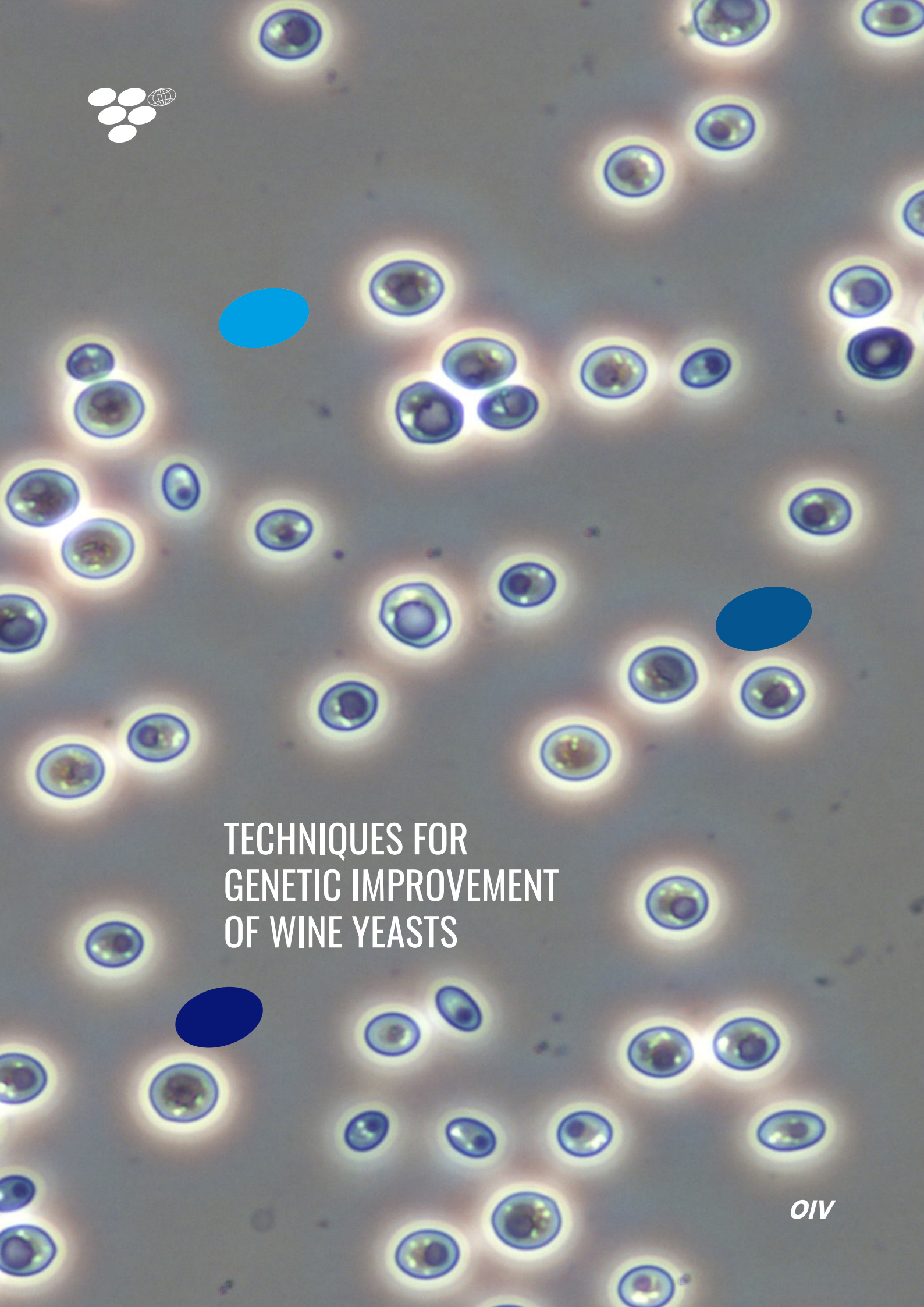

TECHNIQUES FOR GENETIC IMPROVEMENT OF WINE YEASTS

OIV

AUTHORS

Patrizia Romano (IT)
Microbiology group of experts

COORDINATOR

Guido Baldeschi, OIV Oenology Head of Unit

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Charlotte Delebarre, Communication Assistant – layout

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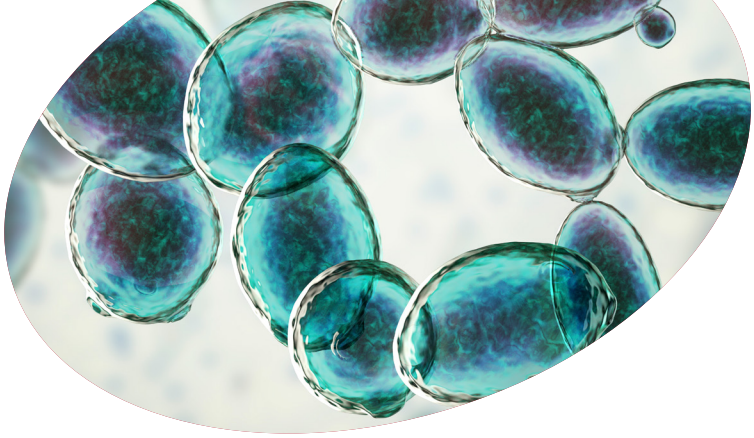
OIV - International Organisation of Vine and Wine
35, rue de Monceau
F-75008 Paris - France
E-Mail: oenologie@oiv.int

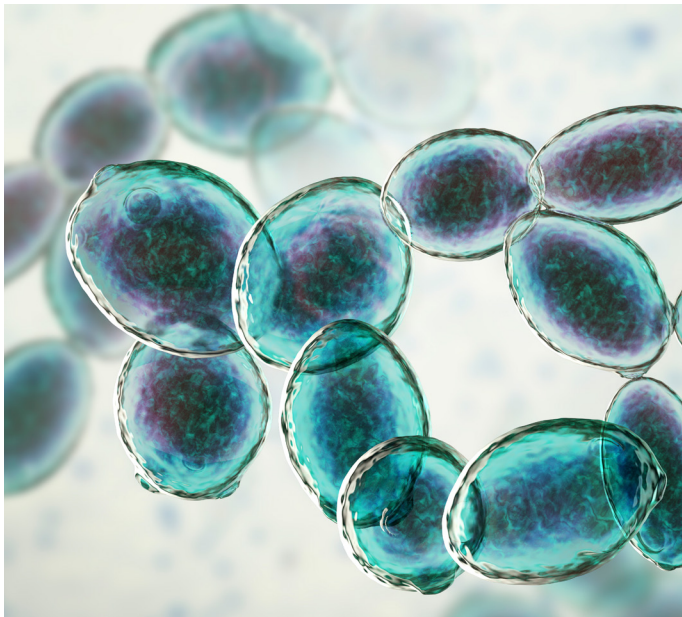


1 • CLONAL SELECTION

Clonal selection is based on the screening of large libraries of yeast samples isolated from winemaking associated environments (grapes, grape must, wineries) and leads to the achievement of a limited number of hits presenting biotechnological properties of interest for the wine industry (possible starters). The selection characters, listed by different authors (Jolly et al. 2014; Mannazzu et al. 2002; Pretorius 2000) cover different aspects including:

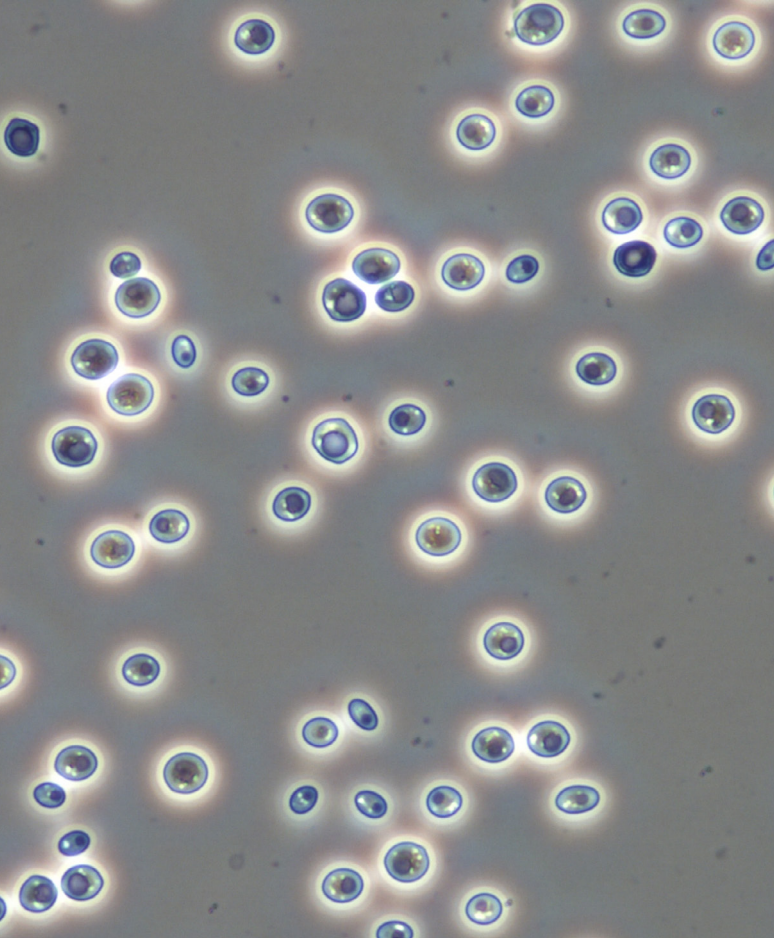
• Genetics, i.e. species identification, unicity of the selected strain among the already existing ones (OIV-OENO 408 2011);
• Physiological/metabolic characteristics relevant to take into account on a technological point of view / Management of the fermentation process (OIV OENO 370 2012);
• Microbial interactions during grape must fermentation (killer character, activity on *Oenococcus oeni* and on non-*Saccharomyces* yeasts);
• Feasibility of the production under the form of a starter complying with the OIV monography specifications (viable population levels, genetic stability, low contaminants...). (OIV-OENO 576A, 576B 2017).

Although referring mainly to the species *Saccharomyces cerevisiae*, all these characters can be evaluated in non-*Saccharomyces* yeasts (Jolly et al 2014). Clonal selection while very effective for the exploitation of natural wine yeast diversity, may not be satisfactory to obtain wine strains with peculiar combination of characters.



2 • MUTAGENESIS

The induction of random mutations by chemical (ethylmethanesulfonate, methylnitrosoguanidine...) and physical (UV) mutagens aims at generating genetic variation within a population and is followed by the selection of individuals with the improved phenotype/s. It is generally used for the removal of undesired monogenic characters (Giudici et al. 2005) or to improve a specific trait (Gonzalez et al. 2016). For example, Cordente et al. (2013) utilised it to obtain wine strains with a decreased production of acetic acid in respect to the parental strain. One of the main drawbacks of this technique is that random mutation, although improving certain traits, can affect other characters and negatively impact on the general fitness of the resulting mutants. Moreover, since wine strains are often homothallic and at least diploid, mutations on a single allele may not be easily detected in the resulting mutants. For this reason mutagenesis should be better applied to haploid vegetative cells and spores (Romano et al. 1983). However, recent work, using natural and chemical mutagenesis followed by selection on a toxic metabolite has shown the feasibility on a diploid wine yeast (Cordente et al. 2018).



3 • DIRECTED EVOLUTION

Directed evolution, also named adaptive evolution or evolutionary engineering, is aimed at the generation of wine yeast strains harboring a combination of phenotypic traits that might not frequently occur in wild type cells. To achieve these results, wild type should be maintained for many generations under continuous selective pressure for the phenotype of interest. This process, that mimics natural selection, leads to the appearance and the enrichment of fit genetic variants with the desire trait/s within the starting cell population (Steensel et al. 2014) and is suitable to the selection of polygenic phenotypes. Cadiere et al. (2011, 2012) applied directed evolution to a commercial wine strain to obtain evolved variants characterised by higher fermentation rate, reduced production of acetate and increased production of higher alcohols and esters. Mezzetti et al. (2014) and Bonciani et al. (2018) utilised it to obtain wine strains with improved glutathione production and fermentative fitness. López-Malo et al. (2015) obtained evolved strains with improved growth and fermentation at low temperature in respect to the parental strain. Tilloy et al. (2014) used the adaptative evolution combined to breeding to generate wine yeast strains with a lower sugar to ethanol yield, a higher glycerol and 2-3 butanediol production and a lower acetate production.

4 • Hybridisation

4.1. • Conventional breeding, or intra-specific hybridisation

Hybridisation has been commonly used for improvement of diploid wine yeast strains since many years (Romano et al. 1985). For heterothallic strains, hybrids can be easily selected by micromanipulating the zygotes formed between meiotic segregants with complementary mating types. For homothallic strains, hybridisation can be accomplished by mixing sporulated cultures or by spore-to-spore pairing using a micromanipulator (Marullo et al. 2007). However, hybrids are obtained with low frequency and are difficult to identify. Methods based on killer factor or spontaneous mutants to antibiotics have been used to increase the success of hybridisation and selection of hybrids (Ramirez et al. 1998).

4.2. • Directed hybridisation

Most of the enological traits of interest are governed by multiple loci and present a continuous variation in a population. Thanks to recently growing genetic tools, the study of the genetic determinant is becoming easier and QTL (Quantitative Trait Loci) mapping can be performed using molecular markers or whole genome sequencing. QTL identification typically involves the generation of a recombined population from two parents with divergent phenotypes, the quantification of the trait of interest in the offspring's and the construction of a genetic map through genotyping or sequencing.

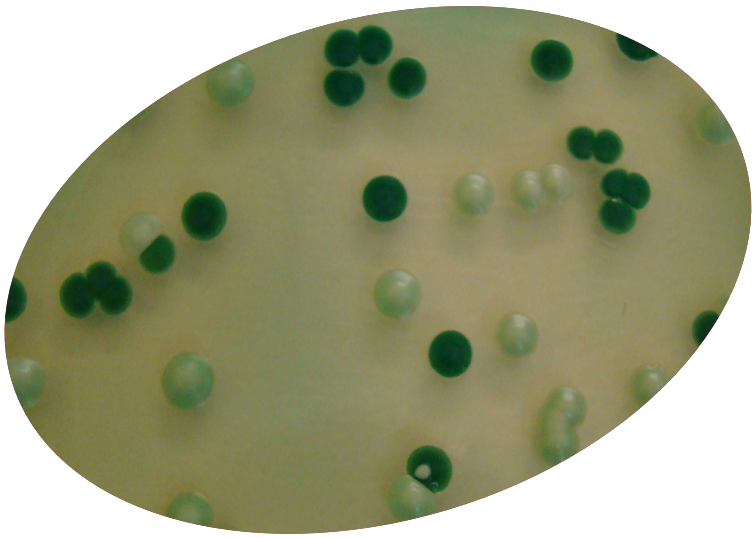
Region(s) of the genome that are linked to the phenotype are identified thanks to a statistical analysis. QTL dissection consists then to the identification and the validation of the impact of the alleles of the candidate genes identified in the region(s). Once the alleles of interest are known, they can be transferred from one strain to another using introgression. Introgression, also called backcrossing assisted by molecular markers, consists in recursive hybridisation between a strain possessing the allele of interest and a strain to improve (Marullo et al, 2009; 2019). Introgression aims at restoring the properties of a strain of interest while adding new properties carried by the identified alleles. The molecular markers or directly the mutations identified in the alleles can be easily followed using allele-specific PCR. Several cycles of backcrossing are often performed to increase the percentage of genome of the receptor strain. Each cycle divides the percentage of the donor genome by 2. The first cross results in a hybrid possessing 50% of the genome of the two parental strains, the second 75% of the receptor etc... 4 cycles resulting in more than 93% of the receptor strain genome. The production of H_2S, lag phase, and POF character (Marullo et al. 2007), volatile thiol release (Dufour et al. 2013), or SO_2, H_2S, and acetaldehyde (Noble et al.2015) have been improved using this approach.

4.3. • Inter-specific hybridisation

In the *Saccharomyces sensu stricto* clade, yeast species showed a severe reproductive isolation (less than 1% of viable spores) (Naumov 1987). In addition, the most important genetic barriers among *Saccharomyces* species are postzygotic. However, interspecific hybrids among species in the *Saccharomyces* genus are frequently detected in anthropic habitats. Indeed, interspecific hybrids between S. *cerevisiae* and psychrophilic species S. *uvarum* or S. *kudriavzevii* have been isolated in natural environment and wine (Masneuf et al. 1998; Le Jeune et al. 2007; Lopandic et al. 2007, Nguyen and Boekhout 2017). These natural hybrids have technological properties differing from those of their respective "parental" species (da Silva et al. 2015). Since their genesis, these natural hybrids may have undergone genomic modifications that can drastically affect their phenotype because of such gross chromosomal rearrangement (Piotrowski et al 2012; Peris et al. 2012), loss of heterozygosity particular mitotypes, aneuploidies and introgressions. However, interesting physiological features frequently observed in chimerical strains, which are generally intermediate among their parental phenotypes, have attracted the interest of both the scientific community and industry.

Indeed, hybrids can also be obtained easily in the laboratory and different methodologies can be utilised to mimic the natural hybridisation phenomena under controlled laboratory conditions to obtain novel yeast strains. These include i) sexual hybridisation through: a) crosses of individual spores (spore-to-spore or direct mating (Bonciani et al. 2015), b) mass mating, c) rare mating; ii) asexual hybridisation through d) cytoduction; f) protoplast fusion-hybridisations (Pérez-Través et al. 2012; Steensels et al. 2014). The choice of the methodology to generate hybrids is related directly to the aim of the hybridisation. Spore-to-spore mating is the only one implying gametes crossing from dissected tetrads. The efficiency of rare and mass mating is based on complementation of auxotrophies and on the improvement of strong selectable characters. Depending on several factors, including the involved strains, the hybridisation mechanism and stabilisation conditions, hybrids that bear differential genomic constitutions, and hence phenotypic variability, can be obtained.

As a direct result of the selected hybridisation methodology, genome stabilisation of recently generated hybrids could be required to guarantee yeast culture soundness (Bonciani et al. 2015). Regarding the hybridisation of haploid strains, a generally stable diploid hybrid is formed and the stabilisation process is quite simple. However, some interesting characteristics present in parental strains could be lost during the sporulation which generates haploid strains. Methods that involve crosses among diploid strains, like rare mating, have been demonstrated to be advantageous (Bonciani et al. 2015). Inter-specific hybridisation allows whole-genome modifications that can be exploited to obtain global improvements in industrial traits, such as those involved in the winemaking industry. In particular, the hybrids between *Saccharomyces cerevisiae* and cryotolerant species *Saccharomyces uvarum* or *kudriavzevii* have been generated in different laboratories (Bonciani et al. 2015; Origone et al. 2018; Perez-Torrado et al. 2015).



5 • Genetic engineering

The genetic engineering, or recombinant DNA technology, exploits a set of molecular tools and approaches in order to manipulate the genetic characteristics of living organisms. In comparison to conventional breeding that transfers a large number of both specific and nonspecific genes to the recipient, genetic engineering only transfers a small block of desired genes; thus, this strategy is less time consuming and yields more reliable products. Briefly, this technology involves the insertion of DNA fragments from a variety of sources, having a desirable gene sequence via appropriate vector. Enzymatic cleavage is applied to obtain different DNA fragments using restriction endo-nucleases for specific target sequence DNA sites followed by DNA ligase activity to join the fragments to fix the desired gene in vector. The vector is then introduced in a host organism, and finally clones containing relevant DNA fragment are selected and harvested. In general, with these methods it is possible to modify specific metabolic pathways by: decreasing or blocking the expression of endogenous genes of a limiting reaction (bottleneck), the overexpression of one or more genes, and the introduction of new genes and regulatory elements.

5.1. • Genome editing by CRISPR-Cas9

The genetic engineering of industrial yeasts is currently undergoing major changes due to the development of a marker-free, high-throughput, and multiplexed genome editing approach inspired to the bacterial immune systems: the "Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) immune system" (Bhaya et al., 2011; Jinek et al., 2012).

CRISPR/Cas9 allows the knockout or the tuning of expression of specific targeted genes and pathways (Carroll, 2012, Horwitz et al 2015). It is based on the utilisation of the single bacterial nuclease Cas9 (CRISPR-associated protein 9), that recognises the region to be cut thanks to the association with a guide RNA (gRNA), which drives the nuclease to a target site. These cutting damages on both strands of the DNA generate double strand breaks (DSB) that can be repaired by two different methods: non-homogenous recombination (NHEJ) or homologous recombination (HR).

In particular, the former consists in a simple ligation of the two broken ends, but it can generate point mutations (both insertion and deletion of nucleotides). HR is the most commonly used repair mechanism in genome engineering, and it consists of repairing the damaged genome thanks to a counterparty fragment called donor-DNA at both ends of the site in which the cut took place (Gratz et al., 2013; Mahfouz et al., 2014).

The first application of CRISPR in yeast was described in 2013 by DiCarlo and collaborators; they transformed S. *cerevisiae* strains by two different plasmids bringing the Cas9 and the gRNA, respectively. Results suggested that yeasts could be a useful biological system where the CRISPR/Cas9 genome editing approach can be implemented for health, agriculture, and conservation purposes (DiCarlo et al., 2013). In 2018, Raschmanová and collaborators tested the CRISPR-Cas9 system in non-*Saccharomyces* yeasts. The successful implementation of CRISPR-Cas9 system in non-conventional yeasts was accompanied by the development of innovative expression strategies (Raschmanová et al., 2018).

Several methods have been described to simultaneously modify multiple genes using CRISPR/Cas9 system (Stovicek et al., 2017), based on vectors containing up to two gRNA expression boxes and selection markers: i) individual cloning of each cassette into the p426-SNR52p-gRNA.CAN1.Y-SUP4t vector and then they are merged thanks to the Gibbson assembly (Mans et al., 2015); ii) application of the homology-integrated CRISPR strategy (HI-CRISPR), that involves the use of a single plasmid (pCRCT) containing the Cas9 sequence, gRNAs organised as arrays in interspaced CRISPR RNA (crRNA) and their donors (Bao et al., 2015); iii) co-transformation of cells already equipped with Cas9 with different plasmids carrying each of them a different gRNA and a different selection marker or, alternatively, with the linearized plasmid 2μ containing the Nat selection marker, the linearised cassettes of the gRNAs flanked by plasmid counterpart fragments to allow recombination and the addition of a USER (Uracil Specific Excision Reaction) containing a single guide RNA (Horwitz et al., 2015); iv) amplification of the gRNAs with primers containing the restriction enzyme cutting site and transformation using simultaneously all the cassettes, inserting them into the plasmid carrying the USER (Jakočiūnas et al. 2015).

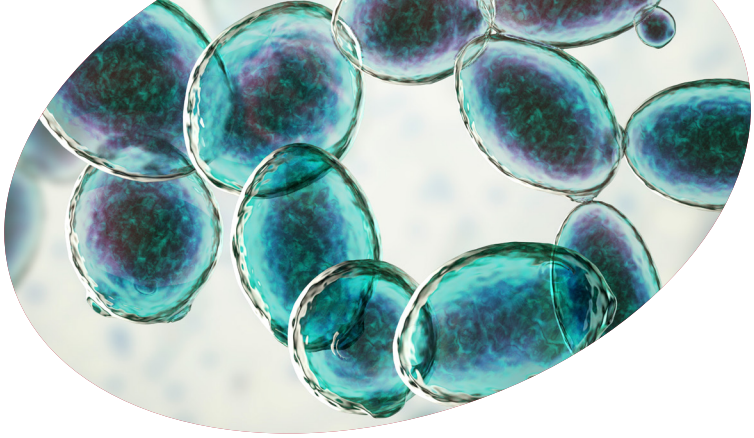
Aside from the molecular advantage of producing quick genome changes by using a unique gene-editing approach, the CRISPR/Cas9 system has the potential to soon become the gold standard technique to produce novel microorganisms suitable for the food industry. This system produces marker-free mutants and has been applied in many eukaryotic organisms (Komor et al., 2017) such as mammalian cell lines (Lee et al., 2015), insects (Gratz et al., 2013), and yeasts (DiCarlo et al., 2013; Ryan and Cate, 2014; Jakociunas et al., 2015). A great advantage of the CRISPR system is the use of a single Cas9 nuclease, in fact it is able to modify multiple genes in the presence at once of different RNAs, each carrying a sequence of gene to modify and donors containing sequences to insert (Wang et al., 2016).

The wine research field has already become aware of the potential of the CRISPR-mediated DNA technologies for the genetic improvement of yeasts (Pretorius 2017). The CRISPR/Cas9 system was firstly successfully established in S. *cerevisiae* commercial strains to generate yeasts with a reduced urea release, thus limiting the potential production of ethyl carbamate under oenological conditions (Vigentini et al 2017). Other examples refer to the investigation of the glycerol metabolism; the use of the CRISPR molecular tool allowed both the modification of *STL1* gene, involved in the glycerol uptake pathway during icewine fermentations (Muysson et al., 2019), and the overexpression of *GPD1* and *ATF1*, implicated in glycerol and acetate ester production respectively (van Wyk, 2021). Recently, the CRISPR/Cas9 system has been exploited to shed new light to the function of genes involved in yeast nitrogen requirements (Su et al 2021). A recent review covered the recent applications of CRISPR/Cas9 based genome editing in wine yeast (Vilela et al., 2021).

5.2. • Synthetic Biology

Finally, the advances in the technology for synthesizing large pieces of DNA have led to a dramatic cost-reduction in gene synthesis, opening new frontiers in the use of Synthetic Biology for the genetic improvement of eukaryotic microorganisms. The *in silico* design of a synthetic DNA allowed the incorporation of unique tailored features, such as the removal of introns, stop codons, and the silent mating loci and functional clustering of tRNA genes. Perhaps the feature with most biotechnological applications, is the addition of loxP recombination sites directly downstream of non-essential genes. The exposure to a recombinase would lead to a rapid rearrangement of the genome as these non-essential genes could be deleted, inverted or duplicated leading to populations with extensive genomic and concomitant phenotypic diversity. This method called "SCRAMBLE" has already been utilized to increase the yeast's ability to degrade cellulose or produce carotenoids. A collection of scientific works on the major findings implementing SCRAMBLE can be found at: https://www.nature.com/collections/dhppvlvxxb/content/scramble.

In this context, synthetic biology has revolutionized large-scale genome editing, with the Yeast 2.0 initiative (http://syntheticyeast.org) being one of the biggest showcases of the possibilities, through the custom design and de novo synthesis of each of the 16 chromosomes of S. *cerevisiae* laboratory strain S288c. Exploiting the excellent recombination capability of S. *cerevisiae*, large synthetic DNA fragments, called megachunks (30-60kb), have been subsequently introduced to replace, and functionally substitute, the corresponding "native" chromosomes. The eventual goal would be to completely replace the original genome with a synthetic version. Using Yeast 2.0 design principles, it is also possible to build a neochromosome which contains S. *cerevisiae* genes -codifying for new functions- that are missing in the S288c strain. To date the SCRAMBLE methodology has not been applied to a wine yeast; however, some industrially-relevant phenotypes have been improved by this approach such as the tolerance to different stress sources (ethanol, heat and acetic acid) (Luo et al., 2018). Key to the successful implementation of SCRAMBLE-like experiments is an appropriate screen. The majority of the current screens are reliant on being high-throughput as billions of genetically non-identical yeasts need to be tested. This requires innovative thinking in order to utilise this "directed evolution on steroids" method to find superior wine yeast. However, as a pioneering work, Lee et al. (2016) were able to engineer -synthetically- a wine yeast to produce, de novo, the raspberry ketone aroma in a white wine, via pathway engineering and synthetic enzyme fusion including genes from other yeast and plant species.



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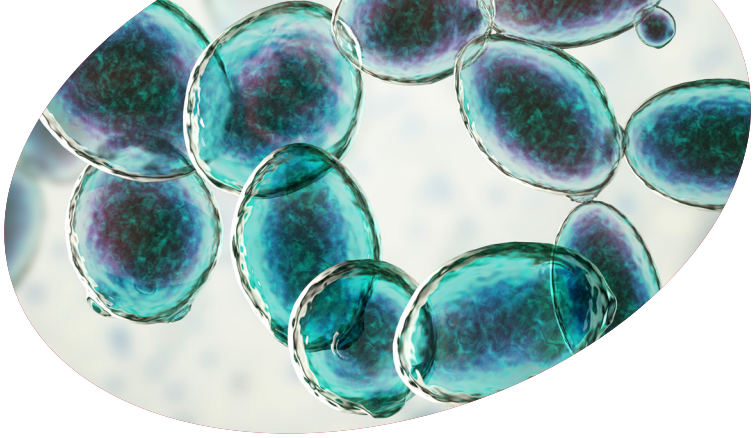

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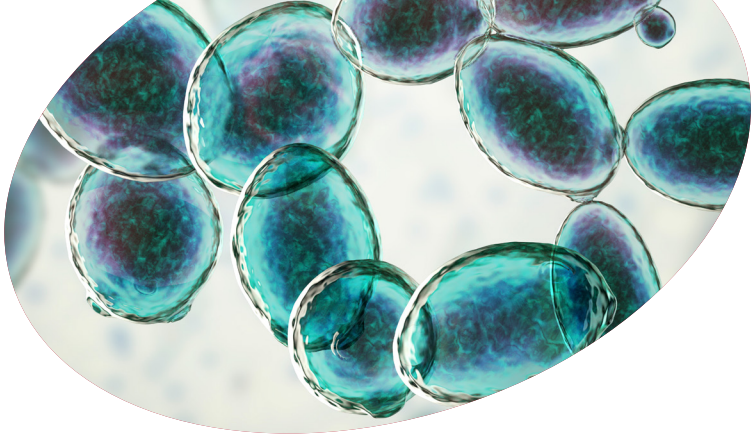

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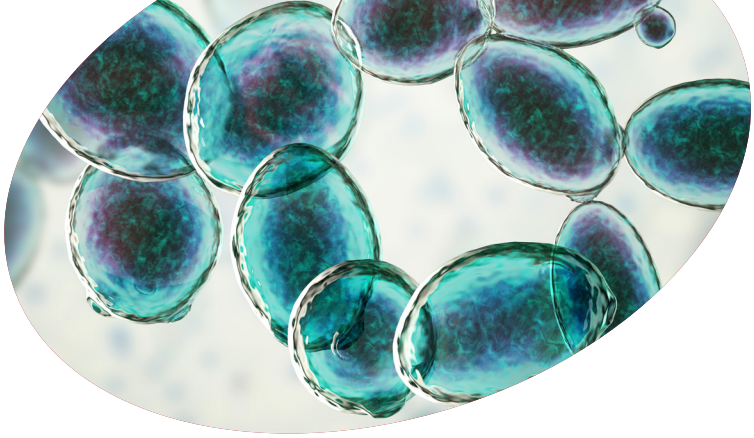

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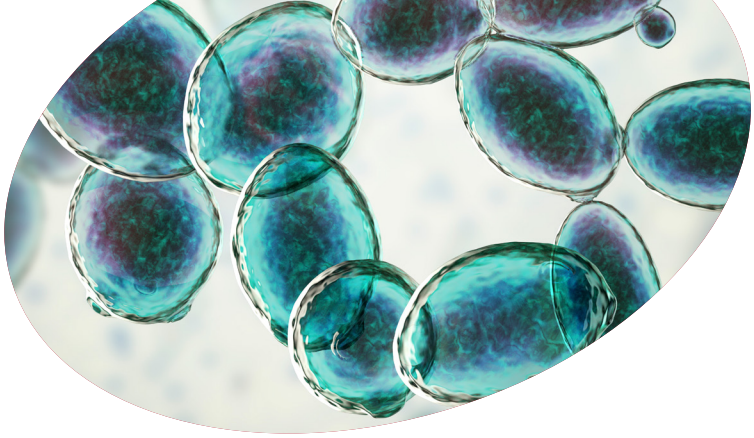

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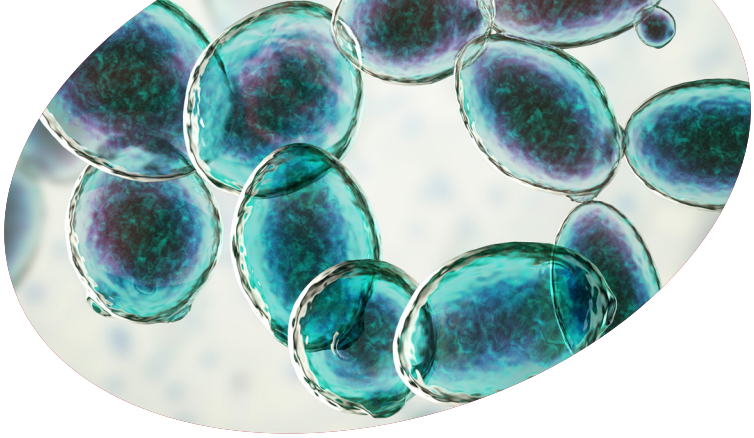

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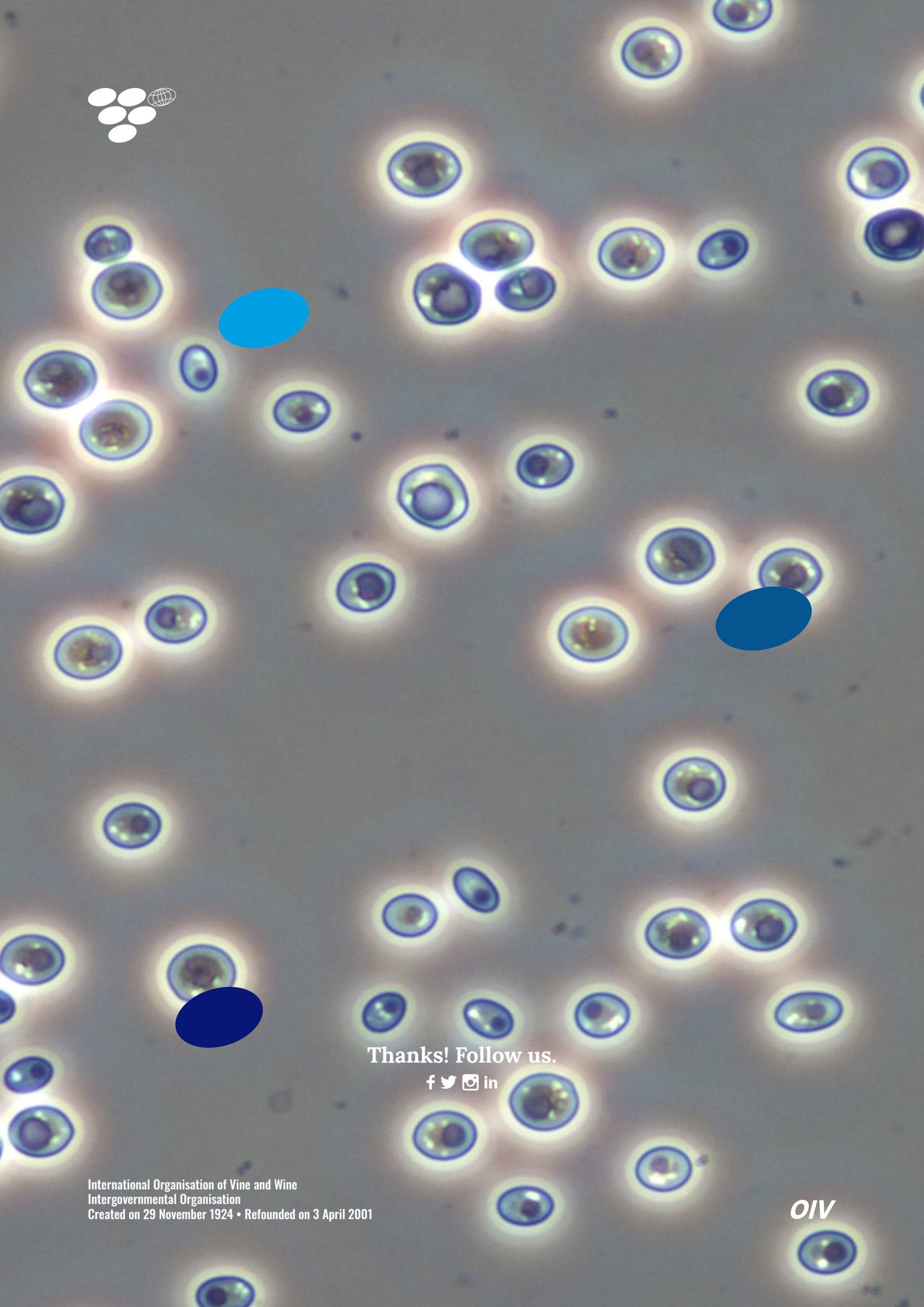
"This document does not concern the Genetically Modified Organisms (GMOs); the definition of these GMOs and the regulatory specifications associated should be considered at country level."



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