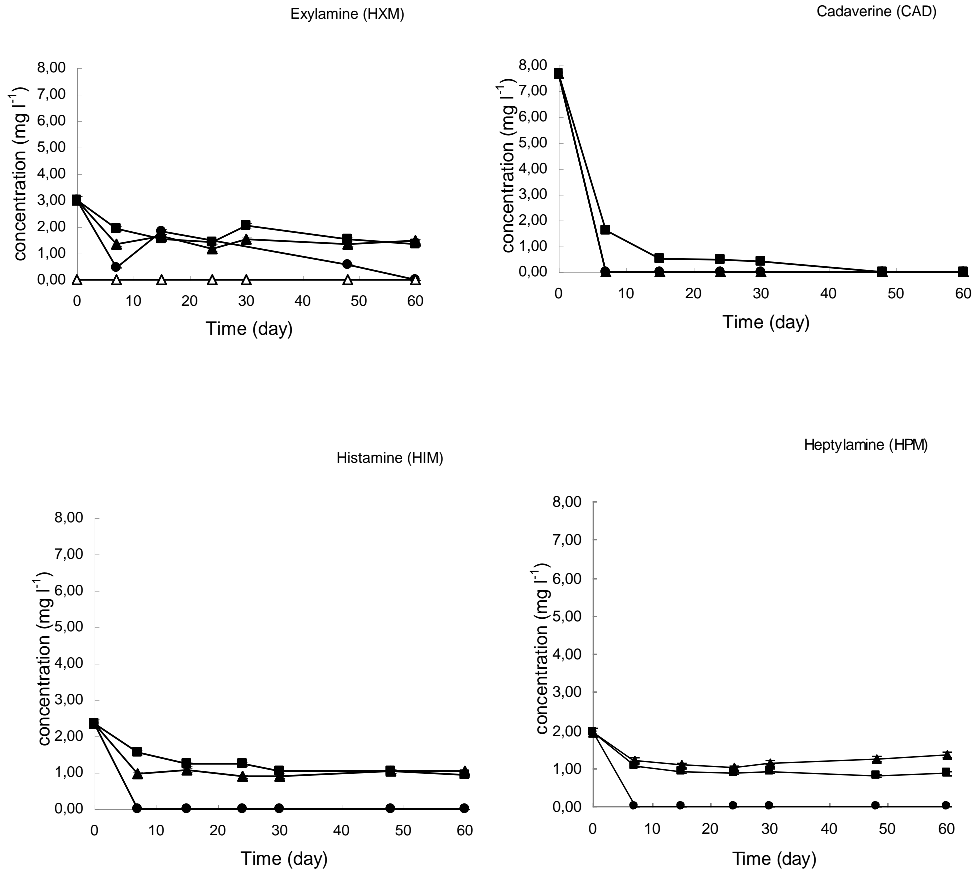

Figure 25

Kinetic of biogenic amines accumulation under the EWA conditions: (▲) control test (wine + cells); (■) wine + cells + SO_2 80 mg/l; (●) wine + cells + LEC 200 mA. A standard error of 5% has been applied; (△) witness, (only wine).

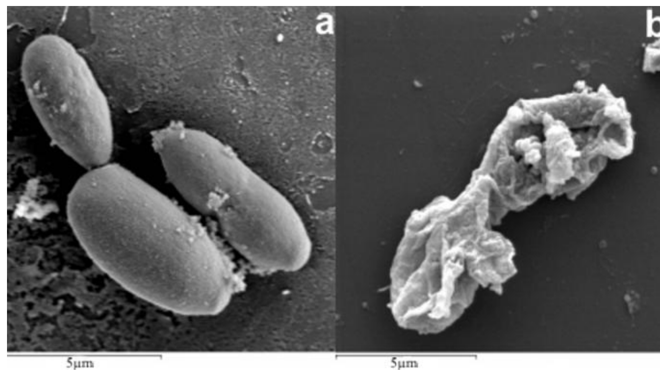

Figure 26

Scanning electron microscope observation of *D. bruxellensis* 4481 strain, under the EWA conditions. Morphological changes of 200 mA electric current application using MMO electrodes. (a) Untreated; (b) Low electric current, LEC-treated (72 h).

3.3.1.3 Discussion (3a)

A wine industry goal is to reduce the risk of wine being spoiled by microbial activity. Recent evidences suggest that 'Brett spoilage', a recurring problem, requires further analysis and the implementation of control measures (Coulter et al., 2004). To completely understand the prevention and control of wine spoilage, it is important to undertake a critical analysis of the many factors associated with the actual development of spoilage. Such investigations should include a review of the various types of spoiler yeasts, as well as an investigation into the winemaking conditions that increase the ability of such micro-organisms to flourish (Kramer & Noonan, 2004). In the present study, we focussed our attention on the effect of LEC on the viability, survival and production of metabolic compounds of *Dekkera* in wine. A pure culture of *D. bruxellensis* strain CBS4481, was adopted for our investigation as it has already been investigated in terms of its physiological behaviour (Vigentini et al., 2008). Microbiological, chemical and biochemical enzymatic assays were used to confirm the role of the LEC effect on yeast spoilage. Indeed, the effects of LEC in controlling yeast spoilage were found to be comparable with those of adding SO_2. The results from the monitoring of the laboratory scale experiments indicate a similar effect from LEC treatment as with SO_2 processing, both treatments resulting in reduced microbial cell survival in the studied red wine. Further the total ATP content, as a descriptive and predictive bio-indicator, confirmed these effects, mainly in short-term application (Ranalli et al., 2002). Recent studies have reported the effects of LEC on yeast cells. Lustrato et al. (2003) demonstrated that LEC (10, 30, 100 mA) could reduce the ATP content and viability of both *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* cultures. In addition, LEC treatment on *S. cerevisiae* resulted in a loss of integrity of the cytoplasmatic membranes. The morphological and cytological changes in *D. bruxellensis* yeast after application of electric current, by scanning electron microscope, were observed. The changes are in accordance with many studies reporting that treatment of bacteria with high intensity current causes an irreversible loss of membrane function, such as a semi permeable barrier between the yeast cell and its environment (Kekez et al., 1996). With regard to the inhibition of wild yeast spoilage, yeasts being known to be prolific acetic acid producers (Gerós et al., 2000; Fugelsgan & Zoecklein, 2003; Lourreiro & Malfeito-Ferreiro, 2003; du Toit et al., 2005), the volatile acid amounts in the trials conducted for comparison purposes were comparable with those obtained both

by combining electric current and SO_2, and separately. This aspect appears particularly important as it highlights the possible role of electric current as an antiseptic in winemaking when applied in red wine production. The present study suggests the importance of applying an appropriate LEC treatment that limits wine deterioration in terms of off-flavours synthesis. However, the detected concentration of the ethyl derivates was always lower than the perception threshold (Chatonnet, 1992) probably because of an initial low concentration of the precursors (hydroxycinnamic acids) in the tested wine. Moreover, the kinetics of volatile phenol accumulation confirms that under our conditions the use of an electric field is adequate to hinder the yeast spoilage. As regards the formation of biogenic amines, the results in the same trials suggest that LEC treatment promotes the disappearance of cadaverine, heptylamine, hexylamine and histamine from wine; further investigation is required to understand this phenomenon. This activity could be of great interest for human health as histamine is the most dangerous amine found in foods (D'Andrea et al., 2004). However, in our research we addressed a single yeast *D. bruxellensis* strain CBS4481, in one type of red wine, so the possibility that differences in strain and in wine composition could lead to different results cannot be ruled out. Therefore, it is important that future research be conducted to extend to other strains of *Dekkera/Brettanomyces* and to include fermentation both in wines derived from different grape varieties and sources and in the presence of other factors such as oxygen exposure. In conclusion, our findings show that the inactivation of wine spoilage yeasts *D. bruxellensis* (CBS 4481) using LEC treatment was obtained. The use of this technological process could improve the quality of wine without recourse to chemical additives, offering a new point of reference for the production of organic or 'biological' wines.

3.3.1.4 References (3a)

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3.3.2 Resistance to exogenous sulphite in *Dekkera/Brettanomyces bruxellensis* (3b)

D./B. bruxellensis species is characterised by a high degree of intraspecies polymorphism. Recently, this is thought to be due to a fusion event among different genomes or to an increased mutation rate resulting in the yeasts asexual state (Vigentini et al., 2011; Hellborg & Piškur, 2009). A consequence of such genetic polymorphism is the ability of this yeast to survive and grow in the extreme environment encountered in wine where sulphite is often used both to control the winemaking process and to stabilize the final product. Actually, sulphur dioxide is the main preservative used in the wine industry and it may be added as potassium metabisulphite (PMB) aqueous solutions (Ribéreau-Gayon et al., 2006). Studies about its effect on *D./B. bruxellensis* inactivation are often contradictory; it is reported that this yeast is recovered mainly from wines not protected by sulphur dioxide (Heresztyn, 1986) and some authors refer its sensitivity to values higher than 30 mg/l of free sulphur dioxide (Gerbaux et al., 2002; Chatonnet et al., 1992, 1993; Ribéreau-Gayon et al., 2006).
Few works are available in literature referring to the ability of *D./B. bruxellensis* to tolerate SO_2, they are mainly aimed to evaluate effect of sulphur dioxide on *D./B. bruxellensis* growth and viability (Barata et al., 2008) or to investigate the cell culturability taking to account the production of volatile phenols and biogenic amines (Agnolucci et al., 2010). Barata et al. (2008) showed that *D./B. bruxellensis* is not more resistant than *Saccharomyces cerevisiae* to major inhibitors like ethanol and sulphur dioxide. The mechanisms underlying the capability to survive in nutritionally-poor environments explain the proliferation of this yeast when the environmental conditions become less stressing. Their study demonstrate that the control of populations of *D./B. bruxellensis* growing in red wine can only be achieved under the presence of relatively high doses of molecular SO_2 (about 1 mg/l of molecular SO_2). Moreover, they did not observe the existence of a viable but nonculturable population (VBNC) after the addition of SO_2 as shown by du Toit et al. (2005) and Agnolucci et al. (2010). These latter reported that SO_2 induced a VBNC state and that the greater percentage of VBNC cells was identified at molecular SO_2 concentrations of 0.2-0.4 mg/l. Thus, SO_2 is a chemical stressor inducing VBNC state in *B. bruxellensis* grown in synthetic wine medium. In particular, vinyl phenols were detected in media containing VBNC or not viable *D./B. bruxellensis*, suggesting that its spoilage metabolism could be maintained during wine storage. Nowadays, no data are available on the metabolic and molecular mechanisms that control the SO_2 resistance in *D./B. bruxellensis*. As concern *S. cerevisiae*, sulphite is also a normal metabolite produced during reductive sulphate assimilation pathway. The question of how endogenous toxicity is avoided can be linked to an efficient regulation system that would minimize pools of intermediates, sulphite among them. Such resistance has been studied at the molecular level in laboratory strains to understand how SO_2 acts by modulating the transcription of some genes of the sulphur, adenine and acetaldehyde metabolism. At high SO_2 concentrations *S. cerevisiae* shows an increased level in the expression of several genes as *SSU1* or those involved in its positive regulation as *FZF1* (Aranda et al., 2006). *SSU1* is a housekeeping gene that encodes a plasma membrane protein with a central role in a network of proteins conferring sulphite tolerance; this system is involved in the extrusion of sulphites from the cytoplasm (Avram & Bakalinsky, 1997; Divol et al., 2005). Knock-out mutants lacking to *SSU1* pump are less resistant to SO_2 in comparison to those strains where the protein is regularly produced (Park & Bakalinsky, 2000). An important feature of sulphite is its chemical reactivity, particularly with carbonyl groups (Liu & Pilone, 2000). This fact explains why a high production of acetaldehyde by a yeast strain leads to increased sulphite resistance (Casalone et al., 1992; Xu et al., 1994). Acetaldehyde and sulphite react to form a stable and nontoxic product, 1-hydroxyethane sulphonate. As a marker of acetaldehyde metabolism, the main cytosolic enzyme is the aldehyde dehydrogenase encoded by *ALD6* gene. The opposite is also true, acetaldehyde increases transcription of sulphur metabolism

coding genes, such as *MET16* (Aranda et al., 2006), establishing another link between both compounds. Regarding sulphur metabolism, it is worth mentioning that methionine addition decreases the yeast thermo-tolerance (Jakubowski & Goldman, 1993), suggesting that sulphur metabolism plays another role in stress resistance. Transcription of genes encoding proteins involved in metabolism strongly depends on media composition in *S. cerevisiae*. Regarding sulphur metabolism, yeast is able to import sulphate from the medium and reduce it first into sulphite and then into sulphide to finally incorporate it into sulphur amino acids such as methionine and cysteine (Thomas & Surdin-Kerjan, 1997). However, whenever a source of organic sulphur is present, this sulphate assimilation pathway is switched off. Methionine is the key regulator of this event (Thomas & Surdin-Kerjan, 1997). *MET* genes transcription is repressed in laboratory strains when methionine is present in the medium at a concentration of over 0.05 mM. In a similar way, adenine in the media represses its own biosynthetic pathway (Daignan-Fornier & Fink, 1992), acting on the first step of the path, the enzyme phosphoribosylpyrophosphate amidotransferase coded by *ADE4*.

3.3.2.1 Materials and Methods (3b)

Strain collection

One hundred and nine isolates of the UC Davis Viticulture and Enology Culture Collection belonging to *B. bruxellensis* species were analysed in this study. UCD and other collection designations and isolation sources are given in Table 15. Strains were maintained at -80 °C in YM medium (Dextrose 10 g/l, Peptone 5 g/l, Malt extract 3g/l, Yeast extract 3g/l) with glycerol 20% (v/v). Yeasts were revitalised in YM for 3-5 days at 30 °C before inoculation of metabolomic and physiological assays.

SO_2 resistance screening

To find strains able to grow at high levels of sulphur dioxide, the whole yeast collection was screened in *Brettanomyces* medium (BM) obtained from a laboratory defined minimal medium (Conterno et al., 2006) added with 5% (v/v) ethanol and acidified to pH 3.6 with orthophosphoric acid 85% (v/v). Growth tests were done in 96-well microplates with 0.2 ml of medium at 23˚C in static condition (Figure 27).

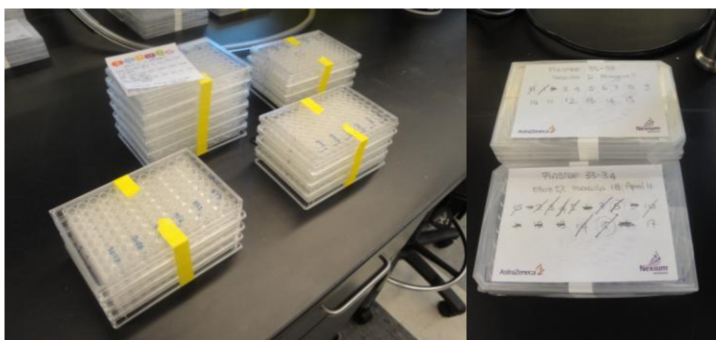


Figure 27

Microplate experiments performed during SO_2 resistant strains screening.

Cells at about 0.4 OD_{650nm} in BM were inoculated at 1% (v/v) in each well. Each microplate carried more than one strain; to test possible cross-contaminations a column of wells with uninoculated BM separated wells containing strains. Six different concentrations of molecular (mol.) SO_2 (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/l) were tested. Potassium metabisulphite (PMB) was prepared as a 20 g/l stock solution and it was added to BM immediately before dispensing it into the wells. Free and combined SO_2 concentrations were measured by an automatic iodometric titration (I_2 0.05 M ; Carlo Erba, Italy) with Ripper method (Iland et al., 1993) (Titromatic 2S, Crison). The differences between added and assayed free SO_2 were negligible. Each strain and condition was carried out in four replicates. Cell growth was monitored for 21 days following the optical density increase at 650 nm using an Emax precision microplate reader (Molecular Devices, Inc., California, USA). Data were collected by SOFTmax software. Positive growth was assigned if 75% replicates reached $OD_{650nm} \geq 0.05$.

Metabolome sampling and quenching

Two different UCD strains were grown in BM without and with 0.2, 0.4 and 0.6 mg/l mol. SO_2 for metabolomic analysis. Cells were inoculated at 1% (v/v) in hermetic and static bottles maintaining a 1:8 ratio between cellular culture and headspace volume. Incubation temperature was at 23˚C (Figure 28). Seven replicates were prepared for each growth condition; six of them were used to collect the cells for the metabolic profiles and one was used as control to follow the cell growth by OD_{650nm} measurement. At about 1.0 ± 0.1OD_{650nm}, cells were collected for the metabolomic analysis. One millilitre of yeast samples pulled from the bottom of the bottles was added to 1 ml of 100% (v/v) cold (-80 °C) methanol to arrest metabolism instantaneously. Then, cells were briefly vortexed, and immediately pelleted in a refrigerated microfuge (4 °C) at 10.000x*g* for 2 min. Supernatants were discarded and pellets were resuspended in 1 ml extraction buffer of methanol/water (1:1, v/v, -23 °C) and transferred in a new refrigerated tube that was pre-weighed for the purpose of determining cell dry weight. Samples were centrifuged at 4 °C for 2 min at 10.000x*g*, and the supernatants discarded. Pellets were stored at -75˚C until all samples were ready to be collected. Before to submit the samples to the Metabolomics Core facility in Davis (CA, USA) for extraction, derivatisation, and GCMS-TOF analysis, pellets were dried in a speed vacuum microfuge for at least 4 hours, weighed, and stored in the ultra-cold freezer until they were

analysed.

Figure 28

Hermetic bottles used for metabolomics trials

UCD #	Source	SO_2 resistance (mg/l)	UCD #	Source	SO_2 resistance (mg/l)	UCD#	Source	SO_2 resistance (mg/l)
605	Beer	0.1	2091	Wine, France	0.1	2494	Wine, California	0.2
615	Wine	0.1	2092	Wine, Germany	0.1	2409	Wine	0.1
734	Wine	S	2093	Wine, New York	0.2	2503	Wine, South Africa	S
752	Wine, France	0.2	2094	Wine	0.1	2504	Wine, South Africa	0.2
860	Unknown	S	2347	Wine, Chile	S	2505	Wine, South Africa	S
2027	Beer, South Africa	0.2	2254	Wine, Chile	0.4	2506	Wine, South Africa	S
2029	Wine	0.1	2255	Wine, California	0.4	2507	Wine, Uruguay	S
2030	Wine	0.1	2385	Wine, Washington	0.1	2508	Wine, Uruguay	0.1
2041	Wine, Thailand	S	2386	Wine, Washington	S	2509	Wine, Uruguay	0.4
2043	Wine, California	0.2	2387	Wine, Washington	0.2	2511	Wine, Virginia	0.2
2049	Wine, New Zealand	0.1	2388	Wine, California	0.2	2512	Apple Cider, Vermont	S
2047	Wine, California	0.1	2398	Wine, Portugal	0.1	2513	Wine	0.1
2050	Wine, New Zealand	S	2399	Wine, Portugal	0.2	2549	Wine, Spain	0.2
2048	Wine, California	0.2	2400	Wine, Portugal	S	2551	Beer	0.2
2046	Wine, California	0.2	2401	Wine, Portugal	0.2	2618	Wine, South Africa	0.1
2054	Wine, California	0.2	2402	Wine, Portugal	0.1	2619	Wine, Chile	0.2
2053	Wine, California	0.1	2403	Wine, Washington	0.2	2620	Wine, Pennsylvania	0.2
2051	Wine, California	0.1	2404	Wine, Portugal	0.1	2748	Wine	0.2
2052	Wine, California	0.2	2405	Wine, Portugal	0.2	2749	Wine	0.2
2058	Must	0.1	2406	Wine, Portugal	S	2750	Wine	0.2
2059	Wine	0.2	2407	Wine, Washington	S	2751	Wine	0.4
2060	Wine, Chile	0.4	2408	Wine, France	0.4	2752	Wine, Italy	0.1
2067	Wine, California	0.2	2396	Wine, Portugal	0.1	2753	Wine, Italy	0.2
2065	Wine, Italy	0.1	2397	Wine, Portugal	0.1	2754	Wine, Italy	0.2
2062	Wine	0.2	2420	Wine, Oregon	0.4	2757	Wine, France	0.4
2063	Wine	0.2	2421	Wine, Oregon	0.4	2759	Wine, South Africa	S
2075	Wine, California	0.2	2422	Wine, Mexico	0.4	2760	Cider, Vermont	0.1
2076	Wine, British Columbia	0.2	2423	Wine, Mexico	0.6	2758	Beer, Chile	S
2077	Wine, Chile	0.2	2426	Wine, Italy	0.6	2776	Wine, California	0.4

UCD #	Source	SO_2 resistance (mg/l)	UCD #	Source	SO_2 resistance (mg/l)	UCD#	Source	SO_2 resistance (mg/l)
2078	Beer, Belgium	0.2	2456	Wine California	0.2	2777	Wine, California	0.2
2080	Wine	0.2	2459	Wine, California	0.1	2807	Wine, France	0.1
2081	Wine	0.2	2484	Wine, Colorado	S	2809	Wine, Australia	0.4
2082	Wine	0.1	2485	Wine, Carolina	S	2810	Wine, Australia	0.2
2083	Wine	0.4	2486	Wine, Thailand	S	2821	Must, California	0.2
2084	Wine	0.4	2490	Wine, California	0.2	2826	Wine, California	0.1
2085	Wine	0.4	2492	Wine, California	0.4			
2066	Wine, Italy	0.2	2493	Wine, Colorado	0.2			

Table 15

D./B. bruxellensis strains collection with levels of molecular SO_2 resistance. S = sensitive strain.

Metabolomic data analysis

Metabolic data were processed using MetaboAnalyst software, a web-based platform for the analysis of quantitative metabolomics data (Xia & Wishart, 2011). After normalisation of the areas of the acquired peaks against the dry weights of the corresponding cell samples, the data sets were submitted to a preliminary statistical analysis for the removal of the outliers. The “Outliers detection” model that uses Random Forest classification method for variable selection (Breiman, 2001) was used. Then, statistical analyses were performed using both univariate and multivariate methods. For the univariate analysis, Analysis of Variance (ANOVA) was chosen when two or more than two groups of data were compared each other. Threshold value $p < 0.05$ was considered significant. Multivariate data analysis was carried out by Principal Component Analysis (PCA) and Partial Least Square-Discriminant Analysis (PLS-DA). Results from PLS-DA model were confirmed by cross-validation indicating the components that best explained the data distribution. In particular, it shows if the separation among groups is statistically significant or is due to random noise. Permutation test (100 permutations) was applied on PLS-DA model to obtain a final validation of the results; in this case it points out if the separation between clusters is statistically significant or due to random noise. Finally, the important features (or compounds in our case) were identified by Variable Importance in Projection (VIP) score which is given as average across all the PLS-DA selected components. Metabolite Set Enrichment Analysis (MSES) was run choosing the Quantitative enrichment analysis (QEA). QEA is based on the GlobalTEST algorithm (Aittokallio et al., 2006), which uses a generalized linear model to estimate the association between concentration profiles of a matched metabolite set and the conditions under study. Metabolic Pathway Analysis (MetPA) is a high-level functional interpretation of data that allows the identification of metabolic pathways mainly involved in the condition under study. MetPA was run choosing the *S. cerevisiae* pathway library, setting GlobalANCOVA and Out-Degree Centrality algorithms for the Pathway Enrichment and the Pathway Topology analyses, respectively (Aittokallio et al., 2006; Hummel et al., 2008).

Physiological trials

Assays were carried out to identify supplements that could increase the SO_2 resistance. Experiments were performed in microplates. Proline (1.7 g/l), or glycine (3.5 mg/l), or adenine (15 mg/l), or methionine (20 and 40 mg/l), or casamino acids (0.18 g/l) (BD Bacto™ Casamino Acids, Vitamin Assay, Oxford, UK) were added to BM from sterile stock solutions immediately before to dispense it into the wells. Proline, glycine and methionine concentrations were obtained from Amerine & Ough (1974), whereas adenine and casamino acids amounts were chosen as suggested by Aranda et al. (2006) and Vigentini et al. (2008). Depending on the strain under study, an opportune amount of PMB stock solution 20 g/l was added to BM as described in the "screening experiment" section. In this case each strain and condition was carried out in eight replicates. Growth conditions were set as described for the screening experiment.

3.3.2.2 Results and discussion (3b)

SO_2 resistance in B. bruxellensis

A first screening was carried out at 5% (v/v) ethanol concentration and using increasing mol. SO_2 amounts (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/l) (Table 15). Approximately 18% of the yeast isolates was sensitive to the presence of sulphur dioxide in the medium and unable to grow within 21 days from the inoculation., On the other hand, about 25% of strains grew at 0.1 mg/l mol. SO_2, 40% at 0.2 mg/l and 15% at 0.4 mg/l after 15 days of inoculation. Only two strains (UCD2423 and UCD2426) grew at 0.6 mg/l mol. SO_2; figure 29 shows the kinetics of growth of the most resistant strains. Moreover, some strains grew in BM containing mol. SO_2 concentrations higher than 0.6 mg/l after 21 days from the inoculation, but in this case the growth was not consistent with the increasing of sulphur dioxide concentration or even among the replicates. To test if this behaviour could indicate a cell adaptive mechanism to survive at a high SO_2 level, strains proliferating at 1 mg/l mol. SO_2 were re-inoculated in BM containing 1 mg/l mol. SO_2, after a growth step in a rich medium. No growth was observed within 6 months.
Another screening of the yeast collection in BM containing 10% (v/v) ethanol at pH 3.6 at 0.5 and 1 mg/l mol. SO_2 was performed. All isolates failed to grow on both conditions. The ethanol concentration seems to increase the sensitivity to sulphite suggesting that a membrane system, such as the sulphite efflux pump of *S. cerevisiae*, could be present in this species. With the aim to reveal the presence of the *SSU1* pump also in this species, PCR reactions and Southern experiments were carried out using specific primers and probes designed for conserved regions of homologous and putative *SSU1* sequences in *S. cerevisiae* and phylogenetically related yeast (*Debaryomyces hansenii*, *Yarrowia lipolytica*, *Candida* spp., and *Kluyveromyces lactis*). No significant results were obtained suggesting that, if present, *SSU1* gene has a low similarity with that of the tested yeasts (data not shown).

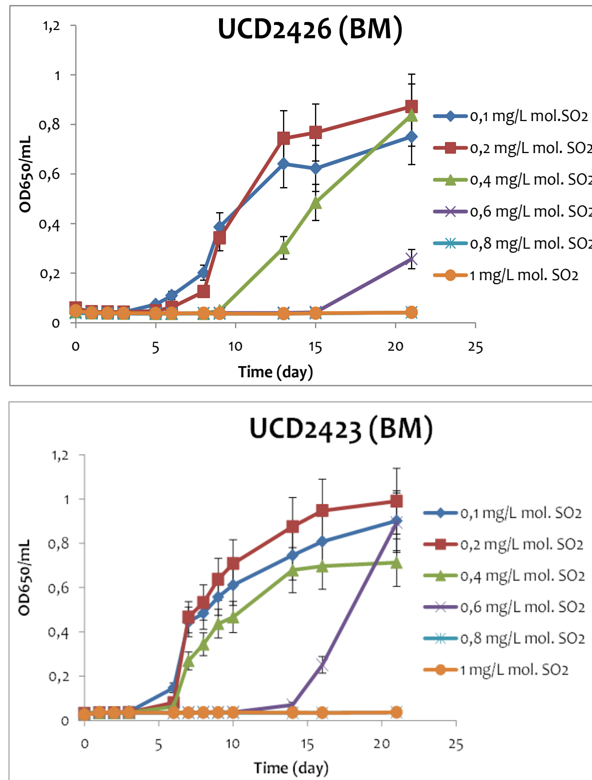


Figure 29

Kinetics of growth of *D./B. bruxellensis* UCD2423 and UCD2426 strains at different molecular SO_2 concentrations.

Metabolomic profiling

The experimental design for metabolomic analyses involved those isolates that were able to grow up to 0.6 mg/l mol. SO_2 during the screening tests. Nevertheless, probably due to oxygen limitation encountered during the incubation into hermetic flasks, these strains were unable to proliferate at the highest concentration investigated. However, the metabolomics profiles of the two strains were generally similar in the tested growth conditions. In particular, UCD2423 and UCD2426 grew in BM containing up to 0.2 and 0.4 mg/l mol. SO_2, respectively. In total, 254 intracellular metabolites were detected and quantified; 133 of them were classified as unknown compounds. Among the known metabolites we found compounds belonging to different classes such as alcohols, amino acids, organic acids, carbohydrates, phosphorylated intermediates and fatty acids (Table 1, Table 2, and Appendix B).

Univariate and multivariate analyses of the data

First of all, samples were grouped on the basis of the growth condition: samples that were grown in BM without sulphur dioxide were enclosed in the "group 1", whereas the ones that were grown at 0.2 and at 0.4 mg/l mol. SO_2 were gathered in "group 2" and "group 3", respectively. Before to submit the metabolomic data to statistical analysis a pre-processing step, consisting in an exploratory analysis of the whole pool of compounds, was performed to remove the outliers. Actually, samples selection is a critical task in identifying a subset of relevant features for the subsequent analysis; for example, PCA is very sensitive to outliers and, therefore, their removal is usually needed in order to obtain good distribution results (Xia & Wishart, 2011). Thirty samples were pre-processed (each including 247 compounds) by a preliminary multivariate analysis; 12 samples of the group 1, 12 samples of the group 2 and 6 samples of the group 3. This step allowed the identification of 5 outliers that were removed from the following data elaboration (Figure 30).

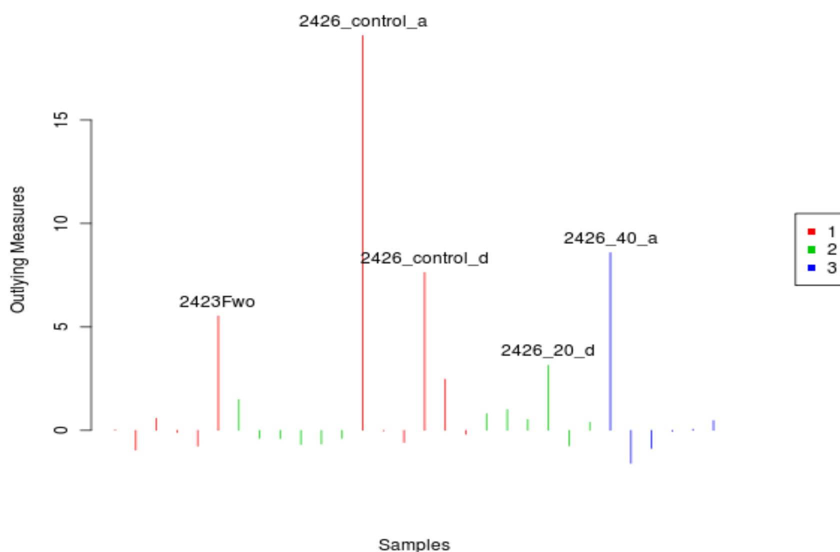


Figure 30

Detection of the outliers among the 30 samples that were analysed. Control or wo = growth condition without sulphite; 20 = growth condition at 0.2 mg/l mol. SO_2; 40 = growth condition at 0.4 mg/l mol. SO_2. Letters refer to replicates. 1= red, growth condition without SO_2; 2 = green, growth condition at 0.2 mg/l mol. SO_2; 3 = blue, growth condition at 0.4 mg/l mol. SO_2.

In total, the statistical analysis started with the identification of the significant features among 25 samples (9 samples of the group 1, 11 from the group 2 and 5 samples from the group 3). Firstly, an univariate analysis method that describes the variation of a single variable (compound) between two or more class labels (experimental conditions) was applied. In this case we selected one-way ANOVA that provides a preliminary overview about features that are potentially significant in

discriminating the conditions under study. Setting the *p* value threshold at 0.05, 143/254 compounds appeared to be significant features indicating that approximately 56% of the data set was influenced by the SO_2 factor (Figure 31).

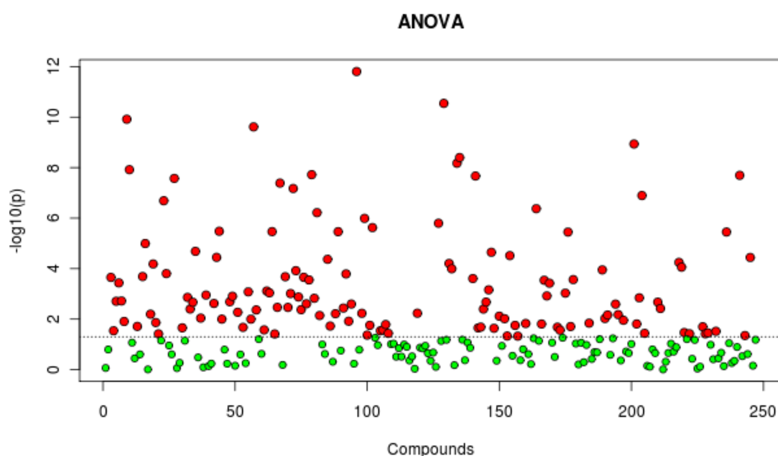


Figure 31

Important features identified by ANOVA plot with *p* value threshold 0.05.

Secondly, a multivariate analysis for the simultaneous observation of more than two variables was performed. It is ideal in analysing metabolomic data that usually consist of dozen of features (compounds), many of which change as a function of the experimental conditions. The PCA plot showed that about 94% of the variation was explained by the first two principal components. Moreover, samples corresponding to replicate growth measurements grouped together (Figure 32a). Figure 32b displays the score plot using PC1 and PC2; an almost complete overlapping between samples belonging to group 1 and 2 was observed. Thus, PLS-DA model was applied to resolve this dispersion. Although a better separation was obtained with PSL-DA compared with the PCA, a partial covering between the two groups was still present (dispersion validated by permutation tests ($p < 0.01$)) (Figure 33a and 33b). This result suggested that a mol. SO_2 concentration ≤ 0.2 mg/l was not enough to significantly influence the metabolism of both resistant strains. PLS-DA loading plots showed that most variables have similar weight in the three groups under study (Figure 33c); in particular, VIP score displayed that among the known compounds arabitol, fructose 6-phosphate, and ribitol were the biomarkers responsible for the clusters separation (Figure 33d). In general, statistical analysis indicates that a sulphite resistant strain if growing in presence of a low concentration of mol. SO_2 does not change own intracellular metabolism. On the other hand, we obtained a good separation patterns (PCA and PLS-DA) of the data from the condition at 0.4 mg/l mol. SO_2 (group 3) showing that distinctively metabolic characteristics appeared under this stress condition.

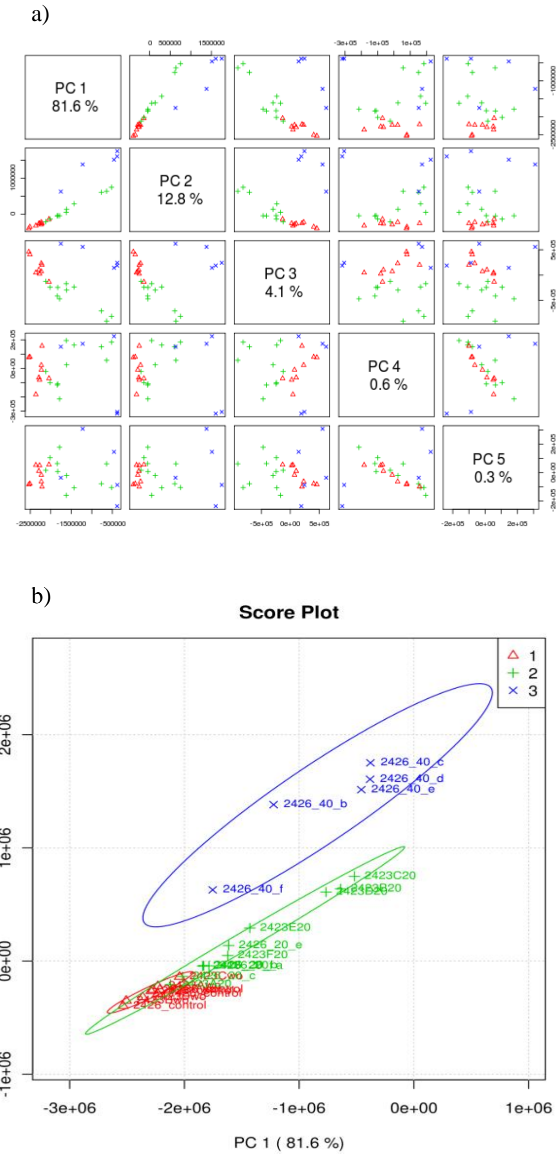


Figure 32

PCA of the samples from group 1 (red, growth condition without SO_2), group 2 (green, growth condition at 0.2 mg L-1 mol. SO_2) and group 3 (blue, growth condition at 0.4 mg/l mol. SO_2): a) pairwise score plots between the selected PCs. The explained variance of each component is shown in the corresponding diagonal cell; b) score plot between PC1 and PC2. The explained variances are shown in brackets.

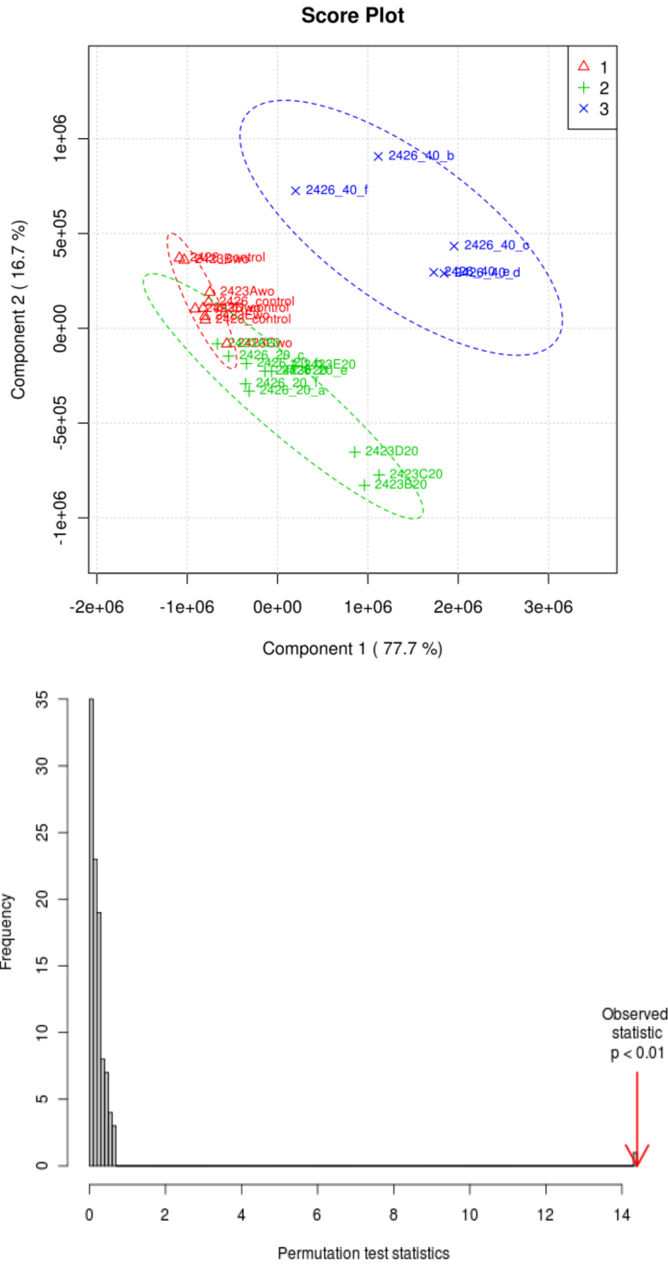
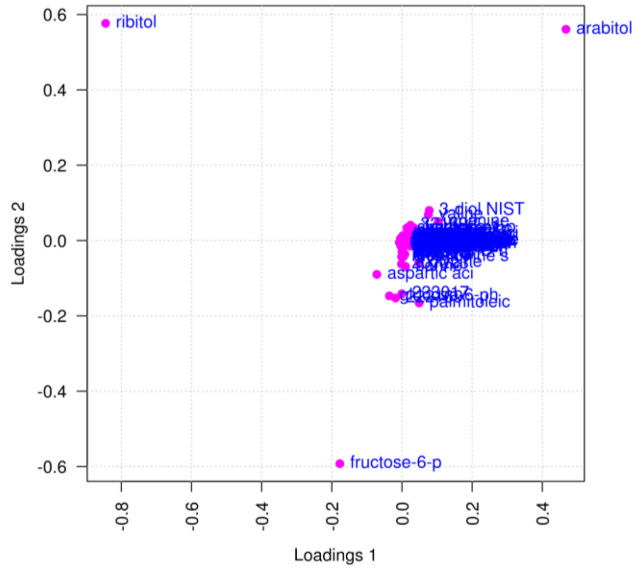


Figure 33

PLS-DA model applied on samples from group 1 (red, growth condition without SO_2), group 2 (green, growth condition at 0.2 mg/l mol. SO_2) and group 3 (blue, growth condition at 0.4 mg/l mol. SO_2): a) score plot for the selected PCs. The explained variances are shown in brackets; b) PLS-DA model validation by permutation tests based on separation distance. The p value based on permutation is $p < 0.01$.



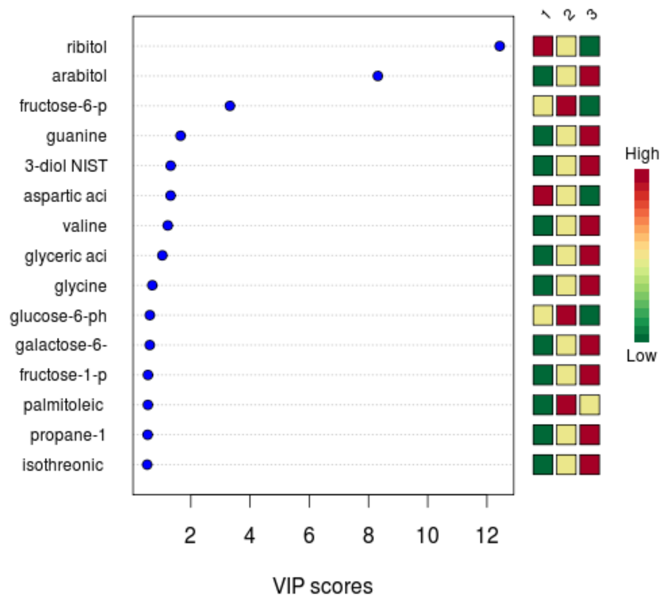


Figure 33 (continue)

PLS-DA model applied on samples from group 1 (red, growth condition without SO_2), group 2 (green, growth condition at 0.2 mg/l mol. SO_2) and group 3 (blue, growth condition at 0.4 mg/l mol. SO_2): c) loading plot for the selected PCs; d) important features identified by PLS-DA. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

Arabitol, fructose 6-phosphate, and ribitol were indicated as potential biomarkers by statistical analysis. Arabitol and ribitol are both involved in pentose and glucuronate interconversion (Figure 34); arabitol can be converted in D-xylose, from xylitol, which enters in the starch and sucrose metabolism or processed to arabinose to go in the amino sugar and nucleotide metabolism. Ribitol is introduced in the pathway from riboflavin metabolism to supply pentose such as D-xylulose, starting from D-ribulose. With the increasing in the SO_2 concentration, we observed a decreasing in the amount of the xylose, xylitol and ribitol intermediates, a stable trend in the arabitol values, and an increasing of the arabinose concentration, suggesting that the pentose and glucuronate interconversion pathway is directed toward the amino sugar and nucleotide metabolism. This scenario could be linked to the starch and sucrose metabolism, that provides xylose by glucose 1-phosphate, since the metabolomic measurement showed a strong increasing of glucose 1-phosphate at 0.2 mg/l mol. SO_2 followed by a decreasing at 0.4 mg/l mol. SO_2. These data suggested that, yeast actively promotes the conversion of the carbohydrate intermediates into amino sugars under the presence a high concentration of mol. SO_2. In fact, an increasing of UDP-N-acetyl-D-glucosamine and fructose 6-phosphate was also observed; the former is the central hub metabolite of amino sugar and nucleotide metabolism and it can be used as a precursor to fructose 6-phosphate involved in fructose/mannose metabolism.

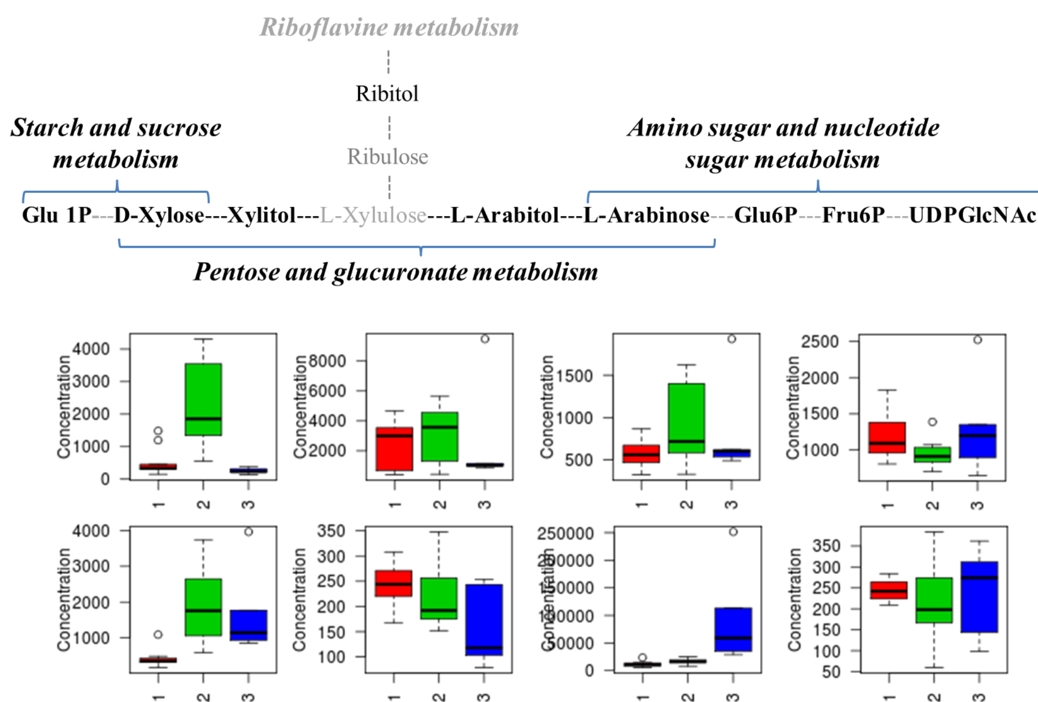


Figure 34

Schematic representation of pentose an glucuronate, starch and sucrose, and amino sugar and nucleotide sugar metabolisms. Intermediates in light grey were not identified during metabolomics analysis. The compound graphs show the concentration distribution of the corresponding metabolite calculated by ANOVA (error bars show the range of data $p < 0.01$). 1: growth condition without SO_2; 2: growth condition at 0.2 mg/l mol. SO_2; 3: growth condition at 0.4 mg/l mol. SO_2. Boxplots order follows the same compound order from left to right.

Metabolic response under SO_2 exposure by MetPA

To look for metabolic pathways involved in SO_2 stress response, all known compounds were analysed by MetPA selecting the *S. cerevisiae* pathway library as a model since it is the closest available database to *B. bruxellensis*. Forty four metabolic pathways resulted to be affected by exogenous sulphite concentration (Table 16). Metabolome view showed that most pathways, characterised by high X and Y values, matched with amino acids (alanine, aspartate, glutamate; arginine/proline; glycine, serine, threonine; β-alanine; cyanoamino acid), carbohydrates (glyoxylate/dicarboxylate; fructose/mannose; pentose/glucuronate; starch/sucrose), and energy (nitrate) metabolisms, and the aminoacyl-tRNA biosynthesis pathway, which is involved in the translation process of genetic information(Figure 35). The highest Pathway Impact value (X) was attributed to alanine, aspartate and glutamate metabolism, whereas arginine and proline pathway obtained the highest weight in terms of compound concentration (Y) (Figure 35, Table 16).
As regards alanine, aspartate and glutamate metabolism, a decreasing trend of alanine amount was measured while aspartate concentration was stable. Glutamic acid increased at 0.2 mg/l mol. SO_2 to fall down at a very low concentration at 0.4 mg/l mol. SO_2 (Figure 36). Glutamate is directly processed in several other metabolisms such as arginine and proline, butanoate, cyanoamino acids, glutathione, nitrogen, and porphyrine that here were found to be involved in SO_2 stress response. Among them, arginine and proline metabolism received the highest Y value in the metabolome view (Figure 35). This result could suggest that glutamate conversion into proline has a relatively high impact on the yeast metabolism. In *S. cerevisae*, proline can be produced by glutamate or by the ornithine transamination . Our data showed the there was an increased proline production and a decreasing in ornithine concentration under SO_2 exposure (Figure 36 and 37).

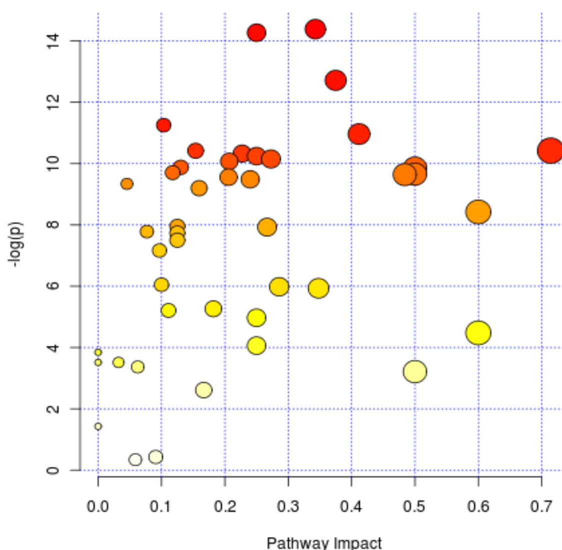


Figure 35

Summary of Metabolomic Pathway Analysis (MetPA). The panel displays all the matched pathways as circles. The colour and size of each circle is based on *p* values and pathway impact values, respectively. Y coordinates (-log(p)) originated from the *p* values calculated with the Enrichment Analysis algorithm, that takes into account the compound concentration values, whereas X coordinates (Pathway Impact) were calculated from the Pathway Topology Analysis algorithm, that considers the links among the molecules in the biological pathways.

	Total Cmpd	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Arginine and proline metabolism	37	11	5.66E-07	6.25E+00	2.49E-05	1.40E-05	0.34
Amino sugar and nucleotide sugar metabolism	24	5	6.35E-07	6.20E+00	2.73E-05	1.40E-05	0.25
Aminoacyl-tRNA biosynthesis	67	17	3.01E-06	5.52E+00	1.26E-04	4.41E-05	0.37
Pyruvate metabolism	23	2	1.30E-05	4.89E+00	5.31E-04	1.43E-04	0.10
Fructose and mannose metabolism	17	5	1.73E-05	4.76E+00	6.93E-04	1.53E-04	0.41
Alanine, aspartate and glutamate metabolism	20	7	2.98E-05	4.53E+00	1.16E-03	1.69E-04	0.71
Butanoate metabolism	17	3	2.99E-05	4.52E+00	1.16E-03	1.69E-04	0.15
Valine, leucine and isoleucine biosynthesis	24	4	3.27E-05	4.49E+00	1.21E-03	1.69E-04	0.23
Pantothenate and CoA biosynthesis	16	3	3.56E-05	4.45E+00	1.28E-03	1.69E-04	0.25
Valine, leucine and isoleucine degradation	16	3	3.91E-05	4.41E+00	1.37E-03	1.69E-04	0.27
Citrate cycle (TCA cycle)	20	5	4.22E-05	4.37E+00	1.44E-03	1.69E-04	0.21
Glutathione metabolism	23	4	5.14E-05	4.29E+00	1.69E-03	1.78E-04	0.13
Nitrogen metabolism	8	5	5.26E-05	4.28E+00	1.69E-03	1.78E-04	0.50
Porphyrin and chlorophyll metabolism	20	2	6.10E-05	4.21E+00	1.89E-03	1.79E-04	0.12
Glyoxylate and dicarboxylate metabolism	14	4	6.41E-05	4.19E+00	1.92E-03	1.79E-04	0.50
Glycine, serine and threonine metabolism	26	6	6.51E-05	4.19E+00	1.92E-03	1.79E-04	0.48
Cysteine and methionine metabolism	33	5	7.08E-05	4.15E+00	1.98E-03	1.83E-04	0.21
Pentose phosphate pathway	18	4	7.59E-05	4.12E+00	2.05E-03	1.85E-04	0.24
Phenylalanine, tyrosine and tryptophan biosynthesis	22	3	8.81E-05	4.06E+00	2.29E-03	2.04E-04	0.05
Purine metabolism	60	10	1.01E-04	3.99E+00	2.53E-03	2.23E-04	0.16
Cyanoamino acid metabolism	10	5	2.20E-04	3.66E+00	5.28E-03	4.61E-04	0.60
Methane metabolism	11	2	3.53E-04	3.45E+00	8.11E-03	6.91E-04	0.12
Glycerolipid metabolism	15	3	3.61E-04	3.44E+00	8.11E-03	6.91E-04	0.27
Sphingolipid metabolism	11	1	4.18E-04	3.38E+00	8.77E-03	7.66E-04	0.08
Pyrimidine metabolism	35	4	4.38E-04	3.36E+00	8.77E-03	7.70E-04	0.12
Nicotinate and nicotinamide metabolism	9	1	5.53E-04	3.26E+00	1.05E-02	9.36E-04	0.12
Glycolysis or Gluconeogenesis	24	2	7.73E-04	3.11E+00	1.39E-02	1.26E-03	0.10
Tyrosine metabolism	19	2	2.36E-03	2.63E+00	4.01E-02	3.71E-03	0.10
Galactose metabolism	17	5	2.53E-03	2.60E+00	4.04E-02	3.83E-03	0.29
Starch and sucrose metabolism	18	6	2.65E-03	2.58E+00	4.04E-02	3.89E-03	0.35
Lysine degradation	16	2	5.18E-03	2.29E+00	7.25E-02	7.35E-03	0.18
Tryptophan metabolism	27	1	5.47E-03	2.26E+00	7.25E-02	7.52E-03	0.11
Propanoate metabolism	14	2	6.95E-03	2.16E+00	8.34E-02	9.26E-03	0.25
beta-Alanine metabolism	7	3	1.14E-02	1.94E+00	1.25E-01	1.47E-02	0.60
Lysine biosynthesis	19	4	1.72E-02	1.76E+00	1.72E-01	2.17E-02	0.25
Biosynthesis of unsaturated fatty acids	42	3	2.14E-02	1.67E+00	1.93E-01	2.62E-02	0.00
Fatty acid metabolism	28	1	2.98E-02	1.53E+00	2.38E-01	3.45E-02	0.03
Fatty acid biosynthesis	37	1	2.98E-02	1.53E+00	2.38E-01	3.45E-02	0.00
Inositol phosphate metabolism	19	1	3.45E-02	1.46E+00	2.38E-01	3.90E-02	0.06
Pentose and glucuronate interconversions	12	5	4.02E-02	1.40E+00	2.38E-01	4.43E-02	0.50
Sulfur metabolism	13	2	7.35E-02	1.13E+00	2.94E-01	7.88E-02	0.17
Phenylalanine metabolism	7	1	2.40E-01	6.20E-01	7.20E-01	2.51E-01	0.00
Glycerophospholipid metabolism	26	2	6.51E-01	1.86E-01	1.00E+00	6.66E-01	0.09
Terpenoid backbone biosynthesis	15	1	7.12E-01	1.47E-01	1.00E+00	7.12E-01	0.06

Table 16

The table shows the detailed results from the pathway analysis. In particular, the Total is the total number of compounds in the pathway; the Hits are the actually matched number from the user uploaded data; the Impact is the pathway impact value calculated from pathway topology analysis. The Raw *p* is the original *p* value calculated from the enrichment analysis. Since many pathways at the same time were tested, the statistical *p* values were further adjusted for multiple testings, such as the Holm-Bonferroni and the False Discovery Rate (FDR) methods (Holm, 1979; Benjamini & Hochberg, 1995).

Figure 36

Arginine and proline metabolism in *S. cerevisiae*. Box plots on the left describe the variation of: a) glutamic acid; b) proline; c) ornithine

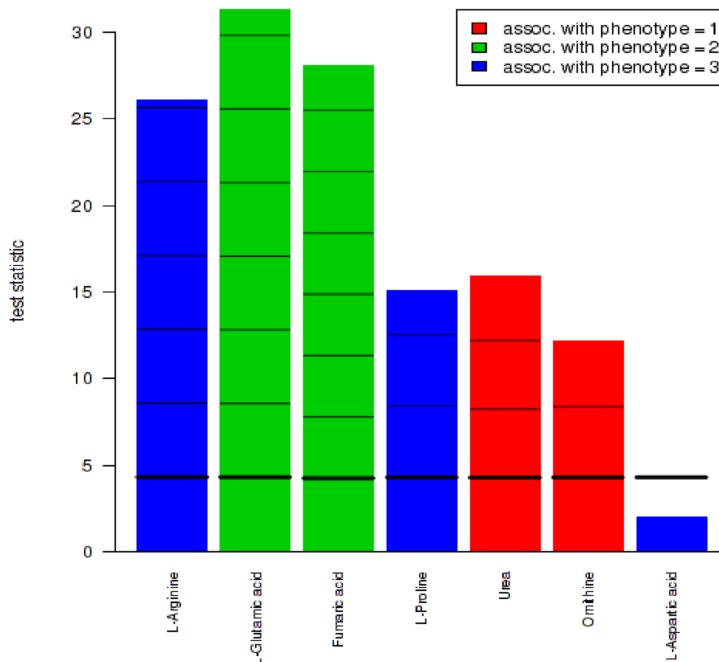


Figure 37

Set of metabolites that matched with the arginine and proline metabolism ranked by their *p* values. Phenotype 1: growth condition without SO_2; phenotype 2: growth condition at 0.2 mg/l mol. SO_2; phenotype 3: growth condition at 0.4 mg/l mol. SO_2. Vertical axis values were obtained performing the QEA.

β-alanine pathway was described by MetPA as a potential pathways in the SO_2 resistance obtaining a relatively high score in terms of pathway impact (X value) (Figure 35). It is defined in the global map of metabolism as a network comprising amino acid derivates and β-alanine represents the central metabolite that can mainly be obtained from aspartate or polyamine degradation. Thus, β-alanine is transformed into pantothenic acid that goes in the pantothenate and Coenzyme A biosynthesis for energy production. Results showed a β-alanine increasing with the SO_2 concentration, whereas pantothenic acid showed a stable trend. Since an increasing in the aspartic acid production during the SO_2 exposure was observed, we suppose polyamine degradation, such as biogenic amines, could be the preferential way to obtain energy in a stressful condition. Actually, biogenic amines degradation was observed in *D./B. bruxellensis* growing in synthetic wines (Vigentini et al., 2008; Agnolucci et al., 2009) but without SO_2.
Finally, taking into account those potential stress response metabolites like glycerol or myo-inositol, MetPA revealed that the lipid metabolism was marginally involved in the sulphite tolerance. In particular, glycerol was overproduced under the highest SO_2 concentration but its pathway, the glycerolipid metabolism, received a modest score in the metabolome view (Figure 35). Myo-inositol failed to be produced.

SO_2 tolerance modulation: effect of proline

Among the mechanisms that are involved in the protection to sulphite, some amino acids, in particular proline, could have an important role modulating the SO_2 tolerance. Preliminary results suggest that intracellular proline may reduce the SO_2 stress. In literature it is reported that proline has a stress-protecting activity on yeast and it can act against oxidative stress in fungi, plants, and mammalian cells. How proline metabolism regulates intracellular redox homeostasis and protects against oxidative stress is not known but it should work as a membrane stabilizer by preventing protein denaturation (Takagi, 2008). In the growth conditions investigated, proline increases the sulphite resistance in sensitive or low SO_2 tolerant strains (Figure 38 and 39) and this effect proved to be a strain dependent character. The phenomenon was not observed in high SO_2 tolerant strains where probably other effective protecting mechanisms could be activated.

Other well-studied metabolic mechanisms in *S. cerevisiae* are probably not used or ineffective in *D./B. bruxellensis*; adenine and methionine (data not shown) do not increase the SO_2 resistance and the sulphite toxicity in the investigated strains, respectively (Aranda et al., 2006).

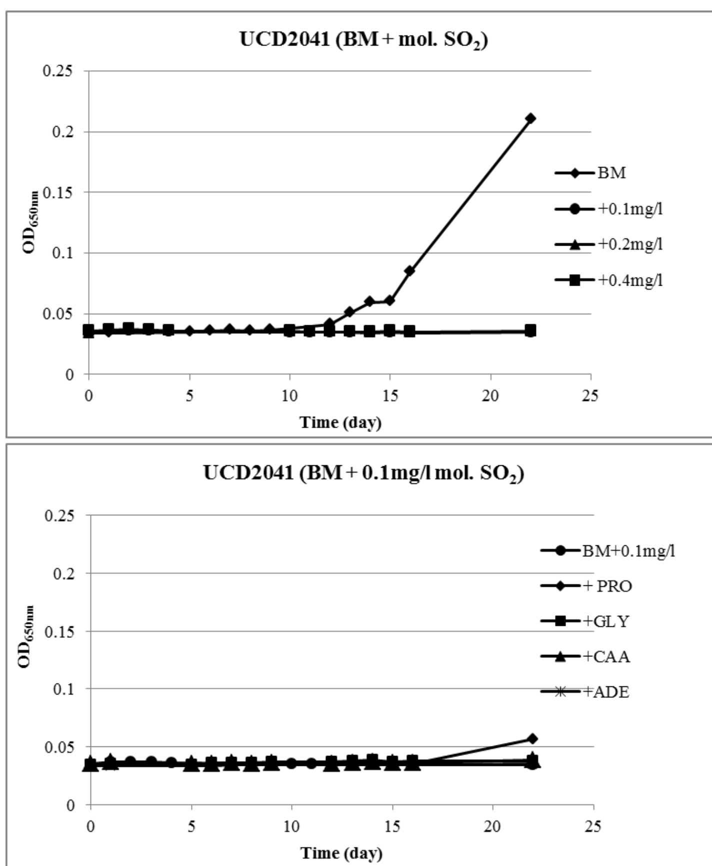


Figure 38

D./B. bruxellensis UCD2041 kinetic of growth in BM: left) SO_2 resistance; right) at 0.10 mg/l mol. SO_2 in presence of different supplements. Data are reported as the average values of eight replicates.

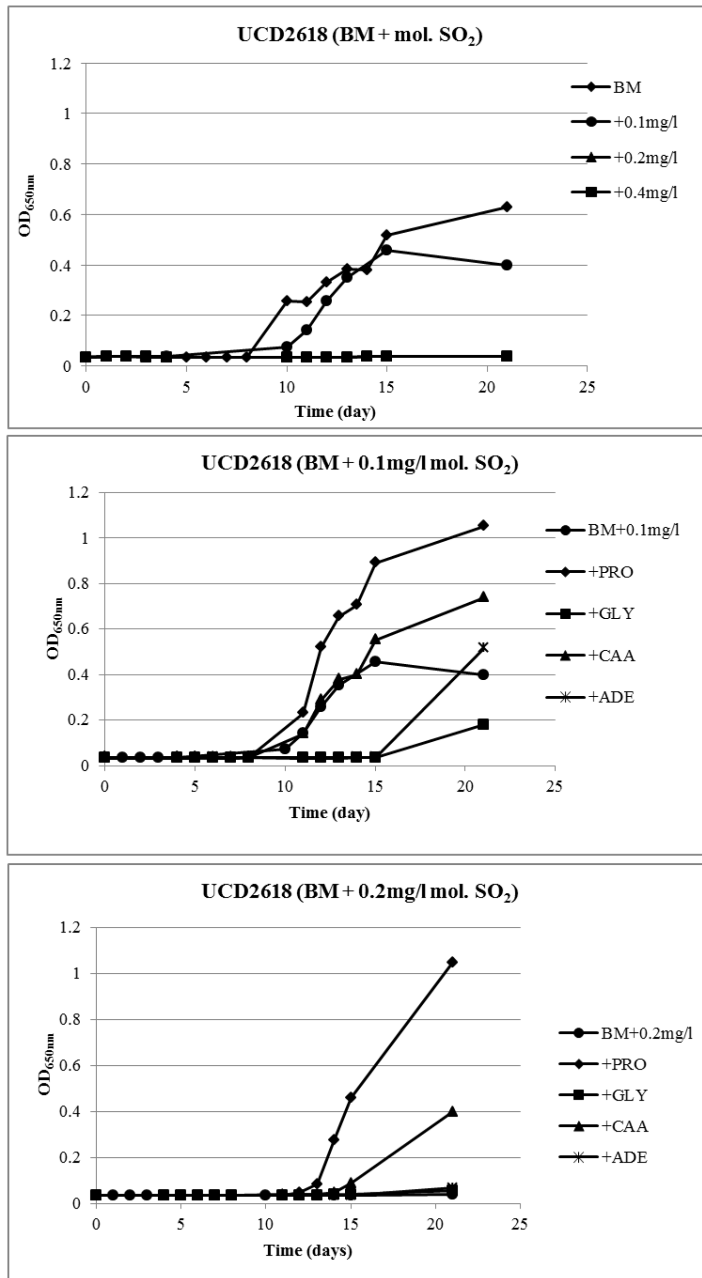


Figure 39

D./B. bruxellensis UCD2618 kinetic of growth in BM: top) SO_2 resistance; left) at 0.1 mg/l mol. SO_2 in presence of different supplements; right) at 0.2 mg/l mol. SO_2 in presence of different supplements. Data are reported as the average values of eight replicates.

3.3.2.3 References (3b)

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3.3.3 Conclusions (3)

Food and beverage spoilage by yeasts has become of increasing importance because of modern food processing technologies, the great variety of new food and beverage formulations, the tendency to reduce the use of preservatives, particularly those effective against yeasts (e.g. sulphur dioxide, SO_2), and the use of less-severe processing. Sulphur dioxide is widely used in the food industry as it is a good antioxidant agent and an excellent antimicrobial compound. It is known that it has an influence on human health, although neither carcinogenicity nor mutagenic effects have been found. Nevertheless, it would be important to reduce the quantity of SO_2 in wine to avoid a cumulative effect since it is a very common additive in many food products. Only a few methods can help to prevent wine spoilage *by Dekkera/Brettanomyces* (adequate hygiene of cellars and barrels, ageing at low temperatures, wine filtration, etc.). In this study we observed that LEC (200 mA) could represents an alternative strategy to the SO_2 addition during wine storage. The LEC effect on both cell activity and microflora viability was assessed. LEC decreased significantly the survival viable cells and increased the death rate of *D. bruxellensis* strain CBS4481 yeast suggesting the importance of an appropriate LEC treatment which limits wine deterioration in terms of off-flavours synthesis. The results demonstrated that the growth of undesirable *Dekkera* can be inhibited by low voltage treatment; LEC was shown to be useful to prevent wine spoilage and has the potential of being a concrete alternative method for controlling wine spoilage.
However, the capability of *D./B. bruxellensis* to survive and to grow in barrel aging wine can be partially ascribed to its high resistance to SO_2, but no data are available on the mechanism that controls this metabolic feature. Since this yeast can be isolated along different fermentation phases, a better control in the SO_2 manage during winemaking could avoid the yeast spoilage. From a screening experiment for SO_2 resistance using a collection composed by 119 *D./B. bruxellensis* strains, sensitive, low, medium and high SO_2 resistant strains were recognised. Actually, some strains can adapt their growth to high levels of sulphur dioxide whereas others have an innate resistance to SO_2. This adaptation could be linked to few cells inside a same population that have developed, during yeast evolution, alternative mechanisms or different gene regulations to tolerate strong stress. Among the mechanisms that are involved in the protection to sulphite, some amino acids, in particular proline, could have an important role modulating the SO_2 tolerance. Future results from metabolomics experiments will allow determination of the molecular mechanisms and metabolic pathways that this yeast can activate in order to survive and grow at high concentration of exogenous sulphur dioxide.

APPENDIX A

Sequencing results

> *D./B. bruxellensis* strain VRANACB 26S ribosomal RNA gene, partial sequence

GCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGGACTATAAAAAGG
AGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGTAGAGGGTGAGAGCCCCGTGAA
TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAATTTTGGAAAGAGAGTGAAATAGTACGTG
AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGACATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCCCCAAGGATGCTGGCATAACGA
GCACATACCACCCGTTCTTGAAACACGGACC

> *D./B. bruxellensis* strain VRANACA 26S ribosomal RNA gene, partial sequence

GCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGGACACTAAAGAGG
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TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG
AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGACATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAAACACGGACC

> *D./B. bruxellensis* strain ML11 26S ribosomal RNA gene, partial sequence

GCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGGACACTAGAGAGG
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TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
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GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGACATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAAACACGGACC

> *D./B. bruxellensis* strain ML23 26S ribosomal RNA gene, partial sequence

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TAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAACA
AGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTGAA
ATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCCTC
GTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATACGG
GGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGACATCTTGTG

GCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACGAGC
AAATACCACCCTTCTTGAAACACGGACC

> *D./B. bruxellensis* strain VL58 26S ribosomal RNA gene, partial sequence

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TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG
AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGACATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAAACACGGACC

> *D./B. bruxellensis* strain VL61 26S ribosomal RNA gene, partial sequence

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TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
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AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGGCATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAAACACAGACC

> *D./B. bruxellensis* strain CMR62/1 26S ribosomal RNA gene, partial sequence

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AGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGTAGAGGGTGAGAGCCCCGTGAA
TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG
AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGGCATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTTCTTGAAACACGGACC

> *D./B. bruxellensis* strain VL67 26S ribosomal RNA gene, partial sequence

GCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGGACACTAGAGAGG
AGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGTAGAGGGTGAGAGCCCCGTGAA
TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG

AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGGCATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAAACAACGGACC

> *D./B. bruxellensis* strain VL68 26S ribosomal RNA gene, partial sequence

GCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGGACACTAGAGAGG
AGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGTAGAGGGTGAGAGCCCCGTGAA
TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG
AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGACATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAAACAACGGACC

> *D./B. bruxellensis* strain VL69 26S ribosomal RNA gene, partial sequence

GCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGGACACTAGAGAGG
AGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGTAGAGGGTGAGAGCCCCGTGAA
TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG
AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGACATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAACCCACGGACC

> *D./B. bruxellensis* strain VL70 26S ribosomal RNA gene, partial sequence

GCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGGACACTAGAGAGG
AGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGTAGAGGGTGAGAGCCCCGTGAA
TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG
AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGGCATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAACCAACGGACC

> *D./B. bruxellensis* strain VOLO 26S ribosomal RNA gene, partial sequence

GCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGGACACTAGAGAGG
AGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGTAGAGGGTGAGAGCCCCGTGAA

TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG
AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGACATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAAACACGGACC

> *D./B. bruxellensis* strain SABA1109 26S ribosomal RNA gene, partial sequence

GCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGGACACTAGAGAGG
AGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGTAGAGGGTGAGAGCCCCGTGAA
TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG
AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGGCATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAAACACGGACC

> *D./B. bruxellensis* strain SABA1309 26S ribosomal RNA gene, partial sequence

GCCCAAATTTGAAATCGGGCAACCGAGTTGTAATTTGGAGACGGGACACTAGAGAGG
AGGAAGGCGATTAAGTGCCTTGGAACAGGCTGCCGTAGAGGGTGAGAGCCCCGTGAA
TCGCTGGAGACCGATCAATTAGTGCCCGCCGAAGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAGTGGGTGGTATATTCCATCTAAGGCTAAATATTAGCGAGAGACCGATAGCAAA
CAAGTACAGTGATGGAAAGATGAAAAGAACTTTGGAAAGAGAGTGAAATAGTACGTG
AAATTGTTGAAAGGGAAGGGTATTTGATCCGACATGGTGTTTAGCAGCGGCCCGTTCC
TCGTGGATGGGTGCACCTGGTTTACACTGGGCCAGCATCGGTTCTGGGAGCCATATAC
GGGGTTCGTGAATGTGGCCCTTCGATTCTGTCGGAGGGTGTTATAGCGCGGGCATCTT
GTGGCTAGCCGGGACCGGGGACTGCGGTGACTTGTCACCAAGGATGCTGGCAGAACG
AGCAAATACCACCCGTCTTGAAACACGGACC

> *D./B. bruxellensis* strain ML11 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA, partial sequence

AAGGATAAAAATACATTAAATTTATTTAGTTTAGTCAAGAAAGAATTTTAAAACTTTC
AACAATGGATCTCTTGGTTCTCGCGTCGATGAAGAGCGCAGCGAATTGCGATACTTAA
TGTGAATTGCAGATTTTCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCTCTGGT
ATTCCGGAGGGCATGCCTGTTTGAGCGTCATTTCCTTCTCACTTTTTAGTGGTTATGAG
ATTACACGAGGGTGTTTTCTTCAAAGGAAAGAGGGGAGAGAGAGGGGATAGAGATTT
AGGGTTTCGGCCGTTCTTTATTTTTTCTTCTCCCCCATTTATCATGTTTGACCTCATATC
GGGTAAGAAGACCCGCAGAC

> *D./B. bruxellensis* strain ML23 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA, partial sequence

AGGATAAAAATACATTAAATTTATTTAGTTTAGTCAAGAAAGAATTTTAAAACTTTCA
ACAATGGATCTCTTGGTTCTCGCGTCGATGAAGAGCGCAGCGAATTGCGATACTTAAT
GTGAATTGCAGATTTTCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCTCTGGTA
TTCCGGAGGGCATGCCTGTTTGAGCGTCATTTCCTTCTCACTATTTAGTGGTTATGAGA
TTACACGAGGGTGTTTTCTTCAAAGGGAAGAGGGGAGTGAGGGGATAATGATTTAAG
GTTTCGGCCGTTCATTATTTTTTCTTCTCCCCCAGTTATCAAGTTTGACCTCAAATCAGG
TAGGAGGACCCGCTGAAT

> *D./B. bruxellensis* strain CMR62/1 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA, partial sequence

AACGGCCGAAACCTTAAATCATTATCCCCTCACTCCCCTCTTCCCTTTGAAGAAAACAC
CCTCGTGTAATCTCATAACCACTAAATAGTGAGAAGGAAATGACGCTCAAACAGGCAT
GCCCTCCGGAATACCAGAGGGCGCAATGTGCGTTCAAGAACTCGATGATTCACGAAAA
TCTGCAATTCACATTAAGTATCGCAATTCGCTGCGCTCTTCATCGACGCGAGAACCAA
GAGATCCATTGTTGAAAGTTTTAAAATTCTTTCTTGACTAAACTAAATAAATTTAATGT
ATTTTTATCCTTGCTTATCCACGTGTCTGCACGGGCTTATGCCCAGCATCCTGTAATGA
TCCTTCCGCAGGTTCACCT

> *D./B. bruxellensis* strain VL58 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA, partial sequence

TAAGGATAAAAATACATTAAATTTATTTAGTTTAGTCAAGAAAGAATTTTAAAACTTT
CAACAATGGATCTCTTGGTTCTCGCGTCGATGAAGAGCGCAGCGAATTGCGATACTTA
ATGTGAATTGCAGATTTTCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCTCTGG
TATTCCGGAGGGCATGCCTGTTTGAGCGTCATTTCCTTCTCACTATTTAGTGGTTATGA
GATTACACGAGGGTGTTTTCTTCAAAGGAAAGAGGGGAGAGTGAGGGGATAATGATT
TAAGGTTTCGGCCGTTCATTATTTTTTCTTCTCCCCCAGTTATCAAGTTTGACCTCAAAT
CAGGTAGGAGGACCCGCT

APPENDIX B

Metabolomic data

Compound	2426woa	2426wob	2426woc	2426wod	2426woe	2426wof	2426_20a	2426_20b	2426_20c	2426_20d	2426_20e	2426_20f	2426_40a	2426_40b	2426_40c	242640d	2426_40e	2426_40f
xylose	1730	4205	683	965	4677	2852	2343	611	641	769	2007	434	938	1036	881	1157	992	9476
xylitol	883	395	327	471	561	668	331	700	512	567	426	658	1338	492	621	601	538	1928
xanthine	128	129	152	116	124	214	129	112	121	430	107	138	320	229	546	432	292	166
valine	131487	26877	34368	37567	30343	39717	75955	75764	47250	34198	80998	41592	30827	99453	355509	446037	113002	125275
uridine	2205	646	518	64	212	229	401	1226	636	468	269	936	70	138	254	239	245	151
urea	14458	16137	19684	36409	16031	45996	12280	30875	16752	15461	13765	14992	10901	4160	353	1888	1753	736
UDP GlcNAc	228	242	259	240	264	284	60	179	198	512	268	242	289	144	274	362	312	98
tyrosine	70577	22698	15061	31951	27093	20837	41205	41349	29510	20186	37273	17436	31217	39301	59084	53203	104491	46316
tryptophan	9967	2529	1838	6092	4487	3283	8030	7493	5343	1459	7472	4820	7747	14368	11869	5796	25620	3634
trehalose	12369	9421	5311	11140	7273	10261	2923	5790	4761	1913	4809	2653	8241	204	753	878	486	354
thymine	582	456	309	238	345	425	237	307	305	813	195	252	314	1005	961	1051	679	791
threonine	30552	7579	7669	12377	6915	8171	15875	18009	10742	13104	19158	13425	19639	41796	33892	46011	33324	22803
sulfuric acid	10996	1055	13055	1457	1523	214	8490	5574	4306	9739	8885	3203	2400	5928	8153	9678	7534	5939
sucrose	388	204	330	431	602	1787	111	1058	245	38	185	536	275	35	221	464	111	125
succinic acid	9895	5278	4483	5297	4640	2792	4023	5830	5477	7088	7282	5574	16618	156483	3292	4982	72499	14429
stearic acid	31641	35831	36901	30016	32473	45307	32160	29647	20741	42421	22142	22912	36605	17812	66881	68607	35262	26381
sorbitol	16897	5186	2992	5420	5762	2258	5311	10719	9341	2682	7523	10406	16934	3005	2355	5039	8011	1207
sophorose	253	217	203	61	159	41	262	171	150	512	138	170	117	90	195	247	190	108
serine	108699	16474	13567	57757	42275	27228	61380	50604	43678	40723	68099	42442	37298	15039	23150	25191	29073	13394
saccharopine	355	155	289	235	240	225	180	198	212	413	162	260	213	93	455	522	214	525
ribose-5-phosphate	1583	1325	210	1167	1095	1230	1690	700	987	2726	982	897	1010	234	482	2722	923	444
ribose	14231	9010	9014	1905	2974	2104	5648	13171	4911	11823	3385	10240	1134	673	2132	3068	908	1334
ribitol	12022	2098106	2156059	1965513	2127003	2531031	1453139	1576109	1766683	280562	1327891	1529278	379547	976829	1054	19454	93072	1647005
pyrophosphate	2018	1704	3749	3242	3234	3190	931	1912	2942	3529	1696	3426	5274	1886	7649	13848	3095	3867
pyrazine 2,5-dihydroxy NIST	593	257	324	371	211	567	306	249	408	687	454	214	1540	320	979	599	2017	492

propane-1,3-diol NIST	653	1036	787	768	683	907	542	818	436	827	546	472	726	236	1503	2116	577	668
proline	36885	1353	4980	10808	3447	6182	18288	11644	11715	4550	19070	9021	10103	115057	76832	79725	63342	82650
phosphoric acid	133020	72696	89473	51068	64267	26544	86096	108967	67050	202525	99008	126195	113205	104083	462157	449205	160926	183071
phenylalanine	51569	18288	10752	21495	17061	14940	30520	35249	17674	32951	29577	14987	13800	9443	11356	15879	42975	12771
pelargonic acid	2980	5832	4367	5252	13614	4055	4340	5123	2336	8390	3788	3616	2172	3632	4109	13967	4459	4135
pantothenic acid	8972	4062	2533	3437	3150	1056	3535	4131	3931	1735	3652	5105	16104	8390	14238	11156	16135	6941
palmitoleic acid	1120	340	472	358	281	516	200	362	398	1530	326	263	1582	272	745	605	781	316
palmitic acid	3522	3754	3562	3244	3883	4784	3791	3534	2301	6169	2697	2841	4287	1756	6862	7560	3572	2818
oxoproline	445294	158180	128314	342142	287678	118696	112036	172622	153343	138795	342603	254224	259582	252553	91196	199076	242558	267920
oxalic acid	5775	5052	6775	4760	6968	11684	4866	5603	4814	15905	4981	3930	6153	4455	9755	17744	8731	5969
ornithine	27985	13649	15518	34904	25419	17102	10054	7871	15460	7423	18363	8705	41837	28938	35572	44798	49243	22829
oleic acid	32	132	163	99	131	211	115	91	95	437	76	93	178	65	79	303	84	88
N-methylalanine	1546	614	4131	953	772	4070	874	1053	3388	3935	1158	1023	1837	2406	5027	12133	2223	9952
nicotinamide	826	497	813	1129	1056	1030	473	340	420	1001	697	283	1961	647	1500	1501	2023	928
N-acetylornithine	3040	1776	1774	3588	2440	1682	1217	674	844	1489	1435	395	7549	6206	3389	3192	9571	5250
N-acetylglutamate	15213	6857	4498	7281	7620	4162	3957	2425	2483	4817	6353	2281	9885	8685	2527	2347	9440	6995
N-acetyl-D-mannosamine	200	202	218	158	168	199	188	235	233	420	188	121	204	59	245	255	185	118
N-acetyl-D-hexosamine	29420	10575	4292	2836	3627	1989	9492	21076	13216	8779	7433	16431	1984	570	797	968	5262	651
myristic acid	514	412	573	546	466	695	515	516	388	1127	377	409	685	235	608	1001	468	475
mevalonic acid NIST	33082	12135	2818	54009	35777	14568	43202	21972	33254	26495	82958	16545	57284	1160	1852	15224	30190	5267
methylhexadecanoic acid	720	598	989	609	733	902	479	544	647	1465	371	546	627	212	1067	1738	619	678
methionine sulfoxide	6434	3210	1627	4257	3164	2494	2050	3315	4178	875	3490	789	1395	4467	1405	980	4479	471

methionine	1033	231	226	376	296	174	139	213	281	407	444	217	986	1740	512	800	2081	533
mannose	39390	34012	45098	20656	28332	13931	24225	64469	27651	13370	32085	33136	45187	1637	994	542	5644	858
mannitol	18659	6055	3776	13538	11137	4498	21299	21311	18335	17453	23038	21193	11863	2809	3296	2941	4289	927
maltotriose	148	130	176	710	151	1287	235	193	256	430	151	102	136	53	219	199	82	111
maltose	12334	9414	4966	11191	6483	285	336	5653	4828	567	4941	2417	268	67	228	52	154	108
malic acid	8369	4177	3800	3391	5137	2407	3792	6721	5449	4106	5308	10411	16855	9023	789	1581	14909	6828
maleimide	1162	531	1211	814	727	1368	1021	953	1261	4393	844	476	875	348	976	1169	867	392
lyxose	361	339	214	204	364	312	151	222	193	427	171	151	337	431	406	581	269	597
lysine	24764	5655	5916	15009	16514	7437	15719	9915	9506	1216	17866	6453	6286	18436	90623	53382	25913	15919
levoglucosan	6486	3337	1248	18270	1123	3182	7809	3022	7765	19670	786	1093	710	243	364	1519	442	17039
leucrose	997	579	324	467	454	365	494	424	455	666	566	431	1306	154	276	311	615	226
leucine	161494	26963	42594	38001	25885	45075	98029	83655	48455	38605	89504	37925	12416	50484	82674	124717	63918	73544
lauric acid	1301	1014	1472	1763	1983	1157	911	948	609	4110	845	842	4293	725	3641	2341	4744	1461
lactic acid	20134	12749	19701	29003	9972	37891	10070	27744	14468	24777	14686	20390	9325	26318	17200	21113	39171	7655
isothreonic acid	982	505	383	708	437	445	275	614	288	280	443	535	337	1568	1476	1921	1214	1417
isonicotinic acid	2240	1590	1144	1292	995	1147	957	1475	756	3341	994	928	629	1256	1686	1250	1367	919
isoleucine	75908	17492	12888	26496	21925	21893	46765	52511	25608	34253	49664	18685	15676	88820	109244	119524	105630	86382
inositol myo-	2339	565	636	2600	2112	509	2115	999	1138	827	1753	1161	562	380	773	484	235	155
hydroxylamine	9367	14554	12564	10714	12166	14419	7419	11420	8074	24650	8896	8005	9585	5505	21044	30776	7661	10580
homoserine	2024	665	1012	897	751	1188	921	831	888	1202	1190	878	1714	402	1045	1567	598	587
guanosine	556	306	247	342	264	372	423	651	371	738	377	446	237	122	190	303	232	167
guanine	5700	2946	2489	799	777	833	1738	2790	1312	1459	907	2145	532	320	1144	476	362	243
glycolic acid	2338	1422	918	2297	1554	1705	1766	2408	2049	5455	1771	1783	428	1219	1827	2202	731	453
glycine	105336	44604	33977	66299	47799	39658	40859	70956	50993	44262	67571	69189	225862	317528	367329	255841	391215	121287
glycerol-alpha-phosphate	155110	68960	89943	68415	52184	40383	79193	66591	57993	115467	63508	83760	238400	58368	118029	145631	147688	70884
glycerol	279504	133708	139831	127861	82185	62120	103048	210947	140340	209180	166644	226274	262083	127579	248002	306648	165700	125707
glyceric acid	7360	2876	2005	4766	4083	2836	5085	5597	5322	6566	6459	9081	3559	1396	1790	1828	2556	1181

glutamine	128532	96460	71126	153675	136968	85798	22255	21451	94594	8861	93442	3540	470282	78865	8091	10837	165722	12346
glutamic acid	80509	39529	23319	51166	41709	28802	78085	46434	50716	60205	84420	42259	254823	76342	287621	113696	314164	95198
glucose-6-phosphate	330	592	542	914	498	418	514	362	733	731	793	801	1674	105	192	251	363	165
glucose-1-phosphate	6126	2880	2561	4036	5361	2132	9685	7869	8188	7539	14696	7471	19097	5520	1995	1864	11264	6063
glucose	191533	114334	79335	91731	103972	85988	285884	177716	205400	391949	204864	228375	24478	197	2519	1587	434	624
gluconic acid	288	179	244	212	271	308	348	281	189	830	152	209	119	79	254	243	118	103
galactose-6-phosphate	992	330	311	1893	1484	1196	1361	549	1157	895	1853	1608	1049	139	217	227	378	314
galactinol	19510	15210	12903	15582	13289	12574	13703	11227	14418	11748	18436	7562	38940	3353	1887	3640	16135	4919
GABA	54202	26537	15993	17217	21622	4415	54770	28857	22735	55597	45709	27825	19204	46256	258774	94158	16529	99132
fumaric acid	4971	2132	1938	3638	3978	1562	3617	5916	5708	4127	5932	7656	9004	3570	948	1268	6791	2569
fructose-6-phosphate	3641	7460	6776	6106	5184	4612	4947	2416	4321	6955	6334	5965	5353	157	484	597	819	586
fructose-1-phosphate	1889	708	1140	2826	2677	1120	2556	985	1738	1804	2596	2208	2328	113	259	340	170	149
fructose	812635	634079	667072	352415	440451	364446	1029394	775304	869154	1824963	695997	940174	729070	1865	3709	3480	14289	1749
ethanolamine	29011	23462	14481	16006	13658	8603	7256	18635	16468	11485	15818	25160	67131	35252	113020	251494	59100	28862
ethanol phosphate NIST	24011	18801	15588	21058	19869	10037	24102	25341	19987	46947	25584	32544	7611	7741	16204	15598	9443	7547
erythrose	285	277	240	252	279	346	782	429	294	2343	385	375	331	77	245	293	170	142
erythritol	5325	2036	1235	3932	1832	1455	733	1489	1289	516	2504	1358	12404	2049	2366	10728	2886	633
cytidine-5'-diphosphate	903	633	318	618	375	546	537	650	385	1332	386	369	1114	1361	597	374	2517	467
cysteine-glycine	281	140	174	216	154	248	189	146	261	413	173	113	125	67	234	317	134	139
cyano-L-alanine	310	161	241	205	172	417	221	155	245	956	200	176	584	1784	1107	763	1201	408
citric acid	23472	6366	8270	8234	10544	1902	5537	11219	8735	7358	12012	27960	14262	3834	8285	5975	7809	2127
citramalic acid	166	143	167	133	132	248	156	152	117	413	117	63	239	209	610	1563	308	226
capric acid	757	665	1084	669	771	1352	353	570	774	1961	393	558	692	314	1288	1053	483	581
butane-2,3-diol NIST	2687	1611	2232	983	1387	1499	2049	1832	1715	4830	2061	2042	1774	2028	2190	4157	3285	1556

beta-alanine	653	1067	427	1170	908	532	267	295	438	584	390	489	3430	3855	2097	4301	5872	934
benzoic acid	65654	90539	100271	64026	76435	97883	59843	78506	53943	165642	61460	53965	58336	33354	124611	196416	50448	66171
aspartic acid	56235	19066	20094	30497	28704	15204	30878	25252	21936	25279	39817	29536	60050	10474	15925	25422	37402	12708
asparagine	18333	6163	4936	10679	6692	5627	11822	5852	6783	7809	10880	5830	8142	42392	45618	40888	11714	12431
arginine + ornithine	19158	8631	11499	30969	22064	14619	6312	4548	12451	1609	13575	4159	26290	21023	28269	27379	41879	12434
arabitol	255682	365136	346559	333090	370809	401075	281599	277996	299752	327703	246392	278023	143905	81389	17540	4362	273211	278919
arabinose	3513	2222	1512	2618	2306	1826	1593	1676	1379	2620	1070	1337	2610	3960	1622	1455	3117	390
aminomalonic acid	206	97	143	176	133	227	145	149	134	372	129	167	353	238	356	440	342	258
alanine	466414	93605	143553	162039	126955	113555	193306	194860	173632	90550	299947	173176	608236	1620466	1578915	1417644	1431624	947729
adipic acid	1152	867	1381	1784	1386	1829	755	1064	849	2429	822	703	838	649	1350	2521	1198	896
adenosine-5-phosphate	620	428	352	2092	1090	487	1105	588	1759	1158	1998	769	5184	849	937	1770	3965	1144
adenosine	2570	609	376	2890	1340	647	1998	2955	4008	1179	3155	6292	858	468	460	731	1060	1383
adenine	112026	58382	44966	20491	19568	23786	33389	53724	30324	35452	25054	40499	23813	12280	30832	39506	13802	13404
aconitic acid	815	296	198	314	255	222	194	276	189	348	261	388	1324	414	793	597	1231	311
5'-deoxy-5'-methylthioadenosine	319	173	210	250	225	312	136	128	168	376	183	177	311	262	499	699	1235	351
4-hydroxybutyric acid	1195	563	462	521	430	473	362	647	585	666	574	665	1551	1053	1186	2475	1602	980
3-hydroxypropionic acid	1361	819	760	886	800	1048	688	1302	1269	1834	1397	1241	810	841	1007	2887	1420	661
3-hydroxybutanoic acid	1485	1068	1306	751	933	1248	923	622	900	2859	716	1000	2228	360	482	2833	1047	1497
3-hydroxy-3-methylglutaric acid	625	326	407	946	771	553	373	472	482	728	539	329	486	2028	9216	9989	3113	2833
2-isopropylmalic acid	468	280	316	201	191	211	197	263	213	560	202	230	582	273	985	812	586	282
2-hydroxyglutaric acid	4990	2455	2588	1445	1798	794	1967	3995	2461	3344	2079	5522	8838	61894	6253	1856	11704	8658

2-(4-hydroxyphenyl) ethanol	214	171	243	133	155	195	142	199	177	564	133	260	439	3121	3811	2674	2566	773
1-deoxyerythritol	1153	415	657	1287	423	656	818	1870	1134	1086	1313	1309	972	1164	5247	10124	1685	1380
400494	24216	12237	2921	19998	12548	6950	16272	19276	33948	19625	43662	22583	22861	6278	17719	48870	15410	7279
388098	2937	3861	3572	2864	3243	4094	2905	3280	2107	5664	2653	2415	2984	1728	5981	7998	2478	3124
382318	3893	6262	5294	4444	5291	5789	3514	4874	3235	10771	3628	3104	4065	2173	8606	12036	3112	4336
294511	480	670	598	388	603	819	451	427	326	448	361	350	442	233	809	942	395	351
272849	31677	57411	43789	43530	47568	82638	30049	46880	36565	92610	34596	27490	41221	16487	82736	116100	31228	33072
270999	3814	3363	5231	1910	2170	1980	2466	3635	2982	5872	4284	4955	1720	1361	5762	7375	1457	2268
270066	19335	28084	28135	18277	24124	27721	18742	21640	15470	50787	17228	17040	18177	10194	36336	51510	15587	19755
268585	30798	51249	38203	36809	44917	63616	31178	41228	29950	100839	27356	26757	37662	16390	76222	105808	28785	30503
234717	3128	3181	3223	2761	2952	4070	2447	2835	2061	5052	2425	2353	3166	1619	4295	6385	3024	2880
226330	1911	2377	2182	1538	1706	968	1433	1902	1123	3003	1415	1485	1045	567	2028	4944	733	1090
216428	2892	10325	1603	3065	2739	2060	3203	3827	4201	7041	2498	1387	6712	911	3319	4135	7676	1271
208812	20292	8663	3545	21142	11394	8455	8071	7548	10199	7334	12577	6900	15700	12778	39919	38053	23318	9219
268313	8188	13526	10180	10099	11500	14279	7368	11964	7374	23571	8895	7581	8809	4940	19931	29187	7943	9434
242417	2780	3704	3612	2431	3041	3805	2535	3317	2159	5657	2569	2323	2445	1729	4923	7679	2319	2553
231652	208	191	93	223	217	2191	208	144	116	58	129	79	232	66	252	267	44	153
367932	9772	11262	10783	8924	10320	10860	7727	10042	6026	17869	8187	8481	7612	5400	15945	23462	7113	9465
301399	450	530	766	406	458	608	494	460	292	854	390	409	254	307	250	1021	236	448
356938	376	269	420	186	301	542	404	259	267	827	281	241	308	144	495	532	268	172
330609	4413	3115	1470	3188	3030	1693	1573	1733	1621	1179	2267	1836	3699	3436	802	920	3458	3458
321692	1348	1536	1238	758	1145	904	983	1608	614	762	1301	870	618	584	1485	3317	739	1200
233017	34540	35702	30529	17172	17304	21244	18359	19099	16050	23175	14217	9362	3937	975	1354	2059	3129	926
231654	170	169	265	112	124	1781	218	71	138	588	149	85	212	46	172	239	61	84
204344	72935	90002	84850	79916	76101	124326	55239	79139	60266	157580	67689	54028	76786	39196	324566	234704	53390	68438
199203	112093	30353	16397	86009	66925	20261	133344	140799	110832	85286	152747	128624	10374	992	826	585	3768	810
319168	1524	618	3152	1245	1145	2051	1417	857	687	762	1211	1432	569	555	1131	1895	515	1756

317187	4854	1067	680	9363	8140	5050	6852	3265	6166	3577	10563	7521	5610	375	364	476	1566	1129
229959	3058	1102	1390	3455	3185	1437	2883	1653	2636	2053	3269	2865	2872	113	259	340	192	149
211972	2969	4136	864	5746	4884	2664	1520	4379	1909	2535	3114	2177	2487	1458	8930	7803	3666	3496
317072	216	155	105	118	311	2419	51	119	84	85	119	68	252	137	479	585	196	220
314770	14775	8493	6223	7946	8585	5626	13806	13370	12732	23923	14006	17345	1245	108	258	253	199	189
310052	9452	4318	3305	5777	4958	2380	5792	6670	8119	3279	8740	10933	15278	193	733	504	1463	167
235965	4827	8299	13596	4959	4056	8196	6890	4548	4219	14491	8129	5424	33729	5926	16518	11150	7278	5024
217783	5240	2376	9319	5867	4831	8508	2663	63994	1649	14211	3945	4399	2323	1585	7841	2598	3833	3852
367944	1566	586	496	985	612	588	327	253	441	690	785	280	2386	221	407	1780	1391	209
356954	813	871	887	802	872	1312	566	758	587	1421	651	743	560	542	1308	2503	603	784
299447	1826	1062	488	1283	821	801	864	1240	1230	786	1433	1258	2823	100	247	255	1424	407
362130	18918	15807	15238	11521	11871	8229	27599	23593	17436	50568	22977	23453	37420	39353	48212	52313	45507	24685
237138	820	561	768	517	453	746	384	626	434	1151	875	1419	1293	502	1606	2118	947	1005
207223	310	1187	497	942	1149	1347	191	530	316	1366	207	421	929	180	1823	1131	663	303
202573	4981	2115	1292	2271	2121	1620	997	2191	6643	2890	7296	4057	24133	623	720	767	2196	445
202572	8682	4260	2289	3210	3234	2324	2263	4713	14107	6289	14350	8888	36610	1468	775	840	7074	884
374786	25479	12987	7544	14060	14112	9766	12214	17089	54612	41577	51930	31746	18388	481	303	422	1175	592
371632	30933	5965	1002	18964	11100	6407	13737	18346	17163	7259	16927	13315	3835	947	446	538	5152	753
343526	4745	1269	580	1261	842	1684	2395	2973	2361	2275	1389	3549	3741	2610	769	1077	4389	1486
204448	728	713	621	610	972	1140	956	660	665	1636	475	593	833	286	1140	1684	471	506
199337	608	652	665	374	310	619	326	338	438	331	275	152	114	78	269	315	191	121
360205	12560	1060	864	11517	4641	3275	1359	1342	1217	1701	9865	1738	8379	764	3884	6282	68667	971
274174	456	537	750	1221	1612	746	934	670	534	1547	726	419	545	359	703	607	575	368
236890	3955	2475	1281	2031	1589	635	1404	1298	1686	1824	1848	840	6199	1037	486	460	4481	516
212251	1093	334	204	653	658	666	1185	465	873	728	974	650	1057	94	265	267	223	313
301006	369	381	990	496	457	1260	394	504	679	1708	318	322	311	401	1085	2120	350	436
232087	23365	6161	7958	8339	10635	2262	5409	11144	8830	6822	11949	27688	14066	3938	7753	5766	7601	2045
224574	547	822	653	485	922	945	190	413	381	820	440	565	133	296	499	1483	219	557

213253	35637	29201	6285	25720	22208	11780	21746	49070	15870	60481	26593	10172	15956	9343	47151	61605	35688	14351
273925	4251	5775	4314	2776	4803	2054	4640	4614	195	1264	3341	3449	4204	2204	10349	11496	1496	3667
222541	2998	2725	1447	3021	2185	1423	547	1989	6800	1606	7556	4662	14019	1778	398	406	10735	858
357010	593	845	705	700	603	1087	618	464	587	963	511	627	578	336	1356	992	647	455
228959	581	533	199	1379	1009	486	218	514	743	516	400	763	2061	881	718	587	2142	318
367914	3521	2535	1585	3652	1876	2804	2252	1690	2632	1486	2437	1052	95206	9664	17425	6847	124697	1952
318770	10128	5160	3615	4628	4647	3777	16726	9270	9031	22447	9799	9854	1557	276	250	229	1093	334
300919	9954	12308	13934	11347	11647	14394	8346	11062	5827	27168	8078	8337	10749	2845	20189	21613	9659	7356
237133	246	282	258	188	197	337	119	199	196	738	155	604	264	172	798	1258	203	524
400671	39443	7245	2005	27921	17742	9468	21710	24945	23447	9582	27755	16451	3146	557	1323	1949	6381	326
362109	1992	713	407	962	715	633	683	1087	702	451	1322	223	526	184	287	295	392	174
231796	1702	875	689	814	863	622	930	1018	1185	950	1249	2258	2742	2766	1314	2168	1262	2024
225896	746	1031	498	844	939	952	254	327	281	1120	396	313	410	1415	442	346	568	896
206022	1605	1581	1667	1513	1002	1985	1049	1349	1047	2340	1001	1024	1793	856	2746	3757	1179	1146
354038	387	736	571	640	702	1009	326	297	234	646	281	258	383	162	782	691	285	416
268306	460	210	467	373	439	457	669	695	294	523	351	459	342	81	375	436	165	174
218784	1677	2631	2293	2225	2410	3380	1746	2302	132	704	1682	1433	1956	915	4733	5609	1576	1852
202095	1184	559	1725	426	793	1531	707	872	1174	1592	913	871	2236	2000	8725	11767	1840	2354
400644	1032	601	569	338	341	397	205	447	343	502	319	311	2826	6723	4386	12715	18059	4379
239332	1767	830	735	275	505	542	447	1294	1102	2197	564	1766	3275	17728	2194	1099	4134	2428
223675	1823	1198	1051	660	665	654	476	629	704	1329	647	604	5460	27716	13915	28502	86707	11264
199239	582	207	312	205	206	298	285	379	299	847	289	415	301	165	329	528	222	187
371802	740	2108	463	551	1091	3134	586	1160	2073	1052	1300	294	549	492	1226	6975	759	1421
362148	2312	1056	438	798	637	537	463	1790	634	1001	612	1766	379	703	427	1151	487	707
356985	2036	635	1769	3087	1741	2099	993	770	2307	875	600	951	4096	599	1635	1722	3727	779
309642	685	1564	1577	1686	1226	2028	558	1389	1087	2217	637	743	676	472	1067	3648	818	634
303839	1761	361	505	647	345	1005	349	680	754	471	871	692	1386	165	312	291	1624	231
269335	10950	4980	3141	5726	5129	3371	2058	1452	1600	2350	4005	303	6334	5539	1182	647	8303	4605

231657	385	758	491	726	851	521	406	713	551	1476	240	406	217	268	522	1734	366	428
225867	129	110	164	76	156	225	97	105	181	352	215	49	1789	1362	616	1081	1928	307
223120	471	364	336	539	509	443	416	425	332	936	561	508	487	86	194	281	577	158
202841	186	139	163	75	192	209	99	143	391	437	216	239	149	62	216	213	153	144
331041	8393	3973	3249	1105	1183	1248	2962	5247	2264	5353	1521	4652	3120	507	904	631	3309	1253
323686	3329	1166	902	1375	928	984	1020	1419	1563	2671	1669	1083	1187	470	477	733	554	538
310985	931	276	178	340	323	232	409	336	502	482	584	445	1009	135	265	313	1083	218
242565	2300	1611	2232	974	1648	1499	1686	1832	1007	1588	1800	1621	2090	1871	2190	4157	3099	1111
231659	36	251	179	180	117	656	144	164	182	656	159	144	153	30	334	418	162	171
229113	3738	6468	4029	4505	4957	4972	2557	5394	2198	7177	3311	3471	3403	291	2136	7530	2167	3178
216571	638	666	826	642	706	819	401	776	461	1100	578	363	326	111	616	723	260	425
213386	412	226	365	419	401	319	293	221	395	717	892	779	17019	1411	1441	3235	10913	831
328652	2812	1951	2411	522	1721	1742	1421	2197	1188	3481	1765	1854	467	398	3935	3982	633	1684
299421	436	295	408	348	271	351	238	287	240	680	338	356	377	167	656	790	510	321
233455	261	364	493	279	268	619	388	286	241	748	160	201	314	144	508	611	192	256
223830	246	221	181	526	333	468	113	155	225	366	310	111	1661	150	232	344	575	122
222169	234	258	305	1889	235	498	228	208	834	553	437	163	224	82	375	386	343	245
222115	1819	2979	2451	2156	2389	3446	1527	2352	1601	3911	1592	1745	2109	1047	3599	5324	1513	1579
213697	382	228	247	274	283	374	135	243	236	448	293	329	411	1520	848	1987	1333	1341
202571	139957	95691	85793	108299	103914	96451	152872	159464	132654	318425	159335	141400	59050	3834	1045	1621	13628	4466
362120	240	177	199	735	410	229	366	161	473	646	1014	1101	25839	1197	210	225	3481	1807
328420	781	311	287	448	412	294	321	280	293	622	418	429	326	1311	676	1089	557	626
318471	27443	6120	3904	13741	10327	3759	16016	26223	19901	8837	19312	23001	2614	173	444	362	823	264
310006	170	184	161	162	124	186	151	144	146	345	153	74	200	645	1217	2349	273	276
303956	728	325	385	855	708	484	245	238	527	946	235	402	599	356	1038	575	794	423
295269	162	1086	5949	762	951	1881	746	358	3358	1407	761	631	789	455	881	11058	318	361
295226	1286	1066	1479	740	1036	6593	186	1079	4517	1278	152	716	687	440	1080	1939	402	1098
237174	261	180	257	141	171	234	157	167	176	465	216	144	193	161	270	406	138	218

228018	255	147	160	319	252	395	137	99	295	362	206	76	1950	158	219	263	743	110
227774	1351	661	226	936	793	292	419	410	375	598	619	322	1245	1057	519	466	1671	494
215088	1768	1708	2104	947	1231	2116	1940	1424	1355	12780	1325	987	1176	676	3851	4609	1676	424
200421	17036	15147	21308	13843	17013	24398	17878	2462	12717	31848	3048	12170	13183	2363	4618	28189	9667	2190
199942	742	638	855	555	584	829	518	557	493	1206	412	515	2118	771	1007	1688	2545	516
356987	1658	760	1594	2712	1973	1955	402	482	2085	611	700	290	3224	923	767	705	2917	247
310667	1232	2285	1619	1713	2131	3914	794	1693	2429	2781	1247	1322	825	511	2439	7281	774	1317
309730	2004	1136	1670	2139	743	4607	1866	1337	1787	2296	2972	1086	4813	1969	3354	5031	3513	1140
304770	495	162	411	223	184	222	312	228	401	451	385	200	306	411	422	788	303	333
303163	627	678	271	562	507	367	175	1044	952	523	407	247	1566	1016	300	283	1819	293
294349	3760	5724	5516	1256	5626	1950	4073	4905	526	1349	3882	3871	5043	2194	9326	12649	3805	3548
280930	1816	983	562	814	739	622	1092	818	1038	950	1327	2332	3001	2819	1615	2549	1262	2098
270594	2531	10213	5302	359	794	1599	2268	4006	549	1247	788	921	1830	721	4218	4442	731	1698
234525	4020	6754	5000	4671	5626	7864	4724	5425	556	3013	4275	3873	5043	2272	8248	10923	3805	4289
227591	466	318	266	273	363	335	365	331	488	506	523	480	462	173	314	422	293	227
225043	2785	2579	3363	2597	2947	4073	2463	2593	1530	6508	1613	2251	2588	1386	5155	5820	1949	2561
200522	1384	622	675	546	409	459	398	457	540	721	507	877	1496	360	415	430	1418	237
199596	2109	2979	2451	1972	2184	3446	1717	2352	1601	4451	1380	1551	1911	1181	3599	5324	1513	1771
359483	1117	634	564	488	501	535	959	630	430	547	782	364	970	396	548	607	1131	535
322226	1793	880	927	850	914	650	530	448	643	605	871	842	1342	1975	422	227	1433	563
289052	187	179	303	219	160	323	141	168	244	366	222	195	282	420	334	617	556	415
269776	5917	6512	12115	3871	4650	4208	4367	9971	4755	13394	6298	11960	6482	4573	18267	27351	4689	7886
239565	884	377	214	612	357	337	260	315	982	690	856	618	2208	313	400	307	1879	249
235327	3826	1101	3711	958	1098	1326	780	719	583	2880	631	2279	830	2248	1922	1529	426	2916
228605	1087	537	545	365	327	447	233	443	291	386	277	764	12956	852	970	1191	1154	881
228249	710	314	256	221	348	308	369	449	412	495	314	347	209	55	223	231	134	98
226910	343	264	169	223	212	245	210	199	228	400	168	163	715	1053	216	247	1227	251
224632	244	188	216	330	148	199	137	200	176	488	188	260	124	292	488	844	229	381

224627	325	293	231	261	242	1320	218	228	31	652	220	79	329	24	382	378	128	172
221574	2241	3096	2440	2416	2723	2442	2170	2660	1802	5479	2053	1998	2699	1315	4554	5503	1770	2325
215397	984	9804	1730	470	1535	1239	0	13013	408	0	8662	1258	0	516	0	16285	0	1079
204994	486	282	327	144	366	443	410	475	743	704	704	559	174	125	380	448	141	184
352849	692	419	542	355	341	314	341	364	251	837	164	395	212	158	632	476	197	137
305055	7677	3637	94216	3732	5405	87686	6099	6766	62621	3925	6123	2934	3407	2363	19306	182967	7176	5093
301584	300	558	3346	602	490	3446	514	493	222	871	673	381	704	386	457	1720	841	787
268539	863	1021	857	643	998	787	352	802	538	1701	690	657	700	308	1487	2674	653	824
234592	19035	15578	15376	11614	11973	8229	27737	23306	17525	50968	23066	23681	37059	39409	48002	52775	45598	24925
228311	1354	650	485	420	346	340	402	431	498	1148	369	496	1030	248	360	579	747	187
227270	4618	8534	13984	5643	4482	7795	6028	4370	4415	13562	8298	5629	32069	6019	14377	11725	6846	5191
224849	360	184	213	271	316	386	260	134	267	591	262	285	324	161	208	633	250	249
223531	653	571	627	746	720	613	336	665	357	2002	493	679	431	253	1255	2228	504	500
218683	165	221	218	186	247	222	196	166	225	724	213	180	531	172	490	619	446	237
213956	2735	10662	6041	452	1069	2510	2550	1933	787	1428	390	1174	1809	986	3571	4364	867	1814
203264	725	630	470	755	646	959	369	166	453	827	419	168	1140	1360	347	842	478	631
200942	7523	662	9514	1416	404	1471	2231	3578	6571	6132	2515	7739	20725	789	1834	1264	8326	366

Table 1

Metabolomics profiles obtained by *D./B. bruxellensis* UCD2426. Compound identification and quantification was obtained by MS/GC-TOF. wo = growth condition without sulphite; 20 = growth condition at 0.2 mg/l mol. SO_2; 40 = growth condition at 0.4 mg/l mol. SO_2. Sample replicates are indicated by letters a-f.

compuond	2423Awo	2423Bwo	2423Cwo	2423Dwo	2423Ewo	2423Fwo	2423A20	2423B20	2423C20	2423D20	2423E20	2423F20
xylose	3037	415	3545	2999	422	1121	4785	4327	4097	5630	5637	3577
xylitol	672	559	676	866	471	735	718	1540	1624	1439	1267	1359
xanthine	127	156	117	169	135	359	228	231	520	398	453	246
valine	53480	46975	54564	31101	52016	3673	32586	54428	96437	73126	55970	24657
uridine	1361	679	1601	875	816	437	194	187	438	356	352	347
urea	28844	10720	15078	13904	47199	687	21363	13840	8575	24537	19306	7443
UDP GlcNAc	217	209	224	276	237	114	161	384	173	317	151	280
tyrosine	34021	20502	31354	25633	22626	5342	27805	35557	54521	49395	37689	30779
tryptophan	5063	2748	3506	3689	2602	1030	4883	7526	10055	10431	7651	7017
trehalose	6532	4374	7584	6699	3981	4278	12795	37531	18773	40011	31427	20314
thymine	406	354	296	504	350	440	262	406	343	424	274	258
threonine	12471	2709	3137	7203	2305	2678	6392	14692	23197	19363	16266	9320
sulfuric acid	2184	4302	6310	2496	3543	45301	962	15430	12904	21297	14961	3355
sucrose	465	101	207	54	646	584	221	198	111	428	343	78
succinic acid	5608	4486	5329	6154	3638	3721	8233	11824	15782	12449	13327	12973
stearic acid	31263	40981	27645	44610	32624	106128	32067	33597	62360	30922	28469	26255
sorbitol	5782	2156	13208	4033	6831	15763	12224	18782	17826	13378	14484	13268
sophorose	163	169	190	245	236	702	34	228	153	99	83	203
serine	30291	20880	17892	14188	23339	4101	53501	107007	128594	110452	107447	85997
saccharopine	213	239	207	227	172	325	151	220	200	188	176	203
ribose-5-phosphate	849	1448	930	1246	859	6143	496	237	1285	1472	1092	1308
ribose	11183	5365	12110	9280	9527	18523	4856	3176	6669	3812	3071	2760
ribitol	2138793	2480882	1823122	2239151	2112082	76783	1917768	99205	9389	307242	1158504	1396817
pyrophosphate	2029	2320	2904	3497	2340	5767	2205	2462	2120	2159	1565	4287
pyrazine 2,5-dihydroxy NIST	265	292	240	274	184	437	344	1110	466	1044	1303	970
propane-1,3-diol NIST	1068	761	671	1390	870	1615	271	691	795	560	532	798

proline	3656	5568	8480	2019	4255	473	6083	68404	48049	61531	20807	5697
phosphoric acid	79815	38911	88686	90203	69798	331696	54418	89455	77124	75653	62343	94479
phenylalanine	33586	19302	26710	23149	19943	3275	20570	21979	45221	36714	31855	23848
pelargonic acid	6061	13143	5863	8057	3810	9066	5898	4689	7603	6611	4248	4586
pantothenic acid	1821	1737	2588	1786	1253	2383	2593	4350	4986	3127	4145	4716
palmitoleic acid	496	605	469	514	458	877	414	1690	576	672	212	552
palmitic acid	4011	4881	3293	5417	3802	12552	4181	4063	5950	4294	3922	3439
oxoproline	146901	160097	200947	176397	132806	4709	169079	380444	543196	489299	444116	371124
oxalic acid	6409	8741	5503	8046	5892	15185	9029	7927	6353	5853	5181	5106
ornithine	11035	6785	14440	11083	10394	3380	12087	29285	23525	45261	26412	26231
oleic acid	146	234	123	149	157	331	131	30	136	121	111	104
N-methylalanine	713	1105	1709	2260	1061	3061	1642	6370	1566	1634	1337	814
nicotinamide	364	554	544	336	387	883	411	1110	930	960	736	648
N-acetylornithine	761	1039	888	1183	689	807	901	2425	1703	3832	2382	1244
N-acetylglutamate	5445	6772	6164	7393	2911	3182	5228	8346	15130	31468	21797	9145
N-acetyl-D-mannosamine	314	243	205	249	315	485	150	396	324	260	96	225
N-acetyl-D-hexosamine	15789	3645	17724	7935	10883	18668	4057	9926	15867	7345	6401	7464
myristic acid	416	557	405	441	564	1443	567	669	667	641	617	491
mevalonic acid NIST	24278	28649	23296	29757	11333	3519	27088	39231	57652	38244	53202	54049
methylhexadecanoic acid	543	1047	491	783	611	2025	616	591	1332	736	1360	615
methionine sulfoxide	3197	1448	3279	2329	1976	542	3225	3412	4121	2980	1594	3347
methionine	287	1180	316	274	410	325	163	662	660	445	412	293
mannose	9995	3008	7725	30067	5468	13660	30700	49629	70222	67219	59479	41467
mannitol	7920	8083	9578	8565	1811	23681	20237	28442	11045	33101	4697	19654
maltotriose	511	251	120	177	175	419	476	186	161	1001	687	147
maltose	6376	4261	6644	6961	80	804	12335	38105	19015	36954	31731	20064
malic acid	5056	3389	5763	5725	3687	8737	3696	7728	9943	6595	8850	7394
maleimide	1060	783	884	825	742	1274	646	916	724	867	744	768

lyxose	248	231	259	238	237	365	316	353	296	399	415	236
lysine	9503	4787	6473	8647	5835	702	6933	10050	21431	23648	17142	14777
levoglucosan	10921	7044	996	7766	19902	20108	3669	21000	16136	419	454	16323
leucrose	412	468	450	449	396	539	507	1347	1219	1677	1363	1078
leucine	50997	73066	91381	34192	78126	1443	32200	58197	121862	69376	40541	19007
lauric acid	2213	1501	1356	2182	1310	5152	1168	2088	1925	1635	1606	1254
lactic acid	32778	12472	20681	15492	37427	48426	20938	11484	11972	30187	24957	10343
isothreonic acid	503	242	463	418	366	738	502	2028	1500	1342	1373	1806
isonicotinic acid	903	1155	1326	1034	994	2904	518	878	901	974	796	659
isoleucine	40869	21077	27837	21564	24548	2323	29097	28657	61786	44850	31470	23866
inositol myo-	734	490	486	662	725	1190	2244	10968	10835	12047	8666	6108
hydroxylamine	14602	16615	9555	18777	13550	19325	14343	10155	10365	10058	231	11047
homoserine	718	732	609	759	713	1265	695	2151	2107	873	733	890
guanosine	345	340	321	615	244	428	229	316	210	366	352	347
guanine	3501	1936	4576	3153	3312	1591	630	844	1125	662	561	911
glycolic acid	1600	1202	1390	1759	1154	2941	2700	2267	2043	2688	1910	1922
glycine	85072	31243	53581	57072	57208	7159	92905	189220	214727	157477	156588	178285
glycerol-alpha-phosphate	61864	61572	96708	56290	72674	205472	45621	87882	113282	115442	122946	110149
glycerol	129070	91916	120831	97674	120347	191715	118016	206711	252605	237544	231341	175450
glyceric acid	1876	938	1942	1705	886	3221	8663	9653	10729	11867	8907	11179
glutamine	26417	13335	49102	38207	13737	6526	82055	172734	80956	99571	53301	118230
glutamic acid	35609	17027	26603	32233	13958	8050	35628	113915	85184	100638	109241	109918
glucose-6-phosphate	403	833	559	1219	332	919	799	2521	1852	2784	2752	2578
glucose-1-phosphate	3073	3077	2429	3620	2973	6445	4827	12016	24439	16489	15376	4870
glucose	105222	95430	164761	167564	148552	505376	160568	212106	155254	174019	125278	108915
gluconic acid	220	239	167	263	274	693	192	286	231	161	191	161
galactose-6-phosphate	451	325	164	416	149	337	1332	4313	3303	3636	3484	3608
galactinol	9834	6638	9383	9677	5419	7568	14297	33640	19600	29540	31048	29391

GABA	25917	13527	15574	3164	9128	8807	19903	41622	50320	62313	73760	53785
fumaric acid	3497	2089	2790	3253	2226	2962	3686	6672	5795	4409	5705	8227
fructose-6-phosphate	4930	10464	7052	15633	3661	9852	4993	15797	11888	13590	13009	12375
fructose-1-phosphate	1168	1117	1168	2320	454	1880	1642	6073	4594	4359	2515	4935
fructose	471612	394097	780372	769903	727181	2446989	754839	909497	690862	699631	520148	507007
ethanolamine	12314	5674	8359	10999	9013	4682	11139	16228	19849	13653	14949	20984
ethanol phosphate NIST	19968	17157	17529	22250	12073	47799	24725	33372	37086	34296	28130	33304
erythrose	329	251	193	270	223	2871	212	433	445	448	296	208
erythritol	2025	1997	2461	2157	1147	1521	3915	10451	8215	13659	8990	5829
cytidine-5'-diphosphate	1107	493	405	771	578	1717	457	1526	897	1429	876	524
cysteine-glycine	204	256	261	177	157	316	179	411	251	318	528	314
cyano-L-alanine	168	236	154	146	188	362	180	1011	364	239	165	112
citric acid	7377	2895	8382	8467	6675	2214	4253	16292	12153	8245	11444	19326
citramalic acid	164	190	119	134	195	443	111	166	139	132	114	95
capric acid	664	877	475	600	646	1630	531	1425	703	571	550	469
butane-2,3-diol NIST	1665	1208	1148	1657	1575	3191	1252	1454	1551	2445	1034	1382
beta-alanine	302	169	270	423	205	407	628	636	415	323	231	1001
benzoic acid	90038	107283	69131	118084	83373	186572	96226	75031	73712	75792	55655	73166
aspartic acid	21002	11385	20134	18254	16198	4546	15589	35112	46113	33105	35854	34668
asparagine	5380	3180	5118	5305	3112	1775	7087	24294	23585	21500	21160	17482
arginine + ornithine	6277	4130	8253	7338	6094	479	9023	21026	12535	40666	16934	16010
arabitol	361679	392938	322327	59891	337122	188018	340134	366563	316105	275268	199026	370735
arabinose	1673	740	1806	1795	1260	2452	2462	2282	2137	2921	2955	1846
aminomalonic acid	256	218	142	141	214	425	159	263	359	272	173	228
alanine	170193	145591	178316	104316	146526	15326	124080	482397	534165	486518	389945	185800
adipic acid	960	1092	807	1362	1038	2477	886	1073	912	1005	1388	925
adenosine-5-phosphate	339	320	238	421	174	554	1063	3743	1060	2129	3163	3185
adenosine	765	434	288	652	258	334	1330	4133	3165	2774	4614	4526

adenine	44050	29188	58116	43982	34726	30523	25682	32846	53171	43224	33560	27592
aconitic acid	195	242	235	224	217	443	385	1057	901	1168	855	625
5'-deoxy-5'-methylthioadenosine	188	175	138	183	139	371	118	232	193	175	175	268
4-hydroxybutyric acid	354	437	420	327	354	506	451	612	595	665	627	398
3-hydroxypropionic acid	863	911	651	777	802	1178	1155	1299	1416	1491	1054	1002
3-hydroxybutanoic acid	1284	1379	962	1306	1105	3637	856	1327	1126	469	142	189
3-hydroxy-3-methylglutaric acid	1634	3291	1084	1714	623	795	4244	7678	7713	7170	5557	5039
2-isopropylmalic acid	201	264	211	255	190	536	199	267	317	279	247	254
2-hydroxyglutaric acid	1716	1125	2271	1904	1872	5580	1446	3686	5283	3804	2858	3301
2-(4-hydroxyphenyl)ethanol	189	178	145	217	207	482	144	154	167	130	102	122
1-deoxyerythritol	564	516	451	401	592	1058	1019	2570	5115	4173	6086	3416
400494	12727	8991	18953	14143	6756	4953	17719	19549	28106	15707	15717	20576
388098	3795	4322	2868	4830	3535	5733	3792	3007	3331	3078	2208	2980
382318	6089	6540	4160	7609	5247	10747	6154	4003	4609	4149	3156	4511
294511	537	495	415	814	373	1651	617	551	742	509	355	399
272849	63014	55420	35745	66464	46206	98867	55636	37455	38738	41387	30380	43520
270999	3733	2670	3563	3159	3869	7086	1661	993	1468	1874	1389	1138
270066	28025	30868	20832	32896	24356	55066	26528	20018	23277	21898	15713	20845
268585	54548	50690	31815	65870	42545	96165	52681	34968	34941	33905	24167	40745
234717	3309	3845	2921	4982	2936	9584	3933	3144	3868	4188	2959	3098
226330	2096	3391	2333	3987	2373	3501	2349	2089	2119	2201	1130	1225
216428	2509	5744	3267	4932	2740	3380	6741	2580	6793	3343	1389	6302
208812	6714	4443	7450	10961	3500	2311	10445	30184	18050	33610	20239	15189
268313	14581	14819	9431	17383	11567	7764	13923	8863	11714	9287	7280	10506
242417	3592	4045	2921	4378	3708	7873	3656	2862	2975	3150	2283	2847
231652	169	190	162	283	200	820	189	197	215	196	226	263
367932	11897	14811	10088	14728	12543	24055	11572	8893	10855	10152	7108	9387
301399	436	843	419	712	577	1280	448	592	527	616	517	364

356938	316	351	306	402	452	765	352	447	363	287	253	312
330609	1329	905	1590	1046	1019	1329	1734	4249	4314	3466	2280	2590
321692	1904	1546	1325	2585	1310	1928	1721	1171	1353	1734	1230	1858
233017	17561	14727	20394	21990	16726	26043	10965	21264	8707	13781	12747	13946
231654	157	134	161	258	46	524	219	154	149	198	158	191
204344	94404	99369	75998	122983	91802	177877	98913	64985	76318	80816	61934	73593
199203	47065	45338	50754	45618	32293	129107	129782	143749	209637	203070	154799	122685
319168	707	5970	2781	840	5255	2591	816	1948	1853	1562	908	322
317187	932	967	335	1581	368	446	7671	22954	18553	20354	18010	19390
229959	1458	1276	1432	2457	495	1555	1989	7754	5993	5563	3395	6093
211972	6770	4475	2388	7236	2854	672	8530	3908	4442	5364	2926	5879
317072	136	360	202	288	184	551	232	42	219	229	224	178
314770	8011	8286	13276	14097	12119	28101	12870	14912	12653	13774	10001	8661
310052	1988	855	4171	3681	3147	1540	8535	13172	8976	7877	6962	8945
235965	11896	12735	6764	10716	4864	25170	5135	6695	3331	6775	6066	8558
217783	6853	6120	5928	62218	5260	16318	54422	3034	6237	5606	4625	51095
367944	659	521	825	505	348	482	1065	2535	2280	3205	2476	1634
356954	1155	1287	958	1146	846	1720	1019	758	901	984	612	857
299447	560	434	800	625	313	765	959	2815	2040	3011	2958	2264
362130	18145	9718	12858	16472	9326	69004	17546	26550	24953	26541	23314	25249
237138	1410	1626	1142	1517	1793	3317	378	585	526	590	505	467
207223	826	811	486	1123	879	458	1177	328	286	165	78	705
202573	6969	3486	3109	10142	4793	401	14985	29005	10740	21751	19702	21206
202572	9554	7351	8426	16538	9230	1256	26019	41505	15349	35154	27333	25772
374786	39982	38803	21855	66841	30160	18683	83973	130926	50294	107488	82843	91862
371632	6366	1809	2210	4701	1349	464	24813	37188	47027	56844	51392	35909
343526	727	346	640	504	627	353	610	2486	1593	1104	1250	1030
204448	1044	1027	661	957	693	1696	736	626	952	751	486	813

199337	350	373	331	311	306	976	140	501	272	339	204	194
360205	3194	1606	1060	883	957	2082	1722	2059	1589	2776	1993	1535
274174	1932	2152	1046	1236	1412	1964	1534	145	1209	1013	678	452
236890	2089	1395	2809	2631	2040	3371	2661	8987	6924	7083	6108	4752
212251	343	357	264	322	228	518	722	1700	1361	2239	1514	1486
301006	526	523	498	815	516	816	817	980	707	483	250	336
232087	7629	2895	8634	8802	6555	2214	4171	16176	12044	8380	11396	19264
224574	756	1228	614	1088	867	328	952	630	617	724	508	640
213253	57076	11681	14633	34881	10799	1148	30248	10184	25837	32553	30583	34333
273925	6452	5791	4097	6702	4588	13862	5454	1463	3965	3688	2687	4144
222541	2836	4037	4193	5918	4735	1030	9614	13054	6720	16492	16478	8873
357010	800	768	513	647	602	1998	443	958	717	531	455	472
228959	1324	493	632	1136	637	353	2260	1476	956	590	235	3984
367914	1599	730	1319	1251	1006	1392	2040	23362	5251	3727	3173	7535
318770	5075	4173	7087	7517	6272	28692	7717	9472	7804	9503	6802	5472
300919	16696	14944	9413	16640	13635	34133	13256	10189	11063	10196	6798	10218
237133	361	601	376	388	507	1615	171	207	209	184	115	105
400671	8050	2114	2411	5921	1290	750	41202	23081	78374	95609	31033	52486
362109	708	454	702	378	421	431	952	1189	2485	1938	1622	894
231796	707	507	1108	929	690	958	354	886	1022	752	1076	904
225896	289	292	382	339	653	702	372	752	589	373	365	391
206022	1376	1843	1277	2105	1542	3612	1844	1784	2070	1705	1247	1340
354038	388	456	457	653	480	973	776	331	450	286	243	275
268306	631	565	468	549	606	1630	485	269	402	357	204	236
218784	3120	3205	1975	3337	2605	5815	2789	1088	2180	2263	1519	1999
202095	547	1035	1150	1128	860	2314	771	1628	1015	647	490	393
400644	323	334	450	342	457	660	393	714	991	889	907	545
239332	930	1114	708	472	451	1561	305	1295	2347	1462	922	1671

223675	907	760	927	885	600	1280	1278	2588	3469	3049	2537	1528
199239	354	627	523	235	462	2335	375	758	481	843	585	576
371802	2482	1450	979	3585	1698	943	2368	3096	776	1676	1053	1721
362148	590	540	434	540	389	684	404	859	1015	1009	843	950
356985	468	1646	1459	930	1326	575	896	2855	1648	749	570	578
309642	1089	2595	1171	1448	1972	3847	1042	1497	982	1089	721	872
303839	328	229	295	294	422	735	408	1607	1634	1878	1883	1218
269335	3549	5201	4497	4416	1996	744	4048	6852	9945	25932	16526	6109
231657	476	1177	604	926	747	841	593	452	523	510	426	487
225867	160	148	167	132	179	301	151	326	101	223	158	100
223120	268	329	150	437	282	542	266	437	376	455	242	225
202841	176	187	186	187	168	467	88	305	134	281	224	165
331041	4066	2687	4244	3206	3371	1913	1529	1811	2527	1987	1741	1303
323686	1475	889	1799	1241	1352	2386	1204	2627	2027	1913	1792	1404
310985	223	234	193	286	216	449	219	1327	967	1267	1182	637
242565	1665	1208	1148	1657	1575	3191	1252	1386	1261	1505	888	1197
231659	213	275	175	96	230	292	99	241	239	96	118	95
229113	6205	4446	4184	6869	3751	10012	5767	2834	4167	3941	2823	4669
216571	973	1150	783	1551	1035	1461	1150	555	697	779	588	588
213386	202	485	385	255	259	413	222	275	428	265	275	243
328652	2173	2397	1680	2242	1963	4293	712	4867	721	492	285	475
299421	512	516	254	361	417	898	269	275	253	267	226	213
233455	385	451	208	362	304	880	312	437	238	331	135	285
223830	156	226	133	261	114	304	196	508	223	137	159	216
222169	331	356	418	2522	313	512	1305	1795	3548	197	3124	446
222115	2468	3157	2050	3226	2445	4676	2567	2131	2067	1849	1550	2028
213697	275	331	317	310	370	368	263	335	467	322	281	329
202571	99763	104002	102195	125135	121110	322052	167172	185438	265364	243121	187908	150923

362120	195	195	123	177	157	506	783	798	1747	1987	1289	371
328420	321	384	339	349	313	681	326	774	821	806	917	688
318471	6324	2362	10520	5101	3741	14182	20694	34817	46148	44796	40114	27637
310006	122	117	91	138	149	452	140	252	217	121	77	43
303956	262	816	560	485	587	368	335	468	706	293	225	329
295269	801	1242	1557	392	1050	1232	2070	5591	998	606	764	780
295226	808	1425	551	773	1391	3423	390	8281	1136	1297	792	797
237174	100	434	157	319	281	850	138	129	151	141	107	101
228018	183	170	133	204	130	280	191	483	238	139	137	283
227774	421	250	355	591	369	479	253	924	898	1818	1307	872
215088	2695	2735	1753	2157	1479	5887	1700	1989	1942	1179	1095	1160
200421	23890	23696	15297	24819	20592	45341	19261	4082	2992	4594	2672	15341
199942	624	768	544	881	728	1537	695	767	628	430	656	517
356987	471	591	758	672	539	826	1193	2517	1135	1352	428	1181
310667	2463	2707	1337	3177	1136	2302	2607	3247	1364	1114	868	1826
309730	2059	1456	1502	3498	967	3338	1385	2716	1960	1705	2735	3010
304770	209	286	341	182	277	422	198	931	588	669	532	186
303163	404	272	209	378	180	410	593	662	380	304	282	783
294349	7057	7900	4680	8064	5773	11720	6191	777	5301	4885	3078	5029
280930	489	793	910	805	690	958	442	886	1113	827	985	1010
270594	8924	7654	8296	6927	10319	39297	339	801	491	163	151	347
234525	7057	7900	4104	7414	6559	14151	5428	3831	5301	4485	3412	3836
227591	758	855	1320	1351	1082	931	405	732	996	1224	804	910
225043	3380	3275	2877	3896	3516	8276	2567	2641	3209	2504	1641	1968
200522	411	343	537	535	336	666	404	1000	681	797	782	661
199596	2715	3157	2050	2929	2713	5583	2394	2131	2225	1997	1550	1867
359483	685	534	736	777	461	578	432	657	795	701	539	642
322226	956	874	829	856	1138	792	1086	1320	1373	1622	1481	1069

289052	218	248	210	293	193	298	215	291	610	668	468	294
269776	7155	7790	6804	7536	6845	15061	3645	3421	3747	3949	3213	2896
239565	234	215	431	322	213	470	659	1164	861	743	671	1034
235327	1326	1604	919	2883	3964	2597	911	564	2442	926	1280	912
228605	1280	540	1251	719	1048	362	703	1291	902	1837	637	574
228249	398	270	343	340	311	557	283	564	477	440	265	336
226910	246	254	279	341	235	340	294	510	452	414	348	248
224632	227	279	141	225	167	313	162	268	100	296	255	142
224627	126	346	189	117	244	937	231	238	98	267	242	253
221574	2605	3165	2470	3497	2788	7457	3297	2271	2675	2508	1724	2213
215397	10005	2110	2857	11866	2089	0	9008	777	2279	12135	5792	10686
204994	251	334	183	294	235	578	543	1014	683	684	838	672
352849	537	605	720	507	627	2639	246	422	607	644	456	424
305055	1905	1720	19688	27783	2121	23091	23996	61314	7457	7541	4976	4161
301584	625	813	1144	459	605	1627	1238	2875	722	583	135	523
268539	913	1170	856	1308	660	1329	1047	816	593	1051	520	632
234592	18026	10038	12945	16609	9601	70239	17386	26675	25069	26628	23377	25249
228311	577	337	547	363	347	603	389	709	935	583	699	499
227270	12103	13053	6961	10999	5089	23784	5331	7416	3813	6334	5903	8251
224849	349	300	293	266	341	762	285	368	321	314	182	275
223531	1002	947	762	1174	784	1018	835	558	836	734	561	619
218683	193	231	171	176	230	374	197	242	278	308	244	201
213956	9568	8340	8725	4037	11519	41219	355	448	484	208	109	176
203264	183	463	424	237	388	322	306	2608	639	361	284	174
200942	1426	565	4010	5606	528	4143	1704	9936	3820	6582	10474	8864

Table 2

Metabolomics profiles obtained by *D./B. bruxellensis* UCD2423. Compound identification and quantification was obtained by MS/GC-TOF. wo = growth condition without sulphite; 20 = growth condition at 0.2 mg/l mol. SO_2. Sample replicates are indicated by letters a-f.

APPENDIX C

Scientific products

Vigentini I. (2010)
Molecular characterisation, stress responses and specific enzymatic activities of *Dekkera/Brettanomyces bruxellensis* wine strains.
15th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Naples, Sep 15-17
2nd Annual PhD Report

Molecular characterisation, stress responses and specific enzymatic activities of *Dekkera/Brettanomyces bruxellensis* wine strains

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Tutor: Prof. Roberto Foschino; Co-tutor: Dr. Claudia Picozzi

The aim of this PhD research project is to develop strategies in order to analyse and control the wine spoilage linked to Dekkera/Brettanomyces bruxellensis *species. The present work describes the main issues that have been investigated during the second year of this PhD project: i) the development of a multiplex PCR protocol of typing using intronic sequences; ii) the setting up of a spectrophotometric assay for the dosage of the vinylphenol reductase activity, involved in volatile phenols production; iii) the yeast response to a low electric current treatment (LEC).*

Caratterizzazione molecolare, risposte a stress e attività enzimatiche specifiche di ceppi vinari di *Dekkera/Brettanomyces bruxellensis*

Lo scopo di questo progetto di tesi di dottorato è sviluppare strategie per l'analisi ed il controllo dell'alterazione del vino legato alla presenza di lieviti della specie *Dekkera/Brettanomyces bruxellensis*. Il presente lavoro descrive le principali attività che sono state condotte nel corso dei primi due anni di questo progetto di dottorato: i) sviluppo di un protocollo di PCR multiplex per la tipizzazione che sfrutta le sequenze introniche; ii) messa a punto di un saggio spettrofotometrico per il dosaggio dell'attività della vinilfenolo reduttasi, coinvolta nella produzione di fenoli volatili; iii) risposta del lievito allo stress da campo elettrico a bassa intensità.

Key words: *Dekkera/Brettanomyces bruxellensis*, ISS-PCR, volatile phenols, low electric current treatment.

1. Introduction

Dekkera/Brettanomyces bruxellensis is a major cause of red wine spoilage since it develops off-flavours (4-ethylguaiacol and 4-ethylphenol) in wine through a specific reductive metabolism where vinylphenol reductase (VPR) is the crucial enzyme. Recent observations suggest that "Brett" spoilage is strictly strain-dependent and therefore, a rapid and reliable identification at strain level becomes strategic (Vigentini, 2008; Agnolucci, 2009). This poster communication reports the main results achieved during the first two years of PhD research project: A1) the development of a new method for molecular typing; A2) the setting up of a protocol for the measurement of the VPR activity A3) the yeast response to an electric current treatment.

2. Materials and Methods

A1) *Molecular characterisation of* D. bruxellensis *wine strains*

A total of 17 *D. bruxellensis* strains belonging to the international CBS collection were analysed in this study (Fig. 1, 2). Genomic DNAs was extracted as described by Querol *et al.* (1992). The primers employed in the amplification reactions were EI1, EI2, LA1, LA2 (De Barros et al. 1996) and DbEI1 (5'-CTGGCTTGGTGTAAGT-3'). Amplifications were carried out following the protocol described for *S. cerevisiae* (de Barros, 1996), setting the annealing temperatures at 46°C

and 47°C when EI1/LA2 or DbEI1/LA and EI2/LA1 or DbEI2/LA1 pairs of primers were used, respectively. Multiplex amplifications were performed as described above using EI1, DbEI1 and LA2 oligonucleotides. The digitalized gel images were analysed using Quantity One version 4.6.2 (Bio-Rad). Indexes of discrimination among ISS genetic profiles were calculated as described by Hunter and Gaston (1988) according to the repeatability percentage.

A2) ***VPR activity of* D. bruxellensis *wine strains***

The same *D. bruxellensis* CBS collection was tested for VPR activity. Cells in exponential phase were harvested, washed in phosphate buffer 0.05 M pH 7 and subsequently centrifuged. Pellets were resuspended in the same buffer, added with dithiothreitol 0.1 mM and PMSF 2 mM. Cells were then disrupted by subsequently steps of icing and vortexing. Total proteins content was measured by Bradford assay. The determination of the specific activity of VPR was calculated by means of a spectrophotometer at 340 nm. Enzyme trials were performed as described in Table 1.

Pipette into cuvette	**Blank**	**Sample**
phosphate buffer 0.05 M pH 7	0.927 ml	0.927 ml
NADH 10mM	0.020 ml	0.020 ml
4-vinylguaiacol 0.3 M	0.003 ml	-
Cell extract	-	0.050 ml
Mix and read absorbances of the solution for 5 minutes. Then, start the specific reaction adding into the cuvette:		
4-vinylguaiacol 0.3 M	-	0.003 ml
Mix and read absorbances of the solution for 5 minutes.		

Table 1

A3) ***Stress response of* D. bruxellensis *to LEC treatment***

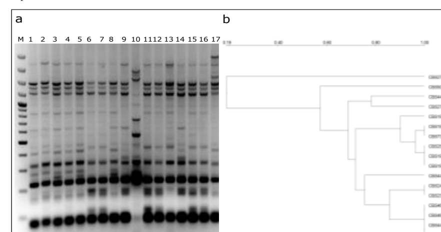
The experiments were performed applying to wine a 200 mA current intensity (Lustrato, 2010) using *D. bruxellensis* CBS4481. Cellular viability was evaluated by plate counts and ATP determination (Ranalli, 2002). Sample treatment was performed as follows: i) control (wine + cells), ii) wine + cells, added with SO_2 (80 mg l^{-1}), iii) wine + cells, subjected to 200 mA. Measurements of pH, titrable acidity, ethanol and sugar concentrations were carried out. Cell morphology was observed using a scanning electron microscopy (SEM) (Ranalli, 2002). Detection of volatile phenols (vinyl- and ethyl-phenols) was evaluated as described by Vigentini *et al.* (2008).

3. Results and discussion

A1) Primers that anneal to specific conserved motifs, such as those designed for ISS-PCR, have been already described for the inter- and intraspecific characterisation of *S. cerevisiae* (de Barros Lopes, 1996). On the other hand, no reliable results have been shown for the discrimination of *D./B. bruxellensis* species at strain level when yeasts are analysed with the primer EI1 alone (Oelofse, 2009). Primers EI1/LA2 and EI2/LA1 were preliminary employed in pair on *D. bruxellensis* collection and results showed that different indexes of discrimination were generated (98.5% and 93.0%, respectively). Considering that *D. bruxellensis* has an intron content similar to the one of *S. cerevisiae* and that the most represented 5’ motif seems to be GTAAGT instead of GTATGT as reported for *S. cerevisiae* (Woolfit, 2007), a new primers pair DbEI1/LA2 was tested (Fig.1). Since different patterns were generated from the same strain, a multiplex PCR was applied on the whole collection using primers DbEI1, EI1 and LA2 (Fig. 2), with an increasing of the discriminatory power up to 99.3%. This work suggests a simple and reliable method for strain typing of *D. bruxellensis* species.

Fig. 1

ISS profiles of *D. bruxellensis* strains using a) DbEI1/LA2 pairs of primers. Lanes: 1, CBS73; 2, CBS1940; 3, CBS1941; 4, CBS1942; 5, CBS1943; 6, CBS2336; 7, CBS2499; 8, CBS2547; 9, CBS2796; 10, CBS2797; 11, CBS4459; 12, CBS4480; 13, CBS4481; 14, CBS4482; 15, CBS4601; 16, CBS4602; 17, CBS5006; M, 100bp DNA ladder (Fermentas, Vilnius, Lithuania); b) Dendrogram built by UPGMA analysis comparing ISS profiles obtained after DbEI1/LA2 amplifications.



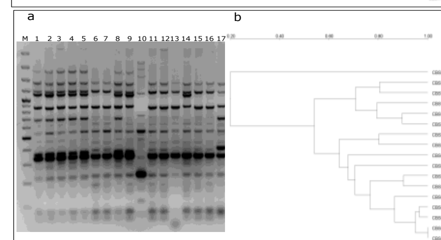
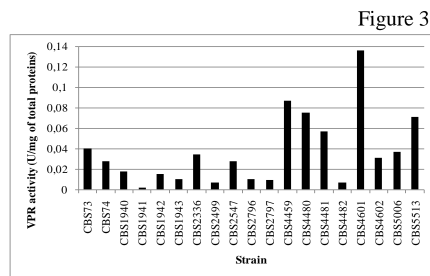


Fig. 2

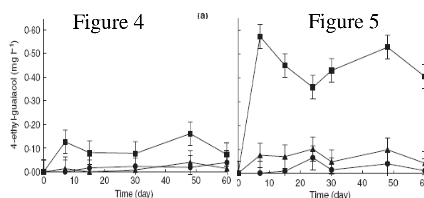
ISS profiles of *D. bruxellensis* strains using a) Multiplex PCR with DbEI1, EI1 and LA2 primers. Lanes: 1, CBS73; 2, CBS1940; 3, CBS1941; 4, CBS1942; 5, CBS1943; 6, CBS2336; 7, CBS2499; 8, CBS2547; 9, CBS2796; 10, CBS2797; 11, CBS4459; 12, CBS4480; 13, CBS4481; 14, CBS4482; 15, CBS4601; 16, CBS4602; 17, CBS5006; M, 100bp DNA ladder (Fermentas, Vilnius, Lithuania); b) Dendrogram built by UPGMA analysis comparing ISS profiles obtained after DbEI1, EI1 and LA2 amplifications.

A2) The specific activity of the VPR enzyme was preliminary measured in presence of NADPH as coenzyme. The low decreasing in the kinetics of reaction obtained for all the strains (A_{340}/min< 0.1), confirmed the low affinity of VPR to NADPH. On the contrary, the use of NADH led to quantifiable values of VPR activity, especially for CBS4601 strain (Fig. 3). These data indicated that VPR is a NADH-dependent enzyme and that a reliable UV method, here described for the first time, can discriminate the strains for their physiological aptitude to produce off-flavours. Tests, performed in triplicate, showed a good repeatability (standard error of 3%).

Figure 3



A3) No significant variations in the viable cell count and in the ATP content were observed between the LEC and SO_2 treatments, showing an inhibition effect due to electric current. Kinetics of off-flavours production, under various conditions, are shown in Fig. 4 and 5. In the control (i/■) higher amounts of volatile phenols were produced compared to tests (ii/▲) and (iii/●). The LEC treatment (200 mA at 72nd h) caused alterations in the morphology and integrity of the cells. Our findings show that the inactivation of *D. bruxellensis* CBS 4481 using LEC treatment was obtained. The use of this technological process could improve the quality of wine without recourse to chemical additives, offering an alternative for the production of organic wines.



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DiSTAM Dipartimento Scienze e Tecnologie Alimentari e Microbiologiche

UNIVERSITÀ DEGLI STUDI DI MILANO

Molecular characterisation, stress responses and specific enzymatic activities of *Dekkera/Brettanomyces bruxellensis* wine strains

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Introduction

Dekkera bruxellensis yeast species can develop off-flavours in wine through a specific reductive metabolism where vinylphenol reductase (VPR), recently purified and characterised, is the crucial enzyme (Godoy, 2008; Tchobanov, 2008). In particular, volatile phenols (4-ethylguaiacol and 4-ethylphenol) are often produced in amounts that are higher than the perception threshold with a loss in product quality. Nowadays, the physiological studies have clarified that this spoilage activity, due to the production of ethyl-phenols, is a strain dependent character (Vigentini, 2008; Agnolucci, 2009; Oelofse, 2009). Although some applications to contrast yeast development in wine have been studied (Lustrato, 2010; Goretti, 2009) their application to wine production is still not well known. For this reason, the identification at strain level throughout the research of new specific molecular targets becomes a strategic activity (Vigentini, 2010). Recently, about 40% of *D. bruxellensis* genome have been sequenced (Woolfit, 2007); when the entire genome will be available, more consistent protocols that possibly correlate genetic and physiological aspects to a single strain could be arranged. Thus, the availability of comparable results among laboratories throughout databases could represent an important goal to face the increasing problem represented by *D. bruxellensis* in the oenological field. The main results achieved during the first two years of this PhD research project include the following activities :

A1) the development of a new method for molecular typing;

A2) the setting up of a protocol for the measurement of the VPR activity;

A3) the evaluation of the yeast response to an electric current treatment.

Research activities

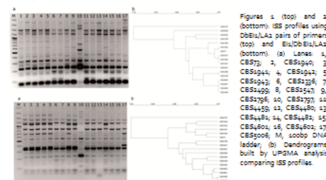
A1) *Molecular characterisation of* D. bruxellensis *throughout Intron Splice Site (ISS) analysis*

METHODS:

A total of 17 *D. bruxellensis* strains belonging to the international CBS collection were analysed in this study (Fig. 1, 2). Genomic DNAs was extracted as described by Querol *et al.* (1992). The primers employed in the amplification reactions were EI1, EI2, LA1, LA2 (de Barros Lopes, 1996) and DbEI1 (5'-CTGGCTTGGTGTAAGT-3'). Amplifications were carried out following the protocol described for *S. cerevisiae* (de Barros Lopes, 1996), setting the annealing temperatures at 46°C and 47°C when EI1/LA2 or DbEI1/LA2 and EI2/LA1 pairs of primers were used, respectively. Multiplex amplifications were performed as described above using EI1, DbEI1 and LA2 oligonucleotides. The digitalized gel images were analysed using Quantity One version 4.6.2 (Bio-Rad). Indexes of discrimination among ISS genetic profiles were calculated as described by Hunter and Gaston (1988) according to the repeatability percentage.

RESULTS AND DISCUSSION:

Primers that anneal to specific conserved motifs, such as those designed for ISS-PCR, have been already described for the inter- and intraspecific characterisation of *S. cerevisiae* (de Barros Lopes, 1996). On the other hand, no reliable results have been shown for the discrimination of *D./B. bruxellensis* species at strain level when yeasts are analysed with the primer EI1 alone (Oelofse, 2009). Primers EI1/LA2 and EI2/LA1 were preliminary employed in pair on *D. bruxellensis* collection and results showed that different indexes of discrimination were generated (98.5% and 93.0%, respectively). Considering that *D. bruxellensis* has an intron content similar to the one of *S. cerevisiae* and that the most represented 5' motif seems to be GTAAGT instead of GTATGT (Woolfit, 2007), a new primers pair DbEI1/LA2 was tested (Fig.1). Since different patterns were generated from the same strain, a multiplex PCR was applied on the whole collection using primers EI1, DbEI1 and LA2 (Fig.2), with an increasing of the discriminatory power up to 99.3%. This work suggests a simple and reliable method for strain typing of *D. bruxellensis* species. As already reported for *S. cerevisiae* (Mattick,1994), further investigation of the fragment sequences could lead to interesting implications on *D. bruxellensis* genome such as its structure, gene regulation and sequence evolution.



Figures 1 (top) and 2 (bottom): ISS profiles using DbEI1/LA2 pairs of primers (top) and EI1/DbEI1/LA2 (bottom). (a) Lanes: 1, CBS73; 2, CBS1940; 3, CBS1941; 4, CBS1942; 5, CBS1943; 6, CBS2336; 7, CBS2499; 8, CBS2547; 9, CBS2796; 10, CBS2797; 11, CBS4459; 12, CBS4480; 13, CBS4481; 14, CBS4482; 15, CBS4601; 16, CBS4602; 17, CBS5006; M, 100bp DNA ladder; (b) Dendrograms built by UPGMA analysis comparing ISS profiles.

A2) *VPR activity of* D. bruxellensis *wine strains*

METHODS:

The same *D. bruxellensis* CBS collection was tested for VPR activity. Cells in exponential phase were harvested, washed in phosphate buffer 0.05 M pH 7 and subsequently centrifuged. Pellets were resuspended in the same buffer, added with dithiothreitol 0.1 mM and PMSF 2 mM. Cells were then disrupted by subsequently steps of icing and vortexing. Total proteins content was measured by Bradford assay. The determination of the specific activity of VPR was calculated by means of a spectrophotometer at 340 nm. Enzyme trials were performed as described in Table 1.

Pipette into cuvette	Blank	Sample
phosphate buffer 0.05 M pH 7	0.927 ml	0.927 ml
NADH 10mM	0.020 ml	0.020 ml
4-vinylguaiacol 0.3 M	0.003 ml	-
Cell extract	-	0.050 ml
Mix and read absorbances of the solution for 5 minutes. Then, start the specific reaction adding into the cuvette:		
4-vinylguaiacol 0.3 M	-	0.003 ml
Mix and read absorbances of the solution for 5 minutes.		

Table 1: UV determination of VPR activity

RESULTS AND DISCUSSION:

The aim of this work was to understand if a functional form of the VPR could be extracted and measured using a simple UV method. To a biochemical and genetic characterisation, this activity could represent a useful assay during the step of the enzyme isolation from a crude cellular extract. The specific activity of the VPR enzyme was preliminary measured in presence of NADPH as coenzyme. The low decreasing in the kinetics of reaction obtained for all the strains (A_{340}/min< 0.1), confirmed the low affinity of VPR to NADPH. On the contrary, the use of NADH led to quantifiable values of VPR activity, especially for CBS4601 strain (Fig.3). These data indicated that VPR is a NADH-dependent enzyme and that a reliable UV method, here described for the first time, can discriminate the strains for their physiological aptitude to produce off-flavours. Tests, performed in triplicate, showed a good repeatability (standard error of 3%).

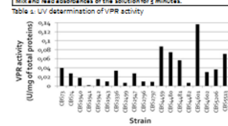


Figure 3: Measurement of VPR activity using NADH as coenzyme.

A3) *Stress response of* D. bruxellensis *to LEC treatment*

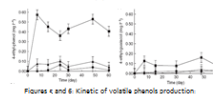
METHODS:

The experiments were performed applying to wine a 200 mA current intensity (Lustrato, 2010) using *D. bruxellensis* CBS4481 (Fig.4). An experiment with adaptation to ethanol at 10% (v/v) was performed according to Vigentini *et al.* (2008). Cellular viability was evaluated by plate counts and ATP determination (Ranalli, 2002). Sample treatment was performed as follows: i) control (wine + cells), ii) wine + cells, added with SO_2 (80 mg l^{-1}), iii) wine + cells, subjected to 200 mA. Measurements of pH, titrable acidity, ethanol and sugar concentrations were carried out. Cell morphology was observed using a Scanning Electron Microscopy (SEM) (Ranalli, 2002). Detection of volatile phenols (vinyl- and ethyl-phenols) was evaluated as described by Vigentini *et al.* (2008).

Figure 4: Vessels and LEC equipment.

RESULTS AND DISCUSSION:

No significant variations in the viable cell count and the ATP content were observed between the LEC treatment and the one where SO_2 was added to wine (data not shown). Thus, the yeast performance was inhibited by the electric current. Kinetics of off-flavours production, under various conditions, are shown in Fig. 4 and 5. In the control (i/■) higher amounts of volatile phenols were produced when compared to tests (ii/▲) and (iii/●).



Figures 5 and 6: Kinetic of volatile phenols production: 4-ethylguaiacol (left) and 4-ethylphenol (right)

The LEC treatment (200 mA at 72h) applied to yeast culture caused alterations in the morphology and integrity of the cells as showed by SEM observations (Fig. 7). In conclusion, our findings show that the inactivation of *D. bruxellensis* CBS 4481 using LEC treatment was obtained. The use of this technological process could improve the quality of wine without recourse to chemical additives, offering a new point of reference for the production of organic wines.

Figure 7: Morphological modifications observed using LEC application: (a) Untreated; (b) LEC-treated (72 h).

Work in progress

1) **Intron investigation on *D. bruxellensis* genome.** More than 50 *D./B. bruxellensis* strains have been amplified using new primers for ISS-PCR designed on *D. bruxellensis* genome and the genetic profiles have been resolved by capillary electrophoresis. Results have shown a high reproducibility among the experiments and the ability to discriminate at strain level.

2) **Identification of genes involved in the response to SO_2 stress in *D. bruxellensis*.** As concern the SO_2 resistance, it is known that *S. cerevisiae* survives throughout increasing in the expression of some genes such as *SSU1* (involved in the extrusion of sulphites) or *FZF1* (involved in the positive regulation of *SSU1*). Up to now we designed and tested primers for PCR and probes for Southern analysis of homologous genes.

3) **Metabolomic analyses of *D. bruxellensis* under different growth conditions** (Davis, CA, USA).

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Molecular characterisation, stress responses and specific enzymatic activities of
Dekkera/Brettanomyces bruxellensis wine strains.
16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Lodi, Sep 21-23
3rd Annual PhD report

Molecular characterisation, stress responses and specific enzymatic activities of *Dekkera/Brettanomyces bruxellensis* wine strains

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The aim of this PhD research project was to develop strategies in order to analyse and control the wine spoilage linked to Dekkera/Brettanomyces bruxellensis *species. As concern the first issue, spoilage microbial analysis, the present work describes i) intraspecific variations of* D./B. bruxellensis *genome using both traditional molecular methods and new protocols for PCRs and electrophoresis separation; ii) species biodiversity trough the study of some physiological and biochemical traits such as the assimilation of carbon compounds, the H_2S production, and the vinylphenol reductase activity, involved in volatile phenols production. Besides, the yeast response to a low electric current treatment (LEC) and to the exposure to sulphite were the topics of the of the microbial spoilage control.*

Caratterizzazione molecolare, risposte a stress e attività enzimatiche specifiche di ceppi vinari di *Dekkera/Brettanomyces bruxellensis*

Lo scopo di questo progetto di tesi di dottorato è stato di sviluppare strategie per l'analisi ed il controllo della presenza di *Dekkera/Brettanomyces bruxellensis* nel vino. Per quanto riguarda il primo punto, ossia l'analisi microbiologica dell'alterazione, il presente lavoro descrive i) la studio della variazione intraspecifica del genoma di *D./B. bruxellensis* sia attraverso l'uso di tecniche molecolari classiche che tramite la messa a punto di nuovi protocolli di PCR e di separazione elettroforetica; ii) la valutazione della biodiversita' della specie legata sia ad attivita' enzimatiche specifiche, come la vinilfenolo reduttasi, che alla capacita' di utilizzo di fonti di carbonio alternative al glucosio e alla produzione di idrogeno solforato. Il controllo dell'alterazione del vino si e' basato sulla risposta del lievito agli stress sia da campo elettrico a bassa intensità che dovuti all'esposizione alla solforosa.

Key words: *Dekkera/Brettanomyces bruxellensis*, ISS-PCR, volatile phenols, low electric current treatment, metabolomics.

4. Introduction

In oenology, "Brett" character refers to the wine spoilage caused by the yeast *Dekkera/Brettanomyces bruxellensis* and its production of volatile phenolic off-flavours (4-ethylguaiacol and 4-ethylphenol) through a specific reductive metabolism where vinylphenol reductase (VPR) is the crucial enzyme. However, the spoilage potential of this yeast is strain-dependent and therefore, a rapid and reliable identification at strain level becomes strategic (Vigentini, 2008). The main results achieved during the PhD research project are described: A1) the development of a new method for molecular typing; A2) the exploration of the phenotypic biodiversity degree; A3) the yeast response to sulphite.

5. Materials and Methods

A1) Intraspecific variations of the Intron Splice Site (ISS) in *Dekkera/Brettanomyces bruxellensis* genome studied by capillary electrophoresis separation

A collection of 50 yeasts belonging to *D./B. bruxellensis* species was analysed in this study (Fig. 1). Genomic DNAs was extracted as described by Querol *et al.* (1992). Strain identification was obtained by partial amplification and sequencing of the 26S rDNA D1/D2 domain using primers NL1 and NL4 (Spirek, 2003). Primers used for the ISS-PCRs were Db1EI1, DB1EI2, LA1and LA2. All oligonucleotides are resulting from primers for *Saccharomyces cerevisiae* (de Barros Lopes, 1996) with the exception of Db1EI1 and Db1EI2 that were designed on *D. bruxellensis* genomic survey sequences (gss) (Woolfit, 2007) and 5'-dye-labelled with 6-Carboxyfluorescein (6-FAM, Primm, Milan, Italy). Db1EI1 (5'-CTGGCTTGG*aata*GTAAGT-3') and Db1EI2 (5'-CTGGCTTGCT*tactt*ACTTAC-3') are longer than EI1 and EI2 (de Barros Lopes, 1996), and contain the "GTAAGT" 5' splice site (5'ss) instead of "GTATGT". Amplifications were carried out following the protocol described for *S. cerevisiae* (de Barros Lopes, 1996) and the annealing temperatures were 46°C and 47°C when Db1EI1/LA2 or Db1EI2/LA1 primer pairs were used, respectively. For fragment size determination, PCR products were run in an ABI Prism 310 Genetic Analyzer (Applied Biosystems – Life Technologies), using POP-4 polymer, 310 Genetic Analyzer Buffer with EDTA, and a 47 cm x 50 µm capillary (Applied Biosystems – Life Technologies). Samples were injected for 20 s at 1.5 kV and separated at 8 kV for 80 min with a run temperature of 60°C. DNA sizing was performed with GeneScan-1200 LIZ (Applied Biosystems – Life Technologies). Digital profiles were visualised using ABI PRISM GeneMapper 3.7 (Applied Biosystems – Life Technologies) software. Fragments between 50 and 1200 bp were scored. Peaks having an amplitude threshold value less than 100 were not scored. The strain CBS 74 was used as an internal standard in the different capillary electrophoresis runs. Electrophoretic patterns generated by GeneMapper 3.7 software were employed to create presence/absence (1/0) matrices. Binary matrices were imported into NTSYS-pc 2.1 package for cluster analysis. Genetic similarity matrices among genotypes were calculated for dominant multilocus markers according to Dice's similarity index, using the SIMQUAL routine. Distance matrices were analysed using UPGMA (unweighted pair group method with arithmetic means) clustering algorithms, through the SAHN-clustering program, and dendrograms were displayed by TREE PLOT program. Bootstrap analysis was assumed to evaluate the reliability of dendrograms. The bootstrap values were calculated by WinBoot package. To estimate the repeatability error (RE) of ISS-PCRs, the genomic DNA, extracted from five distinct *D. bruxellensis* strains (CBS74-1942-1943-2797-5206) was used as template in three independent PCR reactions with Db1EI1/LA2 and Db1EI2/LA1 primer pairs. Percentage of genetic similarity between replicates of each isolates was calculated and the RE threshold value was assigned on the basis of replicates having the lowest percentage of genetic similarity.

A2) *Dekkera/Brettanomyces bruxellensis* physiological biodiversity: is this yeast the "Rubik's Cube" of the oenology?

D. bruxellensis CBS strains (Tab. 1) were tested for VPR activity. Cells in exponential phase were harvested, washed in phosphate buffer 0.05 M pH 7 and subsequently centrifuged. Pellets were resuspended in the same buffer, added with dithiothreitol 0.1 mM and PMSF 2 mM. Cells were then disrupted by subsequently steps of icing and vortexing. Total proteins content was measured by Bradford assay. The determination of the specific activity of VPR was calculated by means of a spectrophotometer at 340 nm. The assay mixture contained phosphate buffer (pH 7) 0.05M, NADH 10 mM, and approx. 250 mg of total proteins. The reaction was triggered with 4-vinylguaiacol 0.3M. Assimilation of carbon compounds was tested using the API/ID32C kit (bioMérieux SA, Marcy-L'Etoile, France) according to the manufacturer's instructions with few modifications.

Before testing, cultures were activated by means of a culture steps in YPD liquid medium (Yeast extract 1% (w/v), Peptone 2% (w/v), Glucose 2% (w/v)) under aerobic conditions at 25°C for 48-72 h. In a final volume of 0.18 mL, about 10^3 cells were inoculated in each well. Test strips were incubated at 25˚C for 72 hours. Positive growth results were attributed by the increasing in turbidity. H_2S production was assessed by spotting 10^6 cell/mL of YPD culture on BiGGY medium Petri dishes (BD, Le Pont de Claix, France).

A3) Adaptation and resistance to exogenous sulphite in *Dekkera/Brettanomyces bruxellensis*
One hundred and nine isolates belonging to D./*B. bruxellensis* species in the UC Davis Viticulture and Enology Culture Collection were screened to find out strains able to develop at high levels of sulphur dioxide in a laboratory defined medium, called *Brettanomyces* medium (BM) (Conterno, 2006), containing 5% (v/v) ethanol and adjusted at pH 3.6. Cells were grown at OD/mL>0.4; then, an inoculum at 1% (v/v) in 96-well plates without shaking at 23˚C was carried out. Six different concentration of potassium metabisulphite (PMB) (10, 20, 40, 60, 80, and 100 mg/L) were tested. Cell growth was monitored for 21 days following the optical density increasing at 650nm. Physiological tests on sensitive and resistant strains were carried out to identify some supplements that could modify the SO_2 toxicity. In particular, proline (1.7g/L), glycine (3.5 mg/L), adenine (15 mg/L), and methionine (20 and 40 mg/L) were added to the medium. Growth conditions and monitoring were set as above described. Enzymatic assays were also carried out to study the involving of specific metabolic pathways in the SO_2 resistance mechanism; acetaldehyde dehydrogenase and sulphite dehydrogenase activities were assessed as described by Postma *et al.* (1989) and Park and Bakalinsky (2000), respectively. In particular, the H_2S production was colorimetrically estimated using traps containing a rolled filter paper adsorbed with 300 µL of 3% lead acetile. Cell extract (>1.5 mg of total proteins) was dispensed into a 50 mL glass tube and the final volume was adjusted to 4 ml with the reaction mixture. Tube was close with a perforate silicon stopper with a H_2S trap inside, and it was incubated at 30°C for 24 h. Two resistant strains, able to grow at up to 60 mg/L of PMB in microplates were used for a metabolomics analysis with and without SO_2 using GCMS-TOF. Cells were inoculated at 1% (v/v) in hermetic bottles in the above mentioned defined medium and growth conditions. Experiments were performed in six replicates and a control was used to follow the cell growth. At OD/mL $\geq$ 0.9, cells were collected for the metabolomics analysis. One millilitre of yeast samples pulled from the bottom of the bottles was added to 1mL of 100% (v/v) cold methanol, vortexed, and immediately pelleted in a refrigerated microfuge for 2 minutes at about 10.000 rcf. Supernatants were discarded and pellets were resuspended in 1mL of 50% (v/v) cold methanol and transferred in a new refrigerated tube that was pre-weighed for the purpose of determining cell dry weight. Samples were centrifuged for 2 minutes at about 10.000 rcf and the supernatants discarded. Pellets were stored at -75˚C until all samples were taken. Then, to submit the samples to the Metabolomics Core facility in Davis (CA, USA) for extraction, derivatisation, and GC analysis, pellets were dried in a speed vacuum microfuge for at least 4 hours, weighed, and stored in the ultra-cold freezer until they were analysed.

Results and discussion

A1) Primers that anneal to specific conserved motifs, such as those designed for ISS-PCR, have been already described for inter- and intraspecific characterisation of *S. cerevisiae* (de Barros Lopes, 1996). On the other hand, no reliable results have been shown for the discrimination of *D./B. bruxellensis* species at strain level when yeasts were analysed with the primer EI1 alone (Oelofse, 2009). In the past, the use of specific primers that recognise the 5'ss -GTATGT- has allowed the identification and discrimination at strain level of a large number of yeast species (de

Barros Lopes, 1996 and 1998). Recently, the setting up of a multiplex PCR protocol throughout the use of modified oligonucleotides that targeted 5'ss -GTAAGT- has confirmed a high polymorphism among *D. bruxellensis* genomes (Vigentini, 2011). In the present work, to improve the primers specificity and to understand if non-random sequence patterns are conserved in *D. bruxellensis* genome, adjacent sequences to 5'ss -GTAAGT- were analysed. As regards the nucleotides that were detected at the 5' exon/intron boundary, the motif -AATA- was the most frequent, instead of the -AAG- observed in other hemiascomycetous yeasts such as *S. cerevisiae* and closely related species; in particular, among the deposited sequences with the 5'ss, 36.5% contained these four nucleotides. An analogous situation has been reported for *Debariomyces hansenii* (Bon, 2003). However, the high similarity of the above mentioned two patterns supports the hypothesis that, also in *D. bruxellensis*, they can both favour the interaction with the T-rich loop inside the spliceosome and allow a recognition mechanism between exons and spliceosome (Ares, 1995). On the other hand the downstream region of the 5'ss was characterised by a sequence of five nucleotides -AAGTA- with a frequency of 76.3%. As concerns this site, we found more frequently an adenine, as already observed in *D. hansenii*, *Yarrowia lipolytica* and *Pichia angusta*, instead of thymine or guanine detected in other hemiascomycetous species (Bon, 2003). Actually, an analysis *on D. bruxellensis* mtDNA has suggested that this species is more closely related to *Saccharomyces*/*Kluyveromyces* genera than to *D. hansenii* and *Candida spp*. lineages (Prochàzka, 2010). However this is in disagreement to what reported by Woolfit *et al.* (2007) which found that approx. 24% of the ascomycete proteins with orthologs in *D. bruxellensi*s, (but not in *S. cerevisiae*) belonged to *D. hansenii* species. Further investigation will be required to confirm the 5'ss boundary of *D. bruxellensis*; anyway, a non-random vocabulary around the 5'ss could be maintained during evolution to preserve the spliceosome activity as happens for the splice sites themselves.
The separation of the amplified fragments was obtained throughout the setting up of a capillary electrophoresis protocol (Fig. 2).

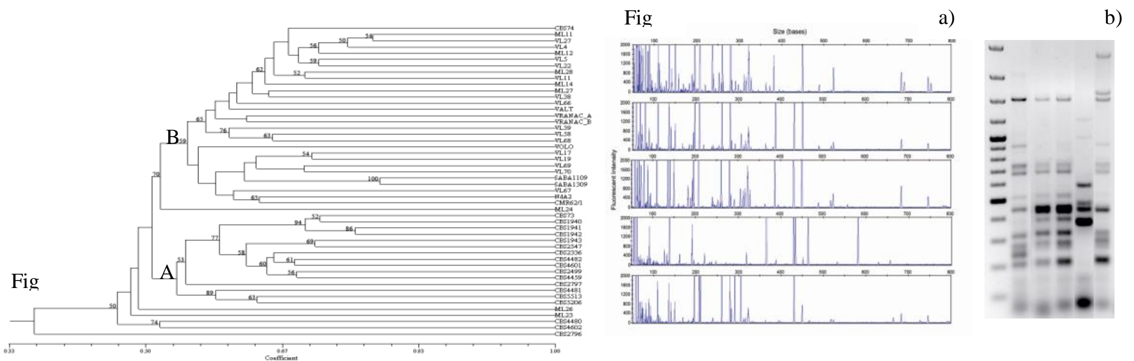

Figure 1 *Dendrograms generated by the combination of ISS-PCR patterns of wine yeasts using Db1EI1/LA2 and Db1EI2/LA1 primer pairs. The coefficient of similarity among the genetic profiles is indicated along the horizontal axis. Only bootstrap values ≥50 (500 replicates) are given.*
Figure 2 *Example of ISS-PCR profiles obtained using Db1EI1/LA2 primer pair: a) analysed by capillary electrophoresis. The molecular sizes (expressed in base pairs) and fluorescent intensity are respectively displayed across the horizontal and the vertical axes. From the top: CBS74, CBS1942, CBS1943, CBS2797 and CBS5206 strains. b) analysed by agarose gel. From the left: DNA marker (100bp DNA Ladder, Fermentas, Vilnius, Lithuania) and CBS strains (in the above mentioned order).*

In the experimental conditions, the lowest value of similarity between replicates of the same sample evidenced in a UPGMA-based dendrogram was 94%. Yeasts biodiversity among the isolates of the whole collection was assessed throughout the analysis of the ISS-PCR profiles that resulted from

the amplification with Db1EI1/LA2 and Db1EI2/LA1 primer sets. In this case, the bootstrap analysis indicated that the best molecular characterisation was achieved drawing together all the ISS-PCR profiles (Fig. 1); the combination of the ISS profiles led to an increasing in the rate of significant nodes (58%) in comparison to 44% and 46% that were found processing the ISS profiles from Db1EI1/LA2 and Db1EI2/LA1 primer pairs, respectively (data not shown). Most of the strains grouped into two main clusters at a similarity level of approximately 54% (A) and 52% (B), respectively. Cluster A included almost all the CBS strains without showing any relationship between the ISS-PCR patterns and the geographical area of isolation. However, the French strains CBS73 with CBS1940, CBS1941with CBS1942, and CBS1943 with CBS2547 shared a similarity higher than 70%. Cluster B grouped most of the wild isolates collected in this work, with the exception of CBS74, the type strain of *D. bruxellensis* isolated from Lambic beer. No evident correlation between genetic profiles and the origin of the samples was found. Nevertheless, Italian strains ML11, ML12, VL4 with VL27, VL5 with VL22, and VL17 with VL19, which were collected from wines produced in the same geographic area (Lucca) in 2006, showed a level of similarity higher than 70%. Moreover, a strong relation was detected for strains SABA1109 and SABA1309 both isolated from the same wine sample. Five strains were positioned outside the main clusters (A and B); two of them derived from Tuscan wine samples whereas the others belonged to CBS collection. It should be noted that CBS2796 showed the lowest similarity within all the investigated yeasts.
As regards the genomic diversity of *D./B. bruxellensis*, this study confirms the high level of intraspecific heterogeneity. From an ecological point of view the main result is that, in comparison to other works carried out in relatively big geographical areas (Curtin, 2007; Agnolucci, 2009), no predominant genotypes were found. This could be due to the high resolution of the technique that comprise only two work steps (amplification and separation) rather than other more complex analyses where multiple approaches are needed. Since our collection was composed both by CBS strains and wild yeasts isolated both inside or outside Italy, some consideration could be done on yeast's geographic (inter- and intra-area) and time dispersion. In disagreement to what observed in *S. cerevisiae* (Richards, 2009), we showed that a specific territory as Italy does not harbour a distinct population of *D./B. bruxellensis* and that different areas in the country (north, middle and south) does not harbour different communities. The cluster analysis describes an almost complete separation between strains isolated before (from CBS collection) and after (wild strains) the year 2000. This observation could support the assumption that *D./B. bruxellensis* represents a model organism to evaluate yeast evolution (Hellborg and Piškur, 2009) or to reinforce the idea that the laboratory cultural conditions change the evolutional rates.

A2) Three different ranges of VPR activity were used to assign the physiological profiles. About 74% of the yeasts showed a low activity of VPR (0-0.040 U/mg of total proteins, profile 1 in Tab.1), 21% were strains with a medium activity (0.041-0.080 U/mg of total proteins, profile 2 in Tab.1), whereas only one strain (5.3%) was classified as a yeast with a high VPR activity (>0.081 U/mg of total proteins, profile 3 in Tab.1). These data indicated that this simple UV- method can discriminate the strains for their physiological aptitude to produce off-flavours. Tests, performed in triplicate, showed a good repeatability (3% standard error).
Using the API/ID32C kit 10 different assimilation profiles were obtained for the 19 CBS strains investigated. All isolates were capable of glucose and esculin assimilation, whereas between 85-75% of the isolates assimilated saccharose, metil-αD-glucopyraniside, and palatinose, 74-65% melezitose, maltose, trehalose and cycloheximide, 64-55% galactose and cellobiose, about 37% N-acetil-glucosamine, between 30-15% glycerol, sorbose, and raffinose, about 5% gluconic acid, levulinic acid, mannitol, and glucosamine. Lactic acid, arabinose, potassium 2-ketogluconate,

xylose, ribose, erythrose, melibiose, glucuronic acid, lactose, inositol, and sorbose were not assimilated by any of the isolates.

In order to assess the H_2S production of the CBS strains, they were all plated on BiGGY agar, and the color of the colonies was evaluated. BiGGY agar uses bismuth as an indicator for the production of sulfide; the more sulfide is produced, the darker the colonies are due to the precipitation of bismuth sulfide. The production of sulfide in this medium is thought to be correlated with the basal level of activity of sulfite reductase. Low producers strains were the ones that showed a colony color ranging from white to light tan (profile 1, Tab.1), medium producers cells had colonies from tan to light brown (profile 2, Tab.1), and high H_2S producers yeasts formed colonies from brown to black (profile 3, Tab.1).

D. bruxellensis **CBS number**	**Physiological profiles**			**Phenotypes**
	VPR activity	**Carbon compounds assimilation**	**H_2S production**	
73	1	1	3	**I**
74	1	1	3	**I**
1940	1	1	3	**I**
1941	1	3	3	**II**
1942	1	4	3	**III**
1943	1	2	3	**IV**
2336	1	3	3	**II**
2499	1	3	3	**II**
2547	1	1	3	**I**
2796	1	1	3	**I**
2797	1	5	3	**V**
4459	2	1	1	**VI**
4480	2	6	1	**VII**
4481	2	7	2	**VIII**
4482	1	8	3	**IX**
4601	3	6	1	**X**
4602	1	9	1	**XI**
5206	1	4	2	**XII**
5513	2	10	1	**XIII**

Tab 1

Combining the results from the three phenotypic experiments, about 70% of the CBS collection can be discriminated at strain level. If compared with genotypic analysis, this result proved that the genomic differences of *D./B. bruxellensis* have a strong impact on the phenotype. The present work suggested that the chromosomal rearrangement occurred in this species (Hellborg and Piškur, 2009) have generated strains with a high physiological polymorphic state. Further investigations will be focused on the understanding of the real intraspecific edges in *D./B. bruxellensis*.

A3) Conditions of high stress, including high acidity, oxygen limitation, high SO_2 and ethanol concentrations, nitrogen starvation, etc., could play an important role in promoting frequent genome rearrangements. A consequence of the genetic polymorphism of *D./B. bruxellensis* is its ability to survive, adapt and grow in the extreme environment encountered in wine where sulphite is often used both to control the winemaking process and to stabilize the final product. Although new technological processes have been developed to improve the quality of wine without have recourse to chemical additives (Lustrato, 2010), sulphur dioxide addiction is still widespread due to its antimicrobial and antioxidant effect. The literature refers to the ability of *D./B. bruxellensis* to tolerate SO_2 (Barata, 2008) but no data are available on the mechanism that controls this metabolic feature. Our current work shows that some strains of *D./B. bruxellensis* have an innate resistance to SO_2, whereas others can adapt their own metabolism to higher levels of sulphur dioxide. The screening results in presence of increasing SO_2 concentrations showed that a rate of about 30% of the yeast collection was sensitive to the presence of any amount of sulphur dioxide and unable to growth, under our conditions; a quite similar percentage of strains could grow at 10 mg/L of PMB. About 33% and 15%

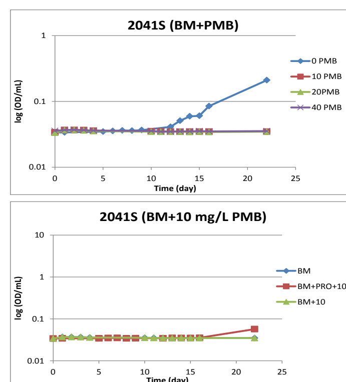

Figure 3 *Proline promotes the growth of the sensitive strain* D./B. bruxellensis *UCD2041 at 10 mg/L PMB after 21 days from the inoculum. (1% standard error).*

of the strains grew at 20 and 40 mg/L PMB, respectively. Only two strains (about 2% of the whole collection) grew at 60 mg/L PMB. Some strains grew in BM containing PMB concentrations higher than 60mg/L between 15 and 21 days from the inoculum. Most of the times, the optical density increasing was not homogeneous neither with the PMB concentration (10, 20, 40, etc.) nor inside the four replicates. This behavior could indicate that SO_2 works as a selective marker giving an advantage to those cells that have a different/multiple mechanism to survive at a high SO_2 level. Physiological data displayed that some amino acids are involved in increasing SO_2 tolerance; in particular, proline can increase the sulphite resistance in sensitive strains on BM (Fig. 3). Other well-studied metabolic mechanisms in *S. cerevisiae* are probably not used or ineffective in *D./B. bruxellensis*. Unlike what was observed in *S. cerevisiae*, neither adenine nor methionine modified the toxicity level of SO_2 under laboratory conditions (Aranda, 2006). The ethanol concentration seems to increase the sensitivity to sulphite suggesting that a membrane system, such as the sulphite efflux pump of *S. cerevisiae*, could be present in this species (Park and Bakalinsky, 2000). Preliminary results from both physiological and the enzymatic assays suggested that H_2S production could be an alternative mechanism to detoxify cells from sulphite in *D./B. bruxellensis*. Results from metabolomics experiments will help us to understand which the metabolic pathways are conferring sulphite tolerance in *D./B. bruxellensis*.

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2° International Symposium "MICRO SAFETY WINE”, Martina Franca, Nov 19-20

Intron splice site PCR analysis to differentiate *Dekkera bruxellensis* at strains level

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D. bruxellensis yeast can develop off-flavours in wine. In particular volatile phenols are often produced in amounts higher than the perception threshold with a loss in product quality. Recent observations suggest that "brett spoilage" is strictly strain-dependent and, therefore, a rapid and reliable identification at strain level of *D. bruxellensis* becomes a key point for an efficient prevention (Vigentini et al, 2008). Among the techniques used to analyse DNA regions with high rate of sequence evolution, introns splice site amplification (ISS-PCR) has allowed to detect polymorphisms in commercial yeast strains of *S. cerevisiae* (De Barros et al, 1996). Recently, the genome of a wine strain of *D. bruxellensis* has been sequenced and the results have shown that about 2% of the *D. bruxellensis* genes contain introns, a value similar to that found in other hemiascomycetes (1% in *D. hansenii*; 4% in *S. cerevisiae*). Moreover, the *D. bruxellensis* introns have 5', 3', and branch site motifs that are very similar to the consensus motif in *S. cerevisiae* (Woolfit et al, 2007). It was reported that the use of 5' intron-exon spice site as target for ISS-PCR in *D. bruxellensis* did not allow the discrimination at strain level (Oelofse et al, 2009), but an optimization of primers that are complementary to the ISSs of this yeast could permit the development of a consistent tool for the typing of the species.

In the present study, 17 *D. bruxellensis* strains belonging to the international CBS collection has been investigated for the ISSs employing specific oligonucleotides. *D. bruxellensis* contains two 5' consensus sequences: GTATGT (like *S. cerevisiae*) and GTAAGT (De Barros et al, 1996; Woolfit et al, 2007). Preliminary results have shown that most yeast collection was discriminated at strain level by the use of different combinations of primers (up to 80-90%). Therefore, to simplify the approach a multiplex PCR that generated stable genetic profiles was set up.

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Intron splice site PCR analysis to differentiate Dekkera bruxellensis at strains level

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Introduction

Investigation on genome variability among individuals represents an important tool to asses their biodiversity and to study their environmental diffusion. Safeguard of microbial strains with technological interest is becoming a strategic activity in food and wine industries. Nevertheless, many species marginally implicated in productions are still poorly studied and rapid and reliable protocols for their recognition and typing are not well described. This is the case of the yeast *Dekkera bruxellensis* that in wine can cause spoilage and increasing in economical looses. During last years, basic studies on *D. bruxellensis* phenotypic and genetic aspects have been published; at the moment, the physiological studies have clarified that the spoilage activity, due to the production of ethyl-phenols, is a strain dependent character and the enzymes involved in this production have been isolated [1]; around 30% of *D. bruxellensis* genome have been sequenced [2]. Since no significant remedies to remove the off-flavours from the wine are available yet, some applications to contrast the yeast development in wine have been studied [3]. However, only few references reported methods to distinguish *D. bruxellensis* at strain level [4]; the protocols to type appear useful but time consuming or need sophisticated instrumentations. The set up of molecular probes designed on genes responsible of the off-flavour production for a rapid detection of dangerous strains could be a partial solution since an ethyl-phenols production around 2 mg/L is considered a benefit for wine [5]. When the entire genome will be available, more consistent protocols could be arranged possibly correlating genetic and physiological aspects to a single strain. Thus, the availability of comparable results among laboratories throughout databases could represent an important goal to face the increasing problem represented by *D. bruxellensis* in the oenological field.

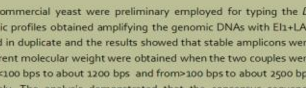
Aim of the work

Introns are DNA regions not linked to any gene function that can vary with a low control; changes as nucleotide substitutions, deletions, or insertions can occur in an intron structure. For this reason they are considered to be good evolution indicators in studies of genome relatedness. However, short conserved sequence for the spliceosome assembling are required during the synthesis of mRNA: in *S. cerevisiae*, the lariat branch point TACTAAC and the 5′ motif GTATGT consensus sequences are strictly conserved [6]. A recent work identified introns in 40 *D. bruxellensis* genes whose *S.cerevisiae* orthologs; moreover, eight introns were specific to *D. bruxellensis* [2]. In this work, Intron Splice Sites PCR (ISS-PCR) was applied to a collection of 17 *D. bruxellensis* strains using primers similar to those used for *S. cerevisiae*. The main goal was to test and to improve the method on *D. bruxellensis* species since it is simple, rapid, and potentially accessible to industrial laboratories with limited molecular expertise and resources.

Merthods

Yeasts were grown overnight in liquid YPD medium with shaking. DNA from a 5 ml culture was extracted as described by Querol *et al.* (1992) [7] using 500 μg ml^{-1} of Zymolyase 100T (USBiological, Massachusetts, USA) as lytic enzyme. The primers employed in the amplification reactions were EI1, EI2, LA1, LA2 [6] and DbEI1, DbEI2 designed on *D. bruxellensis* genome. Concentrations of reagents were: 80-100 ng of DNA, 0.75 μM each primers, 200 μM dNTPs, 1x reaction buffer ($MgCl_2$ free), 2.5 mM $MgCl_2$, 2U *Taq* polymerase (Roche, Germany) in a final volume of 25 μl. Amplification was performed followed the protocol described for *S. cerevisiae* increasing the number of cycles to 40 [6]. PCR products were visualised on agarose gels 1,5% (w/v).

Results and discussion



Primers used for strain discrimination of *S. cerevisiae* commercial yeast were preliminary employed for typing the *D. bruxellensis* collection [6]. Figure 2 and 3 show the genetic profiles obtained amplifying the genomic DNAs with EI1+LA2 and EI2+LA1 pair of primers. The analyses was performed in duplicate and the results showed that stable amplicons were produced in *D. bruxellensis* yeasts. Fragments with a different molecular weight were obtained when the two couples were applied on the same strain; in particular, templates from <100 bps to about 1200 bps and from>100 bps to about 2500 bps were generated with EI1+LA2 and EI2+LA1, respectively. The analysis demonstrated that the consensus sequence 5'GTATGT and the branch motif TACTAAC present into primers, clustered the *D. bruxellensis* collection into few groups. Only two main clusters were produced both using EI1+LA2 and EI2+LA1: group A (CBS73, CBS1940, CBS1941, CBS1942, CBS1943 and CBS2547) and group B (CBS2336, CBS2499, CBS4459, CBS4480, CBS4481, CBS4601 and CBS4602). Strains CBS2796, CBS2796, CBS4482 and CBS5206 were characterised by single genetic profiles (Figure 2 and 3).

To optimise the PCR reaction on *D. bruxellensis* genome, new primers named DbEI1 and DbEI2 were designed. 5′ motifs and the branch motif were used as targets for the introns sequences but the tails of EI1 and EI2 primers were elongated adding from 4 to 6 nucleotides frequently found into the sequences deposited in GenBank with accession numbers EI011584 to EI026443 [2]. Since PCRs performed with a single primer can yield to contradictory profiles in the amplicon length, band intensities and occasional unspecific binding [8], the reproducibly of the amplifications with DbEI1 and DbEI2 used singularly and DbEI1+LA2 or DbEI2+LA1 were tested. Results confirmed what already reported for the use of single primers but the PCRs gave stable profiles when primers were used in couple. Figure 4 reports an example of repeatability that was done on four strains with DbEI2+LA1.

Thus, the PCRs were carried out on the whole collection and results are shown in Figures 5 and 6. The genetic patterns obtained with the primers DbEI2+LA1 and DbEI1+LA2 generated the same two main groups of strains observed using the primers EI1+LA2 and EI2+LA1 (group A and B) with the exception of CBS1940 which appeared with a characteristic profile when amplified with DbEI2+LA1.

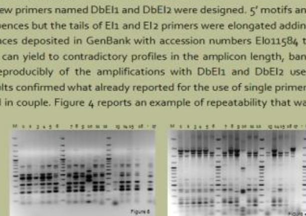


Figure 5: PCR amplifications using DbEI1+LA2 primers
Figure 6: PCR amplifications using DbEI2+LA1 primers
M: marker 100bp; 1: CBS73; 2:CBS1940; 3: CBS1941; 4: CBS1942; 5: CBS1943; 6: CBS2336; 7: CBS2499; 8: CBS2547; 9: CBS2796; 10: CBS2797; 11: CBS4459; 12: CBS4480; 13: CBS4481; 14: CBS4482; 15: CBS4601; 16: CBS4602; 17: CBS5206; -: negative control

Figure 1: From reference [7]

Figure 2: PCR amplifications using EI1+LA2 primers
Figure 3: PCR amplifications using EI1+LA2 primers
M: marker 100bp; 1: CBS73; 2:CBS1940; 3: CBS1941; 4: CBS1942; 5: CBS1943; 6: CBS2336; 7: CBS2499; 8: CBS2547; 9: CBS2796; 10: CBS2797; 11: CBS4459; 12: CBS4480; 13: CBS4481; 14: CBS4482; 15: CBS4601; 16: CBS4602; 17: CBS5206; -: negative control

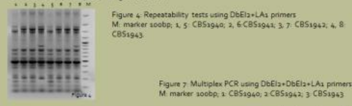


Figure 4: Repeatability tests using DbEI2+LA1 primers
M: marker 100bp; 1, 5: CBS1940; 2, 6:CBS1941; 3, 7: CBS1942; 4, 8: CBS1943.

Figure 7: Multiplex PCR using DbEI2+DbEI2+LA1 primers
M: marker 100bp; 1: CBS1940; 2:CBS1942; 3: CBS1943

A multiplex PCR, at the same mentioned conditions, were applied on strains CBS1940, CBS1942 and CBS1943 using primers DbEI1, DbEI2 and LA1. Figure 7 showed that, as already happened to CBS1940 with DbEI2+LA1, unique profiles were generated allowing the discrimination at strain level. Future improvements will consist on the set up of a multiplex PCR able to distinguish each strain.

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Adaptation and resistance to exogenous sulphite in *Brettanomyces bruxellensis*

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Introduction

Brettanomyces bruxellensis (teleomorph *Dekkera bruxellensis*) is the primary yeast species involved in spoilage of bottled wine. In oenology, "Brett" character refers to a sensory defect linked to off-flavours production by *B. bruxellensis* as it grows in the wine. Studies on the ecological distribution of this species showed a high degree of intraspecies polymorphism probably due to a fusion event among several genomes or to an increased mutation rate resulting in the asexual state (Vigentini *et al*., 2011; Hellborg and Piškur, 2009). In food environments *B. bruxellensis* is mainly associated with fermented beverages, such as wine, beer or cider where growth conditions are highly stressful: high acidity, oxygen limitation, high SO_2 and ethanol concentrations, nitrogen starvation, etc., could play an important role in promoting frequent genome rearrangements. As a consequence of the genetic polymorphism, *B. bruxellensis* is able to survive, adapt and grow in the extreme environment encountered in wine where sulphite is often used both to control the winemaking process and to stabilize the final product.

Materials and Methods

One hundred and nine isolates of the UC Davis Viticulture and Enology Culture Collection belonging to *B. bruxellensis* species were screened to find strains able to grow at high levels of sulphur dioxide in a laboratory defined minimal medium (Conterno *et al*., 2006). This *Brettanomyces* medium (BM) was modified adding 5% (v/v) ethanol and it was acidified to pH 3.6. Cells were grown in YM broth (1% w/v dextrose, 0.5% w/v peptone, 0.3% w/v yeast extract, 0.3% w/v malt extract) for 3-5 days at 30°C. Growth tests were done in 96-well plates with 0.2 mL of medium at 23˚C in static condition. Cells at about 0.4 OD/mL in BM were inoculated at 1% (v/v) in each well. The experimental plan consisted of testing the growth at six different concentrations of potassium metabisulphite (PMB) (10, 20, 40, 60, 80, and 100 mg/L) that was added to BM as a 20 g/L stock solution immediately before dispensing the medium into the wells. Each strain and condition were carried out in four replicates. Cell growth was monitored for 21 days following the optical density increase at 650nm using an Emax precision microplate reader (Molecular Devices, Inc., California, USA). Data were collected by SOFTmax software. Physiological assays were performed for some strains in BM with proline (1.7 g/L), or glycine (3.5 mg/L), or adenine (15 mg/L), or methionine (20 and 40 mg/L), or casamino acids (BD Bacto™ Casamino Acids, Vitamin Assay, Oxford, UK) (0.18 g/L). Proline, glycine and methionine concentrations were obtained from Amerine and Ough (1974), whereas adenine and casamino acids amounts were set as suggested by Aranda *et al*. (2006) and Vigentini *et al*. (2008). Depending on the analysed strain, an opportune

amount of PMB was also added to BM. In this case each strain and condition were carried out in eight replicates. Growth conditions were set as described for the screening experiment and monitoring was extended to 21 days.

Results and Discussion

Literature refers to the ability of *B. bruxellensis* to tolerate SO_2 (Barata, 2008) but no data are available on the mechanism that controls this metabolic feature. The screening results in presence of increasing SO_2 concentrations showed that about 18% of the yeast collection was unable to grow in presence of sulphur dioxide. Fifteen days after the inoculation, about 25% of strains grew at 10 mg/L of PMB, 40% at 20 mg/L and 15% at 40 mg/L. Only two strains (about 2% of the whole collection) grew at 60 mg/L PMB. However some strains grew in BM containing PMB concentrations higher than 60 mg/L between 15 and 21 days from the inoculum. Most of the times, the optical density was not consistent with the increasing of PMB concentration or even among the four replicates. This behaviour could indicate that SO_2 works as a selective marker giving an advantage to those cells that have a different/multiple mechanism to survive at a high SO_2 levels. In our conditions, proline can increase the sulphite resistance in sensitive or low SO_2 tolerant strains (Figure 1 and 2) and this effect was a strain dependent character.

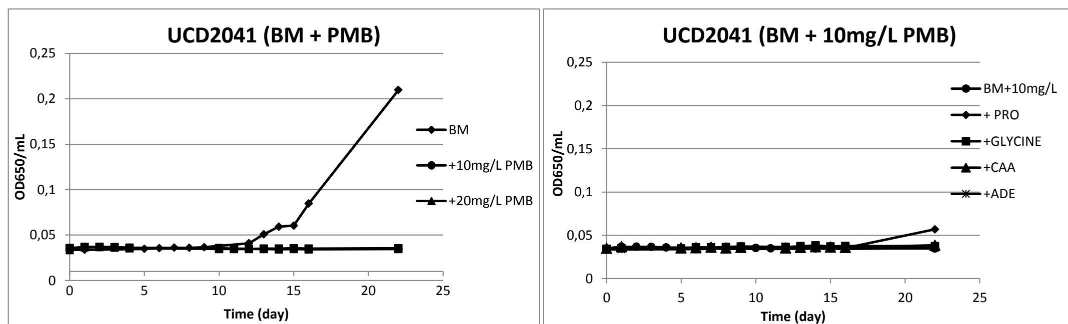

FIGURE 1 *D./B. bruxellensis* UCD2041 kinetic of growth in BM: left) SO_2 resistance; right) at 10 mg/L PMB in presence of different supplements. Data are reported as the average values of eight replicates.

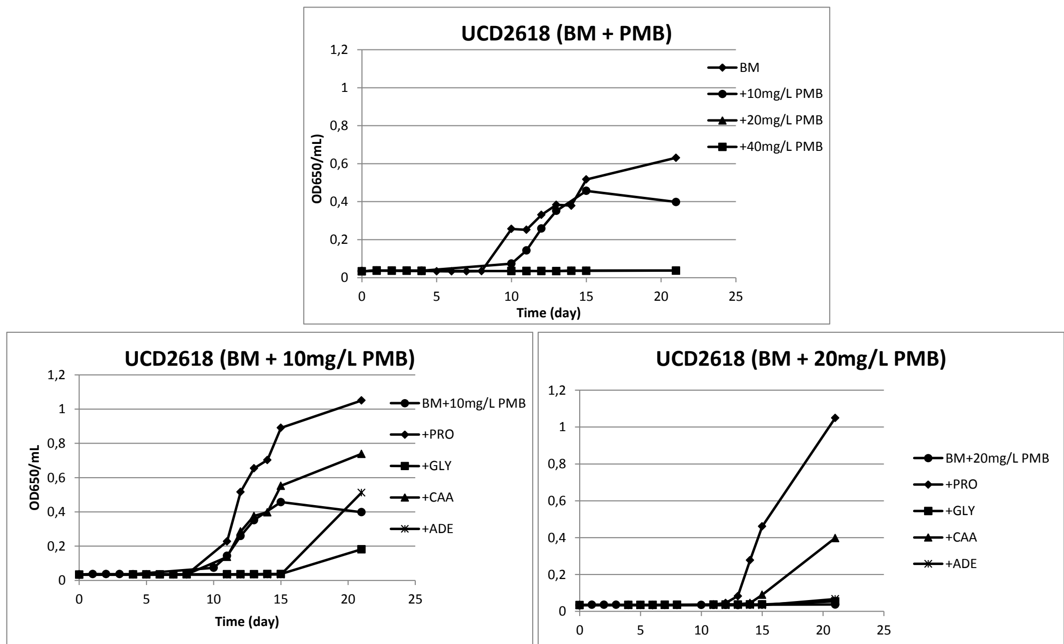

FIGURE 2 *D./B. bruxellensis* UCD261 kinetic of growth in BM: top) SO_2 resistance; left) at 10 mg/L PMB in presence of different supplements; right) at 20 mg/L PMB in presence of different supplements. Data are reported as the average values of eight replicates.

The phenomenon was not observed in high SO_2 tolerant strains where probably other effective protecting mechanisms could be activated. In *Saccharomyces cerevisiae* the main system to detoxify cells from sulphite is a membrane pump, encoded by the *SSU1* gene (Park and Bakalinsky, 2000). In this study, a test performed on the two resistant strains that were able to grow in BM at 5% ethanol with 60 mg/L PMB demonstrated that they could not grow when the ethanol concentration was increased at 10%. The ethanol concentration seems to increase the sensitivity to sulphite suggesting that a membrane system, such as the sulphite efflux pump of *S. cerevisiae*, could be present in this species. With the aim to reveal the presence of the SSU1 pump also in this species, PCR reactions and Southern experiments were carried out using specific primers and probes designed for conserved regions of homologous and putative *SSU1* sequences in *S. cerevisiae* and phylogenetically related yeast (*Debaryomyces hansenii*, *Yarrowia lipolytica*, *Candida* spp., and *Kluyveromyces lactis*). No significant results were obtained suggesting that, if present, *SSU1* gene has a low similarity with that of the tested yeasts.
In contrast to what was observed in *S. cerevisiae* (Aranda *et al.*, 2006), neither adenine (Figure 1 and 2) nor methionine (data not shown) modified the toxicity level of SO_2 under laboratory conditions.

Conclusion

Several works proved that a high biodiversity is present in *B. bruxellensis* species both from the genetic and physiological point of view. This polymorphism was also observed in this work where sensitive, low, medium and high SO_2 resistant strains were recognised. Actually, some strains of *B. bruxellensis* have an innate resistance to SO_2, whereas others can adapt their growth to high levels

of sulphur dioxide. This adaptation could be linked to few cells inside a same population that have developed, during yeast evolution, alternative mechanisms or different gene regulations to tolerate strong stress. Among the mechanisms that are involved in the protection to sulphite, some amino acids, in particular proline, could have an important role modulating the SO_2 tolerance. Our preliminary results suggest that intracellular proline could reduce the SO_2 stress. In literature it is reported that proline has a stress-protecting activity on yeasts; it should work as a membrane stabilizer by preventing protein denaturation (Takagi, 2008). Other well-studied metabolic mechanisms in *S. cerevisiae* are probably not used or ineffective in *B. bruxellensis*; adenine and methionine do not increase the SO_2 resistance and the sulphite toxicity in the investigated strains, respectively (Aranada *et al*., 2006). Future results from metabolomics experiments will allow determination of the molecular mechanisms and metabolic pathways that this yeast can activate in order to survive and grow at high concentration of exogenous sulphur dioxide.

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Genetic diversity and physiological traits of *Brettanomyces bruxellensis* strains isolated from Tuscan Sangiovese wines

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ABSTRACT

Eighty four isolates of *Brettanomyces bruxellensis*, were collected during fermentation of Sangiovese grapes in several Tuscan wineries and characterized by restriction analysis of 5.8S-ITS and species-specific PCR. The isolates were subsequently analysed, at strain level, by the combined use of the RAPD-PCR assay with primer OPA-02 and the mtDNA restriction analysis with the *Hinf*I endonuclease. This approach showed a high degree of polymorphism and allowed to identify seven haplotypes, one of them being the most represented and widely distributed (72 isolates, 85.7%). Physiological traits of the yeasts were investigated under a wine model condition. Haplotypes clustered into two groups according to their growth rates and kinetics of production of 4-ethylphenol and 4-ethylguaiacol. Hexylamine was the biogenic amine most produced (up to 3.92 mg l^{-1}), followed by putrescine and phenylethylamine. Formation of octapamine was detected by some haplotypes, for the first time.

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1. Introduction

Wine alterations of biological origin are caused by the activity of bacteria and yeasts. Advances in wine technology and the implementation of Good Manufacturing Practices throughout the winemaking process have significantly decreased the risk of wine spoilage by bacteria, but spoilage by yeast contamination still remains a potential threat. Indeed, yeasts are so far the most feared contaminants affecting the wine quality. The common spoilage effects are film or sediment formation, cloudiness or haziness, gas production and off-odours and off-tastes at all stages of winemaking. Particularly for products aged in wood barrels, there has been an increased interest in spoilage by yeasts in the genera Brettanomyces/Dekkera.

Brettanomyces bruxellensis (teleomorph *Dekkera bruxellensis*) is the most representative in wines among the species of this genus. *B. bruxellensis* is able to produce 4-vinylphenol and 4-ethylphenol from p-coumaric acid, whereas 4-vinylguaiacol and 4-ethylguaiacol from ferulic acid. Recent studies (Silva et al., 2005; Farina et al., 2007) revealed that *B. bruxellensis* strains varied in their production of phenolic substances in wine. Such volatile substances smell of stable, wet-horse and other stinks (Chatonnet et al., 1997; Gerbaux et al., 2000) that are generally considered strongly detractive for the product. In wines spoiled by *B. bruxellensis* high amounts of acetic acid can be found as well (Romano, 2007; Vigentini et al., 2008). However the behaviour of this yeast species in terms of off-flavour production, at the strain level, is not yet fully understood, both because only few systematic studies on *B. bruxellensis* strains of different origin have been performed and because the cultural media used have been poorly standardized or they had little oenological significance.

Some studies showed that *B. bruxellensis* is able to produce neuro-active and vasoactive amines, mainly phenylethylamine from phenylalanine at an average quantity of 10 mg l^{-1} and lower amounts of other biogenic amines (BA) (Caruso et al., 2002). Moreover the ability of *Brettanomyces* yeasts to generate biogenic amines seems to be strain dependent (Vigentini et al., 2008). These compounds can be metabolically synthesized and degraded in animals, plants and microorganisms, and are found in a wide variety of foods such as fish, meat, cheese, wine, beer and other fermented foods (Silla Santos, 1996). The occurrence of BA (i.e. histamine, tyramine, putrescine, cadaverine, phenylethylamine, spermine and spermidine) in wine has been linked to the amino acid decarboxylase activity of lactic acid bacteria, such as *Oenococcus oeni*, which is used as a starter during malolactic fermentation (Coton et al., 1999). Genetic determinants of some decarboxylases have been identified in *S. cerevisiae* (White Tabor and Tabor, 1985) and in *O. oeni* (Marcobal et al., 2006; Moreno-Arribas et al., 2003) whereas they are unknown for *B. bruxellensis*.

In oenological practices, the isolation and enumeration of *B. bruxellensis* are carried out by using selective/differential microbiological media. However, these protocols have a limited efficacy, due

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ORIGINAL ARTICLE

Inactivation of wine spoilage yeasts *Dekkera bruxellensis* using low electric current treatment (LEC)

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Keywords
Dekkera bruxellensis, ethylphenol, inactivation, low electric current, wine spoilage yeast.

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Abstract

Aims: The objective of this study was to investigate the inactivation of a selected yeast *Dekkera bruxellensis* strain 4481 in red wine by application of low electric current treatment (LEC).
Methods and Results: LEC (200 mA) was applied for 60 days to a red wine, Montepulciano d'Abruzzo, in an alternative strategy to the SO_2 addition during wine storage. The LEC effect on both cell activity and microflora viability was assessed. LEC decreased significantly the survival viable cells and increased the death rate of *D. bruxellensis* strain 4481 yeast. A final comparison was made of the main physico-chemical parameters of the wine after the different treatments. The study suggests the importance of an appropriate LEC treatment which limits wine deterioration in terms of off-flavours synthesis.
Conclusions: The results demonstrate that the growth of undesirable *Dekkera* can be inhibited by low voltage treatment; LEC was shown to be useful to prevent wine spoilage and has the potential of being a concrete alternative method for controlling wine spoilage.
Significance and Impact of the Study: Wine spoilage can be avoided by preventing the growth of undesirable *Dekkera* yeasts, through the effective use of LEC in the winemaking process.

Introduction

Winemaking is the result of the multiplication and metabolism of numerous yeasts and bacteria in grape juice. After the fermentation process, the fermented juice is stabilized by a sulfating process to eliminate the presence of micro-organisms. However, spoilage sometimes occurs during ageing, or even after bottling, because of contamination or the survival of some residual strains. Indeed, yeasts belonging to the genus *Dekkera/Brettanomyces* represent a major problem in the wine industry (Sponholz 1993). Such yeasts are not normal inhabitants of the skin of the grape or of the fermenting must, but they can develop in white and red wines at the end of the alcohol fermentation and during the ageing of the wine in wooden barrels (Ibeas *et al.* 1996; Castro-Martinez *et al.* 2005). Such yeasts, depending on the availability of oxygen (Ciani and Ferraro 1998; Abbott and Ingledew 2005) and carbon and energy sources (Chatonnet *et al.* 1995; Dias *et al.* 2003), can produce unpleasant odours and give the wine a taste that affects the aroma (Kunkee and Bisson 1993; Fugelsgan and Zoecklein 2003). These yeasts are also notorious for their ability to produce acetic acid and their spoilage of bottled wine (Fugelsang 1997; Gerós *et al.* 2000). The metabolic products responsible for wine spoilage by *Dekkera/Brettanomyces* sp. are mainly volatile phenols, acetic acid and tetrahydropyridines and are associated with significant economic losses (Kunkee and Bisson 1993; Fugelsang 1997). The production of the volatile phenols 4-ethylphenol and 4-ethylguaiacol is because of the sequential activity of two enzymes that decarboxylate hydroxycinnamic acids into hydroxystyrenes, which are then reduced to ethyl derivatives (*p*-coumaric acid is the substrate of 4-ethylphenol and

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SHORT COMMUNICATION

Intron splice site PCR analysis as a tool to discriminate *Dekkera bruxellensis* strains

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Abstract *Dekkera bruxellensis* yeast species can develop off-flavours in wine through a specific reductive metabolism. In particular, volatile phenols are often produced in amounts that are higher than the perception threshold with a loss in product quality. Recent observations suggest that "brett spoilage" is strictly strain-dependent, and therefore, a rapid and reliable identification at strain level of *D. bruxellensis* becomes strategic for an efficient prevention. Among the techniques used to analyse DNA regions with high rate of sequence evolution, intron splice site PCR amplification (ISS-PCR) has allowed the detection of polymorphisms in commercial strains of *S. cerevisiae*. Recently, the genome of a *D. bruxellensis* isolated from wine has been sequenced and the results have shown that about 2% of the genes, a value similar to the ones found in other hemiascomycetes (1% in *D. hansenii*, 4% in *S. cerevisiae*) contain introns. Moreover, *D. bruxellensis* introns have 5′, 3′ and branch motifs that are very similar to the consensus motif in *S. cerevisiae*. Although the use of the 5′ intron–exon splice site as a target for ISS-PCR in *D. bruxellensis* did not allow the discrimination at strain level, an optimisation of primers could permit the development of a consistent tool for the typing of the species. In the present study, 17 *D. bruxellensis* strains belonging to the international CBS collection have been investigated for the ISSs, employing specific oligonucleotides containing different 5' consensus sequences: GTATGT (*S. cerevisiae*) and GTAAGT (*D. bruxellensis*). Results have shown that almost the whole yeast collection was discriminated at strain level using different combinations of primers. Therefore, to simplify the approach, a multiplex PCR protocol able to generate stable genetic profiles was developed.

Keywords *Dekkera bruxellensis* · Yeast typing · Introns

Introduction

Investigation on genome variability among individuals represents an important tool to assess their biodiversity and to study their environmental diffusion. The safeguard of microbial strains with technological interest is becoming a strategic activity in food and wine industries. Nevertheless, many species marginally implicated in productions are still poorly studied, and rapid and reliable protocols for their recognition and typing are not well described. This is the case of the yeast *Dekkera bruxellensis* that can cause spoilage and increasing economic losses in wine. During the last years, some studies on *D. bruxellensis* phenotypic and genetic aspects have been published (Conterno et al. 2006). At the present time, the physiological studies have clarified that

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APPENDIX D

PhD Thesis Abstract

Among yeasts responsible for wine spoilage, *Dekkera/Brettanomyces bruxellensis* is the species on which the scientific community has the highest interest. This fact is documented by an increasing in the international publications and by the beginning of the genome sequencing. Recently, it has been traced the evolutional *D. bruxellensis* lineage by the analysis of promoter sequences, which separated from the *Saccharomyces* yeasts more than 200 mya.

Spoilage caused by *D./B. bruxellensis* is mainly due to the following issues:

- ✓ this species remains viable and active in beverages preserved by extreme abiotic stress (anaerobiosis, up to 12-13% ethanol (v/v), minimal amounts of fermentable sugars);
- ✓ the adopted treatments (sulphiting, membrane filtration, transfer of wine to sanitized barrels) are not always effective.
- ✓ the off-flavours produced by *Brettanomyces* include volatile phenols characterised by disagreeable odours.

The aim of this thesis was to develop strategies in order to analyse and control the wine spoilage linked to *D./B. bruxellensis* species. As concern the first issue, spoilage microbial analysis, the present PhD thesis describes:

- ✓ the development of new methods for *D./B. bruxellensis* molecular typing;
- ✓ the phenotypic biodiversity of *D./B. bruxellensis* species.

The topics on the microbial spoilage control were:

- ✓ *D./B. bruxellensis* response to stress conditions.

i) Studies on the natural distribution of *D./B. bruxellensis* have shown an existing high intraspecies polymorphism degree which is probably due to a fusion event among genomes or to the lacking of a sexual state. Moreover, since *D./B. bruxellensis* has been mainly associated to fermented beverages that represent mutagenic environments determining the frequent genome rearrangement of *D./B. bruxellensis*. Genetic variations are usually accumulated with a higher frequency in DNA regions that are not linked to any gene function respect to the coding regions such as introns. Thus, they are considered good indicators in evolutional studies; in *S. cerevisiae*, the lariat branch point TACTAAC and the 5' splice site GTATGT (5'ss) are conserved sequences that were used to build primers for the Intron Splice Site amplification analysis (ISS-PCR) described for inter- and intraspecific characterisation of *S. cerevisiae*. The main goals of this first topic was to develop new methods for *D./B. bruxellensis* molecular typing. The setting up of a multiplex PCR protocol throughout the use of modified oligonucleotides that targeted 5'ss -GTAAGT- has confirmed a high polymorphism among *D. bruxellensis* genomes. Thus, a further optimisation of the primers, a simple capillary electrophoresis protocol that can accurately separates the amplified fragments and clear rules for the ISS profiles elaboration were applied. The results points out that the genetic signatures obtained exploiting the ISS as molecular targets are able to show genetic differences that, up to now, only other laborious technique can put in evidence (Karyotyping, PFGE-RFLP, AFLP). The proposed protocol has proved to be reliable and robust. Moreover, considering that a positive correlation between the extent of non-protein-coding DNA and the eukaryotic complexity degree has been observed, the ISS fingerprinting can represents a useful tool to analyse the evolution rate of a yeast species.

ii) *D./B. bruxellensis* yeasts have evolved numerous developmental options in order to adapt and survive the changing status of the environment. Independent studies showed that distinct

genetic groups of *D./B. bruxellensis* can have different physiological characteristics and strong differences in their ability to produce 4-ethylphenols. The main goal of second topic was to characterise *D./B. bruxellensis* from a phenotipic point of view. In particular, the physiological diversity within *D./B. bruxellensis* strains was investigated studying the growth and the production of volatile phenols and biogenic amines under a wine model condition. Moreover, the carbon compounds assimilation, H_2S production, and vinylphenol reductase (VPR) activity were also analysed. The potential hazard of spoilage when *D./B. bruxellensis* grows in oenological conditions was confirmed since most of the analysed strains were able to produce volatile phenols or showed a detectable VPR specific activity. Actually metabolic traits, as growth rate and off-flavour production, proved to be related and strain-dependent suggesting that an early detection and identification of "fast-growing yeasts" and "fast volatile phenols producers" could be essential to introduce adequate corrective measures. The experiments on carbon assimilation revealed that about 30% of the analysed yeasts has a own pattern in the utilization of carbonious sources. A negative correlation between VPR specific activity and H_2S production was observed. Take into account that volatile phenol production could be used by *D./B. bruxellensis* yeasts to restore the redox balance in anaerobic condition and that in *S. cerevisiae* the liberation of H_2S arises from a reduction of inorganic sulphur throughout the activity of sulphite reductase enzyme, this result could indicate that strains characterized by a low VPR specific activity have evolved other mechanisms to reoxydise equivalents, among these the capability to exploit the sulphite reduction.

iii) A goal of the wine industry is to reduce the risk of wine being spoiled by microbial activity. The main aim of the third topic of this research was to study the response to stress conditions in *D./B. bruxellensis* due to the yeast exposition to an electric current treatment and exogenous SO_2. Results indicated that a similar effect occurred on cells after the current treatment in comparison to the SO_2 exposition; both treatments resulted in a reduced microbial cell survival in the studied red wine. The kinetics of volatile phenol accumulation confirmed that, the use of an electric field could be adequate to hinder the yeast spoilage. As concern the latter issue, the SO_2 resistance, a metabolomic study on the effect of the SO_2 addiction to *D./B. bruxellensis* cultures was carried out too. Results displayed that among the metabolic pathways resulted to be affected by exogenous sulphite concentration, arginine and proline metabolism seem to be involved in the SO_2 tolerance. Unlike what was observed in *S. cerevisiae*, neither adenine nor methionine modified the toxicity level of SO_2 under laboratory conditions. The ethanol concentration seems to increase the sensitivity to sulphite suggesting that a membrane system, such as the sulphite efflux pump of *S. cerevisiae*, could be present in this species.

In conclusion, the main research products of this PhD thesis were:

- ✓ a new PCR protocol to typing *D./B. bruxellensis* that uses specific primers for this yeast species, and a precise and reliable fragment separation protocol by capillary electrophoresis. Actually, this method shows a high reproducibility (94%), it is rapid in comparison to other techniques that in the past allowed a discrimination at strain level of *D./B. bruxellensis* isolates (Karyotyping, RFLP-PFGE, AFLP, etc.), and it represents a useful tool to monitor the yeast evolution rate;
- ✓ the collection of *D./B. bruxellensis* phenotypic features that never have been used to evaluate the biodiversity degree in this species, such as the VPR specific activity, H_2S production, and the assimilation of carbon compounds different from ones found in wine. The compilation of a database collecting both genetic and phenotypic traits of different

D./B. bruxellensis strains is the future perspective to offer an efficient way to counteract this spoilage yeast;

- ✓ a new technology to reduce the survival of *D. bruxellensis* in wine using a low electric current (LEC) treatment.
- ✓ the understanding of some metabolic mechanisms involved in the SO_2 response in *D./B. bruxellensis*. This step will allow the following upgrade toward the study of molecular mechanisms, and metabolic pathways that this yeast can activate to protect itself against the exposure to high concentration of exogenous sulphur dioxide.

PhD Abstract

(Italian)

Ad oggi, tra i lieviti responsabili dell'alterazione del vino, *Dekkera/Brettanomyces bruxellensis* è la specie più studiata. L'alterazione prodotta da *D./B. bruxellensis* è dovuta principalmente ai seguenti aspetti:

- ✓ essa rimane vitale nelle bevande che vengono conservate tramite l'impiego di estremi stress abiotici (anaerobiosi, fino al 12-13% di etanolo (v/v), minime quantità di zuccheri fermentabili);
- ✓ i trattamenti adottati (solfitazione, filtrazione a membrana, il trasferimento del vino in botti sterilizzate) non sempre sono efficaci;
- ✓ la produzione di composti dall'aroma sgradevole da *Brettanomyces* comprende i fenoli volatili.

Lo scopo di questo progetto di tesi di dottorato è stato di sviluppare strategie per l'analisi ed il controllo della presenza di *D./B. bruxellensis* nel vino. Per quanto riguarda il primo punto, ossia l'analisi microbiologica dell'alterazione, il presente lavoro descrive:

i) lo sviluppo di nuovi protocolli per il typing di *D./B. bruxellensis*;
ii) la biodiversità fenotipica nella specie *D./B. bruxellensis.*

Il controllo dell'alterazione del vino si e' basato su:

iii) lo studio delle risposta di *D./B. bruxellensis* a condizioni di stress.

i) Studi sulla distribuzione e l'incidenza di questa specie sono riportati in letteratura; in particolare, essi suggeriscono un'elevata biodiversità dovuta alla fusione tra genomi oppure alla mancanza di una stato sessuale. Poiché *D./B. bruxellensis* viene principalmente associato alle bevande fermentate, si ritiene che le condizioni ambientali estreme possano determinare il frequente riarrangiamento genico di *D./B. bruxellensis*. In *Saccharomyces cerevisiae*, il motivo intronico TACTAAC (lariat branch point) ed il suo sito in 5' di splicing GTATGT (5'ss) sono sequenze conservate e sono state utilizzate nella costruzione di primers per l'analisi "Intron Splice Site amplification" (ISS-PCR). Se da un lato la messa a punto di un protocollo per una multiplex PCR che impiega oligonucleotidi modificati proprio a livello del 5'ss -GTAAGT- ha confermato l'elevato polimorfismo esistente in *D. bruxellensis*, dall'altro una nuova serie ottimizzata di primers, un semplice protocollo di separazione elettroforetica capillare e chiare regole per l'elaborazione dei profili genetici ISS hanno permesso di evidenziare differenze genetiche che, fino ad ora, solo altre tecniche sofisticate, ma laboriose, potevano fare (cariotipo, RFLP-PFGE, AFLP, etc.). Inoltre, considerando che esiste una correlazione positiva tra il DNA non codificante e la complessità dei genomi eucariotici, i profili ISS rappresentano un strumento per analizzare il tasso evolutivo di questa specie di lievito.

ii) I lieviti della specie *D./B. bruxellensis* hanno evoluto numerose opzioni di sviluppo al fine di sopravvivere ai cambiamenti dell'ambiente e distinti gruppi genetici possono avere diverse caratteristiche fisiologiche. L'obiettivo di questa attività è stato di caratterizzare una collezione di *D./B. bruxellensis*, aventi diversi profili genetici, dal punto di vista fenotipico studiando lo sviluppo del lievito e la produzione sia di fenoli volatili che di ammine biogene in un vino-modello. Inoltre, l'assimilazione delle fonti di carbonio, la produzione di H_2S e l'attività della vinilfenolo reduttasi (VFR) sono state valutate. I risultati confermano il carattere ceppo-dipendente di questa specie nel produrre fenoli volatili ed indicano che l'individuazione di ceppi a rapido sviluppo e con cinetiche di produzione di off-flavours elevate può dimostrarsi

efficace per introdurre adeguate contromisure. Il 30% dei ceppi sotto analisi si dimostrava possedere un proprio profilo di assimilazione delle fonti di carbonio. Infine, una correlazione negativa tra l'attività dell vinilfenolo reduttasi e la produzione di H_2S è stata osservata. Poiché si ipotizza che *D./B. bruxellensis* produca venoli volatile per riequilibrare il potenziale redox e in *S. cerevisiae* la liberazione di H_2S avviene attraverso l'attività di una solfito reduttasi, è possibile che ceppi caratterizzati da basse attività della VFR sfruttino altri sistemi per riossidare il NADH, tra questi la riduzione dei solfiti.

iii) Un obiettivo dell'industria del vino è quello di ridurre il rischio iniziale di contaminazione. Durante questa attività è stato studiato l'effetto della corrente elettrica sulla sopravvivenza e sulla produzione fenoli volatili in *D./B. bruxellensis* nel vino. I risultati hanno indicato che un simile effetto si verifica sia sulle cellule dopo il trattamento con la corrente e quelle esposte all'SO_2; infatti, entrambi i trattamenti hanno ridotto la sopravvivenza delle cellule. La cinetica di accumulo di fenoli volatili ha confermato che l'utilizzo di un campo elettrico potrebbe essere sufficiente per impedire l'alterazione del vino. Infine, questa è stato intrapreso lo studio degli effetti metabolici che si scatenano in *D./B. bruxellensis* a seguito dell'esposizione da SO_2. I dati suggeriscono che la via metabolica della prolina sia coinvolta nell'aumento di tolleranza all'anidride solforosa.

In conclusione, i principali prodotti della ricerca di questa tesi di dottorato sono stati:

- ✓ un nuovo protocollo di PCR per la tipizzazione di *D./B. bruxellensis* che usa sia primers specifici per questa specie di lievito che un sofisticato ed affidabile protocollo di separazione dei frammento in elettroforesi capillare;
- ✓ la raccolta la correlazione di dati fenotipici di *D./B. bruxellensis* che non sono mai stati utilizzati per valutare il grado di biodiversità in questa specie, come ad esempio l'attività specifica della VFR, la produzione di H_2S;
- ✓ una nuova tecnologia per ridurre la sopravvivenza di *D. bruxellensis* nel vino grazie all'impiego di una trattamento a bassa corrente elettrica (LEC);
- ✓ la comprensione di alcuni dei meccanismi metabolici coinvolti nella risposta all'SO_2 in *D./B. bruxellensis*.