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POTENTIAL APPLICATION OF INDIGENOUS *PICHIA KLUYVERI* FOR ENHANCED WINE AROMA QUALITY

Jiao Jiang[1], Wenjing Zhang[1], Li Feng[1], Dongqing Ye[1], Yanlin Liu[1,2]*

[1]College of Enology, Northwest Agricultural and Forestry University, Yangling, Shaanxi 712100, China
[2]Shaanxi Engineering Research Center for Viti-viniculture, Yangling, Shaanxi 712100, China

*Corresponding author: yanlinliu@nwafu.edu.cn

Abstract

Aims: In previous work, five indigenous *Pichia kluyveri* strains, GS1-1, FS-2-7, HS-2-1, C730 and C732, were isolated and selected from spontaneous fermented wines from Ningxia and Gansu. The aims of this study were to 1) evaluate resistance of these strains to environmental stressors that may restrict their growth and the progress of alcoholic fermentation; 2) Investigate their fermentation dynamics; 3) Characterise aroma profiles of Cabernet Sauvignon wines made from mixed cultures of *P. kluyveri* and *Saccharomyces cerevisiae*.

Methods and Results: Tolerance assays were conducted in YEPD medium to test resistance of each *Pichia kluyveri* strain to sugar, pH, ethanol, temperature and free SO_2. All strains except FS-2-7 were able to tolerate 60% w/v glucose, low pH of 2.0, 16% v/v ethanol, extreme fermentation temperatures (11˚C and 44˚C), and 500 mg/L total SO_2. Following this, these strains were inoculated into a synthetic grape juice medium to test their fermentation performance and evaluate basic parameters of the final synthetic wine. Strain HS-2-1 was the first to initiate fermentation, and produced significantly higher amounts of total organic acids and less volatile acids compared to other strains. Thus, strain HS-2-1 was chosen for further characterisation in Cabernet Sauvignon fermentation trials co-fermented with *S. cerevisiae* NX11424 at different ratios. Viable yeast cell numbers were determined by plate counting. Yeast-derived volatile compounds of the final wine were analysed using head space-solid phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC/MS). Mixed fermentation did not inhibit HS-2-1 growth, and also produced less volatile acid, and significantly more esters and higher alcohols compared to single fermentation by *S. cerevisiae*. Notably, concentrations of isopentanol, ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl 9-decenoate and ethyl lactate increased in line with the increased proportion of HS-2-1 in the inoculum.

Conclusions: This study shows indigenous *P. kluyveri* HS-2-1 has good resistance to alcoholic fermentation associated common stressors, better fermentation performance, and excellent oenological characteristics when co-fermenting with *S. cerevisiae*.

Significance and Impact of the Study: Chinese wine regions such as Ningxia and Gansu have developed dramatically in recent years. These wine regions are in great need to produce wines with typical regional characteristics. To promote regional typicity, using selected indigenous yeasts could introduce a unique local character or “terroir” during winemaking. *Pichia kluyveri* widely occurs at earlier stages of spontaneous fermentation, however limited research has been done on its oenological characteristics. This study comprehensively investigated the features of indigenous *P. kluyveri* strain(s), and highlighted the potential application of strain HS-2-1 in winemaking by co-fermenting with *S. cerevisiae* for improving the fruity and floral aroma profile of these Chinese wines.

Keywords: *Pichia kluyveri*, stress tolerance, fermentation, volatile compounds, aroma

Introduction

The choice of yeast for winemaking is crucial for obtaining wines with distinctive sensory properties. Thus, non-*Saccharomyces* yeasts have been widely investigated due to their ability to enhance regional characteristics in wines. Like many non-*Saccharomyces* strains, *Pichia kluyveri*'s presence on grapes, was previously considered as a spoilage wine yeast (Jolly *et al.*, 2014). However, recent studies demonstrate *P kluyveri* could positively contribute to wine aroma complexity and increase yields of desirable compounds. For example, fermentation with pure *P. kluyveri* results in greater amounts of ethyl lactate compared to single *Saccharomyces cerevisiae* ferments (Amaya-Delgado *et al.*, 2013). Additionally, co-inoculation of *P. kluyveri* has shown to produce wines with more varietal thiols (Anfang *et al.*, 2009). Both Fernández-González *et al.* (2003) and Escribano *et al.* (2017) reported *P. kluyveri* as a potential producer of glycosidases and esterases, therefore being able to release desirable volatile compounds from non-volatile precursors. Apart from these properties, desirable yeast strains are also expected to display killer phenotype against spoilage microbes (Zagorc *et al.*, 2001), as well as have good resistance to common alcoholic fermentation inhibitors (Lin *et al.*, 2020).

Previous work has shown that *P. kluyveri* was one of the most abundant yeast species on grapes in Northwest China wine regions, and was usually the dominant microbe at the earlier stage of spontaneous fermentation (Wang, 2009). However, limited research has been done on their oenological features.

In this study, five *P. kluyveri* strains isolated from Northwest China were evaluated for their potential as wine starter cultures. Stress tolerance, killer activities and fermentation performance were assessed to determine whether these strains were suitable for winemaking.

Material and Methods

The indigenous *P. kluyveri* strains, GS1-1, FS-2-7, HS-2-1, C730 and C732, collected from uninoculated fermenting wines using grapes sourced from Ningxia and Gansu China, were characterised in this study.

Survival of *P. kluyveri* was examined under environmental stressors in YEPD medium, with *S. cerevisiae* X16 (Laffort) as control. Each tube containing 5 mL YEPD was inoculated with about 1×10^7 cells/mL. Culture density was measured after incubating at 28˚C for 72 h using a spectrophotometer at 600 nm. Killer activity of each strain was tested against the sensitive reference strain *S. cerevisiae* 1296 by the diffusion plate assay (de Ullivarri *et al.*, 2018). Killer activity was detected when a clear zone was observed surrounding the colonies.

Pichia kluyveri strains were then evaluated by fermentations in 250 mL of a synthetic grape juice medium (Triple M, 100 mg/L glucose, 100 mg/L fructose (Spiropoulos *et al.*, 2000)). All ferments were performed in triplicate in autoclaved conical flasks fitted with airlocks, inoculated at a rate of 1×10^6 cells/mL, at 20˚C with shaking at 120 rpm. Fermentation was monitored by measuring weight loss of the ferments every 12 h. The best performing strain was further evaluated via sequential fermentations in 2 L fermentation vessels filled with 1.5 L Cabernet Sauvignon must (2016; Ruina Chateau, Shaanxi). The must was treated with 60 mg/L SO_2 and 600 mg/L dimethyl dicarbonate overnight for sterilisation prior to inoculation of the *P. kluyveri* at 1×10^6 cells/mL. Indigenous *S. cerevisiae* NX11424 was then inoculated at the ratio of 1:1, 1:10 and1:20 after 48 h.

Sugar, ethanol, titratable and volatile acidity were determined following OIV-MA-INT-00-2020. Wine samples were analysed for non-targeted volatile compounds using head space-solid phase microextraction-gas chromatography with mass spectrometry (HS-SPME-GC-MS) (Hu *et al.*, 2019). Plotting of graphs and statistical analysis was performed using GraphPad Prism 8.0 (GraphPad, USA) and/or XLSTAT (Addinsoft SARL, France). A two-sided p-value less than 0.05 was considered statistically significant.

Results and Discussion

Survival of *P. kluyveri* strains under environmental stressors varied among isolates, with values of OD_{600} ranging from 0.10 to 1.98 (Figure 1A). Generally, growth was detected for all *P. kluyveri* strains under typical winemaking conditions, among which, strain HS-2-1 had higher cell density than other strains. Additionally, HS-2-1 was able to inhibit *S. cerevisiae* 1296 (supplement data). Similarly, Labbani *et al.* (2015) reported killer activity of *P. kluyveri* DBVPG 5826 against many spoilage food fungi, including *S. cerevisiae*. Fermentation performance was tested in Triple M fermentations with single *P. kluyveri* cultures (Figure 1B-1C). When comparing sugar consumption during fermentation, as indicated by CO_2 weight loss, HS-2-1 fermented fastest, followed by C730, C720, FS-2-7,

and GS-1-1. All strains except C730 could complete fermentation, with residual sugar dropped below 2 g/L. By contrast, single fermentations often slowed and eventually halted for many other non- *Saccharomyces* yeasts (Lin *et al.*, 2020). Additionally, all *P. kluyveri* strains significantly increased both the titratable acidity and volatile acidity (< 1.2 g/L) of the fermented Triple M compared to X16.

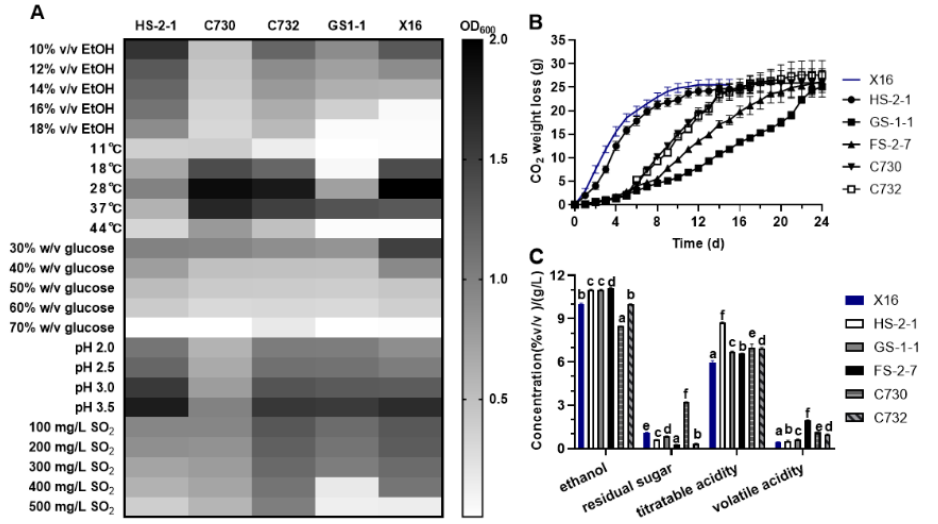


Figure 1:

A) Growth of *P. kluyveri* strains under different levels of stressors;

B) CO_2 weight loss of the Triple M ferments;

C) Key parameters of the finished Triple M ferments.

Values with different letters are significantly

Since HS-2-1 had better resistance to ethanol and pH, it also performed better in the Triple M fermentation (fermented fastest with the highest titratable acidity of the ferment), it was chosen as the best performing strain for further characterisation in sequential Cabernet Sauvignon fermentations (Figure 2). Sugar utilisation was the most rapid for the NX11424 fermentation, finishing in 8 d, followed by mixed fermentations (10 d), and the HS-2-1 monoculture fermentation (11 d). Trends in viability were in accordance with the sugar consumption of these strains. Population of HS-2-1 remained over 10^6 CFU/mL throughout the experiment, whereas a drastic increase in viability was seen for NX11424 in sequential fermentations after inoculation, and peaking at 8 d. In this fermentation trial, higher titratable acidity was also observed in HS-2-1 fermented wines, whilst volatile acidity had decreased significantly in these wines.

Figure 2:

Sequentially inoculated trials.

A) Sugar consumption kinetics;

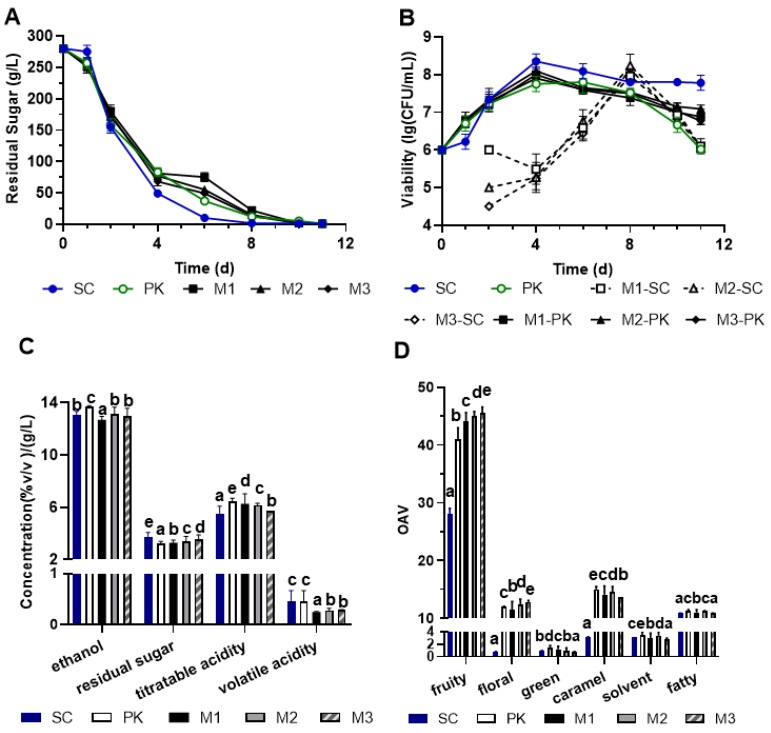
B) Yeast growth kinetics;

C) Key parameters of the finished wine;

D) Aroma profile of the finished wines.

SC: *S. cerevisiae* NX11424; PK: *P. kluyveri* HS-2-1; M1-M3: mixed fermentations with inoculation ratios of 1:1; 10:1 and 20:1.

Values with different letters are significantly different ($p < 0.05$).



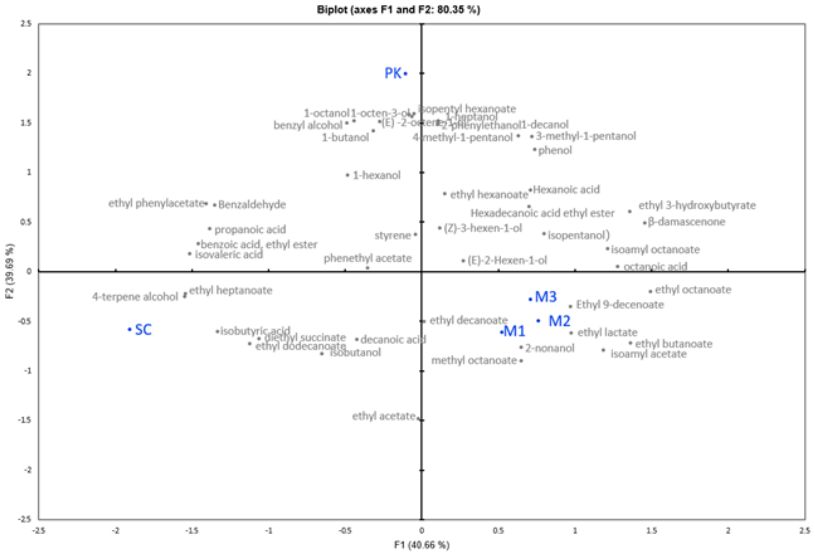
Forty-six volatile compounds were detected by HS-SPME-GC-MS in Cabernet Sauvignon wines (supplement data). Significant differences were observed in the concentration of higher alcohols and esters in these wines. In particular, higher ratio of HS-2-1 resulted in wines with greater amounts of 2-phenylethanol, isopentanol, ethyl butanoate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl 9-decenoate and ethyl lactate, which contributes to fruity and floral aromas of the wine. This was in agreement with studies reported by Chua *et al.* (2018) and Prior *et al.* (2019). Further, Escribano *et al.* (2017) observed esterase activities in *Pichia* sp., indicating *P. kluyveri* has potential to modify the sensory profiles of wines.

To visualise the relationship between inoculation treatments and volatile composition of Cabernet wines, principal component analysis was performed (Figure 3). PC1, accounted for 40.66% of the overall variation, and separated NX11424 and HS-2-1 wines. PC2, which constitutes 39.69% of the variation in the data, separated wines made from pure HS-2-1 and sequentially fermented wines. Separation of PC1 was driven by ethyl esters whilst isopentyl hexanoate, 1-octanol and 3-methyl-1-pentanol were the compounds driving the separation of PC2.

Figure 3:

Principal component analysis of the aroma profiles of mean value for sequential Cabernet Sauvignon fermentations.

Treatments represented in blue and aroma attributes represented in grey.



Conclusion

Our findings demonstrated *Pichia kluyveri* HS-2-1 could be potentially used to enhance wine aroma quality. However, since this study was performed in lab-scale in a sterile medium/must, winery-scale trials are further needed to evaluate HS-2-1 under industrial fermentation conditions.

Acknowledgements

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Data Availability

The supplement data used in this study is available from the corresponding author on request.

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