Phenotypic variations of primary metabolites yield during alcoholic fermentation in the *Saccharomyces cerevisiae* species

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Abstract

Saccharomyces cerevisiae, as the workhorse of alcoholic fermentation, is a major actor of winemaking. In this context, this yeast species performs alcoholic fermentation to convert sugars from the grape must into ethanol and CO_2 with an outstanding efficiency: it reaches on average 92% of the maximum theoretical yield of conversion. Primary metabolites produced during fermentation stand for a great importance in wine where they significantly impact wine characteristics. Ethanol indeed does, but others too, which are found in lower concentrations: glycerol, succinate, acetate, α-ketoglutarate… Their production, which can be characterised by a yield according to the amount of sugars consumed, is known to differ from one strain to another. *S. cerevisiae* is known for its great genetic diversity and plasticity that is directly related to its living environment, natural or technological and therefore to domestication. This leads to a great phenotypic diversity of metabolites production. However, the range of metabolic diversity is variable and depends on the pathway considered. In the aim to improve wine quality, the selection, development and use of strains with dedicated metabolites production without genetic modifications can rely on the natural diversity that already exists. Here we detail a screening that aims to assess this diversity of primary metabolites production in a set of 51 *S. cerevisiae* strains from various genetic backgrounds (wine, flor, rum, West African, sake…). To

approach winemaking conditions, we used a synthetic grape must as fermentation medium and measured by HPLC five main metabolites. Results obtained pointed out great yield differences between strains and that variability is dependent on the metabolite considered. Ethanol appears as the one with the smallest variation among our set of strains, despite it's by far the most produced. A clear negative correlation between ethanol and glycerol yields has been observed, confirming glycerol synthesis as a good lever to impact ethanol yield. Genetic groups have been identified as linked to high production of specific metabolites, like succinate for rum strains or alpha-ketoglutarate for wine strains. This study thus helps to define the phenotypic diversity of *S. cerevisiae* in a wine-like context and supports the use of ways of development of new strains exploiting natural diversity. Finally, it provides a detailed data set usable to study diversity of primary metabolites production, including common commercial wine strains.

Abbreviations

CCM: Carbon Central Metabolism; GM: Genetically Modified; CV: Coefficient of Variation; α−KG: Alpha-Ketoglutarate; MLF: Malo-Lactic Fermentation; TCA: Tricarboxylic Acid

Introduction

Fermented products stand today for a great importance in human society both on an economic and a social point of view. Historically, human and fermentation share a long story: first trace of cereals fermentation has been found in Israel and estimated back to 13000 B.C. (Liu *et al*., 2018) and the first trace of fermented beverage from rice, honey, and a fruit, is known back to 7000 B.C. in China (McGovern *et al.*, 2004). Since then, fermentation uses have expanded to a wide diversity of processes and products, like food, beverages or more recently biofuels. In alcoholic beverages, alcoholic fermentation is the main step of elaboration and is mostly carried by yeast from the *Saccharomyces* genus, especially *Saccharomyces cerevisiae* species. A perfect example is wine, which is the result of the alcoholic fermentation of grapes or grape juice. In a technological point of view, wine fermentation is the biotransformation of glucose and fructose, existing in equal proportions in grapes, in carbon dioxide and ethanol which brings new characteristics to the product: sensory qualities, stability…

Alcoholic fermentation is of high technological interest as well as metabolic importance for *Saccharomyces cerevisiae*. Through the glycolysis, this biological process results in generation of pyruvate and energy in the form of ATP. Pyruvate, which is a central metabolite, is then converted in two steps in ethanol and carbon dioxide, which ensure a quick re-oxidation of enzymatic cofactors used in glycolysis, making alcoholic fermentation the most efficient way to promptly provide energy to the cell (Bakker *et al.*, 2001). Moreover, in typical wine conditions, it is the only way for *S. cerevisiae* to produce ATP, respiration being repressed by the Crabtree effect or impossible due to the absence of dioxygen (De Deken, 1966; Pfeiffer and Morlay, 2014). Both fermentation main products, ethanol and carbon dioxide, are by far the most produced metabolites during alcoholic fermentation and

therefore in wine making (Nidelet *et al*., 2016). A simple way to compare these productions between species, strains or fermentation conditions is to define a yield, mass or molar, of metabolite produced by substrate consumed. Ethanol yield of wine fermentation carried by *S. cerevisiae* is known to be around 0.47 gram per gram of hexoses consumed, which represent 92% of the maximum theoretical yield (calculated as one mole of glucose give two moles of ethanol) (Tilloy *et al*., 2015). The major part of remaining hexoses is used as a carbon source for cell multiplication and production of other metabolites in minor concentrations, such as glycerol, acetate, succinate, acetaldehyde, etc. These metabolites count for largely smaller carbon fluxes, but can stand for significant technological value. Glycerol, which is linked to stress resistance, can impact the mouthfeel of wine from a certain concentration (Albertyn *et al*., 1994; Noble and Bursick 1984). It has been identified as the second most produced metabolite in fermentation and as the flux with the greatest impact on ethanol production (Goold *et al*., 2017). Acetate, which is a way to restore redox balance and a metabolic intermediary, is a major off-flavour linked compound and subject to legal limits (Vilela-Moura *et al*., 2008). It appears that yields of fermentation metabolites like ethanol, glycerol or acetate are linked to domestication degree of strains (Tapia *et al.*, 2018).

For all compounds, yield values differ among strains and environmental conditions of fermentation (oxygenation, temperature, nutrients concentrations, presence of other microorganisms…) (Du *et al*., 2012; Tronchoni *et al*., 2022) but the range of variation stays very limited for ethanol compared to biomass or other metabolites. In their work, Nidelet *et al*., (2016) have compared 43 strains from six different ecological origins and shown that the coefficient of variation of carbon flux toward ethanol synthesis following glycolysis and alcoholic fermentation is only between 2 and 3 %. In a contrasting way, yields of glycerol or acetate have a respective variation around 10 and 30 % while representing a significantly lower carbon flux for the cell (Camarasa *et al*., 2011; Nidelet *et al*., 2016). Generally, global yields are calculated at fixed points of the fermentation: 80% of hexoses consumed, exponential phase… One of the reason of these choices is that ethanol yield is not constant during fermentation and that the flux is difficult to calculate beside the exponential growth phase which is the only stage

with a quasi-steady state (Celton *et al*., 2012; Nidelet *et al*., 2016; Quirós *et al*., 2013). However, in a wine production context, the definition of a yield per strain needs to be done when the fermentation is completed, which means that all hexoses have been used.

Representing a very small percentage of the carbon fluxes in the cell, metabolites with very low concentrations are produced too. However, they can still have a significant impact on the final fermented product, like organic acids, higher alcohols and esters, and so their production is considerably studied (Antonelli *et al*., 1999; Regodón Mateos *et al.*, 2006).

The last thirty years have seen considerable research efforts concentrated on understanding and impacting primary metabolism, mainly with the aim of reducing the final ethanol content of wines. Besides physical or chemical methods, many microbial strategies have been developed to modify ethanol production during fermentation. We can cite here genetically modified yeast strains, hybrids strains, optimisation through adaptive laboratory evolution… (reviewed in Varela and Varela, 2019). Beside this, modulation of the carbon central metabolism (CCM) without disturbing the cell balance still remains complex to operate in wine context, mostly because of the multigenic character of the associated traits (Bro *et al*., 2006; Hubmann, Foulquié-Moreno, *et al*., 2013; Hubmann, Mathé, *et al*., 2013; Salinas *et al*., 2012).

Nevertheless, elaborate strategies to develop *S. cerevisiae* strains with a modified glycerol or ethanol yields in wine fermentation requires to clearly identify the diversity of the CCM metabolism as well as its constraints and trade-offs.

Here we present results from a screening strategy of 51 strains from different origins that aims to identify the variability of yield of primary fermentation metabolites in laboratory wine-like conditions among the *S. cerevisiae* species.

Materials and methods

Strains

51 strains have been used. Information is available in supplementary data (S1).

Strains selection has been made considering results from precedent works of the laboratory, with the aim to have a lot of diversity in fermentation profiles (Camarasa *et al*., 2011; Legras *et al*., 2018; Nidelet *et al*., 2016). EC1118 has been chosen as a reference strain to estimate block effect. Genetically modified (GM) and laboratory evolved strains for precise CCM traits have been included too. Strains were conserved at −80 °C in 20% glycerol YPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) and cultivated on YPD agar plate (YPD + 20 g/l agar).

Genetic groups constitution

Strains from various genetic backgrounds, but all linked to fermented beverages, are represented in the set, known using previous work on *S*. *cerevisiae* genome sequencing (Akao *et al*., 2011; Eder *et al*., 2018; Fay and Benavides, 2005; Liti *et al*., 2009; Marsit *et al*., 2015; Novo *et al*., 2009; Schacherer *et al*., 2009). To classify and organise this intraspecific diversity, two works have been used to define the following genetic groups: wine, rum, West African, sake and flor (Legras *et al*., 2018; Peter *et al*., 2018). Genomic data to establish these groups are available for 39 strains. Strains without information have been labelled as “Unknown”. A supplementary group, labelled as “Miscellaneous”, has been used to gather strains with mosaic, very singular or unclassifiable genomes, but it will not be used as a consistent group like others.

Fermentation conditions

Fermentation conditions have been chosen to ensure a quick and complete alcoholic fermentation. One colony has been grown on an overnight culture of YPD medium as pre-culture. Then 10^6 cells/ml of these pre-culture have been inoculated in a 280 ml fermenter. A synthetic medium that mimics grape must composition has been used containing 90g/l of glucose, 90 g/l of fructose, 425 mg/l of

assimilable nitrogen (as a mixture of amino acids and ammonium) and a set of nutrients reflecting grape juice composition (Bely *et al*., 1990). Fermentations have been carried at 28°C with agitation. Fermenter weight has been measured twice a day to observe fermentation progress. Fermentations carried at the same time represent a fermentation block. Three replicates have been made for each strain (except LMD17, LMD37, LMD39, performed in six replicates due to their use in a parallel project and EC1118 performed in duplicate per block, *i. e*. 28 replicates in total).

Metabolite analysis

Fermentation metabolites concentrations have been measured using high performance liquid chromatography as described in Deroite *et al*. (2018) and analysing chromatograms on OPEN LAB 2X software. Fermentation samples have been centrifuged 5 min at 3500 rpm at 4 °C and kept at -18°C. Before analysis, samples have been diluted to 1/6 with 0.005 N H_2SO_4 and then centrifuged 5 min at 13000 rpm at 4 °C. The supernatant has been kept at -18°C until being analysed. The HPLC method allows to measure concentrations of glucose, fructose, ethanol, glycerol, acetate, succinate, pyruvate and alpha-ketoglutarate. Analyses were performed in duplicate and the mean has been calculated for each sample and used in results analysis.

Quantification has been made with a Rezex ROA column (Phenomenex, Torrance, California, USA) set at 60 °C on a HPLC (HPLC 1260 Infinity, Agilent Technologies, Santa Clara, California, USA). It has been resolved isocratically with 0.005 N H_2SO4 at a flow rate of 0.6 mL/min. Concentration of acetate and pyruvate have been measured with a UVmeter at 210 nm and other compounds with a refractive index detector at 35°.

For each fermentation, two measures have been done: in the must before fermentation (done for each block) and at the end of the fermentation. All analyses have been conducted on finished fermentation, i.e. when combined fructose and glucose concentration fall under 3 g/l, or when fermenter weight stays constant during 24h.

Yield has been calculated for each metabolite as following:

$$Y_{metabolite} = \frac{C_{metabolite}}{C_{glucose+fructose;initial} - C_{glucose+fructose;final}}$$

Each concentration is expressed in g/l or mg/l, leading to yields expressed in g/g or mg/g. When necessary, values of yield are expressed as follows: mean ± standard error.

Statistical analysis

Statistical analysis has been made using R studio software (version: 1.4.1106). The R script used for analysis is available as supplementary data (S3) as well as the raw data set (S2) and the final ones that arose from the analysis (S4 and S5).

EC1118 has been used in each block in order to evaluate a possible block's effect. It has been estimated on EC1118 data using the following model:

$$Y_{lm} = \mu + Block_l + E_{lm}$$

With: Y the phenotype (yield for a given metabolite) for the block *l* (1-51) and the replicate *m* (1-2). μ represent the mean of the considered phenotype and *E* the residual error, with $E \sim N(0, \sigma^2)$.

A block effect has been observed on EC1118 data. This has been corrected by calculating a variation factor on EC1118 metabolite values. This correction (raw value - correction coefficient) has been applied on glucose, fructose, ethanol, glycerol, succinate, acetate and alpha-ketoglutarate concentrations data for all strains following their fermentation block.

The block effect being exempted yields can be expressed with the following model:

$$Y_{ik} = \mu + S_i + E_{ik}$$

$$Y_{jk} = \mu + G_j + E_{jk}$$

$$Y_{ijk} = \mu + G_j + S_i(G_j) + E_{ijk}$$

With: Y the phenotype (yield for a given metabolite) corrected for block effect for the strain *i* (1-51), the genetic group *j* (1-5) and for the replicate *k* (1-28). μ represent the mean of the considered phenotype, *S* the effect of the strain *i*, *G* the effect of the genetic group j and *E* the residual error, with $E \sim N(0, \sigma^2)$.

To express the variation of yield of a metabolite among a group of strains, the variation coefficient has been used (Albatineh *et al*., 2014). A correction according to the number of strains in a group has been applied, allowing us to compare groups of different sizes. The correction has been applied as follows:

$$CV_{corr} = \frac{\sigma}{\mu} \times \left(1 - \frac{1}{4(n-1)} + \frac{1}{n}\left(\frac{\sigma}{\mu}\right)^2 + \frac{1}{2(n-1)^2}\right) \times 100$$

With, for a group of strains and a metabolic yield: μ the mean, σ the standard deviation, *n* the size of the group and CV_{corr} the corrected coefficient of variation, expressed as percentage.

Results

Here we present results obtained for 51 strains following the fermentation of a synthetic grape must. Concentrations have been measured for 5 main compounds from the CCM: ethanol, glycerol, succinate, acetate and alpha-ketoglutarate, determined by HPLC. After a correction of block effect and

yield calculation for each compound (expressed as gram of metabolite measured per gram of hexoses consumed), strains have been compared between each other. All strains have been able to consume entirely glucose and fructose from the must within 5 days.

We detail the analysis of the 51 strains set of the metabolic yields for 5 metabolites a global analysis by PCA, an analysis of correlation between metabolic yields and the impact of strains' genetic origins. Among our set of strains, 5 have been genetically modified or obtained using adaptive laboratory evolution methods aiming to modify the CCM: 5074, LMD13, LMD14, LMD41 and LMD45. These strains will be used as a sort of control and will be discarded in correlation studies between metabolites, the 46 other strains being gathered in a group called "wild".

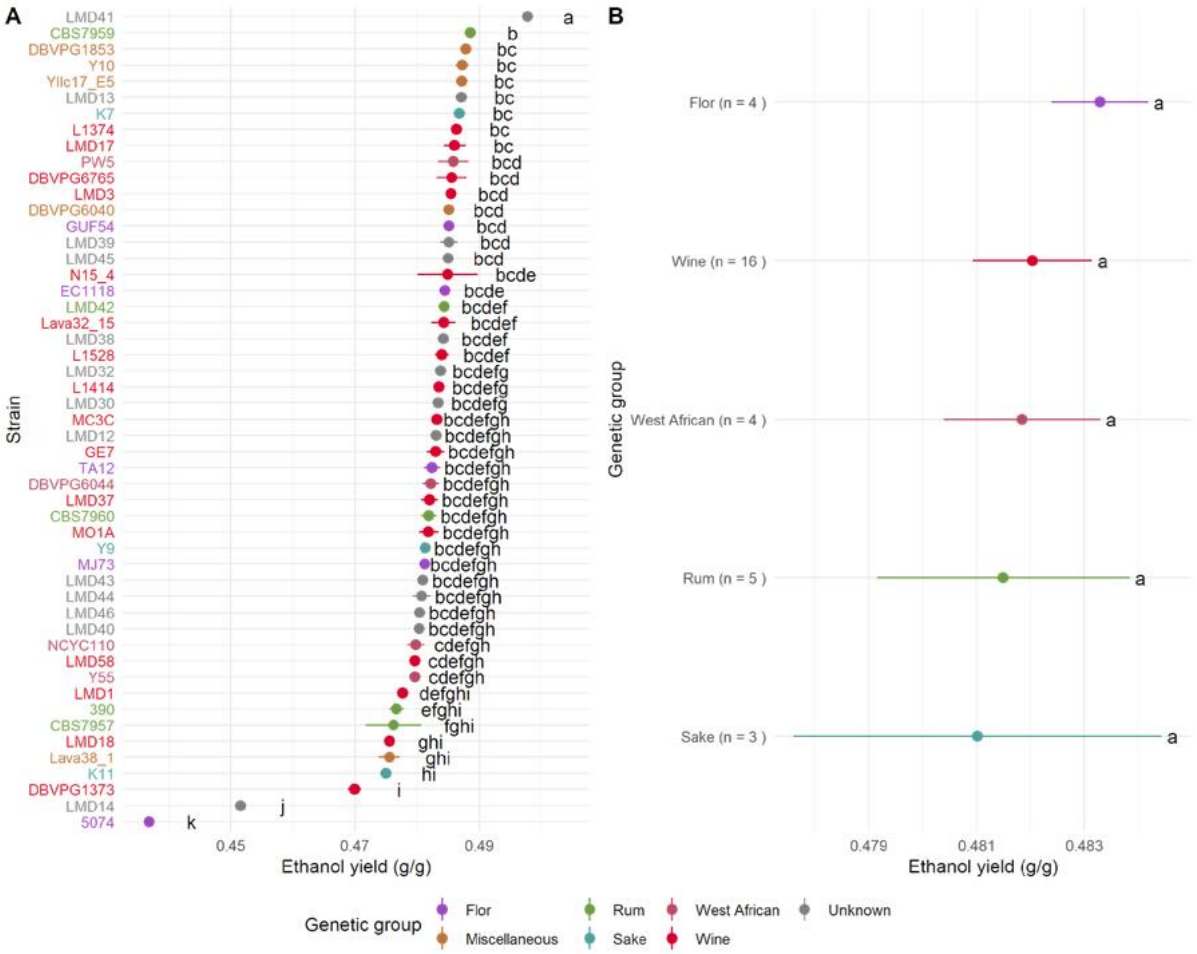
Ethanol

In the first place, we took a look at the major produced metabolite during alcoholic fermentation: ethanol (Figure 1). We observed significant differences of yield between all strains. ($F_{50, 136}$ = 38.8, p-value << 10e-3). The highest producer of ethanol, the strain LMD41, with a yield of 0.4978 ± 0.0005 g/g, is a GM strain optimised to maximise ethanol production while reducing glycerol synthesis in bioethanol context. The lowest ethanol producer is the strain 5074, with a yield of 0.4368 ± 0.0008 g/g, a haploid segregant obtained by an adaptive evolution strategy aiming at reducing ethanol production while increasing glycerol. Results from this evolution have been used to build the second lowest producer of ethanol: the commercial wine strain LMD14, which shows a yield value of 0.4515 ± 0.0007 g/g.

For wild strains, ethanol yield values are all contained in a smaller range: between 0.47 and 0.49 g/g, but still show significant differences ($F_{45, 126}$ = 7.3585, p-value << 10e-3). This range represents a variation inferior to 4%, with concentrations between 80.7 g/l for the lowest producer and 83.9 g/l for the highest.

Correlation between ethanol yield and genetic group belonging of strains has been studied. We used for that only values of strains from defined and homogenous groups: wine, flor, rum, sake and west

249 African. Means of each group have been calculated for each metabolite, using the average values of
250 all strains included in the group.
251 No significant difference of yield between genetic groups has been observed ($F_{4, 27}$ = 0.1448 and p-
252 value = NS).



253

254 **Figure 1 -** Average ethanol yield for each strain (A) and for main genetic groups (B)
255 *Standard error as error bars. Different letters represent significant differences between two means*
256 *(Tukey's test, p<0.05)*

257 **Glycerol**

258 The second more important metabolite by flux in CCM is known to be glycerol. Results can be seen in
259 Figure 2. Here, yield values are distributed between 0.092 and 0.025 g/g considering all strains, and
260 between 0.032 and 0.049 g/g considering only wild strains, with in both cases significant differences
261 between strains (with respectively $F_{50, 136}$ = 504.77 and $F_{45, 126}$ = 75.403 and p-values << 10e-3 for both).

The haploid segregant obtained following an adaptive laboratory evolution aiming to improve glycerol production (strain 5074) is the strain with the highest glycerol yield: 0.0914 ± 0,0005 g/g. It is followed by the commercial high glycerol producing strain LMD14 with a yield of 0.0723 ± 0.0016 g/g.

The genetically modified strain LMD41, with maximised ethanol production and reduced glycerol production, is the one producing the lowest glycerol, with a yield below 0.03 g/g. Another modified strain, LMD45 shows a similar glycerol yield: it has been built for low by-product production, which means low glycerol and acetate yields.

As detailed for ethanol, we looked for correlation between strains' genetic origin and glycerol yield. No significant difference between genetic groups has been observed for glycerol yield ($F_{4, 27}$ = 0.585, p-value = NS).

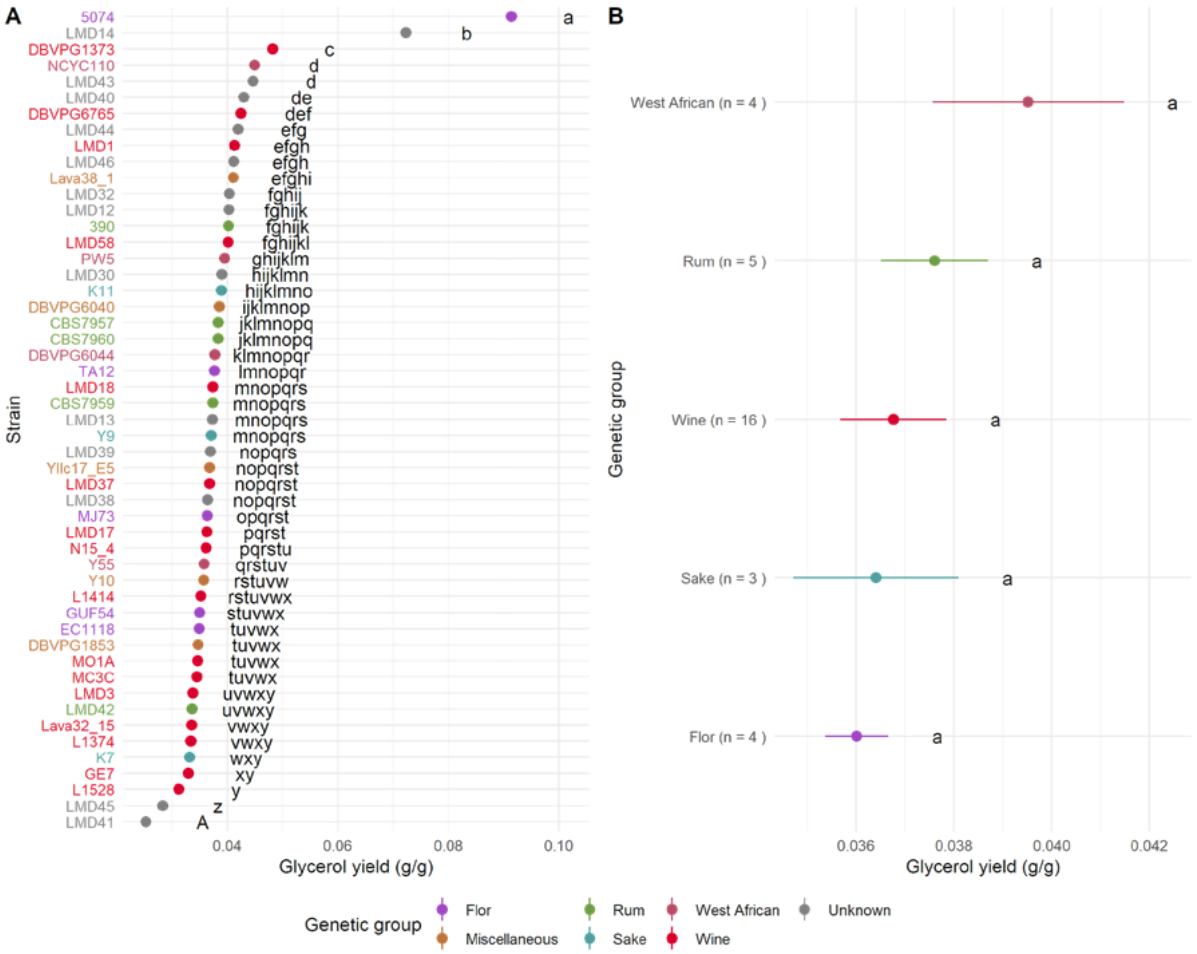


Figure 2 - Average glycerol yield for each strain (A) and for main genetic groups (B)

Standard error as error bars. Different letters represent significant differences between two means (Tukey's test, p<0.05)

Acetate

Acetate is an important metabolite in wine context: it's the third most produced metabolite in fermentation after ethanol and glycerol and, besides its low concentration, it is directly linked to off-flavour and subject to legal limits. Thus, its production is always characterised in wine strains studies. Results can be seen in Figure 3. Significant differences have been observed between strains of our set, all strains taken into account or only wild ones (respectively $F_{50, 136} = 444.66$ and $F_{45,126} = 441.49$, both p-values << 10e-3).

Acetate yields are included in a range of 0.6 to 7.9 mg/g, with a mean of 3.41 ± 0.23 mg/g. We can observe a great diversity for acetate yield among strains, with many strains significantly different from each other. The highest acetate producer is Yllc17_E5 with a yield culminating at 7.85 ± 0.28 mg/g. This yield value corresponds to a concentration of 1.35 g/l of acetate, which places this strain above the maximum limit in wine.

LMD45, known as a genetically modified strain for low fermentation by-product, shows the second lowest acetate yield: 0.68 ± 0.02 mg/g, which is a value more than 10 times lower than the highest acetate producer of the set.

In an interesting way, our two extreme strains for ethanol and glycerol yields are extremely close with a medium acetate yield.

Contrary to previous metabolites, the strains' genetic origins have a significant effect on acetate yield (p-value = 0.019). Indeed, we spotted that flor and west African strains, with an average yield of respectively 4.53 ± 0.53 mg/g and 4.74 ± 0.81 mg/g, have higher acetate yield than wine strains with 2.69 ± 0.32 mg/g.

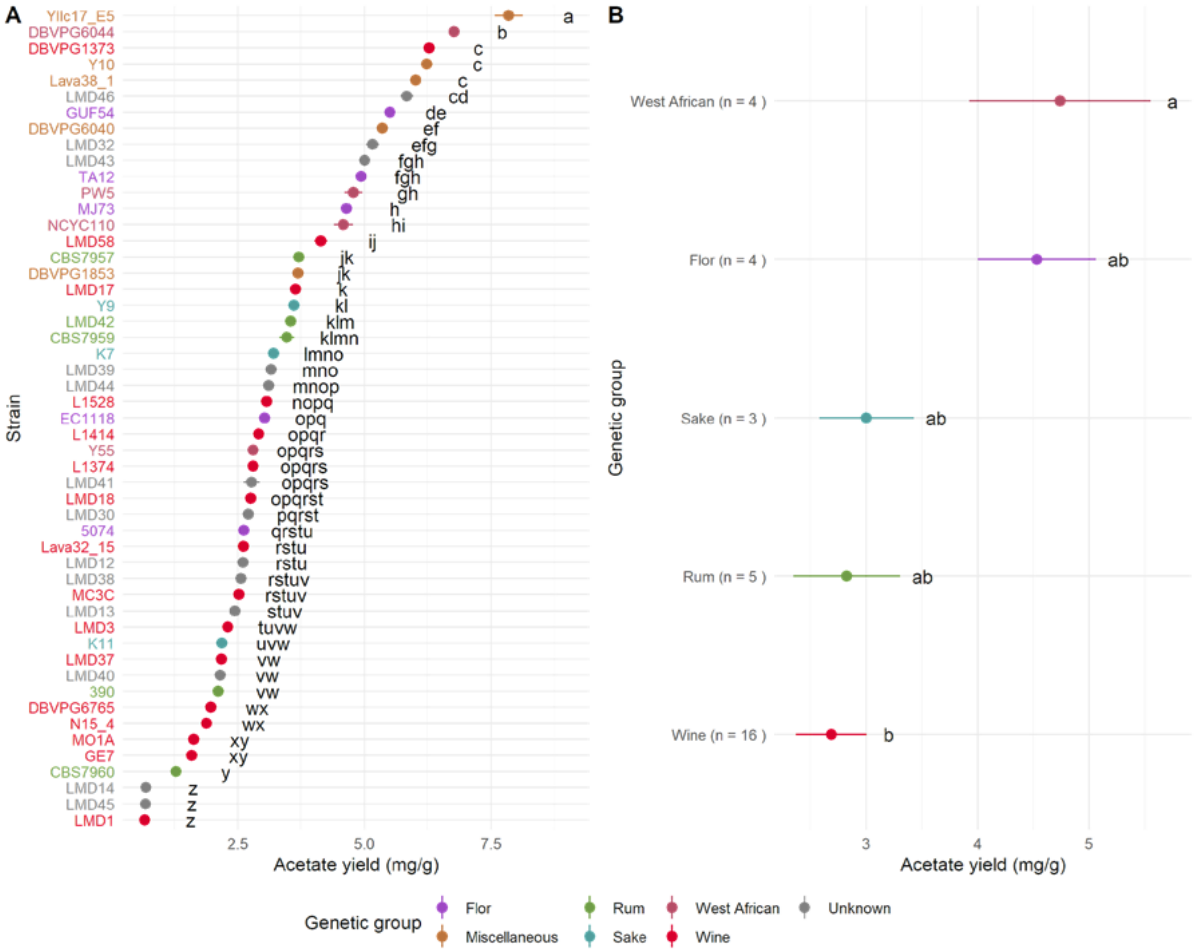


Figure 3 - Average acetate yield for each strain (A) and for main genetic groups (B)

Standard error as error bars. Different letters represent significant differences between two means (Tukey's test, p<0.05)

Succinate

Succinate can be seen as a metabolite with positive impact in wine, but is rarely considered due to its low production by wine yeast.

Succinate yield values show significant differences between strains too ($F_{50, 136}$ = 61.8 with all strains and $F_{45, 126}$ = 61.1 with only wild ones, p-value << 10e-3 for both) (Figure 4).

These values range from 3.1 to 32.8 mg/g, with GUF54 as the lowest producer and LMD41 as the highest. These yield values correspond to concentration ranging from 0.54 to 5.62 g/l.

A large part of the strain set has low and non-significant differences of succinate yield, except the three higher producers, LMD41, LMD44 and LMD43, that show a gap with the rest of the set. The

highest yield measured is LMD41 with 32.8 ± 3.8 mg/g. As for acetate, we also highlighted a significant impact of strains' genetic origins on succinate yields ($F_{4, 27}$ = 4.8, p-value = 0.005). Rum strains, with a yield of 8.4 ± 1.2 mg/g produce more succinate than flor strains that show an average yield of 4.0 ± 0.5 mg/g (p-value = 0.016, obtained with a Welch two sample t-test).

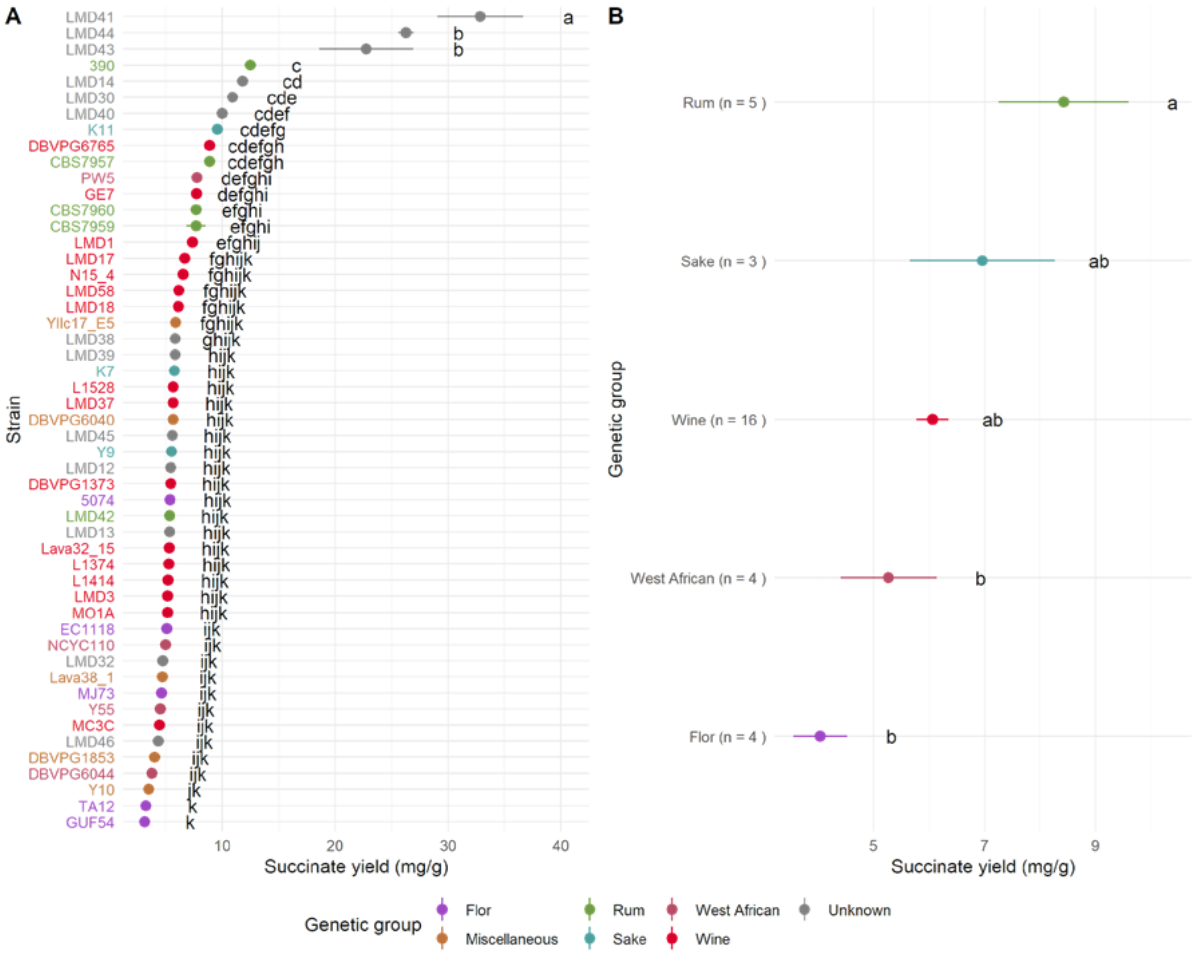


Figure 4 - Average succinate yield for each strain (A) and for main genetic groups (B)

Standard error as error bars. Different letters represent significant differences between two means (Tukey's test, p<0.05)

α–ketoglutarate

α-ketoglutarate (α–KG) is a low concentration metabolite of the CCM produced during fermentation, but stands for a great importance in the nitrogen metabolism, especially in wine fermentation. In our set, significant differences of α-KG yield between strains can be observed ($F_{50, 136}$ = 72.103 for all strains and $F_{45,126}$ = 47.299 for wild ones, both p-values << 10e-3). Values of yield range between 0.08 mg/g

and 2.7 mg/g with the highest producer being by far LMD14 (2.69 ± 0.16 mg/g) and the lowest DBVPG6044 (0.09 ± 0.03 mg/g) (Figure 5).

A correlation between genetic groups and α−KG yield have been spotted too ($F_{4, 27}$ = 6.23, p-value = 0.001). Indeed, wine strains are higher producers of α−KG compared to rum, sake, or flor strains.

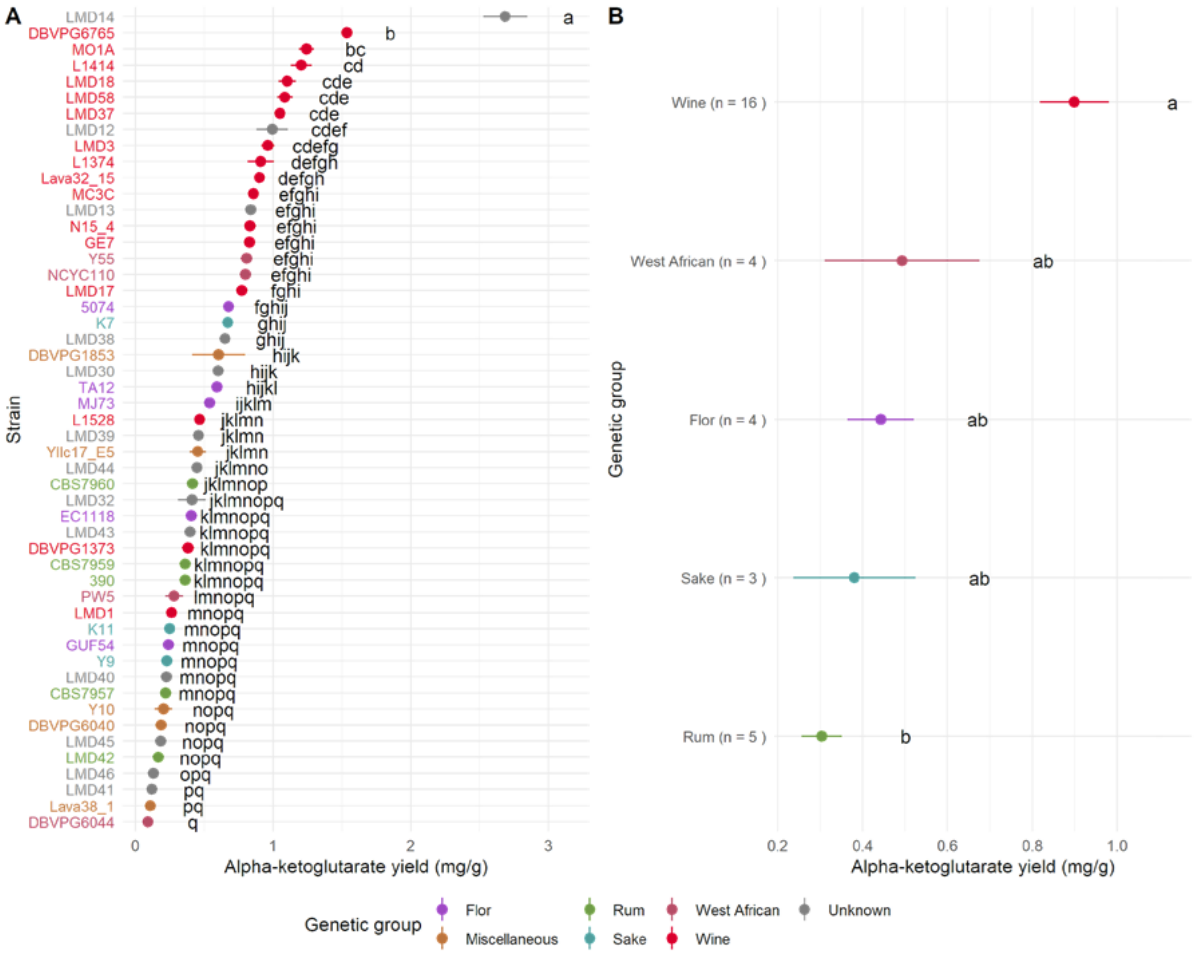


Figure 5 - Average α-ketoglutarate yield for each strain (A) and for main genetic groups (B)

Standard error as error bars. Different letters represent significant differences between two means (Tukey's test, p<0.05)

Comparison of all metabolites yield variation

In the aim to have a better comparison of metabolic yields between them and between strain groups, we decided to calculate the coefficient of variation for each metabolite (Figure 6).

With a variation coefficient of 1.8% when all strains are considered, ethanol is the metabolite with the yield presenting the lowest variation. With only wild strains, the coefficient of variation is even lower,

dropping to 0.9 %. Other metabolites have a more important variation among our selection of strains, with a peak for succinate and alpha-ketoglutarate around 75%. Overall, variation is higher when all strains are considered. Acetate is the only exception, with a similar coefficient of variation for all strains group and wine group (respectively 48 and 47 %).

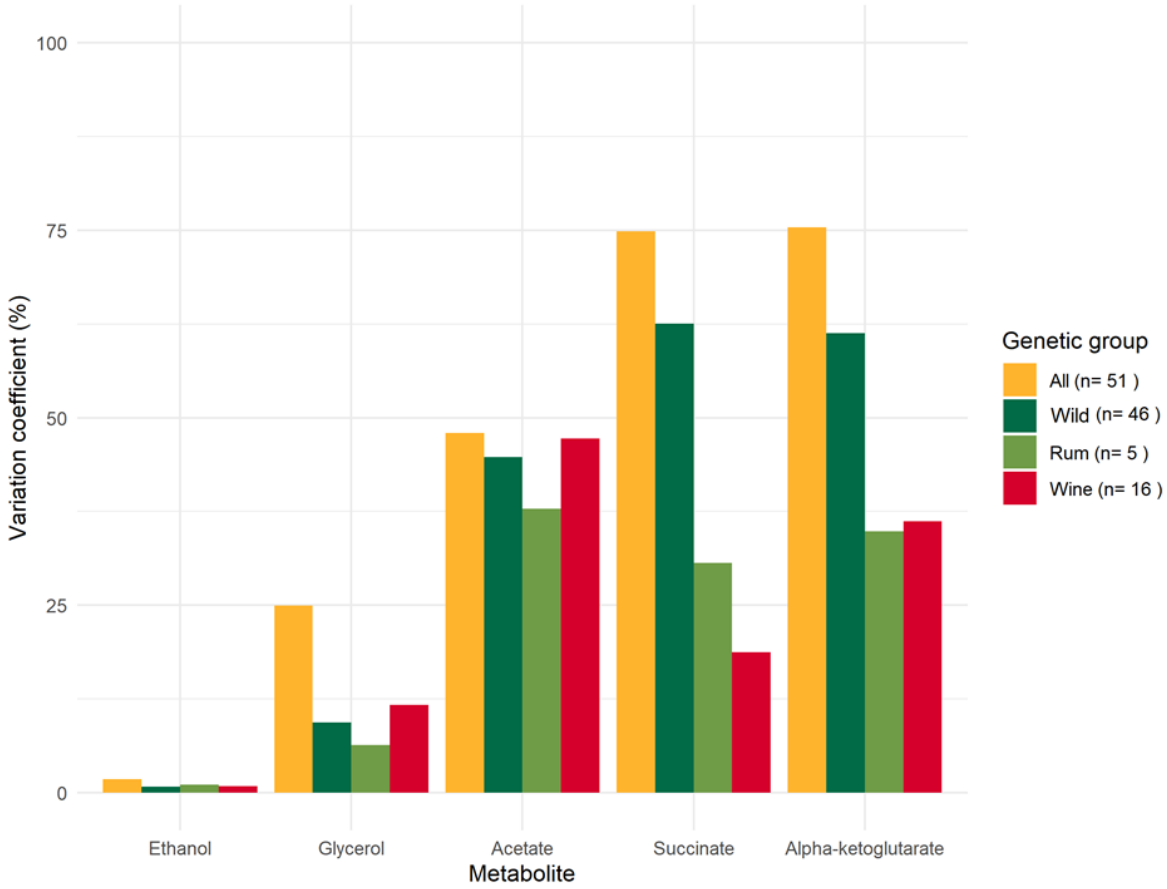


Figure 6 - Variation coefficient of each metabolite for all strains, wild strains, wine strains and rum strains

Calculated using all replicates means of each strain for rum, wine, natural and all strains groups

Metabolic yields correlation

After considering metabolic yields one by one, correlations between them have been looked at. The strongest one is a negative correlation between glycerol and ethanol yields in the complete set of strains ($R^2 = 0.859$, $F_{1,49} = 297.7$, p-value << 10e-3). However, this correlation seems driven by modified

and evolved strains because of their extreme yield values. Without these strains, the correlation is still relevant but with a larger dispersion (R^2 = 0.332, $F_{1,44}$ = 21.86, p-value << 10e-3) (Figure 7).

Correlation have been identified too for others metabolic yields :

- A positive correlation exists between glycerol and succinate yields if we consider only wild strains ($F_{1,44}$ = 9.0559, p-value = 0.0043) but with a very high dispersion (R^2 = 0.1707)
- A negative correlation exists between acetate and alpha-ketoglutarate yields both in all strains and wild strains sets (respectively $F_{1,49}$ = 12.2 and $F_{1,44}$ = 12.55, p-value = 0.00102 and 0.00095), but again with very high dispersion (respectively R^2 = 0.1994 and R^2 = 0.2219).

No other significant correlation has been observed between metabolic yields, considering all strains or only wild ones.

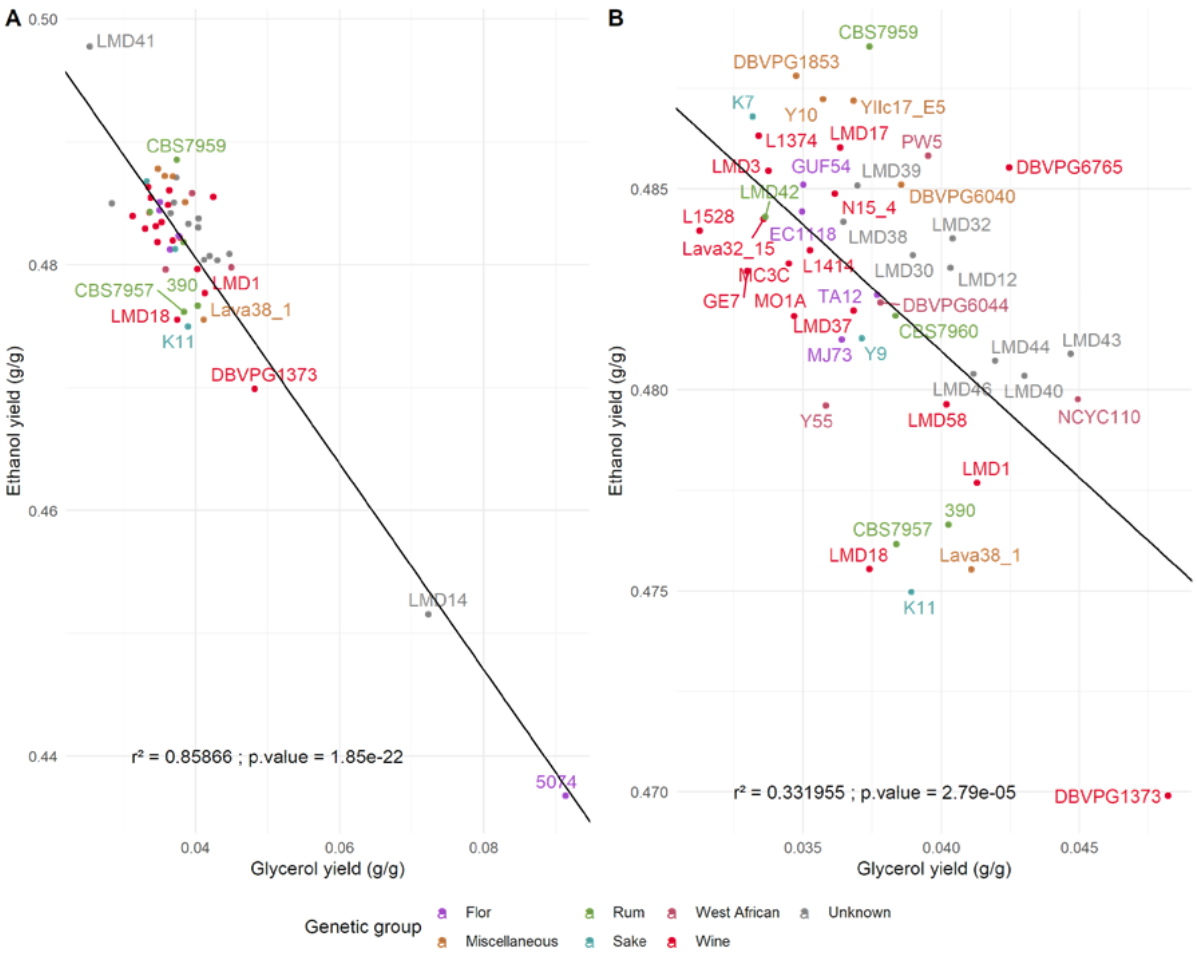


Figure 7 - Relation between ethanol and glycerol yields, all strains considered (A) or only wild strains (B)

Global analysis and hierarchical clustering

To obtain a global view of our data set, a Principal Component Analysis (PCA) has been performed with yields values of ethanol, glycerol, acetate, succinate and alpha-ketoglutarate (Figure 8). This analysis allows us to situate strains in relation to each other and investigate the effect of the genetic background. PCA has been performed with only wild strains to avoid bias induced by GM and evolved strains. We also performed a Hierarchical Clustering on Principal Components (HCPC) on wild strains, allowing us to define 4 clusters of strains. We chose this number because it is the smallest that better represents the distribution. The clustering showed a good superposition with the genetic group and reflects observations already made in metabolite by metabolite analysis. For example, the wine strains group is quite homogenous and seems mainly driven by the alpha-ketoglutarate and acetate yields (except the strain DBVPG1373). The first cluster gathers almost all wine strains (except DBVPG1373). LMD1, which is a wine strain, is located in cluster 3 but is very close to cluster 1 in PCA representation. The second cluster gathers 3 flor strains, EC1118 being located closer to wine strains, in cluster 1. The third cluster represents strains with no relation with each other and which are quite dispersed. The last cluster is only composed of 2 strains, that are characterised by their very high yield of succinate: LMD44 and LMD43. Sake, rum and west African groups don't show any consistency in clustering. Strain LMD12, LMD30, LMD38 and LMD39, which are commercialised for wine fermentation, are clustered with wine genetic strains. The last commercial wine strain, LMD32, is clustered with flor strains.

In variable representation, negative correlation of glycerol and ethanol can be seen. Acetate, succinate and α-KG appear to be not or weakly correlated with glycerol or ethanol, enforcing the idea that their variations are more related to genetic groups specificities than to major CCM fluctuations.

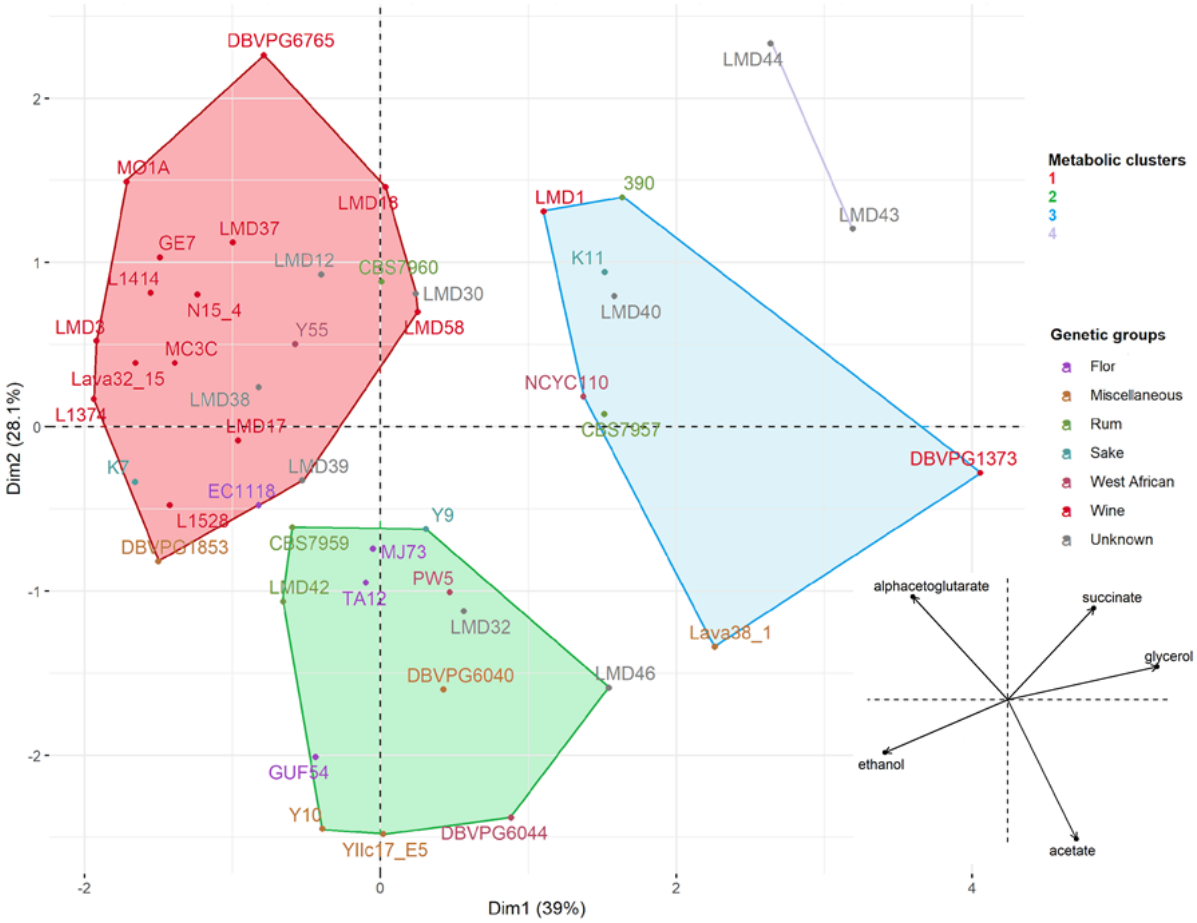


Figure 8 - Principal Component Analysis on natural strains for ethanol, glycerol, acetate, succinate and α−KG yields, individual and variable plot, with Hierarchical Clustering on Principal Components

Coloured points represent strain, tinted by origin. 4 clusters have been defined: 1 (red), 2 (green), 3 (blue) and 4 (purple)

Discussion

Our analyses on a diverse set of strains allow us a broad view of primary metabolic diversity. As it has already been observed, our results confirm our main hypothesis: wild variations exist among the *Saccharomyces cerevisiae* species concerning yields of primary metabolites and our experimental design allows us to assess it. Moreover, this methodology brings more accuracy in metabolic yield assessment and generates robust and standardised data that can be reused in other studies on yeast

metabolism. It allows to precisely define yields in a wine-like context, using synthetic gape must with metabolite assessment at fermentation final stage.

The medium used in this study is a very close imitation of grape must, so it is perfectly suited to study wine strain metabolism, but also for every strain able to ferment a complex medium with high sugar concentrations (Bely *et al*., 1990). The use of an internal reference, the strain EC1118, adds another advantage: new strains can be added in the set as long as the same reference is used in each fermentation block and in the same fermentation conditions.

Time of fermentation being dependent of nitrogen level and temperature, we carried fermentation at 28°C with a must containing a relatively low concentration of sugars and a high concentration of usually limiting nutrients (assimilable nitrogen, vitamins, anaerobic growth factors…) to ensure a quick and total hexose conversion to ethanol (Rollero *et al*., 2015). These conditions allow fermentations to be completed within 3 days.

Our methodology allows a medium throughput screening, which is a good balance between phenotyping a large number of strains and having a high accuracy measure enabling to distinguish traits with low variation.

GM or evolved strains have also been included in the selection as a kind of “controls”. Indeed, these five strains have been selected for defined characteristics linked to the CCM. . We observed that the two strains which show the highest glycerol yield and the lowest ethanol yield among all strains are LMD14 and 5074. Both are strains obtained following an adaptive evolution aiming to reduce their ethanol production while enhancing glycerol (Tilloy *et al*., 2014). At the other end of the spectrum, LMD41, modified to enhance ethanol production while cutting glycerol production, represents the highest value of ethanol yield and the lowest for glycerol. Finally, the last GM strain, LMD45, shows the second lowest acetate and glycerol yields, which is consistent with its modifications aiming to reduce fermentation by-product synthesis. All these features clearly represent the already known characteristics of the selected strains, for which they have been modified or evolved, and validate our methodology.

If we compare metabolites with each other, great differences of yield exist. Ethanol is the most produced compound, with a yield ten times higher than glycerol, which has a yield ten times higher than acetate. α-ketoglutarate has the lowest yield values but is still close to acetate. Significant variations of yield have been assessed between strains, wild or not, for each metabolite, proving that our conditions allow us to discriminate strains between them for their primary metabolite yields. However, variations among yields are not equal and differ depending on the considered metabolite.

Ethanol and glycerol are the most produced metabolites during alcoholic fermentation. With a variation coefficient inferior to 2%, ethanol yield shows a very low variation, and even less considering only wild strains. Glycerol yield varies more than ethanol, showing a variation coefficient around 25%, allowing a better differentiation of strains in a set. The same variation ranking can be observed in Nidelet *et al*. (2016) results, obtained in a similar medium using 43 strains (including 20 common to our set), with ethanol being the most constant flux, followed by glycerol and then acetate, succinate and α-KG as the most variable. This observation can be found in many other different works about CCM too. Tronchoni *et al*. (2022), performed a screening in wine-like media in aerobic conditions using 25 *S. cerevisiae* strains. Ethanol yields are lower than our results, that is consistent with aerobic conditions, but the range of variation is very similar: no great observable differences and significant differences only between strains with extreme values. Another comparable screening can be found in the work of Nieuwoudt *et al*. (2006) on 15 strains (commercial or not) and 19 hybrids. Fermentation media used are natural and synthetic laboratory must. On both media, similar results have been obtained: a higher range of variation is observable for glycerol than for ethanol. As well, Hubmann, Foulquié-Moreno, *et al*. (2013) performed a relevant screening on 52 beer and distillery *S*. *cerevisiae* strains for their ethanol and glycerol yields, on a YPD like medium. All these data present a larger diversity between strains for glycerol than for ethanol.

Even if their yield values and variations are different, literature demonstrates a clear negative correlation between ethanol and glycerol productions or yields and our data show that this correlation is visible but not obvious in a small range of yield, represented here by wild strains. The correlation is

clearer when extreme values from modified and evolved strains are considered, but three values (strains 5074, LMD14 and LMD41) are driving it.

No relation between genetic group belonging and yield of glycerol or ethanol have been found in our data. This goes against precedent observation that states wine strains are defined as high glycerol producers compared to other groups (Camarasa *et al*., 2011). Nevertheless, it is worth noting that in the study of Camarasa *et al*. (2011), groups are based on the environmental origin and our groups on genetic origin, and that these two origins don't always match (as an example, strain Y55 used to be classified as a laboratory strain isolated from a wine environment, but Liti *et al*. (2009) showed that this strain is in fact closer to a West African genetic lineage).

Globally, glycerol, considering its concentration and variation range and the strong negative correlation with ethanol yield, is confirmed to be the best candidate to impact carbon fluxes in the cell.

Succinate is produced in minor concentrations compared to ethanol or glycerol and its production doesn't seem correlated with them. However, it shows a larger diversity of yield. Succinate is one of the metabolites with the widest range of variation according to its yield, but this variation is mainly driven by exceptionally high producing strains LMD41, LMD44 and LMD43, all commercially used in bioethanol production. This result goes against the main goal of maximising ethanol production without by-products, but high succinate production can be explained by the antibacterial character of this metabolite (particularly against lactic acid bacteria). This trait has been selected over-time to limit contamination which can reduce the global yield of the bioethanol production process (the so-called "rum" group contains numerous Brazilian bioethanol strains) (Dorta *et al*., 2005; Dong *et al*., 2015). High succinate yield has been observed on wild strains with known genetic group affiliation, but two of the highest succinate producers are the commercial strains LMD43 and LMD44. Both are used in distillery context, no information about their genome is currently available and therefore they are not classified in genetic groups. If we suppose that they are potentially part of the rum genetic group, this

474 will confirm our hypothesis of a characteristic link between high succinate yield and the rum group. In
475 an interesting manner, the genetically modified strain LMD41 is both the best succinate and ethanol
476 producer of all the set. As far as we know, genetic modifications aimed only to enhance ethanol yield
477 but a side effect on succinate can be considered. This high succinate production can also arise from
478 the original strain which is already used in bioethanol production. On the other hand, wine strains are
479 very low producers of succinate, even if it can be considered as a desirable metabolite in wine,
480 positively-linked to final quality (Chidi *et al*., 2018). This reduced succinate production in the wine
481 group can be explained once again by its inhibitory effect on lactic acid bacteria, which are main actors
482 of the malo-lactic fermentation (MLF). MLF being an important step of wine making to modulate
483 acidity, a possible hypothesis is that wine yeasts have been selected, willingly or not, to be compatible
484 with MLF (Caridi and Corte, 1997; Son *et al*., 2009; Torres-Guardado *et al*., 2022).
485
486 Acetate is responsible for major off-flavour in wine, and so is subject to legal limits (Paraggio and Fiore,
487 2004; Vilela-Moura *et al*., 2008). It presents a large variation of yield among our strain set and no
488 correlation has been found with more produced metabolites ethanol and glycerol. The wine genetic
489 group shows a very low acetate yield, which is most likely a direct consequence of the selection for
490 low acetate produced in wine fermentation. In contrast, flor group strains appear to be high producers
491 of acetate, maybe due to their more oxidative metabolism (Moreno-García *et al*., 2017).
492
493 Another metabolite that showed interesting results is α-KG, especially for its link with genetic groups:
494 wine strains have a higher yield than other groups. One of the main hypotheses is that it is explained
495 by the strong relation between this metabolite and the nitrogen metabolism. Indeed, α-KG is mainly
496 used in the cell to assimilate ammonium and then synthesise glutamate. This amino acid being
497 prominent in grape must (and therefore in the synthetic must we used), α-KG is not used and simply
498 released in the medium (Avendaño *et al*., 1997; DeLuna *et al*., 2001; Camarasa *et al*., 2003; Magyar *et*
499 *al*., 2014). Glutamate synthesis uses NADPH cofactor, which needs to be regenerated subsequently.

One of the ways to produce NADPH from $NADP^+$ is the conversion of acetaldehyde to acetate (Saint-Prix *et al*., 2004). In addition, we observed that strains from the wine groups display a low acetate yield on average (if we exclude DBVPG1373 which shows abnormal values compared to the rest of the group). In the work of Nidelet *et al*. (2016), it has been observed that acetate flux in fermentation is negatively correlated to biomass synthesis, itself negatively correlated to α-KG. Even with no biomass data, the negative link between acetate and α-KG on our set confirms these results. Another explanation can be linked to the low succinate production of wine strains. This metabolite being the final step of the TCA cycle oxidative branch in fermentation conditions, α-KG is then produced and released as succinate is not.

Global tendencies in our data set are consistent with conclusions drawn in other publications, including those used to select our set of strains (Camarasa *et al*., 2011; Nidelet *et al*., 2016, Legras *et al*., 2018). Strains from the west African genetic group (which gathers strains from palm wine and other traditional african beverages making processes) and from the flor group have been identified as very high acetate and low succinate producers. Acetate shows a great diversity, larger than glycerol or ethanol, in our set, which has already been shown by Tronchoni *et al*. (2022). Many comparable tendencies exist between work of Salinas *et al*. (2012) and ours for 5 strains: EC1118, L1374, L1528, DBVPG6765 and DBVPG6044. Like in our results, DBVPG6044 is a very high producer of acetate in fermentation (the second highest in our set) and the other strains have a significantly lower production with very close values. Concerning succinate, EC1118 and DBVPG6044 show low production and DBVPG6765 a higher one. If absolute values of yield or production differ, relative differences between strains seem preserved among experiments.

The metabolite by metabolite approach highlighted interesting correlations and furthermore the PCA confirmed them and proved to be a good tool to group strains by their primary metabolites production. For wine strains, metabolic clusters on the PCA match with the genetic groups except the

strain DBVPG1373. However, although this strain belongs to the wine group, it was isolated from soil which may explain its location. PCA reveals other noticeable results: Yllc17_E5, which is quite aside in the phylogenetic tree (Legras *et al*. 2018), is very isolated in PCA results. EC1118, which is a commercial wine strain, has been identified as an intraspecific hybrid between strains from the wine and flor groups (Coi *et al*., 2017). This particularity can be directly observed in our results: EC1118 is located in the wine cluster but close to the flor cluster, and shows intermediate succinate and acetate yields. LMD32 is a commercial strain with unknown classification mainly used for wine stuck fermentation restart. In PCA, it appears very close to the flor genetic group. The hypothesis that LMD32 is genetically related to the flor group would make sense because it is supposed to be used in the same condition as EC1118, which is known to be related to flor strain and appears very close to cluster 2 where other flor strains are located. Even if it has been identified as an uncommon strain (classified in rum group, but closer to laboratory and Mediterranean oak groups in Legras *et al*. (2018) and considered as part of a "Mosaic beer" group in Peter *et al*. (2018), CBS7957 shows results that bring it close to other rum strains in PCA results.

Even if the capacity of conducting a wine-like fermentation is considerably linked to domestication and genetic origin (strains from bread or from natural environments like oak trees are most of the time unable to perform a wine-like alcoholic fermentation (Camarasa *et al*., 2011; Legras *et al*., 2018; Tapia *et al*., 2018), a complementary set of strains, wider and more balanced between genetic groups, can bring more diversity and enforce our determination of natural yield variations.

This work presents yield values of pure strain in fermentation: we used a pasteurised medium. No competition with bacteria or other yeasts can interfere in fermentation unlike in natural musts, where other species can impact primary metabolites yield (Tristezza *et al*., 2016; Ciani *et al*., 2022). The temperature used, 28°C, is common for red wine-making, but high for white wine standards which are usually fermented at colder temperatures. For strains from other genetic groups than wine, synthetic grape must can represent conditions very far from their usual environment. However, despite these

551 differences that can be overcome in scale adjustment, our methodology gives keys to identify strains
552 with good potentialities for wine-like fermentation.
553 Multiple studies have compared numerous strains for their primary metabolites production in
554 fermentation. However, our study compares strains from different genetic groups on a wine-like
555 media only focusing on complete fermentation concentrations. Here we confirm precedent
556 observations but also provide a robust comparative methodology and a data set easily usable on 51
557 strains from various genetic backgrounds. This screening helps to define and confirm the existing
558 phenotypic variations for wine fermentation products among the *S. cerevisiae* species and set the
559 potential of improvement for these traits.
560 However, we only took a look at five metabolites from primary carbon metabolism so we don't have
561 any data on notable aromatic metabolites, positively or negatively, for wine fermentation
562 (acetaldehyde, esters, higher alcohols, acetoin…). Completing this analysis with other metabolite
563 production information would enforce the clustering and reveal strain relevance for further strain
564 development projects, for wine or other fields.
565
566 To conclude, our screening answers the main question asked: a diversity, weak but significant, exists
567 in ethanol yield among the *S*. *cerevisiae* species. Larger fluxes, like ethanol or glycerol, are the most
568 constraint and not linked to genetic origins, while in contrast smaller fluxes show larger variations and
569 clear links with genetic origin. This represents improvement potentialities of wine strains for these
570 characteristics with non-GM methods (such as adaptive laboratory evolution, positive selection,
571 breeding…).
572 If the two major produced metabolites, ethanol and glycerol, are linked in their production, the yield
573 of minor metabolites is more related to the genetic background of strains which is shaped by selection
574 in a defined environment.
575

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Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

Data, scripts, code, and supplementary information availability

Supplementary data: 10.5281/zenodo.7665200

- Strain table (S1)
- Fermentation data (S2) containing: “read me” file, initial data, final data and strain list used for R analysis
- R script for data analysis and figure generation (S3)
- Final metabolite concentrations (S4) and yields (S5)

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