InCider: Selecting your Yeast
Federico Tondini
Boston (USA) 07/07/1989
Scientific Adviser

Expertise: Microbiology, Food Biotechnology, Fermentation Science

Current Research Topics: Indigenous Yeast, Wine Fermentation, Cell Physiology

Education:
- 2018 - PhD in Wine Science - University of Adelaide, Wine Microbiology and Microbial Biotechnology Laboratory
- 2014 - Master’s degree in Industrial Biotechnologies - Heineken Netherlands / University of Milan-Bicocca
- 2011 - Bachelor’s degree in Biotechnology - University of Milan - Bicocca

Publications:
Saccharomyces
Non-Saccharomyces

Complexity
‘Terroir expression’

No predictability
No reliability

Osmotic stress
Nutrient
Ethanol toxicity

Assess the risk
Predict the outcome
Which Yeast was the most involve in spontaneous Cider fermentation?

The main yeasts found in cider are Saccharomyces yeasts. A study of unpasteurized ciders and cider musts obtained from different cider houses from northwestern regions of France reported 15 yeast species among 208 picked isolates.

Saccharomyces bayanus was the predominant species from the beginning to the middle steps of the fermentation process, accounting for up to 41% of the picked isolates, whereas S. cerevisiae took over the process in the final stages of fermentation.
Family Name: Saccharomyces

Name: Cerevisiae

Nationality: China/Far East Asia

⌀: 3-4 µm

Work: Ale Beer, Wine

most psychrotrophic species

“The origin and adaptive evolution of domesticated populations of yeast from Far East Asia.” Duan et al. (2018)
‘make-accumulate-consume’

- Crabtree effect: most remarkable characteristics of *S. cerevisiae* and closely related species is their ability to produce and accumulate ethanol

- Overflow in sugar metabolism

- Ethanol inhibits the growth of other microbes.

Ethanol prolongs shelf-life, improves digestibility and acts as a euphoriant.
the lager yeasts
*S. pastorianus* (*S. cerevisiae × S. uvarum × S. eubayanus*)

wine, cider and brewing
*S. cerevisiae × S. kudriavzevii*
*S. bayanus* (*S. uvarum × S. eubayanus*)
*S. cerevisiae × S. uvarum*
*S. cerevisiae × S. kudriavzevii × S. uvarum*
• Man’s oldest industrial microorganism

• Man used yeast before the development of a written language

• Ancient Egyptians were using yeast and the process of fermentation to produce alcoholic beverages and to leaven bread over 5,000 years ago
• Early fermentation systems for alcohol production and bread making were formed by natural microbial contaminants

• Microbial flora would have included wild yeasts and lactic acid bacteria that are found associated with cultivated grains and fruits.

• Over the course of time, the use of starter cultures helped to select for improved yeasts by saving a “good” batch of wine, beer or dough for inoculating the next batch

→ DOMESTICATION
DOMESTICATION

• ‘Domestication’ is a term that refers to artificial selection and breeding of wild species to obtain cultivated variants with enhanced desirable features that thrive in man-made environments, often at the cost of suboptimal fitness in natural settings.

“Origins, evolution, domestication and diversity of Saccharomyces beer yeasts”. Gallone et al. (2018)
• 17th century, Antoni van Leeuwenhoek: developed high-quality lenses and was able to observe yeast for the first time.

• 1785, Antoine Lavoisier: French chemist analyzed the mechanism by which sugarcane is transformed into alcohol and carbon dioxide. The experiment provided a clear insight into the basic chemical reactions needed to produce alcohol but nothing about yeast contribution.

• 1857, Louis Pasteur: demonstrate experimentally that fermented beverages result from the action of living yeast transforming glucose into ethanol.
• 1880s, Emile Christian Hansen: developed the first pure yeast culture and wort inoculation was performed some years later.

• 1890, Müller-Thurgau: performed the first inoculation of grape must with a pure yeast culture, but only in the late 1970 it became a wine industry common procedure.

These practices have improved the control and reliability of the fermentation process, limiting microbiological alterations and have largely contributed to increased quality in recent decades.
Although of the entire cider microflora contribute to the cider chemistry, yeasts detain a predominant role, since they promote the AF.

Traditional fermentation occur through a spontaneous process performed by the sequential action of different yeast species/strains.

Proper control of fermentation through chemical and nutrient additions, temperature control, and microflora reduction or inoculation allows for a “clean” and consistent fermentation.
Debate Still Open

The main objection to the use of selected starter cultures is the standardization of quality and avoid stuck and sluggish fermentation, with concomitant production of undesired metabolites.

Autochthonous yeast starters, reflect the biodiversity of a particular area, which support the idea that indigenous yeast strains can be associated with a “terroir”.

Vs

Main objection to the use of selected starter cultures is the standardization of quality, avoid stuck and sluggish fermentation, with concomitant production of undesired metabolites.
Domestication of industrial yeasts

~1600 AD

Beer 1
- Strong domestication
  - Off-flavors ↓
  - Maltotriose utilization ↑
  - Survival in nature ↓
  - Sexual reproduction ↓
  - Genome decay ↑
  - Aneuploidy & CNV ↑

Mixed

Beer 2
- Limited domestication
  - Maltotriose utilization ↓
  - Wine stress resistance ↑
  - Survival in nature ↑
  - Sexual reproduction ↑

Wine

Wild
- No domestication
  - Survival in nature ↑
  - Sexual reproduction ↑

Very little diversity in wine yeast strains
More diversity in brewing yeast
Alternative yeast strains do offer unique profiles
Phenotypic evolution by industry

YEAST DIVERSITY REFLECTS HUMAN HISTORY
During traditional cider making, the yeast faces an increasingly hostile environment. During fermentation the medium rapidly becomes anaerobic and is increasingly laden with ethanol and other potentially inhibitory metabolites.

Yeast cells reproduce (10^6 -> 10^7) and disperse themselves throughout the fermenting juice, converting sugars to alcohols, carbon dioxide and various flavor compounds.
The cells absorb dissolved sugars, simple nitrogenous matter (amino acids, ammonium ions, and small peptides), vitamins, and ions through their plasma membrane. Subsequently, they employ a series of reactions known as metabolic pathways (glycolysis, biosynthesis of cellular constituents, etc.) and use these nutrient materials for growth and fermentation.
Why yeast cells produce these flavor-active molecules?

• Specific cellular building blocks, redox balancing and detoxification reactions

• Fundamental role in the lifestyle of yeast: signaling information to animal vectors, regulation of fungal growth and communication between yeast cells or colonies

(Richard et al. 1996; Bruce et al. 2005; Leroy et al. 2011; Davis et al. 2013)
Christiaens et al. 2014. The Fungal Aroma Gene ATF1 Promotes Dispersal of Yeast Cells through Insect Vectors
The aroma profile of fermented foods and beverages comprises hundreds of compounds.

The flavour profiles of cider can principally be attributed to the biochemical activities during fermentation within the yeast cell.

The proportional volatile fractions of cider: 49% alcohols, 36% esters, and 11% carbonyl compounds.

The need to understand and control aroma compound synthesis is driven by the fact that these compounds play a key role in the sensorial quality of fermented alcoholic beverages.
• Food fermentation is all about increasing the sensory quality for the consumer, and obtaining unique signature flavors that help to distinguish a product from others on the market


• Consumers select products based on taste, preferring to pay more for a refined sensation, rather than less for quantity

1. Involve in the biocontrol of moulds, which influences quality before harvest.

2. Perform alcoholic fermentation of must sugars and transform juice into cider; the de novo biosynthesis of the flavour and aroma compounds.

3. Enzymatic conversion of flavour neutral, grape components into odour-active compounds.

4. Yeast autolysis products.

5. Absorption of juice components.

6. Spoilage during the storage period and even after packaging.

7. Influence growth of other spoilage microorganism, for example, lactic acid bacteria, acetic acid bacteria.
When presented with the appropriate nutrients, yeasts produce complex bouquets of aroma compounds including esters, higher alcohols, carbonyls, fatty acid derivatives and sulfur compounds. Moreover, while not directly synthesized by yeasts, volatile thiols and monoterpenes are sometimes released from odorless precursors by yeast-derived enzymes.
Our understanding of the fermentation process and the associated aroma production by yeast has increased exponentially over the last centuries, from the discovery of yeast cells in 1680, to the sequencing of the entire *Saccharomyces cerevisiae* genome just two decades ago.

In-depth look at the phenotypic and genetic diversity of nearly 200 industrial yeasts profiled the of differences in aroma formation.

1996 Release: Yeast Genome Sequenced
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<thead>
<tr>
<th>Gene</th>
<th>Enzyme</th>
<th>Function</th>
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<tr>
<td>BAP2</td>
<td>Branched chain amino acid permease</td>
<td>Uptake of branched chain amino acids</td>
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<tr>
<td>BAT1</td>
<td>Mitochondrial branched chain amino acid aminotransferase</td>
<td>Branched chain amino acid transaminase activity</td>
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<td>Cytosolic branched chain amino acid aminotransferase</td>
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<td>ATF1</td>
<td>Alcohol acetyltransferase</td>
<td>Acetate ester production</td>
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<td>Acetate ester production</td>
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<tr>
<td>EEB1</td>
<td>Acyl-coenzymeA/ethanol O-acyltransferase</td>
<td>Short-chain esterase activity</td>
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<tr>
<td>EHT1</td>
<td>Acyl-coenzymeA/ethanol O-acyltransferase</td>
<td>Short-chain esterase activity</td>
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</tbody>
</table>

(Procopio, Qian et al. 2011)
From AA to Higher Alcohols
From AA and Lipids to Esters

A

Acetyl-CoA + Ethanol + Isoamyl alcohol + 2-phenylethanol

ATF1 ATP1

Ethyl Acetate

ATF2

Isoamyl Acetate

Phenylethyl Acetate

B

Hexanoyl-CoA + Ethanol + Octanoyl-CoA

EEB1 EHT1

Ethyl Hexanoate

EEB1 EHT1

Ethyl Octanoate
### Esters

**Table of esters and their smells**

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<thead>
<tr>
<th>from the alcohol (first word)</th>
<th>methyl 1 carbon</th>
<th>ethyl 2 carbons</th>
<th>propyl 3 carbons</th>
<th>2-methyl propyl-4 carbons</th>
<th>butyl 5 carbons</th>
<th>pentyl 6 carbons</th>
<th>hexyl 7 carbons</th>
<th>benzyl benzene ring</th>
<th>heptyl 8 carbons</th>
<th>octyl 9 carbons</th>
<th>nonyl 9 carbons</th>
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<td>nonanoate 9 carbons</td>
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<td>cinnamate</td>
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<td>decanoate 10 carbons</td>
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A phenol is an organic compound in which a hydroxyl group (-OH) is bonded to an aromatic hydrocarbon ring (also called a benzene ring).

p-coumaric acid \rightarrow 4-vinylphenol \rightarrow 4-ethylphenol
Given its importance in product quality, much effort has been devoted to fine-tune flavor production by yeast in an industrial setting. Globally, two approaches can be applied to steer the yeast's physiology to alter aroma production: adjusting the fermentation environment or modifying the genotype of the production strain.
GMO? Not accepted by consumers
Generation of artificial diversity
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</tbody>
</table>

- **Parental strain 1**: Starving conditions → Sporulation → Hybrids
- **Parental strain 2**: Starving conditions

A process flowchart illustrating genetic inheritance and hybrid formation.
After crossing a wine strain with *S. eubayanus*, hybrid strains were expected to inherit the more pleasant aroma profile of the former and the reportedly high tolerance to low temperatures of the latter.
Screening of cider yeasts for sparkling cider production (Champenoise method)

Bélén Suárez Valles, Rosa Pando Bedriñana *, Ana Lastra Queipo, Juan José Mangas Alonso
Área de Tecnología de los Alimentos, Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), 33300 Villaviciosa, Asturias, Spain

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ABSTRACT
A total of 350 colonies isolated from a cider cellar in Asturias (Spain) were identified by rDNA ITS-RFLP restriction analysis. Saccharomyces spp. strains were characterized by mitochondrial DNA (mtDNA) restriction analysis. Fifty-four different Saccharomyces spp. strains were identified and tested to ascertain their capacity to carry out secondary fermentation of sparkling ciders. The screening of yeasts to determine their principal enological characteristics (tolerance to ethanol, production of volatile acidity and hydrogen sulphide) was accomplished by means of rapid, non-expensive assays (plate agar). As a result, 13 (24%) of the 54 initial Saccharomyces spp. yeast strains were eliminated. The technological

10 S. cerevisiae strains were found as true flocculants and were able to grow in ethanolic medium and in the presence of 200mg/l of sulphite
New Fermol Yeast strains:

✔️ ✔️ Fermol Elegance: does not produce sulfur compounds

✔️ ✔️ Fermol Glutaferm -1: allows to have very high levels of glutathione in the fermenting must and wine
NOTE TO THE EDITOR

Evolution-based strategy to generate non-genetically modified organisms Saccharomyces cerevisiae strains impaired in sulfate assimilation pathway

L. De Vero, L. Sollieri and P. Giudici

Department of Agricultural and Food Sciences (DiPSAA), University of Modena and Reggio Emilia, Reggio Emilia, Italy

Keywords

evolutionary strategy, sulfate assimilation, sulfite and hydrogen sulfide production, wine yeast strains.

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Abstract

An evolution-based strategy was designed to screen novel yeast strain impaired in sulfate assimilation. Specifically, molybdate and chromate resistance was used as selectable phenotype to select sulfate permease-deficient variants that unable to produce sulfites and hydrogen sulfide (H2S).

Methods and Results: Four Saccharomyces cerevisiae parent strains were induced to sporulate. After tetrad digestion, spore suspensions were observed under the microscope to examine the conjugation of gametes. Then, the ex suspension was inoculated in tubes containing YPD medium supplemented with ammonium molybdate or potassium chromate. Forty-four resistant strain were obtained and then tested in microinfiltration. Three strains with a low sulfite production (SO2 < 10 mg L-1) and with an impaired H2S production in grape must without added sulfites were selected.

Keywords

Saccharomyces cerevisiae, evolutionary strategy, sulfite and hydrogen sulfide production, wine strain.
Bayanus yeast suitable for the fermentation of cider, fruit wines and grape must.
It produces fruity nuances and carries over a regular fermentation with high alcohol tolerance and no H2S production.
Cold & SO2 tolerant and with short lag phase, it guarantees quick dominance over the wild yeast.
Paramenters

- Temperature range
- Nitrogen Requirement
- Fermentation performance
- SO$_2$ tolerance
- Aroma productions
- Flocculation
TEE-BOT high throughput small scale fermentation robot

• This machine is able to assist monitoring of the fermentation progress by autonomously collecting and cold-storing samples for further analysis.
• The Tee-Bot is a highly efficient tool for users who need to run fermentation experiments. 96 flasks of ~100 mL volume can be simultaneously monitored while maintaining temperature and agitation at user defined levels.
• The Tee-Bot has been specifically designed for wine fermentations, but it can be easily adapted for other fermentation media.
Fermentation Facility:

- Platemaster, Gilson
- Tecan 200 spectrophotometer + stacker
- Guava® easyCyte HT Sampling Flow Cytometer, Millipore
- ChemWell® 2910 Automated EIA and Chemistry Analyzer

Its technology has been custom built with a high degree of automation for process reliability, cost-effectiveness and ultimately more robust research outcomes.
Analytical Chemistry facility:

Agilent 7890A GC coupled to 5976C MSD detector
Fermentation performance

Fermentation duration

Fermentation performance

Fermenter duration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Code</th>
</tr>
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<tbody>
<tr>
<td>Feromé* Sauvignon</td>
<td>SB</td>
</tr>
<tr>
<td>GlutaFermONE</td>
<td>G1</td>
</tr>
<tr>
<td>control</td>
<td>Control_G1</td>
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<tr>
<td>enzyme 5 g/l</td>
<td>Enz</td>
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<tr>
<td>Fermod Plus 250 mg/L</td>
<td>F_P_L</td>
</tr>
<tr>
<td>Fermod Plus 400 mg/L</td>
<td>F_P_H</td>
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Increasing demand for innovative products, alternative flavours, and low-alcohol beers has stimulated research into the potential benefits of alternative yeasts; in particular, non-Saccharomyces species have been isolated and characterized for the development of specialty beers.

*M. pulcherrima (LEVULIA PULCHERRIMA)*: 3% ethanol, extracellular enzymes, bioprotection, less acetic acid

*L. thermodurans (LEVULIA ALCOLMENO)*: 7%, different esters production, lactic acid production

*T. delbrueckii (LEVULIA TORULA)*: 9%, different esters production, extracellular enzymes, mannoprotein, less acetic acid
The results from the screening of enzymatic activities in non-Saccharomyces cider yeasts suggest their potential to improve the aroma and flavor of cider used as natural inocula.
How to assess yeast for apple juice fermentation?
DESCRITTORI ORGANOLETTICI

PROPRIETÀ METABOLICHE E ORGANOLETTICHE
How to assess yeast for apple juice fermentation?
Apple Contents

- An apple contains:
  - 80% water
    (varies with irrigation practices and weather conditions)
  - 10% carbohydrate
    • Sugars (mostly fructose, with some glucose—100% fermentable)
    • Fiber/cellulose – removed by pressing
  - 4% vitamins/minerals
  - 6% of:
    • Organic acids (primarily malic acid)
    • Pectin – pectinase highly recommended
    • Polyphenols – flavonoids and, to a varying degree, tannins
    • Very small amounts of proteins (added yeast nutrition is often needed!)

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<th>Sugars (g/L)</th>
<th>Wort</th>
<th>Grape</th>
<th>Apple</th>
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<tr>
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<td>8.3</td>
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<td>Glucose</td>
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<tr>
<td>Phenylalanine</td>
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<tr>
<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
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<tr>
<td>Lysine</td>
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<table>
<thead>
<tr>
<th>Minerals (mg/L)</th>
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<tbody>
<tr>
<td>Calcium</td>
<td>36.4</td>
<td>147.3</td>
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<tr>
<td>Magnesium</td>
<td>106.3</td>
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<td>Sodium</td>
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<td>Potassium</td>
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<td>Zinc</td>
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<td>Copper</td>
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<td>1.12</td>
<td>0.1</td>
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<td>Iron</td>
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<td>Aluminium</td>
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<td>Manganese</td>
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<tr>
<td>Phosphorus</td>
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<td>Silica</td>
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<td>Chloride</td>
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<td>Sulphate</td>
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<tr>
<td>Nitrate</td>
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<td>Free oxalic acid</td>
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<td>4.8</td>
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<tr>
<td>Phosphate</td>
<td>1299.3</td>
<td>352.5</td>
<td>163.9</td>
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</table>
Ctrl-Ferm a patented, unique and innovative system for the detection of H$_2$S and CO$_2$ during alcoholic fermentation (AF). Ctrl-Ferm is easy to mount and simple to use: once positioned the detector tube on the top of the fermenter, the instrument aspires and measures the gases, uploading the data real-time to a website, accessible from the winemaker phone or personal computer. Ctrl-ferm is available in two version, for the measurement of 1 fermentation or up to 5 tanks simultaneously.
How Oxygen Effects Esters

- Strong yeast growth
- Healthy cell membranes
- Low ester production

"The Sweet Spot"

- Poor yeast health
- Lots of esters
- Higher ester production

Yeast Pitch Rate

High Pitch Rate

- Low esters due to fast fermentation

Low Pitch Rate

- Lots of esters but slow fermentation
  (Possibility of stuck fermentation and autolysis)
Temperature

- High temperature results in fruity esters
- Slightly lower temperature makes less esters but more phenols
- Lower temperature still produces very low esters and phenols (ideal for lagers)

Sugar and FANS

Sugars and FANS are yeast food
More sugar = More esters
Too much sugar = Acetaldehyde
Free Amino Nitrogens (FANS)
More FAN = Better yeast health
How to isolate yeasts from various fruit?

• Collected samples should be diced and place in 9 ml of YPD (Yeast extract 1%, Bacteria peptone 2%, Glucose 2%)
Smelling or ‘sniffing’ approach

To make the solid media, 20 g/L agar was added and dissolved into pre-heated apple juice at 80°C, then poured into Petri dishes. All non-conventional strains were streaked onto the agar plates and incubated at room temperature for 2–7 days.

The aim of this screening was to identify diverse aroma compounds typically found in fermented beverages as desired compounds or off-flavours, or simply strains characterised by a strong and novel aroma profile. Plates were ‘sniffed’ directly and classified as: ‘pleasant aroma’, ‘unpleasant aroma’ or ‘no growth’. Additionally, ‘pleasant aroma’ was ranked as ‘intermediate’ or ‘strong’ depending on the level of perception. ‘Unpleasant aroma’ was classified as ‘phenolic’ or ‘acetic’ as both are common undesirable compounds produced by non-conventional yeasts.
- APPLE JUICE
- 1.5 L fermentation
- 3 Replicates
- Temperature controlled
- $5 \times 10^6$ cells/ml inoculum
- Sample daily
- Taste
IC-Gene brings the power of molecular biology in the vineyard and in the cellar. It allows a rapid detection of spoilage microorganisms (i.e. *Brettanomyces*, *Botrytis*). The analysis is based on a new PCR reaction, with improved sensitivity and specificity that reduce time of the assay and false outcome. Thanks to its easy execution and real time results, this method can be done ON SITU, directly from the matrix (wine, berries, waters, etc.). Low cost and the easy reproducibility allow to manage the flow of the winery in complete safety. Ten different samples can be processed simultaneously, and the time required is less than 2 hours.

We recommend IC-gene for:

(i) Identify unnoticed *Botrytis* contaminated must. If recognized, you can avoid negative organoleptic effects.

(ii) Test wine samples, rinsing water and wood surfaces to keep your winery free from *Brettanomyces*.

*(NEW)* Detection of harmful bacteria in storage water.