

***Schizosaccharomyces pombe*: a promising biotechnology to modulate wine composition**

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Abstract

There are numerous yeast species related to wine making, particularly non-*Saccharomyces*, that deserve special attention due to the great potential they have when it comes to making certain changes in the composition of the wine. Among them, *Schizosaccharomyces pombe* stands out for its particular metabolism that gives it certain abilities such as regulating the acidity of wine through maloalcoholic fermentation. In addition, this species is characterized by favouring the formation of stable pigments in the wine and releasing large quantities of polysaccharides during ageing on lees. Moreover, its urease activity and its competition for malic acid with lactic acid bacteria make it a safety tool by limiting the formation of ethyl carbamate and biogenic amines in wine. However, it also has certain disadvantages such as its low fermentation speed or the development of undesirable flavours and aromas. In this chapter, the main oenological uses of *Schizosaccharomyces pombe* that have been proposed in the last years will be reviewed and discussed.

Keywords: *Schizosaccharomyces pombe*; oenological uses; maloalcoholic fermentation; stable pigments; wine safety

Origin and features of *Schizosaccharomyces pombe*

Schizosaccharomyces pombe, also known as fission yeast, was discovered by Lindner in 1983 [1]. The cells of this species have a characteristic rod shape with sizes varying between 3-5 x 5-24 μm (**Figure 1**). However, immediately after cell division the new cells formed have a more rounded shape due to the turgor pressure [2]. It has a peculiar mode of vegetative reproduction by fission (cross-wall formation) instead of budding, what is more common among yeasts [3]. Cells are separated by the formation of a transverse septum. The spores are formed as a result of the sexual reproduction by conjugation of the cells when adverse conditions occur, such as nutrient starvation, and, in the case of *S. pombe*, between two and four (most often) haploid spores originate in the ascus [4].



Figure 1. Optical microscope image of *S. pombe*. The transverse septum formed during the asexual reproduction is indicated by an arrow.

Its growth rate is very slow with a long lag phase and a great vitamins requirement. However, it has a low nitrogen requirement [5]. In normal minimal or complex media the generation time is between 2 and 4 h [6]. Usually, *S. pombe* does not develop properly in most culture media. A selective-differential media based on the resistance of *S. pombe* to actidione (antibiotic) and to benzoic acid (inhibitory agent) has been recently proposed to isolate strains of this genus from media with high sugar content [7]. *S. pombe* strains have been isolated from grape juice, molasses and kombucha tea [1,8]. In addition to glucose, *S. pombe* can also use glycerol, sucrose, raffinose, and maltose as carbon source [9].

Another peculiarity of *S. pombe* is that it can grow in environments with low water activity, that is, it is an osmophilic yeast and therefore can be found in media with high sugar content [1]. It also develops properly in very low pH environments and in a wide range of temperatures [10]. Moreover, it is somewhat resistant to food preservatives, such as sulphur dioxide, actidione, benzoic acid and dimethyl dicarbonate [10,11].

Regarding its fermentative performance, it is able to ferment glucose until an alcoholic degree of around 10-15 % v/v ethanol, depending on the yeast strain and the aeration conditions [10]. As already mentioned, the genus *Schizosaccharomyces* is known for its slow growth rate and excessive production of hydrogen sulphide during fermentation [12]. These two features, together with high volatile acidity, are the main limitations for its use in winemaking. The production of acetic acid is strain dependent, usually ranging between 0.8-1.4 g/L [13]. Nevertheless, through the selection of strains and their use in combination with yeasts of the genus *Saccharomyces*, wines of differentiated quality can be obtained from unbalanced musts with high total acidity.

Currently, thanks to recent research that opens new possibilities for using them, non-*Saccharomyces* yeasts are getting rid of their bad reputation and it is possible to find *S. pombe*

yeasts encapsulated in alginate beads being marketed as an alternative to malolactic fermentation or chemical deacidification [14]. An advantage of using these encapsulated yeasts is that they can be removed from the medium at the desired time and, in addition, the same capsules can be reused in several cycles (up to 5 times), although with a slight loss of degrading activity [15,16]. Regarding sensory properties, the wines obtained by sequential fermentation of *S. pombe* and *S. cerevisiae* resulted full-bodied, with better structure, balance, and length than the controls made without using this deacidification technique [16].

Wine pH modulation

Wine acidity is the main responsible for freshness. After L-tartaric acid, L-malic acid is the second organic acid in wine that contributes significantly to its total acidity. Its average content in wine highly depends on the grape variety and the climate, varying widely between 1-10 g/L [17]. Reaching an appropriate balance between the sugar content and the total acidity of the wine is fundamental to ensure its optimum quality. In addition, excessive amounts of malic acid may cause a microbiological instability in wine. These are the two main reasons to modulate wine pH.

The biological deacidification of wine through the use of *Schizosaccharomyces pombe* has been studied thoroughly [18,19] since its ability to transform malic acid into ethanol and carbon dioxide was discovered in the early 20th century [20]. Thanks to this ability of *S. pombe* to develop the maloalcoholic fermentation (MAF) (**Figure 2**), it is possible to modulate the pH of the wine by the consumption of practically all the malic acid present in the must with the corresponding stoichiometric production of ethanol. Unlike *S. cerevisiae* in which the malic enzyme is located in the mitochondria (organelle in low numbers and dysfunctional under winemaking conditions), *S. pombe* has an active transport system for the

uptake of extracellular malic acid in addition to a malic enzyme located in the cytosol with a very high substrate affinity [21]. The degree of degradation of malic acid is strain-dependent, normally varying between 75-100 % [5,10]. *Issatchenkia orientalis* has been also proved to have this strong malic acid degradative metabolism [22,23]. However, this yeast species is only present in small quantities at the beginning of fermentation due to its sensitivity to ethanol [24,25]. Kim, Hong, & Park, (2008) [26] have also reported the effectiveness of using a mixed culture of *Issatchenkia orientalis* and *Saccharomyces cerevisiae* to reduce the malic acid content during fermentation. Always when trying to improve the quality of the wine through the combination in mixed or sequential fermentation of different non-*Saccharomyces* and *Saccharomyces* yeast species, it is not only important to know the contribution of each species or strain, but also to select the adequate inoculum ratio [27]. Other yeast species, including some *Saccharomyces spp.* (commercially available strains are generally unable to degrade L-malic acid effectively during fermentation), are able to consume malic acid, but to a lesser extent (usually <25%). Another possibility is to use genetic engineering to improve the ability of *S. cerevisiae* to degrade malic acid, for example, through the incorporation of genes responsible for the transport of malic acid in *S. pombe* or the malolactic enzyme from *Oenococcus oeni* [28].

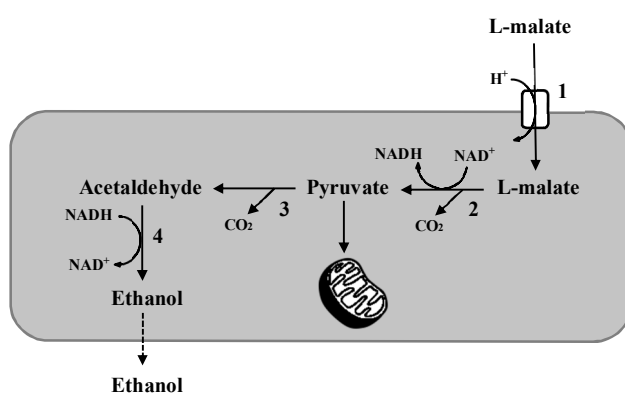


Figure 2. Schematic representation of the malic acid degradation by *Schizosaccharomyces pombe*: maloalcoholic fermentation (MAF) and alternative use of pyruvate in mitochondria for cellular biosynthesis. The enzymes involved in the biochemical transformations of the MAF are the following: 1: Malate permease (active transport); 2: Malic enzyme (malate decarboxylase); 3: Pyruvate decarboxylase; 4: Alcohol dehydrogenase.

Although it has been mentioned on numerous occasions that the aroma produced by the fermentative metabolism of *S. pombe* is not suitable for a quality wine, no particular off-flavour has been described yet. In general, only atypical aroma [19] and a loss in the fruity character [29] have been identified by some authors. This could be directly related to the fact that it is a slow fermenting yeast [12]. Sometimes, more than 30 days are necessary to finish the fermentation [29]. Moreover, immobilization techniques have been developed (e.g. alginate beads) in order to avoid negative side effects such as high levels of acetic acid and other off-flavours [30]. Following this technique, once the desired malic acid content is reached in the wine thanks to the demalication activity of *S. pombe*, this yeast is removed and the fermentation is finished by *S. cerevisiae*. Snow & Gallander (1979) [31] suggested that one or two days of *S. pombe* fermentation is adequate to get a quality wine similar to that obtained with *S. cerevisiae*, thus also avoiding excessive deacidification.

In addition to biological deacidification, either by yeast (MAF) or lactic acid bacteria (MLF), there are also other methods to regulate wine acidity such as blending, carbonic maceration or chemical deacidification using carbonate salts (usually calcium carbonate, CaCO_3 , potassium carbonate, K_2CO_3 , and potassium bicarbonate, KHCO_3) [17].

Improvement of wine colour stability

The colour of the wine is one of the main sensory properties indicative of quality, especially in red wines. It is highly dependent on the winemaking technology, especially influenced by the maceration time and the mechanical processes performed during the same (e.g., punch downs, pump overs, délestage...) [32], but the microorganisms used, both yeast and bacteria, also play an important role in the development of the final colour of the wine and its stability.

First, the fermentative yeast may influence the colour by modifying the pH. In this sense, oenological yeasts usually produce wines with pH between 3.2-3.8 and under this conditions the anthocyanins tend to have a deep red colour, while the higher the pH, there is a bathochromic shift from red to purple and later to blue [33]. In the case of pure fermentations with *S. pombe*, pH control would be essential to avoid changes in colour to some extent, because this yeast, as mentioned above, makes a high consumption of malic acid and therefore the pH of the wine increases slightly.

Second, through the release of secondary metabolites of the fermentation that can react with the grape anthocyanins and thus create more stable forms of colour such as pyranoanthocyanins. The enzymatic activity of the yeast is also fundamental, especially regarding the β -glucosidase and hydroxycinnamate decarboxylase activities. The first enzyme leads to a colour loss because it catalyses the breakage of the bond between the glucose and the anthocyanidin moieties, also known as anthocyanase activity [34]. These same authors reported that no β -glucosidase activity was detected for the four strains of *S. pombe* evaluated.

The formation of pyranoanthocyanins is of great interest to preserve an intense colour in the wine during aging processes, since these pigments originated during fermentation are much more long-lasting due to its greater stability against pH changes and discoloration by SO₂. The strain effect is quite significant when several *S. cerevisiae* are used, so yeast selection is a useful tool to obtain higher amounts of pyranoanthocyanins during fermentation [35]. Certain

strains of *S. pombe* can lead to improvements in the colour stability of red wine thanks to its high formation of vitisin A and derivatives thereof [36]. It is a direct consequence of its high production of pyruvic acid during fermentation (ranging 150-350 mg/L). The higher values in *S. pombe* compared to *S. cerevisiae* (<100 mg/L; [36]) are probably due to the specific maloalcoholic fermentation pathway in which pyruvate is involved as metabolic intermediate (**Figure 2**). The amount of pyruvate released by *S. pombe* can favour the formation of 2-4-folds more vitisin A and acetyl vitisin A than *S. cerevisiae* during the fermentation of red wines (**Figure 3**). Concerning the production of Vitisin B derivatives, it is observed a lower formation of vitisin B with respect to *S. cerevisiae* fermentations [36]. However, the synthesis of vitisin A is more interesting in wines than vitisin B because the latter is a red-brown pigment (495 nm compared with 515 nm vitisin A; [35] and the hue is less suitable for wine colour quality.

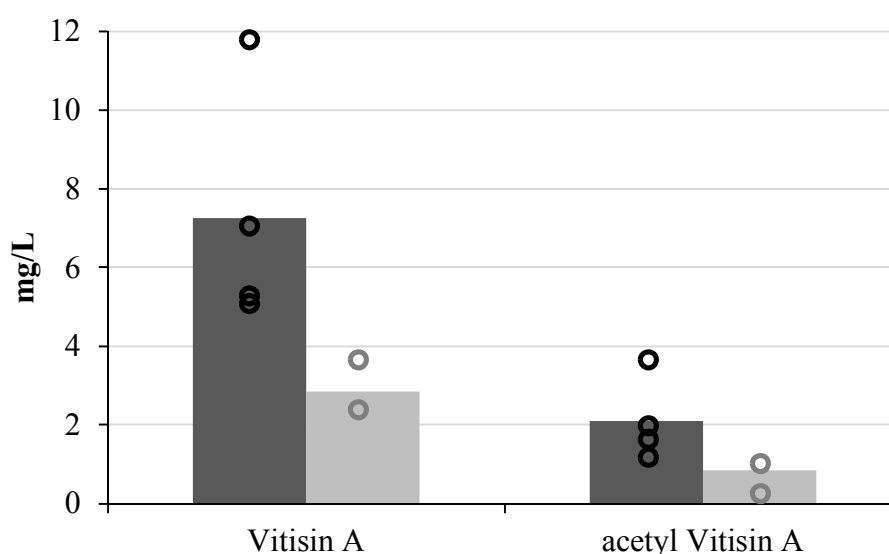


Figure 3. Formation of vitisin A and acetyl vitisin A during the fermentation with *S. pombe* (4 strains; dark grey bars) and *S. cerevisiae* (2 strains; light grey bars). Bars are average concentrations, dots are single values for each strain (Adapted from [36]).

Similarly, some strains of *S. pombe* have also shown the ability to transform the hydroxycinnamic acids present in the must (e.g., *p*-coumaric, caffeic and ferulic acids) into vinyl phenols that can condense with monomeric anthocyanins to form vinylphenolic pyranoanthocyanins [37,38]. These pyranoanthocyanins display same enological properties than vitisins concerning to their stability. Vinyl phenols are formed during fermentation by yeasts with hydroxycinnamate decarboxylase activity (HCDA). It is possible to find this activity in many non-*Saccharomyces* strains [39]. It has been observed that some strains of *S. pombe* exhibit a strong HCDA that can increase the formation of malvidin-3-O-glucoside-4-vinylphenol and malvidin-3-O-(6''-*p*-coumaroylglucoside)-4-vinylphenol by 10-30% compared with *S. cerevisiae* strains selected for their performance in this activity [40]. Therefore, the selection and use of *S. pombe* in fermentation is a powerful tool to increase the formation of stable pyranoanthocyanins both vitisin A derivatives and vinylphenolic pyranoanthocyanins.

Polymeric pigments are formed by chemical condensation between grape anthocyanins and other flavanols in a slow process during ageing that is affected by precursor contents, pH, temperature and oxygen levels. Some of these pigments have red-orange colour but others can absorb at 540 nm or higher wavelength showing red-bluish colours. It has been observed that some yeasts are able to promote the formation of these pigment in a faster process during fermentation [41,42]. *S. pombe* has shown high performance increasing the formation of polymeric pigments derived from malvidin-3-O-glucoside and catechin or procyanidin B2 [41]. Yeast influence can be produced by the fermentative release of acetaldehyde which can favour the formation of ethyl linked dimers [43]. Also some synergic effects were seen when *S. pombe* is used in sequential fermentations with *Lachancea thermotolerans* [41].

And third, yeasts are able to trap significant amounts of pigments in their cell wall [44,45]. During fermentation, yeast cell walls can reach an specific surface of 10 m²/L of must when

population is 10^8 - 10^9 CFU/mL [44], and cell wall adsorbed anthocyanins can represent 1.6-6% of wine content reaching 28% for some derivatives in some yeast strains [45]. The amount of anthocyanins adsorbed to yeast cell walls can be evaluated by using plating media dosed with grape anthocyanins [46]. Alternatively, and with higher precision, it is possible to recover adsorbed anthocyanins from yeast cell walls and analyse the extracts by LC-DAD or LC-DAD-MS [44]. When adsorption of anthocyanins was studied in non-*Saccharomyces* cell walls, a differential adsorption was observed according to yeast species but also according to strain. In a comparative view of adsorption in the plating system with anthocyanins added to the agar formulation, it is possible to see a medium adsorption capacity for *S. pombe* (strain 938) compared with other species with high adsorption (*L. thermotolerans*, formerly *Kluyveromyces thermotolerans*) or the low adsorption of *Metschnikowia pulcherrima* (Figure 4).

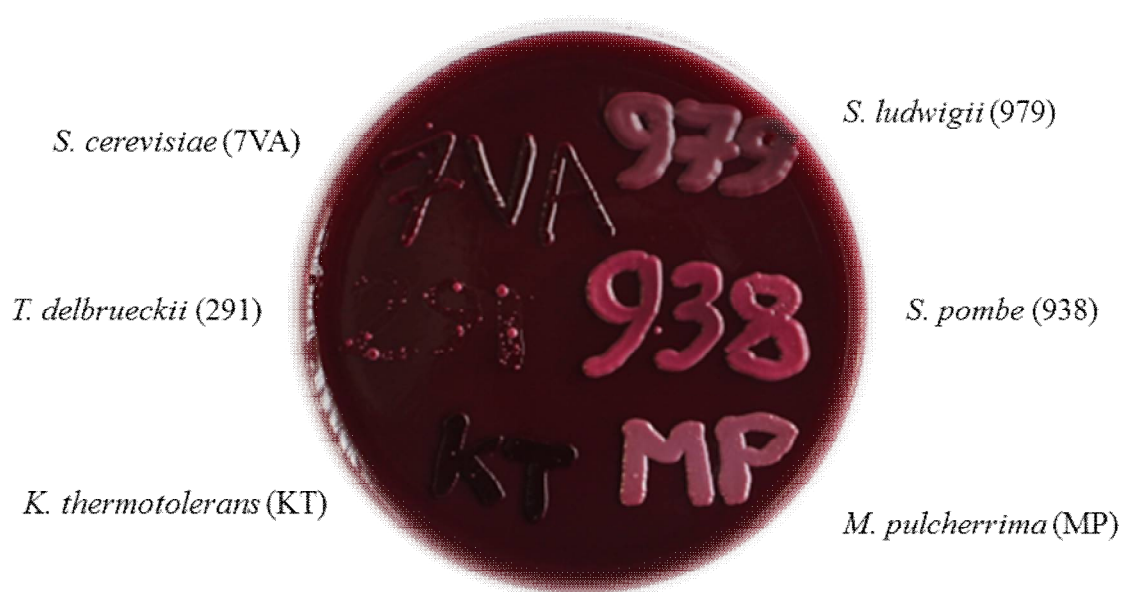


Figure 4. Adsorption of grape anthocyanins in yeast cell walls (*Saccharomyces* and non-*Saccharomyces*) during growth in a specific plating media containing pigments.

The protective effect of ageing on lees (AOL) on wine oxidation by the release of reductive compounds from yeast structures such as glutathione can also produce some preservation on wine anthocyanins. The high release of cell wall compounds from *S. pombe* during ageing on lees [47] can favour and enhance this protective effect, thus better preserving wine colour.

Large release of polysaccharides during ageing on lees

As previously stated, *Schizosaccharomyces* yeast genus physiology and metabolism present some peculiarities. *S. pombe* cell wall carbohydrate composition and distribution of polysaccharides and other wall constituents is quite special; something which shouldn't be surprising bearing in mind we are dealing with an osmophilic yeast that reproduces itself/asexually by binary fission thanks to the formation of a wall, from centre to centre of the cell (**Figure 1**; [48,49]) Early in the seventies some authors far from food research, studied the structure and composition of their cell walls [50]. Indeed, the qualitative composition and formation of cell wall polysaccharides can vary greatly among yeasts, and that differences are useful for taxonomic classification purposes [51,52]. Later electron microscopy studies after enzyme treatments, aiming to deeply understand the molecular organization of *S. pombe* cell walls, highlighted the presence of galacto-manoproteins in the outer layer of the cell wall as its main qualitative discriminating feature [53]. A comparative illustration of the cell walls of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* based on that study permits the observation of those qualitative differences among these species, can be consulted in [47]. Besides those special features of *S. pombe*, a simple optical microscopy let us appreciate its thickened cell walls. That big thickness and that previously mentioned particular molecular organization of biopolymers in *S. pombe* cell walls give them structural strength enough to resist high osmotic pressures.

Ageing over lees is relatively new to red wine production since this technique has been traditionally used to produce white wines [54,55]. The autolytic release of yeast cell wall polysaccharides in wine making and their contribution to aspects such as mouthfeel and tactile properties, wine aroma, body and physicochemical stability has been widely studied, discussed and demonstrated [56]. In particular, mannoproteins act as colloidal stabilizers, having a positive effect on tartrate stability [57], decreasing tannin aggregation and precipitation [58] and protecting wine from protein haze in white wines [59,60]. The production and release of these macromolecules depends on the yeast strain, and according to Vidal, Williams, Doco, Moutounet, & Pellerin, (2003) [61], they can reach up to 35 % of total polysaccharides in wine.

Ageing over lees of red wines have been usually described as a technique that helps to stabilize colour and modulate astringency. Several interactions between yeast lees and phenols could take place simultaneously, and factors such as yeast lees reactivity towards oxygen [62,63], the strain capacity to absorb anthocyanins [44,64,65] or the presence of β -glucosidase activity explains why contradictory results and hypothesis can be easily found in the literature.

The autolytic release of polysaccharides by *Schizosaccharomyces pombe* were studied for the first time in synthetic media by Palomero et al., in 2009 [47]. From the first moment, results showed a high quantity of cell wall polysaccharides. At 28 days both osmophilic yeasts *Schizosaccharomyces pombe* and *Saccharomycodes ludwigii* released cell-wall fragments in concentrations more than ten times greater than those produced by the *Saccharomyces* and *Pichia* strains studied. Two months into the over lees ageing process, the polysaccharide concentrations of the osmophilic yeast autolysates were those that would be reached at six or seven months by *Saccharomyces*. This rapid release could entail an important competitive advantage for wineries due to the reduction of ageing periods. Besides that an early elution

peak were observed in the HPLC-RI chromatograms corresponding to biopolymers of over 788 kDa. These fragments are therefore larger than most of those observed from *Saccharomyces*, and should be studied in order to understand its potential oenological interest to modulate astringency, improve palatability and preserve colour [47]. Domizio, Liu, Bisson, & Barile, (2017) [66] have recently found larger polysaccharide molecules in earlier peaks by HPLC-RI when studying and characterizing *S. pombe* polysaccharides, but were not detected in the chromatograms of the corresponding *S. japonicus*. These authors obtained similar results when studying the release of polysaccharides during alcoholic fermentation. They conclude all the *Schizosaccharomyces* strains studied released a quantity of polysaccharides approximately 3 to 7 times higher than that released by a commercial *Saccharomyces cerevisiae*. Subsequent studies have obtained similar results, underlining the polysaccharide overproduction of yeast belonging to the *Schizosaccharomyces* genus [67].

Bio-tool for ensuring wine safety

The metabolic characteristics of *S. pombe* make it the ideal bio-tool to reduce the content of certain unwanted compounds in musts and wines, either because they are responsible for long-term microbiological instability, because they adversely affect the sensory quality of the wine or because they can lead to toxic compounds [10]. Some examples of compounds that can be controlled by *S. pombe* are malic acid, biogenic amines, gluconic acid and ethyl carbamate.

The ability of *S. pombe* to metabolize malic acid was previously discussed (See section “**Wine pH modulation**” above). This organic acid is not only a source of microbiological instability in the wine, but also contributes a hard and green acidity.

Biogenic amines may pose health problems. An advantage of using *S. pombe* instead of lactic acid bacteria (LAB) for the elimination of malic acid is that this yeast does not promote the formation of biogenic amines, a health issue associated to the traditional malolactic fermentation performed by LAB [68,69]. It is worth mentioning that, unlike wild malolactic bacteria, the available commercial LABs have been selected according to the criterion of low production of biogenic amines and ethyl carbamate. The combination of *Lachancea thermotolerans* and *S. pombe* for the sequential fermentation of a red must with high pH has shown to be effective in the control of the biogenic amines formation, with a reduction in the concentration of histamine of up to four times with respect to the same grape must that underwent a malolactic fermentation [68]. In addition, the organoleptic quality of the wine was not compromised by the employment of two non-*Saccharomyces* yeasts for the fermentation.

Gluconic acid decreases the microbiological stability of the wine, since LAB can metabolize it increasing the acetic acid levels and, therefore, damaging the quality of the wine [70]. High contents of this acid are related to the development of the fungal disease called gray rot. It has been shown that *S. pombe* can metabolize gluconic acid and in this way favour the biological aging of wines by preventing the development of LAB [71].

Despite belonging to the ascomycetes group, *S. pombe* exhibits a strong urease activity [72]. Urease catalyses the hydrolysis of urea into ammonia and carbamate, the latter product is spontaneously hydrolysed to carbonic acid and ammonia under oenological conditions. Ethyl carbamate is a well known carcinogen produced along the fermentation and ageing, and urea is one of its precursors [73]. The safety limits established for ethyl carbamate in wines widely varies between 15-60 µg/L depending on the country and the type of wine, being the dessert wines those with upper limit. Therefore, fermenting with *S. pombe* may prevent ethyl carbamate production.

Sparkling wines and other fermented beverages (ice wines, beers)

In the winemaking of sparkling wines there is a second fermentation in bottle in which it is necessary to use a yeast able to ferment in the presence of around 10 % v/v ethanol (base wine). In addition to complying with this requirement, *S. pombe* can be an interesting species for this process due to its high release rate of polysaccharides during ageing on lees [74]. The higher the polysaccharide content in the wine, the better the mouth-feel sensations (reducing astringency, enhancing sweetness and roundness) and the aromatic persistence and quality [75]. It also has a protective effect on wine colour.

Moreover, the employment of *S. pombe* for the second fermentation and ageing on lees of sparkling wines production was suggested as a way to obtain differentiation [76]. With the use of *S. pombe*, red sparkling wines with higher pyranoanthocyanin concentrations and higher colour intensity were obtained [76]. Concerning the sensory evaluation, both white and red wines were rated as good quality without notable differences in relation to the control of *S. cerevisiae* in taste characters, but with some differences at the aromatic and visual level. *S. pombe* seems more suitable for winemaking in red than in white, losing partially in the latter the fruity and floral character. The red sparkling wines made with *S. pombe* were the ones that obtained the highest score for the colour intensity and aromatic intensity. Although the herbal, buttery and yeasty notes stood out significantly, the wines had a good balance on the nose and no aromatic defects were perceived. However, these results come from a single strain of *S. pombe* (selected for its good fermentative behaviour), it would certainly be interesting to try new strains.

S. pombe was also tested as yeast responsible for the bottle fermentation in brewing [13]. The amount of sugar metabolized in beer production is markedly lower than in wine and,

therefore, the amount of acetic acid synthesized by *S. pombe* should also be lower. *S. pombe* also has potential application in the production of ice wines, especially when it comes to obtaining wines with a better balance of acidity [77].

Conclusions

Schizosaccharomyces pombe is a useful tool for total or partial deacidification of grape musts, achieving a good quality in the final wine when immobilization techniques are used. This species has shown promising results regarding the production of stable colour forms, therefore, ensuring the colour preservation in aged wines. Its use in winemaking can also prevent potential health risks associated with the metabolism of the lactic acid bacteria responsible for malolactic fermentation. As a general conclusion, due to its great resistance to pH, temperature and preservatives, and its ability to ferment in media with high sugar content, *S. pombe* is a strain of great versatility with potential utility not only in oenology, but also in other sectors of the food industry.

Author Contributions: I.L. Literature review, writing and editing; A.M. Literature review, writing and images design; F.P. Literature review and writing; C.G. Critical reading; J.A.S.L. Critical Reading.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflicts of interest.

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