Yeast hybridization to overproduce esters and minimize sulfites in wines

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INTRODUCTION

Consumers are pushing towards the elaboration of higher quality and always **fresher and more expressive and aromatic wines** while paying more and more attention to their **health**. The presented works aims to **offer new tools for winemakers** to help them produce both **intensely fruity and clean wines.** Through the **generation of yeast by hybridization, a multi-step screening** of the strains was elaborated to select a new yeast strain with a **clean and very fruity aromatic profile.**

Acetate esters such as isoamyl acetate and **higher alcohols** such as Isoamyl alcohol and 2-phenyl-ethanol are strong aromatic compounds produced by the yeast and responsible for highly fruity wines (Figure 1). On the other end sulfites and other sulfur compounds are responsible for off-flavors (burnt matches, rotten eggs, cabbage, etc…) and tend to hide the fruitiness in wines. Sulfites are also of health concern and modern winemaking tends to limit its use to the minimum required.. Therefore, the **target of this project was to generate and select a yeast strain that optimizes the production of acetate esters and higher alcohols while minimizes the production of Sulfites.**

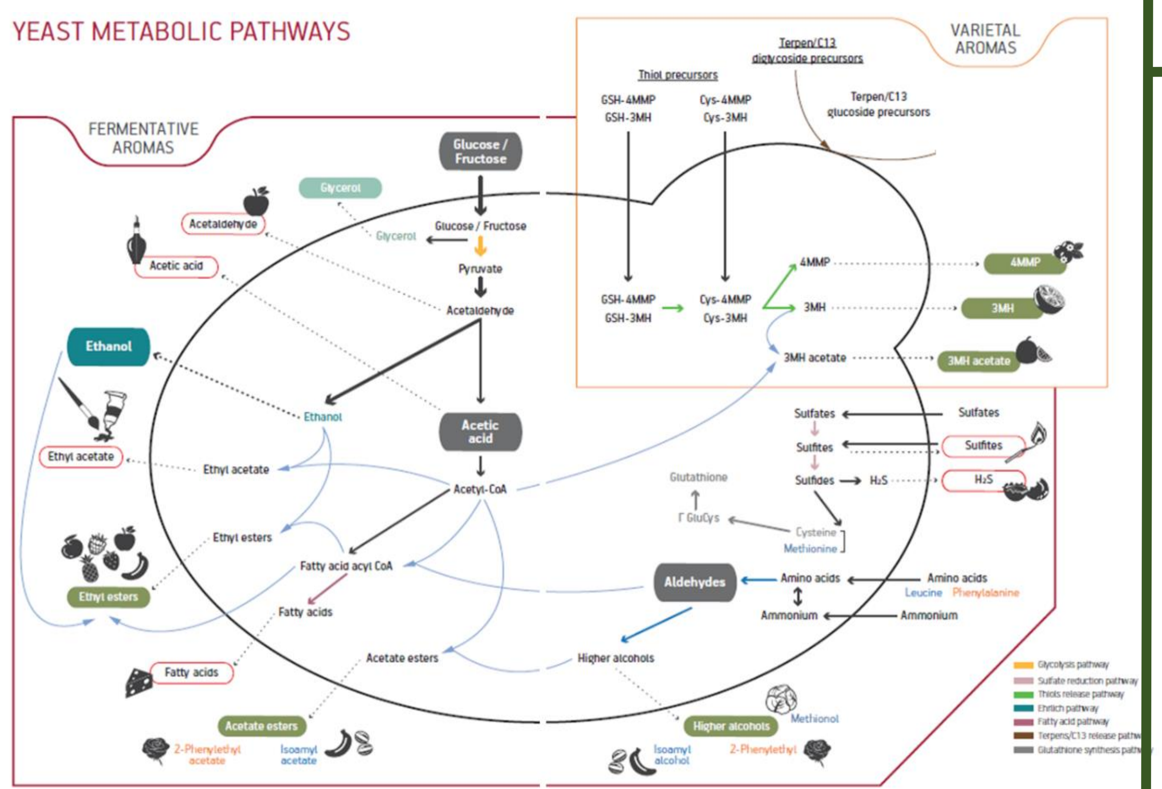


Figure 1: Yeast 's impact on fermentative and varietal aromas

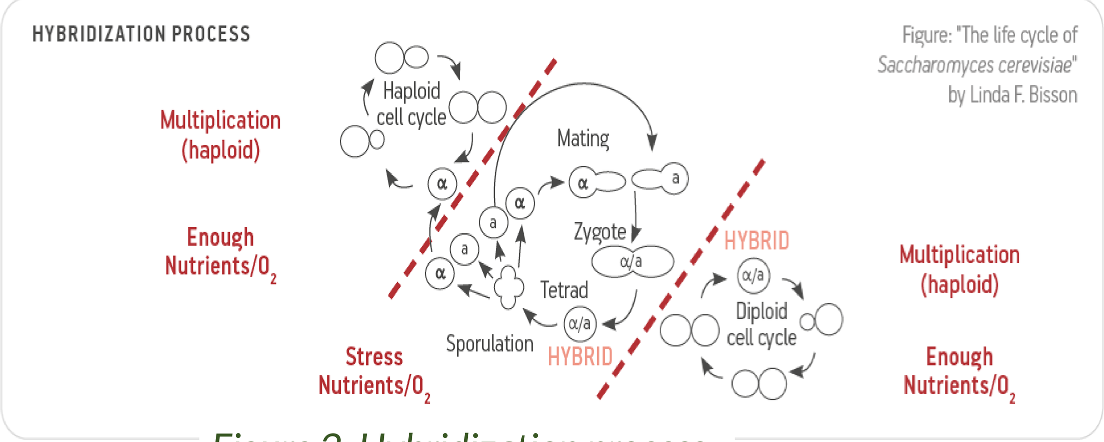


Figure 2: Hybridization process

New diversity in Yeast can be found through 2 major ways:

- **Selection from nature**, vineyards or wineries of different genus or species. This method is simple but does not yield targeted results in selection of strains on specific criteria.
- **Genetic technics** such as Mutagenesis, Directed evolution, Genetic modifications (not yet accepted in the wine industry) or Hybridization. Hybridization is the method selected for this project . It allows to create additional diversity from the existing one in nature and select targeted traits in yeasts.

Hybridization is a widespread natural phenomenon that leads to the production of offspring from the union of two different parents. Like all fungi, yeast has asexual and sexual reproductive cycles. Yeast is most commonly the result of the asexual/budding cycle. Each new cell is therefore the genetically a replica of its mother cell. For in laboratory hybridization, two parent strains are put under the best conditions to produce spores and to organize their "mating" while ensuring a large number of descendants (Figure 2).

MATERIAL AND METHODS

- **2 parents** (Figure 3) with interesting and complementary phenotypes were selected in order to realize a **mass hybridization** that was the method chosen and preferred to micromanipulation to maximize chances of generating an interesting hybrids. The challenge is to created hybrids that will overperform both partially deficient parents especially in terms of **fermentation resistance, sulfur compounds and acetate esters.**

Parents selection

	Parent 1	Parent 2
Overall resistance towards difficult fermentation conditions (low YAN, High S…)	-	+
SO_2 production	-	+
H_2S production	+ (3/5)	+/- (2/5)
Acetaldehyde	+/-	+
Acetic acid production	+	+/-
Acetate esters production	-	+

Figure 3: Parent's selection

1rst micro scale screening

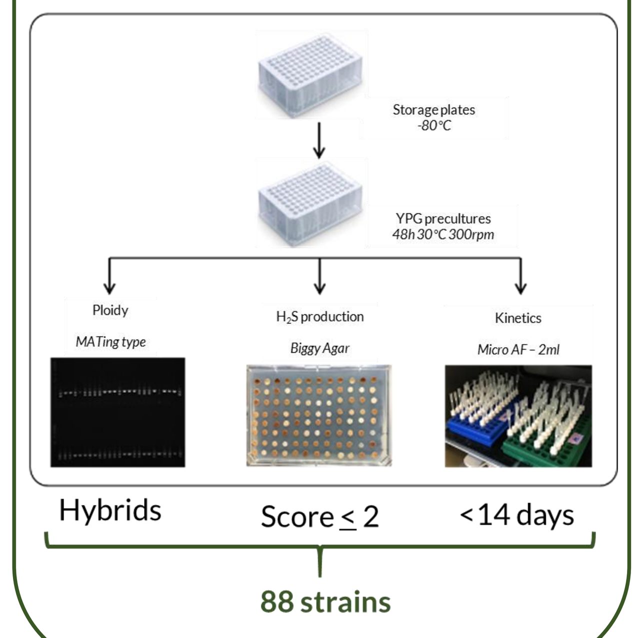
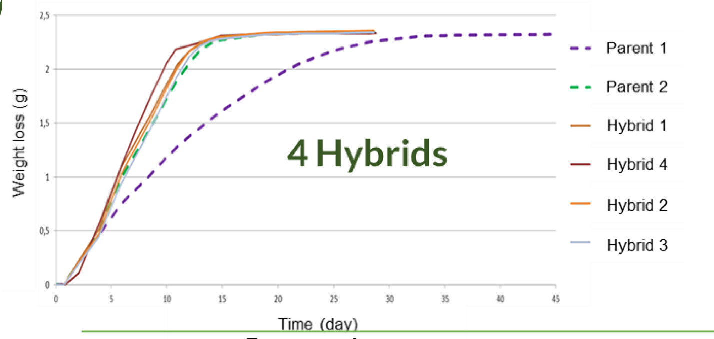


Figure 4: #1 screening

- **616 daughter strains were generated** among those Hybrids, Segregants and Self-diploid strains.
- **A first screening process in three steps at micro scale** (µL) was then performed in order to identify real hybrids, determine their production **of H_2S and their fermentative profile** in synthetic medium compared to their parents **(Figure 4)** . **At the end of this first screening, 88 strains were kept.**
- All of these strains then underwent a **secondary and tertiary screening (Figure 5) at a mini scale (mL)** in order to check their **kinetics and their main oenological features such as acetic acid and SO_2 production. The four best hybrids were selected for microvinification trials (Results section).**

2ndand 3rd mini scale screenings



Synthetic OIV medium (adjusted 250 g/L sugars)

17°C – 100 rpm

Strains	Fermentation duration (days)	Glu+Fru (g/L)	Acetic acid (g/L)	Total SO_2 (mg/L)
Parent 1	46	2.22	2.10	6.0
Parent 2	29	2.92	1.85	8.7
Hybrid 1	29	3.38	0.53	8.0
Hybrid 2	29	2.77	0.59	11.0
Hybrid 3	**29**	**3.11**	**0.38**	**4.0**
Hybrid 4	29	3.05	1.40	18.3

Figure 5: #2 and 3 screenings

RESULTS

1rst Microvinification

- The selected 4 hybrids went through a 1st microvinification screening to **confirm their fermentation abilities, production of acetaldehyde and aromatic profile** compared to the parents **.**
- Partnership with INRA Colmar, FR: Muscat - Inoculated at 10^6 CFU/mL - 25L - 20°C - Adjustment to YAN/S = 0.8 with DAP (Tables 1 and 2)
- Aromatic analyses after bottling with SBSE concentration, thermal desorption then gas chromatography and mass spectrum (SBSE-TD-GC-MS) (Figure 6 and Table 3)

Table 1: Must Analysis Muscat, FR

Sugars (g/L)	195.3
Total acidity (g H_2SO_4 /L)	4.72
pH	3.16
Malic acid (g/L)	2.93
Free SO_2 (mg/L)	2.0
Total SO_2(mg/L)	6.1
YAN (mg/L)	61.0
Turbidity (NTU)	50.1

Table 2: Wines Analysis Muscat, FR

Strains	Fermentation duration (days)	Glu+Fru (g/L)	Acetic acid (g/L)	Malic acid (g/L)	Lactic acid (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Acetaldehyde (mg/L)
Parent 1	47.5	4.0	0.23	1.80	0.277	2	6.3	16.2
Parent 2	26.5	2.2	0.32	2.35	0.011	2	14.5	16.9
Hybrid 3	35.5	1.2	**0.18**	**0.01**	**1.058**	**5**	**5**	**1.9**

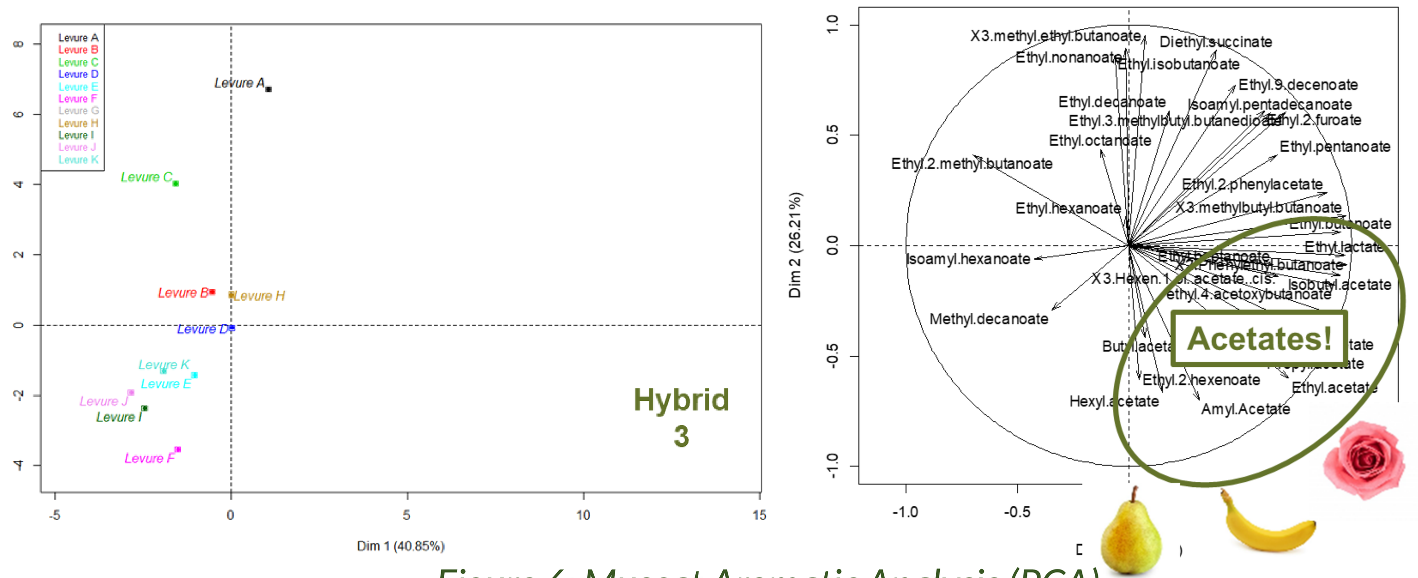


Figure 6: Muscat Aromatic Analysis (PCA)

Table 3: Isoamyl Acetate produced by strain

Strains	Isoamyl acetate (mg/L eq ethyl acetate)
Parent 2	26,69
Parent 1	13,06
Hybrid 3	**121,15**

x 4.5 !!!

- **Hybrid 3 was highlighted as very interesting for its overproduction of isoamyl acetate (Table 3 and Figure 6) , low Acetic acid, low FSO_2 and TSO_2 and Acetaldehyde, favoring MLF (Table 2).**

2nd Microvinification

- The selected hybrids went through a 2nd microvinification **to confirm fermentation abilities & aromatic properties on neutral variety** compared to commercial strains
- Partnership with IFV, FR : Melon de Bourgogne – Inoculated at 2.10^6 CFU/mL - **50L** – 18°C – Adjustment to YAN/S=1 with DAP - Addition of 30g/hl of yeast hulls at yeast pitching (Tables 4 and 5)

Table 4: Must analysis

Sugars (g/L)	185.5
Total acidity (g H_2SO_4 /L)	3.98
pH	3.1
Malic acid (g/L)	3.1
Free SO_2 (mg/L)	0
Total SO_2(mg/L)	12
YAN (mg/L)	62
Turbidity (NTU)	60

Table 5: Wines' analysis

Strain	AF duration (days)	Alcohol (% v/v)	Residual sugars (g/l)	Volatile acidity (g H_2SO_4/l)	Total acidity (g H_2SO_4/l)	Malic acid (g/l)	Total SO_2 (mg/l)
Ref. 1	13	12.30	0.0	0.17	3.60	1.4	27
Ref. 2	30	12.13	2.8	0.27	3.66	1.7	0
Hybrid 3	**20**	12.20	0.0	**0.37**	3.93	2.0	**0**

- **Hybrid 3 was again as the most interesting hybrid with high aromatic intensity toward fruity notes, candy sensation and a decrease of the vegetal hints (Figure 7) combined with no reductive notes - no SO_2/low H_2S (Table 5).**

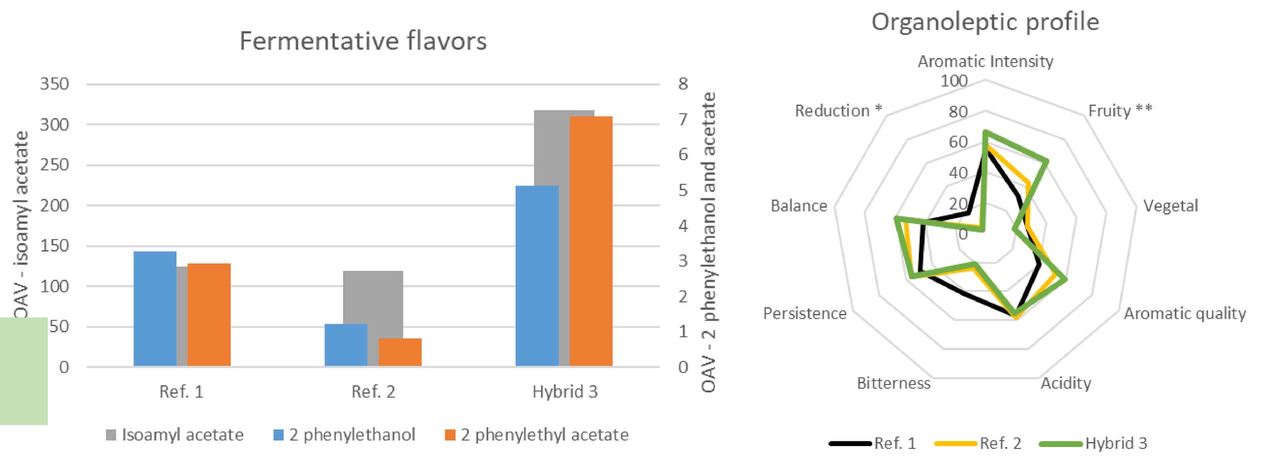


Figure 7: Aromatic analyses (OAV) + Professional tasting (12 panelists)

Field trials

- **To confirm the commercial interest** of the selected dry yeast strain Hybrid 3
- Must and wine analysis , fermentation follow up and yeast implantation control at 2/3 AF of Hybrid 3 and Control Yeasts tanks (Figure 8)
- **43 different trials in 4 different countries (FR, SP, IT, GR)**

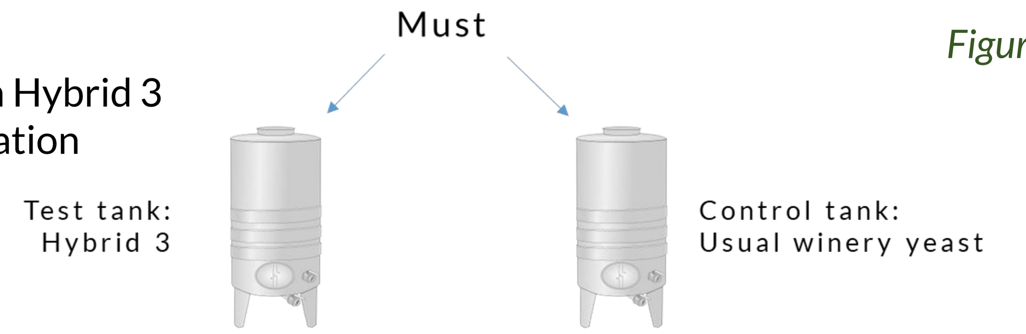


Figure 8: Field trials Protocol

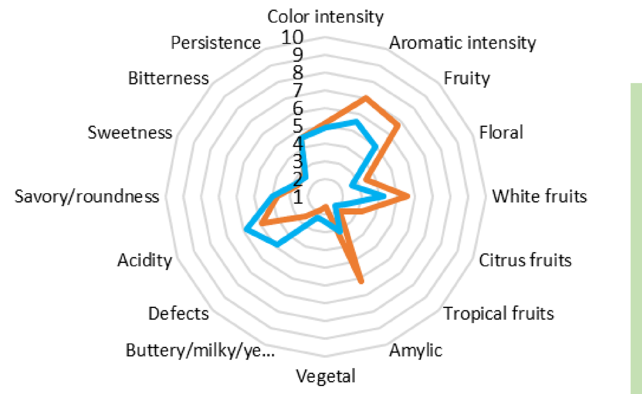


Figure 9: Example of Sensory analysis of a 20% implanted trial

- **Hybrid 3 has a longer lag phase than average yeast (+2 days av) mainly impacted by low temperature but once implanted it has a regular fermentation and no issues at the end of the fermentations. It has a moderate SO_2 sensitivity (between 50 to 75 PPM initial TSO_2).**
- **For implanted trials (Figure 8, 100% at 2/3 AF) it showed dramatically higher intensity and fruitiness, better balance, higher volume and less bitterness.**
- **Even on low implantation rate (Figure 9) this yeast shows stronger fruitiness which makes it a good candidate to enhance a blend.**

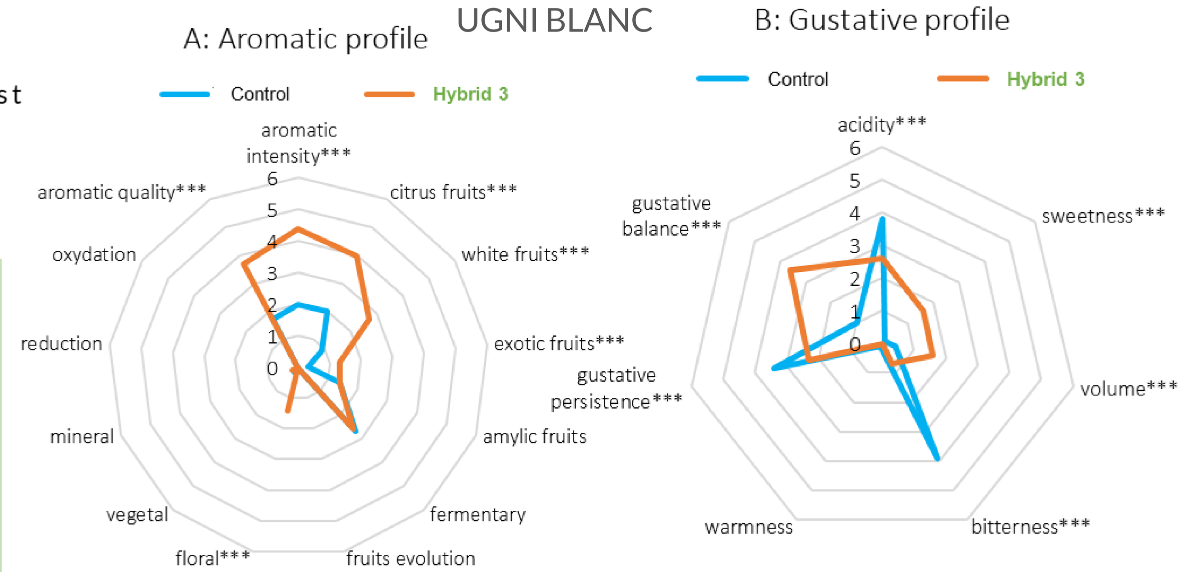


Figure 8: Example of Sensory analysis of a 100% implanted trial

CONCLUSION

- **Hybridization technics** can generate new strains that can **overperform both parents' capabilities** through adapted screening tests.
- **Hybrid** 3 was found with distinctive features looked for in this project with **40% less acetaldehyde and SO_2 than reference strains and 2- 4 x more isoamyl acetate .**
- Identified targeted wines for Hybrid 3:
  - ✓ **Young and technological wines** with strong amylic notes, intense fruitiness/floral notes/ green notes removal/ balanced mouthfeel;
  - ✓ **Nice choice for blend base ;**
  - ✓ **Low SO_2 Wine.**

- **Hybrid 3 is now SafOeno HD A54 in Fermentis Portfolio**