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:

i) the development of new methods for *D./B. bruxellensis* molecular typing;
ii) the phenotypic biodiversity of *D./B. bruxellensis* species.

The topics on the microbial spoilage control were:

iii) *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.