Pichia kluyveri in Wine Technology

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Contributor: Antonio Santos

The surfaces of grapes are covered by different yeast species that are important in the first stages of the fermentation process. In recent years, non-*Saccharomyces* yeasts such as *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, and *Pichia kluyveri* have become popular with regard to winemaking and improved wine quality. For that reason, several manufacturers started to offer commercially available strains of these non-*Saccharomyces* species. *P. kluyveri* stands out, mainly due to its contribution to wine aroma, glycerol, ethanol yield, and killer factor.

Keywords: Pichia kluyveri ; thiols ; higher alcohols ; esters ; fatty acids ; wine ; P. anomala ; P. fermentans ; P. guilliermondii ; P. kudriavzevii ; P. membranifaciens

1. Introduction

The growing interest in *P. kluyveri* is reflected in the number of scientific publications regarding this species. According to the PubMed® database, over a period of 10 years (2009–2019), 33 publications were related to *P. kluyveri* and wine, of which 14 were in the last two years (2018 and 2019). Despite the growing interest, it is still far from the interest shown for other wine-related yeast species. In the same period, 1503 works on *S. cerevisiae* and wine, 114 on *Metschnkowia* spp., 116 on *T. delbrueckii*, and 75 on *L. thermotolerans* were published.

Different *Pichia* species that have been found in must fermentations and are considered to be wine-related are included in the *non-Saccharomyces* group: *P. fermentans, P. membranifaciens, P. occidentalis, P. terricola, P. manshurica, P. kudriavzevii*, and *P. kluyveri.* The frequency of isolation of *Pichia* species from grapes is lower than that of *S. cerevisiae* (28%) and other non-*Saccharomyces* such as *Hanseniaspora uvarum* (44%). The frequency varies from 0.12% for *P. occidentalis* up to 4.7% for *P. anomala*. Other reported *Pichia* species usually isolated from grapes are *P. manshurica* (2.81%), *P. menbranifaciens* (0.98%), and *P. kudriavzevii* (0.85%). Those lower frequencies justify the lack of commercial strains compared to other species, making it difficult to make a proper selection.

Among the wine-related *Pichia* species, *P. kluyveri* is the most studied and is the only one commercially available in the yeast market currently. *P. kluyveri* is characterized by its ability to improve the composition of aromatic compounds such as thiols, terpenes, and fruity esters. Currently, there are only two commercial starters based on *P. kluyveri*: WLP605 (Vintner's Harvest©, Yakima, WA, USA), which is advertised as increasing rose petal and floral aromas, contributing to improve the overall bouquet of wine, and FROOTZEN® (Hansen©, Hoersholm, Denmark), which is advertised as increasing varietal and thiolic aromas. Both are indicated for use in sequential fermentation, first with *P. kluyveri,* and 48 h later with a *S. cerevisiae* strain, which will properly end the alcoholic fermentation.

Pichia species show multilateral buds for asexual reproduction, whereas sexual reproduction is characterized by unconjugated asci; the conjugation occurs between a parent cell and its bud or between independent cells. Asci may be persistent or deliquescent, with usually one to four and more rarely five to eight ascospores. The ascospores are rough or smooth and spherical to hat-shaped, and sometimes they present equatorial or subequatorial ledges. The cell shape is spherical to ovoid and occasionally may appear as pseudohyphae. *Pichia* spp. can ferment glucose but rarely other sugar molecules. The genus assimilates some sugars and is not able to assimilate nitrate as a nitrogen nutrient. The genus produces coenzyme Q-7. The last genus revision described 20 accepted species, among which *Pichia membranifaciens* is considered the type species; only a few of them are considered positive in winemaking.

As far as *P. kluyveri* is concerned, the cells are slightly ovoid and about 2–10 µm, and it is very difficult to distinguish their shape from the shape of *S. cerevisiae* or *S. ellipsoideus* cells. Its ability to produce a film during its development in must is very characteristic and allows us to easily distinguish the species among other yeasts (Figure 1). The ability to distinguish this film formation is very useful at the industrial scale to quickly evaluate implantation success when using commercial products that contain *P. kluyveri*. This species is able to produce pseudohyphae in plate cultures but not in liquid fermentation. It can also produce hat-shaped ascospores. The species only ferments glucose and shows growth in liquid media containing glucose, ethanol, or glycerol. Like other *Pichia* species, *P. kluyveri* resists high osmotic pressure, presenting optimal growth in 10% NaCl or 5% glucose. It has been usually isolated from rooted fruit and green parts of plants, being widely distributed in all type of ecosystems.

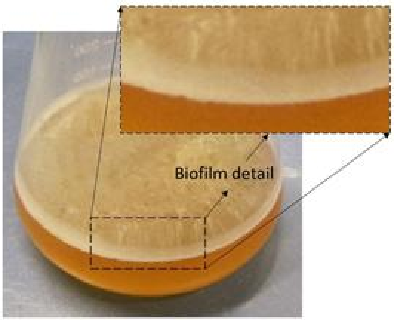

Figure 1. Film produced by *Pichia kluyveri* over grape must at the beginning of alcoholic fermentation.

2. P. kluyveri Impact on Different Wine Quality Parameters

2.1. Ethanol

P. kluyveri is only able to ferment up to 4–5% (*v*/*v*) in ethanol, consuming only glucose and leaving fructose. This fermentation capacity is insufficient to produce regular wines or sparkling base wines but is enough to produce other beverages such as beer of about 3.2% (*v*/*v*) or tequila base.

The ethanol yield of *P. kluyveri* is 22% lower than that of *S. cerevisiae*, producing 0.36 g of ethanol per gram of sugar. Most *Pichia* species have lower yields than *P. kluyveri*, such as *P. fermentans* (0.04 g), *P. membranifaciens* (0.08 g), *P. terrricola* (0.19 g), and *P. kudriavzevii* (0.33 g). However, some *Pichia* species have been shown to have a higher ethanol yield; for example, *P. holstii* yields around 0.43 g of ethanol per gram of sugar.

Since *P. kluyveri* is unable to ferment fructose and consume the full amount of glucose present in grape juice, it must be combined with fermentative yeast such as *S. cerevisiae* to completely ferment sugars and achieve the desired quality parameters. Sequential fermentation involving *P. kluyveri* and *S. cerevisiae* resulted in lower final ethanol content than *S. cerevisiae* controls. The difference increased in fermentations when *P. kluyveri* was present for a longer time during the winemaking process. Sequential inoculation at 48 h resulted in a lower ethanol content of 0.16% (*v*/*v*), while another sequential inoculation at 96 h resulted in 0.25% (*v*/*v*).

The ethanol reduction is due to the oxidative metabolism of non-*Saccharomyces* species that consume glucose without ethanol formation. The sugar that is not converted into ethanol is transformed into other compounds, such as glycerol or acids. Among those species, *P. kluyveri* is the second most efficient among 23 studied species, after *M. pulcherrima*. When it was employed in sequential fermentation, the ethanol content was reduced between 3 and 22%.

With regard to the fermentation kinetics, coinoculation of *P. kluyveri* and *S. cerevisiae* in a 9:1 ratio presumed a final delay of 3 days in alcoholic fermentation compared to the *S. cerevisiae* control. In that study, *P. kluyveri* cells were detected during the first 9 days in an alcoholic fermentation that lasted for 23 days at 14 °C. The sequential inoculation strategy allowed the detection of *P. kluyveri* until 6 days after *S. cerevisiae* inoculation, which occurred 8 days later than the *P. kluyveri* inoculation. However, other studies reported a fast to immediate decrease after *S. cerevisiae* inoculation, which reinforces the importance of selecting a compatible *S. cerevisiae* partner that allows the virtues of *P. kluyveri* to be increased during alcoholic fermentation.

2.2. Glycerol

Glycerol concentration is higher in sequential fermentation involving *P. kluyveri* than in *S. cerevisiae* controls, and the effect increases when *P. kluyveri* ferments longer. A sequential inoculation of 48 h resulted in an increase in glycerol of 0.33 g/L, while another inoculation of 96 h resulted in a higher increase of 1.3 g/L. Other studies reported a decrease of about 48% in coinoculation. This difference could be explained by possible strain variability similar to that reported for other non-*Saccharomyces* species. Although some studies reported positive significant increases in final glycerol concentration related to *P. kluyveri* performance, other non-*Saccharomyces* such as *C. zemplinina* are much more efficient for this purpose, able to produce a final glycerol concentration up to 15 g/L.

Organic Acids

P. kluyveri does not notably influence wine organic acids as other specific non-*Saccharomyces* do. It is reported to slightly consume malic acid in a concentration of about 0.1 g/L. However, that is not enough to significantly influence the pH or achieve malic acid microbiological stability. All studies involving *P. kluyveri* have reported nonsignificant statistical differences in acetic acid production between *P. kluyveri* sequential fermentation and *S. cerevisiae* control.

One study reported increments of some acids derived from the tricarboxylic acid cycle under sequential fermentation involving *P. kluyveri*: α-ketoglutaric acid (24%), oxalic acid (50%), and succinic acid (300%). In this study, the control was a *T. delbrueckii* strain and the fermentative product was durian wine. There are no available data yet for grape wine compared to *S. cerevisiae* control, so further studies must be performed on this topic, as similar results could occur with grape juice fermentation. Succinic acid concentration in wine usually varies from 0.5 to 1 g/L, so final concentrations up to 5 g/L by sequential inoculations reported for *P. kluyveri* could be an interesting alternative to wine acidification. As those concentrations are over the average value for wine, it is probable that they significantly influence its sensorial properties. While citric, L-lactic, L-malic, and L-tartaric acids are described as sour and astringent from a sensorial point of view, succinic acid is described as sour, salty, and bitter. However, the study does not include a sensory analysis to corroborate the possible influence of succinic acid on the final flavor.

2.3. Aroma Compounds

P. kluyveri species showed a remarkable ability to release 3-sulfanylhexan-1-ol acetate (3_SHA) compared to other *Saccharomyces* and non-*Saccharomyces* species. 3-SHA is a pleasant volatile molecule that produces desired aromas in wine described as passionfruit or box tree. A study reported that sequential fermentation involving *P. kluyveri* and *S. cerevisiae* reached notably higher final concentrations of 3-SHA than the control fermented only by *S. cerevisiae*. The increases varied from 10 to 72% depending on the initial inoculation ratio between *S. cerevisiae* and *P. kluyveri*. The optimum reported initial ratio was 1:9 and the final 3-SHA concentration varied from 55 to 72% higher than the *S. cerevisiae* control depending on the *P. kluyveri* strain that performed the fermentation. The increase of 3-sulfanylhexan-1-ol (3-SH) was about 40% for the 1:9 ratio inoculation. Statistically significant differences in thiol release by *P. kluyveri* were reported to depend on the *S. cerevisiae* strain employed to properly end the alcoholic fermentation. Those results suggest that the *S. cerevisiae* partner must be carefully selected to optimize the final total thiol concentration released during alcoholic fermentation when working together with the selected *P. kluyveri* strain. As significant strain variability regarding thiol release is reported for *P. kluyveri*, selecting yeast strains with high β-lyase activity, similar to other non-*Saccharomyces* species such as *T. delbrueckii* or *M. pulcherrima*, could optimize *P. kluyveri* thiol release activity.

P. kluyveri reduces the content of total higher alcohols under sequential fermentation by about 15%, with each higher alcohol affected in a different range (e.g., hexanol, –50%; 2-phenyl-ethanol, −20%; and butanol, −20%). Other studies reported the same results, with variations in different ranges [33] ((Z)-4-decen-1-ol, −9%; (E)-4-decen-1-ol, −8%; 1-decanol, −4%; 1-hexanol, −28%; 1-nonanol, −12%; 2-hepten-1-ol, −32%; 2-methyl-3-buten-1,2-diol, −14%; 3-octanol, −11%; 5-nonanol, −20%; and cyclooctanemethanol,α,α,-dimethyl, −12%). A similar effect was previously observed in other non-*Saccharomyces* such as *Torulaspora*, *Lacchancea*, and *Metschnikowia*. Other studies observed an increase in higher alcohols of around 25% and great variation among them (e.g., hexanol, +12% and 2-phenyl-ethanol, +25%). The latest biotechnology techniques for producing varietal wines tend to reduce as much as possible the production of higher alcohols to values below 350 mg/L because they mask the varietal aroma compounds. The final total higher alcohol concentration reported for sequential fermentation between *P. kluyveri* and *S. cerevisiae* was always below 350 mg/L, varying from 176 to 254 mg/L.

Different studies report a higher production of total esters for sequential fermentation involving *P. kluyveri* than *S. cerevisiae* controls. A study on the presence of different enzymatic activities of oenological impact reported that all studied strains of *P. kluyveri* presented esterase activity, which catalyzes the formation of esters. The highest increase was 25% for 2-phenyl ethyl acetate and 50% for a longer sequential inoculation. Another study reported further increases up to 60% in red wine. The compound 2-phenyl-ethyl acetate is a desirable aromatic compound that increases the perception of aromas such as rose or floral when it appears in concentrations over 0.25 mg/L. The yeast strains employed to ferment neutral varieties such as Airen and Ugni blanc, which did not possess high levels of molecules such as terpenes or thiols, are selected to enhance the final fruity ester concentration. On the contrary, yeast strains employed to ferment varieties with strong varietal characteristics such as Verdejo, sauvignon blanc, and Muscat are selected to produce lower levels of esters in order to not mask the varietal aromas.

Several studies reported no effect of *P. kluyveri* on the final concentration of total terpenes compared to *S. cerevisiae* control. This is mainly because β-glucosidase activity is reported to be not common in *P. kluyveri* strains. Other factors that could affect this phenomenon are the initial sterilization of the grape juice and the performance of varieties with low terpene content. Indeed, differences in specific terpenes are reported. It was reported that *P. kluyveri* sequential fermentation had higher levels of linalool oxide and hotrienol by about double and 40%, respectively, while the concentration of nerol was lower by about 10%.

Total fatty acid content was not influenced by *P. kluyveri* or even decreased, and decanoic acid was the most affected, with decreased concentration by around 18%. These results agree with a report showing the absence of lipase enzymes in *P. kluyveri* species. Specific fatty acids such as isovaleric acid stood out due to an increase of around 25% compared to *S. cerevisiae* control. The production of isovaleric acid should be taken into account for strain selection, as concentrations over 50 mg/L produce undesirable aromas such as rancid cheese. This phenomenon has been previously reported for other non-*Saccharomyces* species such as *T. delbrueckii*.

One study reported lower production of acetaldehyde by nearly 40% compared to *S. cerevisiae* control, although the final values were far below the olfactory threshold of 125 mg/L and related to undesirable oxidative descriptors. This additional effect could increase the impact of other varietal aroma compounds such as thiols and terpenes, as they are less masked for this significant aromatic compound that produces oxidative aromas. The table 1 summarizes the main aroma compounds influenced by *P. kluyveri*.

Table 1. Main aroma compounds influenced by *P. kluyveri,* chemical structure, aromatic descriptor, and perception threshold.

Group	**Aroma Compound**	**Structure**	**Odor Descriptor**	**Perception Threshold (ng/L)**
Higher alcohols	2-methyl butanol		Harsh, nail polish remover	30,000
	3-methyl butanol		Harsh, nail polish remover	30,000
	2-phenyl ethanol		Rose	10,000
Esters	2-phenyl-ethyl acetate		Rose, raspberry	250
	2-methyl-butyl acetate		Banana	5
Terpenes	Linalool		Flowery, fruity	6 for white varieties 15 for red varieties
	Hotrienol		Faintly flowery, elderflower	110
Thiols	3-SHA		Passionfruit, box tree	4
	3-SH		Grapefruit, citrus peel	60

Some studies have reported some off-odor compounds in sequential fermentation with *P. kluyveri* compared to *S. cerevisiae* control. Some studies reported an increase in fatty acid content, such as 3-methyl-butanoic acid (isovaleric acid) and phenylamine, which are linked to undesirable aromas, such as cheese, sweaty feet, or off-putting sourness. *P.*

kluyveri has been reported to produce higher levels of H_2S by about 50% in sequential fermentation, although the final value was below the fault threshold.