# Biological Demalication and Deacetification of Musts and Wines

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Grape must sometimes reveal excess acidity. An excessive amount of organic acids negatively affects wine yeasts and yeast fermentation, and the obtained wines are characterized by an inappropriate balance between sweetness, acidity or sourness, and flavor/aroma components. An appropriate acidity, pleasant to the palate, is more difficult to achieve in wines with high acidity due to an excess of malic acid because the *Saccharomyces* species generally cannot effectively degrade malic acid during alcoholic fermentation. One approach to solving this problem is biological deacidification by lactic acid bacteria or non-*Saccharomyces* yeasts, like *Schizosaccharomyces pombe,* that can degrade L-malic acid. Excessive volatile acidity in wine is also a problem in the wine industry. Free or immobilized Saccharomyces cells have been studied to solve these problems since these yeasts are wine yeasts that show a good balance between taste/flavor and aromatic compounds during alcoholic fermentation.

Saccharomyces cerevisiae | acetic acid | malic acid | mitochondrial carriers | acetic acid metabolism | malic acid metabolism | carboxylic acids transport

## 1. General Introduction

A balanced wine in terms of flavor should be the goal of every winemaker. Much is known about how taste components, such as acidity, sweetness, savoriness (″umami″), and alcohol can work together in coordination or discord on the palate [1][2][3]; regarding organic acid content in grapes, malic acid, and tartaric acid account for up to 90% of all the acids present [4][5]. The organic acid composition of the grape can be influenced by environmental factors such as light, irrigation, humidity, and temperature. However, the cultivar and growing region are also relevant for acid concentration in grapes [6]. In some cases, grape must acidity may need to be corrected. An acidity adjustment involves either acid addition or acidity reduction. In some places, grapes are harvested with a total acidity under ten g/L or higher.

Acetic acid is the prime component of the volatile acidity of grape musts and wines. Among other causes, acetic acid can be formed as a byproduct of alcoholic fermentation or as a byproduct of the metabolism of acetic and lactic acid bacteria, which can metabolize residual sugars to increase volatile acidity. This acid hurts yeast fermentation ability and affects wine quality when present above a given concentration [7].

There are three general methods that winemakers can use to lower high acidity in dry red or white wine production: physical methods (blending and amelioration), chemical methods (bicarbonates), and biological methods (yeast and bacteria). Biological deacidification only affects the malic acid in the total acidity of the wine and does not reduce tartaric acid. Malolactic fermentation (MLF) is the most common biological deacidification or demalication method. Lactic acid bacteria (LAB) can consume the malic acid and convert it to lactic acid [8]. Yeast can also consume malic acid by converting it into ethanol through malo-ethanolic deacidification. This microbiological process can cause a slight increase in the wine's alcohol content. Still, sometimes, this is preferred over the aroma and flavor of lactic acid produced by lactic acid bacteria. It has long been known that yeasts like *Schizosaccharomyces pombe* are incredibly efficient at converting malic acid [9][10].

## 2. Uptake of Malic Acid into the Yeast Cell

Carboxylic acids are organic compounds that participate in many cellular processes. Previous research has been carried out on yeasts to characterize the transport mechanisms of these acids across the yeast cell membrane. Carboxylic acids are weak acids; they partially dissociate in aqueous systems. There is an equilibrium between undissociated, uncharged molecules and their anionic form(s), according to their pKa and the medium's pH. This property is essential as it influences cell behavior and the mechanisms by which the acids can cross the yeast membrane [11].

The transport mechanisms of carboxylic acids can be mediated or non-mediated, passive or active, energy-independent or dependent.

The active transport system is provided by two distinct processes: $B_1$-pumps associated with yeast stress responses. The extrusion of the anion form of the acid accumulated in the cytoplasm occurs and permeases $B_2$-associated metabolic routes of nonfermentable carbon sources [11]. All these transmembrane transporters are currently classified by the Transport Classification Database (http://www.tcdb.org).

The yeast *S. cerevisiae* has long been known as a poor metabolizer of extracellular malate due to the lack of a mediated transport system for the acid [12]. The ability of a yeast strain to degrade extracellular l-malate is dependent, among other reasons, on the efficient transport of the dicarboxylic acid into the cell. *S. cerevisiae* lacks an active transport system for l-malate, and extracellular l-malate enters the cells using simple diffusion. Moreover, the malic enzyme of *S. cerevisiae* has a considerably lower substrate affinity for l-malate (*K*m = 50 mM) [13], and the *S. cerevisiae* malic enzyme is mitochondrial. In contrast, the *S. pombe* malic enzyme is cytosolic [14].

It is also known that S. pombe and Zygosaccharomyces bailii strains can metabolize l-malate in the presence of glucose or another assimilable carbon source [15]. In contrast, *Hansenula anomala*, *Candida sphaerica*, *Pichia stipitis,* and *Pachysolen tannophilus* are yeast species that utilize malic acid as the sole carbon source. However, this skill is repressed in glucose [16]. Low efficiencies in malic acid metabolism have been observed for *S. cerevisiae*, *Candida colliculosa, Candida stellata,* and *Hanseniaspora uvarum*/*Kloeckera apiculata* [13].

As mentioned above, the ability of a yeast strain to degrade extracellular l-malate depends on the efficient transport of the dicarboxylic acid into the cell. The yeasts *C. utilis, C. sphaerica, H. anomala,* and *K. marxianus* can transport malate by a proton symport/induced and glucose-repressed system [13][17][18][19]. In contrast, the transport of this acid is carrier-mediated in *Kluyveromyces lactis* (active transport/strain dependent and glucose repressed [20]). *Z. bailii* can also transport l-malate by facilitated diffusion for a non-dissociated form, which is induced by glucose and repressed by fructose [21]. *Schizosaccharomyces pombe* possesses a proton symport/constitutive transport system [13].

## 3. The Demolition Activity of *Saccharomyces* Strains

The ability of *Saccharomyces* strains to degrade malic acid is strain-dependent [22][23][24]. The low malate consumption can be explained by the absence of an active l-malate carrier, low substrate affinity, and the mitochondrial location of the malic enzyme [25][26][27]. Under fermentation conditions, the malic enzyme is regulated by the fermentative glucose metabolism that causes mitochondrial deterioration [28].

Redzepovic et al. [22] found that the indigenous *Saccharomyces paradoxus* strain RO88 degraded 38% of the malic acid in chardonnay must. The wine produced was of good quality; while the *S. pombe* strain removed a much higher value of malic acid (90%), the wine produced was of poor quality. In the same study, the authors verified that *S. cerevisiae* Lalvin strain 71B, commercially promoted as a “malic-acid-degrading wine strain,” only degraded 18% of the malic acid. However, the malic enzyme gene expression from *S. paradoxus* RO88 and *S. cerevisiae* 71B increased towards the end of fermentation after glucose depletion.

## 4. Acetic Acid Metabolism in *S. cerevisiae*

The aroma threshold for acetic acid in wine depends on the wine variety and style; a concentration of 0.8–0.9 g/L produces a sour aftertaste in wine [29]. According to current legislation [30], the maximum acceptable limit for volatile acidity in red and white table wines is 1.2 g/L of acetic acid [30].

*S. cerevisiae* can metabolize acetic acid during a refermentation process [31][32]. According to the authors and the work done by Vilela-Moura et al. [7][32][33][34] and Vasserot et al. [35], excessive volatile acidity can be removed by re-inoculating (performing a refermentation) with an appropriate *S. cerevisiae* wine yeast. Under aerobic conditions, acetate can be used as a carbon and energy source to generate energy and cellular biomass [36]. In *S. cerevisiae*, acetate transport and metabolism are subject to glucose repression. However, in chemostat cultures of *S. cerevisiae* grown in mixtures of acetic acid and glucose, if the glucose concentration is deficient, the cells will no longer be repressed. They will metabolize acetate concomitantly with glucose [37]. A review by Vilela-Moura et al. [7] considers acetate's cellular uptake/transport into the yeast cell an essential step for its catabolism. In glucose-repressed yeast cells, at low pH, acetic acid enters mainly by simple diffusion [38]. Ethanol will enhance the passive influx of acetic acid, following the first-order kinetics with a constant rate, which increases exponentially with ethanol concentration [39].

In 1999, Casal et al. [40] found that the transport carrier Jen1p is required to uptake lactate in *S. cerevisiae*. This transporter is also able to transport acetate. Later, it was found that the protein Ady2p was vital for acetate transport in acetic acid-grown cells [41]. Being the only available energy and carbon source, acetate is metabolized to acetyl coenzyme A (acetyl-CoA) by one of the two ACS proteins: Acs1p (peroxisomal) or Acs2p (cytosolic). Acetyl-CoA enters the mitochondria and is then oxidized in the TCA cycle. Entering the glyoxylate cycle, acetyl-CoA is also used to produce succinate, refilling the cell with biosynthetic precursors. This action involves the enzymes Icl1p (isocitrate lyase) in the cytosol and Mls1p (malate synthase) located in the peroxisome [7]. Moreover, acetyl-CoA synthesizes macromolecules, requiring active gluconeogenesis [42].

## VA Bio-Reduction by S. cerevisiae Yeasts Strains and its Limitations

Consumption of glucose and acetic acid is a characteristic of indigenous and commercial wine yeasts [33][43]. In these works [33][43], the characterization of indigenous and commercial *S. cerevisiae* strains regarding volatile acidity bio-reduction was performed. Among the strains studied, some exhibited a simultaneous consumption pattern of glucose and acetic acid in refermentation assays of acidic white wines with a synthetic culture medium simulating refermentation with a must (conditions of high glucose/low ethanol concentrations) and refermentation with a marc (conditions of low glucose/high ethanol concentrations). Under limited oxygen conditions, all the *S. cerevisiae* strains studied showed acetic acid degradation efficiencies like those of *Z. bailii* ISA 1307. Curiously, the commercial strain S26 also revealed the capacity to decrease the volatile acidity of wines in aerobic and limited aerobic conditions by 92% and 61%, respectively [33].

Upon inoculation of grape must or wine, a controlled oxygen environment is essential for yeast acetic acid consumption [33]. Moreover, yeast cells must adapt to the hostile fermentation environment, which gradually changes. In fermentation or refermentation, multiple stress conditions are imposed on the yeast cell, such as high osmolarity, low pH, sulfur dioxide ($SO_2$), ethanol toxicity, temperature variations, increasing nitrogen limitation, and elevated acetic acid concentrations. All of these stress conditions may lead to reduced cellular growth, cellular death, and, consequently, stuck fermentations [31][44][45][46].

Controlled wine oxygenation can be accomplished through micro-oxygenation (MO), where oxygen is added by a stainless steel sparger that produces tiny bubbles, promoting oxygen dissolution in the must/wine. The influence of different combinations of the initial concentration of acetic acid, ethanol, $SO_2$, and MO on sour acid removal from wines with excessive volatile acidity was evaluated by two commercial yeast strains (S26 and S29) [32][34]. Both strains (*S. cerevisiae* S26 and S29) reduced by 78% and 48%, respectively, the volatile acidity of an acidic wine with an initial concentration of 1.0 g/L in acetic acid. Sulfur dioxide in the concentration range of 95–170 mg/L inhibited the capacity of both strains to reduce the volatile acidity under the experimental conditions used. Deacetification of wines with the better-performing strain, S26, was related to changes in the concentration of wine volatile compounds. The most pronounced increase was observed for compounds with a fruity flavor: isoamyl acetate and ethyl hexanoate with banana-like taste and apple-pineapple-like flavor, with an 18- and 25-fold increment, respectively; both values above the detection threshold. Furthermore, deacetification led to increased fatty acid concentration but did not negatively impact the wine's sensory properties [34]. However, in the work of

Vasserot et al. [35], the results were not as promising. In their work, a commercial *S. cerevisiae* strain used in champagne winemaking was tested for its ability to metabolize acetic acid during fermentation. The acetic acid metabolized by the yeast strain increased with increasing initial acetic acid concentration. Acetic acid consumption occurred during the second part of the exponential growth phase. However, when the initial acetic acid concentration was higher than 1.0 g/L, independently of the yeast strain used, the final acetic acid concentration could not be reduced to a legally acceptable level [30]. Acetic acid consumption modified yeast metabolism once more acetaldehyde (with a tart flavor reminiscent of green apples) and less glycerol (reduced wine softness) were produced. Considering the reduction of the NADPH/$NADP^+$ (Nicotinamide Adenine Dinucleotide Phosphate reduced/Nicotinamide Adenine Dinucleotide Phosphate oxidized) ratio, resulting in acetic acid consumption, Vasserot et al. [35] proposed that acetic acid modifies yeast metabolism by reducing the Ald6p activity ($NADP^+$ dependent aldehyde dehydrogenase).

As mentioned before, yeast cells need to adapt to a hostile environment during fermentation or refermentation, so cell immobilization by entrapment in beads is a technique that offers some advantages like continuous cell utilization, protection of immobilized cells against inhibitory substances in the fermentation medium; increased fermentation rates; stimulation of production, and protection from shear forces [47]. In 2013 Vilela et al. [48]. Studied the efficiency of acetic acid consumption by the previously characterized commercial S26 strain, but the cells were immobilized in a matrix of alginate-chitosan. Several vital parameters were analyzed, such as initial pH, number/composition of the immobilization matrix, cell concentration, and deacetification process efficiency. Immobilized cells reduced 21.6% of the initial volatile acidity 72 h after inoculation. The initial acidic wine had a concentration of 1.1 g/L of acetic acid, ethanol 12.5% (*v*/*v*), and a pH of 3.5 h. However, deacetification did not change after 168 h and was accompanied by a slight decrease in ethanol concentration and cell leakage. Initial wine pH adjustment to 3.12 and duplication of initial cell concentration led to increased volatile acidity removal up to 61.8%, and no cell leakage from the beads was observed [48].

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