



Up-regulation of Retrograde Response in yeast increases glycerol and reduces ethanol during wine fermentation

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ABSTRACT

Nutrient signaling pathways play a pivotal role in regulating the balance among metabolism, growth and stress response depending on the available food supply. They are key factors for the biotechnological success of the yeast *Saccharomyces cerevisiae* during food-producing fermentations. One such pathway is Retrograde Response, which controls the alpha-ketoglutarate supply required for the synthesis of amino acids like glutamate and lysine. Repressor *MKS1* is linked with the TORC1 complex and negatively regulates this pathway. Deleting *MKS1* from a variety of industrial strains causes glycerol to increase during winemaking, brewing and baking. This increase is accompanied by a reduction in ethanol production during grape juice fermentation in four commercial wine strains. Interestingly, this does not lead volatile acidity to increase because acetic acid levels actually lower. Aeration during winemaking usually increases acetic acid levels, but this effect reduces in the *MKS1* mutant. Despite the improvement in the metabolites of oenological interest, it comes at a cost given that the mutant shows slower fermentation kinetics when grown in grape juice, malt and laboratory media and using glucose, sucrose and maltose as carbon sources. The deletion of *RTG2*, an activator of Retrograde Response that acts as an antagonist of *MKS1*, also results in a defect in wine fermentation speed. These findings suggest that the deregulation of this pathway causes a fitness defect. Therefore, manipulating repressor *MKS1* is a promising approach to modulate yeast metabolism and to produce low-ethanol drinks.

1. Introduction

Food-producing yeasts require rich balanced growth media for optimal performance. Insufficient or unbalanced nutrients can cause stress under biotechnological conditions, which leads to cell growth arrest and delayed fermentations (Orozco et al., 2019). The mechanisms that respond to nutrient abundance and starvation are called nutrient signalling pathways, which have been well-characterised under laboratory conditions for the budding yeast *Saccharomyces cerevisiae* (Conrad et al., 2014). These pathways sense the levels of each nutrient to promote or cease cell growth and stress response in a coordinated way. For instance, the presence of glucose induces cyclic AMP synthesis and Protein Kinase A (PKA) activation, which lead to cell growth and general stress repression, while nitrogen abundance activates the TORC1 complex to promote protein synthesis. Both pathways are coordinated and share common targets to achieve subtle metabolism control. Knowledge

of such pathways under laboratory conditions is useful for understanding industrial yeast's behaviour, but differences in growth media and conditions must be taken into account. A phenomic analysis of a wine strain carrying deletions in the key genes of different signalling pathways has pointed out the relevance of PKA in wine fermentation and the relations between carbon and nitrogen repression mechanisms (Vallejo, Peltier, et al., 2020). However, such pathways' behaviour cannot be taken for granted in industrial environments because early induction during the grape juice fermentation of the markers that are usually linked with carbon and nitrogen starvation, despite no nutrient shortage existing, has been observed (Vallejo, Matallana, et al., 2020).

Retrograde Response (RR) is a pathway that reacts to mitochondrial status and includes a subset of genes that are coordinately expressed and involved in the biosynthesis of the tricarboxylic acid cycle (TCA cycle) intermediate α -ketoglutarate (da Cunha et al., 2015; Jazwinski, 2013). This is a main node for amino acid synthesis because it is used by

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glutamate dehydrogenase to yield glutamic acid, which is the main point for ammonium incorporation. RR does not control all mitochondrial TCA enzymes, only those from oxaloacetate to α -ketoglutarate, plus other enzymes that produce precursors in the cytoplasm, and peroxisomes like cytosolic pyruvate carboxylase *PYC1* and peroxisomal citrate synthase *CIT2*. Their transcriptional activation happens through transcription factors Rtg1/3 when mitochondrial function is low or glutamic acid is scarce. Upstream regulation depends on an activator, Rtg2, and a repressor, Mks1, which interact. Mks1 is described as a pleiotropic negative transcriptional regulator for being originally described as a negative regulator in Ras-cAMP signalling (Matsuura and Anraku, 1993). As it was clearly identified later as a TORC1 complex target (Dilova et al., 2002), it clearly coordinates different signals to better control amino acid metabolism. A transcriptomic analysis has shown the tight control of lysine biosynthetic genes by *MKS1* because most of the enzymes in the pathway from α -ketoglutarate to lysine are up-regulated in the *MKS1* deletion mutant (Dilova et al., 2002).

Understanding metabolism regulation could be useful for improving the production of metabolites of interest (Cambon et al., 2006). Efforts are being made to reduce ethanol content in wine in line with health concerns and consumer preferences, but also because global warming leads to higher sugar levels when grape berries reach maturity, which result in undesirable unbalances of high ethanol and low acidity (Jones et al., 2005). Diverting the glycolytic flux from ethanol to glycerol is one of the best approaches to lower alcoholic levels. Glycerol is involved in maintaining redox balance and is also an osmolyte that prevents hyperosmotic shock. Besides, glycerol is a molecule that has been reported to confer the body unctuousity and helps the perception of other aromas in wine, and also has a positive impact on beer-making (Zhao et al., 2015). Glycerol is produced by diverting some of the triose dihydroxyacetone-P from glycolysis into glycerol-3 P through the action of glycerol-3 P dehydrogenase. That process consumes NADH insofar as the net output becomes a reduced fermentative flux and eventually reduction in ethanol occurs. Overexpression of *GPD1*, the main enzyme that produces glycerol, achieves the expected phenotype. Yet due to redox unbalances, this modification leads to increased acetic acid, which is not a desirable trait in fermented beverages (Remize et al., 1999). Further deletion of acetic acid-producing enzymes is, therefore, required (Cambon et al., 2006). In this work, a deletion of RR repressor *MKS1* increased glycerol without further increasing acetic acid under wine-making conditions. Ethanol also reduced as a direct consequence. A similar output in glycerol production was seen in other strains of biotechnological interest, like brewing and baking yeast. So apparently conserved regulatory mechanism can be manipulated to modulate the amount of carbon fluxes during industrial fermentations.

2. Materials and methods

2.1. Yeast strains and laboratory growth media

Haploid wine strain C9 was a gift from M. Walker (Walker et al., 2003). Wine commercial strains EC1118, T73, 71B and M2 come from Lallemend Co. (Canada). Brewing yeast SafAle US-05 is from Fermentis (Lesaffre, France). Baking yeast Cinta Roja is from AbMauri (Spain). The whole *MKS1* gene was deleted from C9 by homologous recombination using the *kanMX* marker, which was PCR-amplified from the pUG6 plasmid, a gift from J.H. Hegemann (Guldener et al., 1996), using oligonucleotides with 40 bp homology to the flanking regions described in Supplementary Table 1. The complete *RTG2* ORF was deleted from the T73 strain by the same procedure. The *MKS1* CRISPR-Cas9 deletions of the diploid strains were made using plasmid pRCC-K with the *kanMX* marker, which was a gift from Eckhard Boles (Addgene plasmid # 81191) (Generoso et al., 2016). The gRNA for the *MKS1* gene was introduced by PCR into pRRC-K plasmids using primers *MKS1*-Fw and *MKS1*-Rv (Supplementary Table 1) after transformation into *Escherichia coli* competent cells NZY α (NZYTech, Portugal) to obtain the

pRCC-K-g*MKS1* plasmid. Yeast transformations were done by the lithium acetate method (Gietz and Woods, 2002). For the CRISPR-Cas9 deletions, pRCC-K-g*MKS1* was co-transformed with a long oligo (*MKS1* donor) to help to recombine the cut made by Cas9. Gene deletions were checked by PCR with the primers in the promoter (c), ORF (d) and terminator (e) of the gene of interest (Supplementary Table 1).

Yeasts were by default propagated in rich YPD medium (1% yeast extract, 2% bactopectone, 2% glucose). YPS and YPM changed the carbon source by 2% sucrose and 2% maltose, respectively. Solid plates contained 2% agar, and 20 μ g/ml of geneticin for the selection of *kanMX* transformants if required. Minimal medium SD contained 0.17% yeast nitrogen base, 0.5% ammonium sulphate and 2% glucose (Adams et al., 1998). For the growth spot analysis, serial dilutions from stationary cultures in YPD were made and 5 μ l drops were placed on selective media. Growth curves were obtained on a 96-well plate with 200 μ l of each media and inoculated an OD₆₀₀ of 0.1 from a stationary culture in YPD. Plates were read with a Multiskan (Thermo Scientific) plate reader with shaking.

2.2. Fermentations

C9*mks1* Δ standardised fermentations were carried out in a Sauvignon Blanc grape must by the previously described method (Peltier et al., 2018). The 20 ml screwed vials were tightly closed with a screw cap and silicone/PTFE stoppers, and were filled with 11.5 ml of grape must inoculated with 2×10^6 cell/ml, and hypodermic needles were inserted for CO₂ release. The fermentation temperature was maintained at 24 °C by an incubator. Vials were kept static or shaken at 175 rpm throughout fermentation using an orbital shaker. The fermentation kinetics was estimated by manually monitoring (2–3 times/day) the weight loss caused by CO₂ release on a precision balance. The amount of CO₂ produced according to time was modelled to estimate the kinetics parameters: the maximal amount of released CO₂ (CO₂max in g.L⁻¹), the lag phase (lp in h), the time to release 35%, 50% and 80% of the maximal expected CO₂ after subtracting the lp (t35-lp, t50-lp and t80-lp in h), and the average hexose consumption rate between 50% and 80% of CO₂max (V50.80 in g.L⁻¹.h⁻¹). The concentrations of organic metabolites acetic acid, glycerol, malic acid, pyruvate and total SO₂ were measured at the end of fermentation using the respective enzymatic kits (Megazyme, Bray, Ireland) following the manufacturer's instructions. Glucose and fructose were enzymatically assayed (Stitt et al., 1989).

The CRISPR-Cas9-edited commercial wine strains were grown in bobal red grape juice (Bodegas Murviedro, Requena, Spain) in conical tubes with 30 ml of juice at 24 °C (Orozco et al., 2012). Colony Forming Units (CFU) was followed by plating serial dilutions on YPD plates and counting colonies. Reducing sugars were measured with DNS (dinitro-3, 5-salicylic acid) according to Miller's method (Robyt and Whelan, 1972). Ethanol, glycerol, acetic acid and lactic acid were spectrophotometrically measured by the coupled enzymatic reactions linked with the NAD⁺/NADH redox pair using commercial kits (Megazyme Ltd, Bray, Ireland, www.megazyme.com; the K-ETOH, K-GCROL, K-ACET and K-LATE kits). Ethanol was measured by following NADH production by alcohol dehydrogenase. Glycerol was phosphorylated by glycerokinase to the glycerol-3-P that pyruvate kinase transformed into pyruvate, a substrate of lactate dehydrogenase, which consumes NADH to produce lactate. Acetic acid in the presence of ATP and CoA yields acetyl-CoA by acetyl-CoA synthetase action. This acetyl-CoA, plus oxaloacetate, is transformed into citrate by citrate synthase, and oxaloacetate is made from malate by malate dehydrogenase to produce NADH. Lactic acid was transformed directly by lactate dehydrogenase to produce NADH.

For brewing fermentations, a wort-like medium consisting of 180 g/l granulated malt supplemented with 10 g/l yeast extract was used (García Sanchez et al., 2012). Then 15 ml of wort were used to fill screw-cap vials like those explained above at 25 °C at 100 rpm. Fermentation was followed by weight loss. For baking yeast, a model

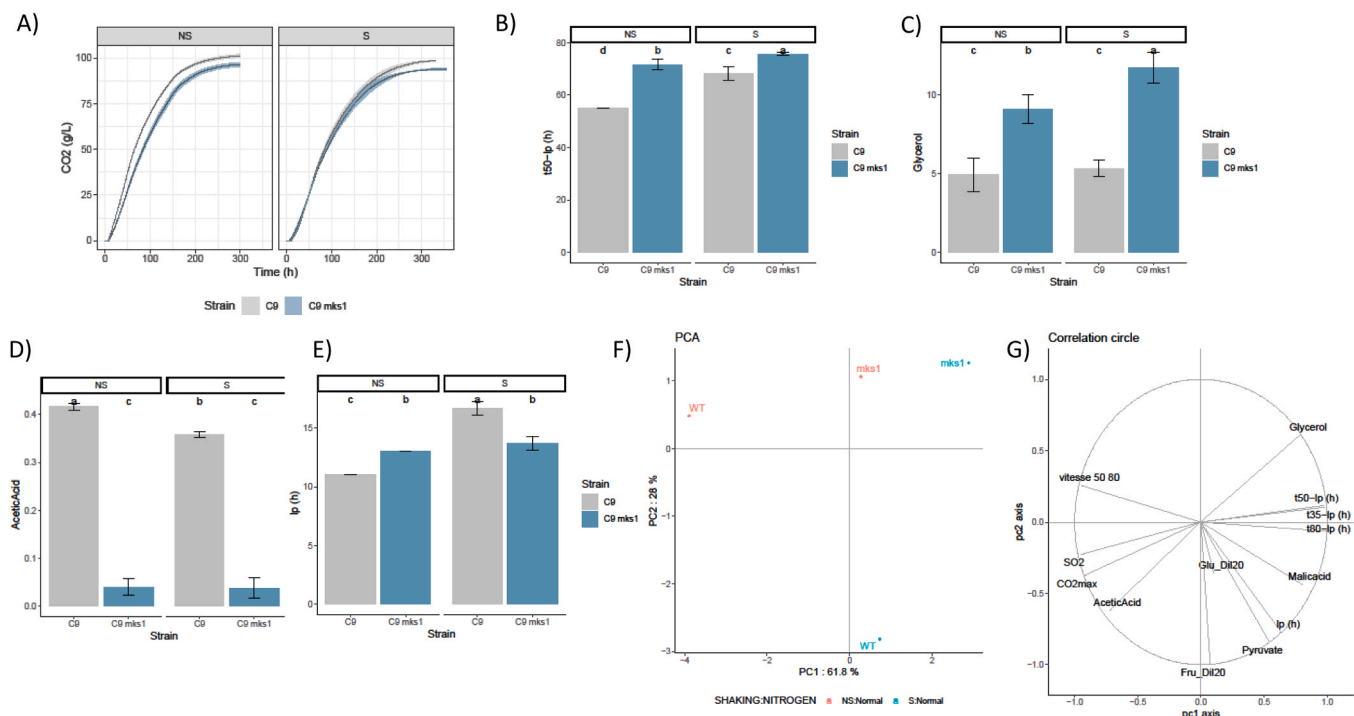


Fig. 1. *MKS1* deletion impacts yeast metabolite production by haploid wine yeast during microfermentations in white grape juice. A) Fermentation kinetics of strains C9 and C9 *mks1*Δ followed as CO₂ production under static (NS) and shaking (S) conditions. B) Time to reach 50% CO₂ production, minus the lag phase. C) Glycerol production at the end of fermentation. D) Acetic acid production at the end of fermentation. E) Lag phase. F) The PCA analysis of kinetic and metabolic parameters of both strains under both conditions. G) Correlation circle. The shown values are the means of three replicates.

liquid dough was used (Panadero et al., 2005) and was inoculated at 30 mg (dry weight) per ml. Fermentation was done in Erlenmeyer flasks at 30 °C with slight shaking (80 rpm).

3. Results

3.1. *MKS1* deletion increases glycerol and reduces acetic acid during winemaking

A phenomic analysis of the nutrient signalling pathways has been previously done by analysing multiple deletions on haploid wine strain

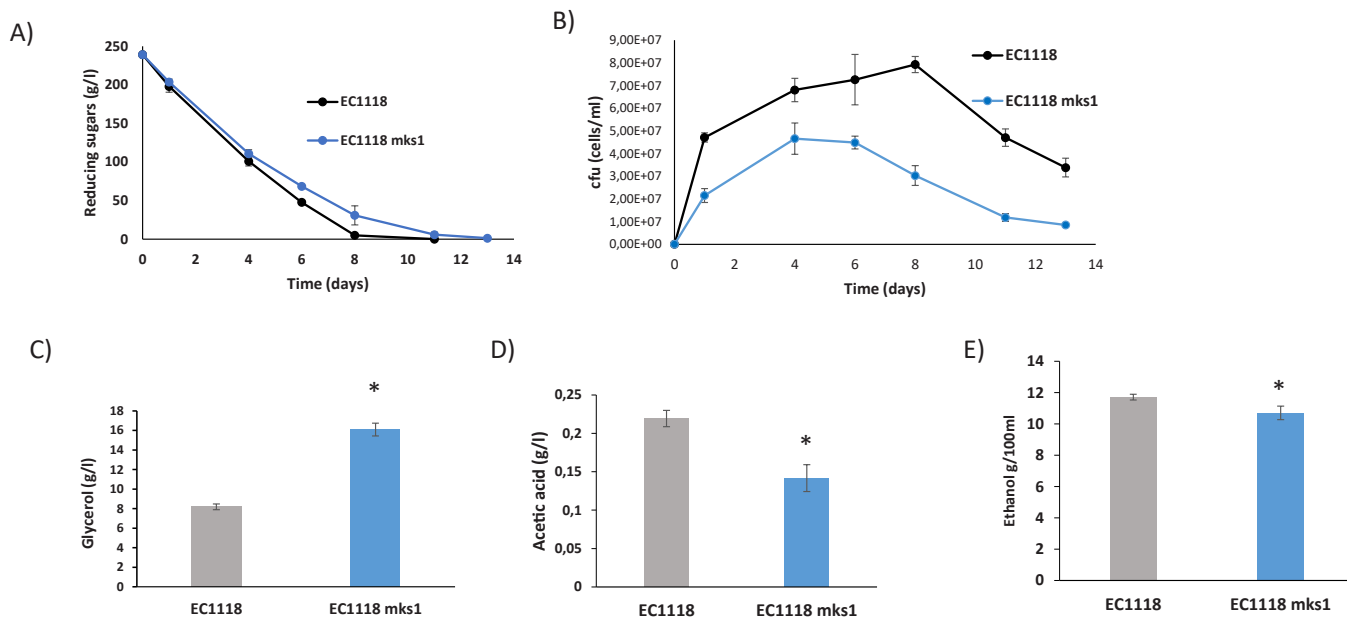


Fig. 2. A CRISPR-Cas9-edited commercial wine yeast lacking *MKS1* increases glycerol and decreases ethanol production. EC1118 and EC1118 *mks1*Δ were grown in red grape juice. A) Total sugar consumption. B) Cell viability measured as CFU/ml. C) Glycerol production at the end of fermentation. D) Acetic acid production. E) Ethanol production. The shown values are the means of three replicates. Error bars represent standard deviation (* $p \leq 0.05$, two-tailed t-test).

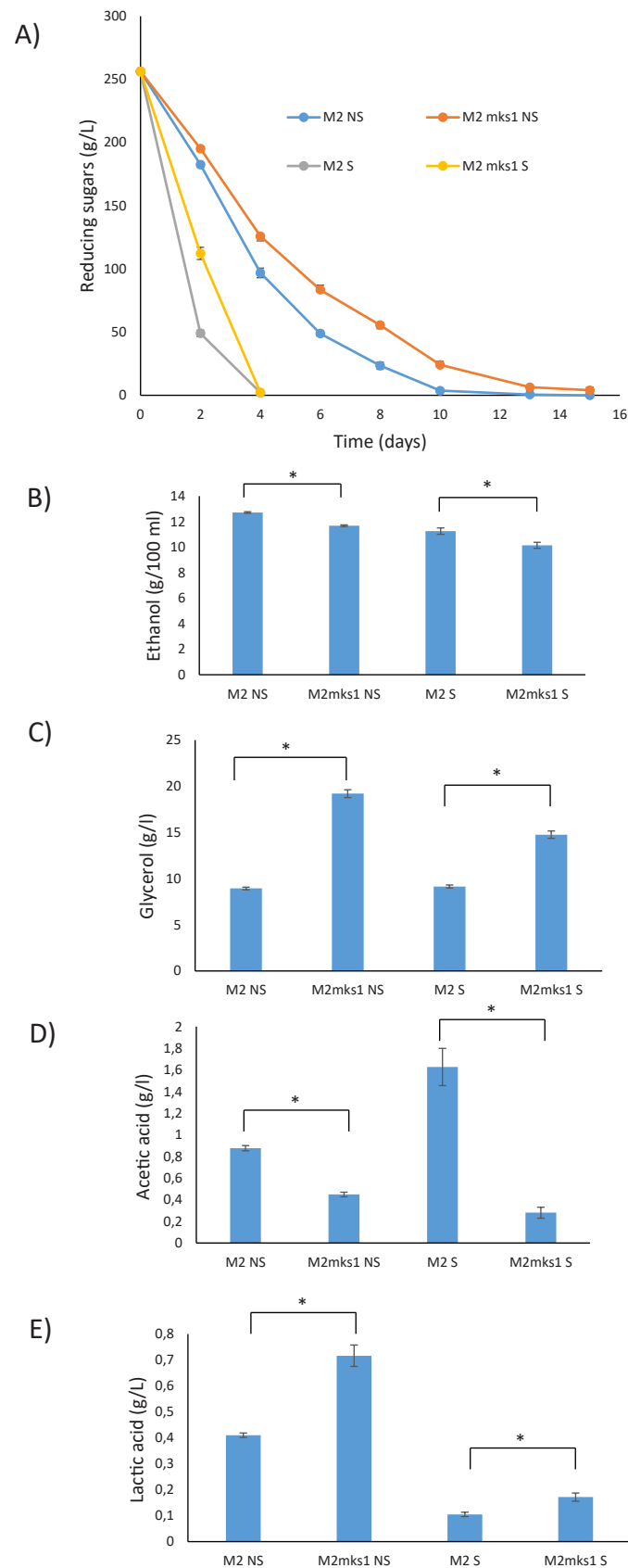


Fig. 3. Full aeration in the presence of *MKS1* deletion does not produce high acetic acid levels. M2 and M2 *mks1*Δ grown in red grape juice in flasks kept static (NS) or vigorously shaken (S). A) Reducing sugars consumption during fermentation. B) Ethanol production at the end of fermentation. C) Glycerol production. D) Acetic acid production. E) Lactic acid production. The shown values are the means of three replicates. Error bars represent standard deviation (* $p \leq 0.05$, two-tailed t-test).

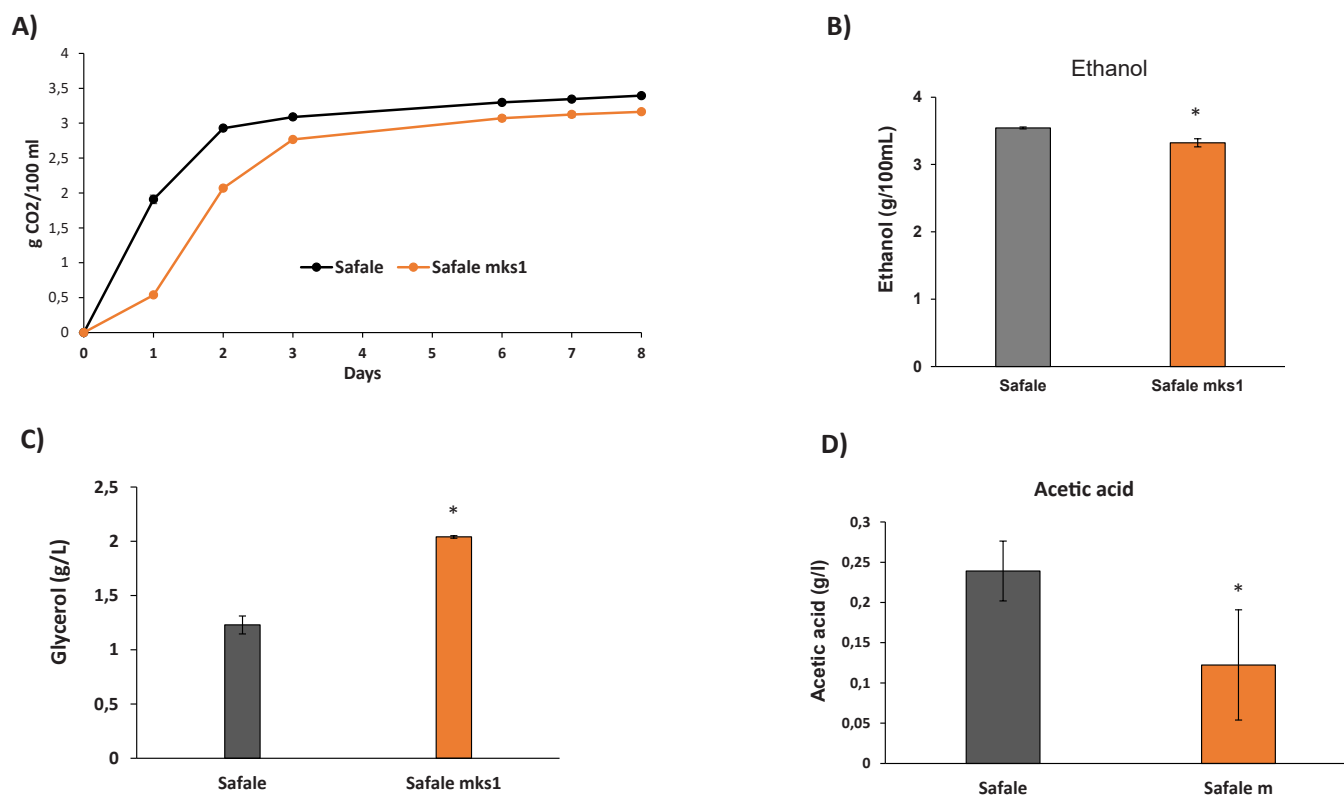


Fig. 4. *MKS1* deletion produces high glycerol levels in brewing yeasts. SafAle US-05 and its M2 *mks1*Δ derivative were grown in malt. A) Weight loss during fermentation. B) Ethanol production at the end of fermentation. C) Glycerol production. D) Acetic acid production. The shown values are the means of three replicates. Error bars represent standard deviation (* $p \leq 0.05$, two-tailed t-test).

C9 (Vallejo, Peltier, et al., 2020). A mutation in RR repressor *MKS1* was performed and tested during the same standardised microfermentations using white grape juice, and by comparing static conditions (NS; no shaking) and shaking conditions (S) to analyse the effect of aeration. Fermentation progress was followed by weight loss (Fig. 1A). The fermentation profile was similar between both conditions, with a slightly slower fermentation speed for the *mks1*Δ strains. This effect was more evident from the beginning under the non-shaking conditions. For instance, the time to reach 50% of CO₂ production minus the lag phase is always longer (Fig. 1B), being lag phase the time observed before the release of the first 2 g·L⁻¹ of CO₂ (Peltier et al., 2018). Metabolites of oenological interest were measured at the end of fermentation. *MKS1* deletion led to an increase in final glycerol (Fig. 1C) and a reduction in acetic acid (Fig. 1D) under both conditions. More glycerol was produced by the mutant strain under the shaking conditions. The mutation-conditions interaction was estimated, and the lag phase time was the parameter that contributed the most to the strain/shaking combination to whole variance, 37% (Fig. 1E). The start of fermentation was delayed under the static conditions, but reduced under the shaking conditions. This finding indicates that this pathway is responsive to oxygenation and influences the start of growth. Fig. 1F shows the Principal Component Analysis (PCA) plot that illustrates the mutants group together, and the correlation circle (Fig. 1G) denotes that their influence on glycerol production contributed to this fact.

3.2. Role of *MKS1* gene deletion in commercial wine yeast strains

As the phenotype caused by *MKS1* deletion was interesting, it was tested under different conditions. Firstly, instead of a haploid derivative, *MKS1* was deleted in a commercial diploid strain, EC1118. This was done by developing a CRISPR-Cas9 approach that enabled us to delete both copies with a single transformation. Having done this,

microvinifications were carried out in natural grape juice. Red must was used this time to test additional growth conditions in open filled-in conical tubes. This allowed to take samples during the process to measure sugar metabolism and to analyse cell viability by plate counts (Fig. 2). Fermentation progress was measured by monitoring reducing sugar consumption (Fig. 2A). Once again, the mutant strain was slower in fermentation speed terms, but eventually reached completion on day 13 versus the 11 days recorded for the parental strain. Regarding cell viability, the mutation caused a delay in growth and a lower maximum cell count (Fig. 2B) suggest that slower fermentation was due to a smaller cell number, and not to lower fermentative power per cell. After reaching its maximum, cell viability decreased in both strains so that no big differences in the chronological life span were observed. Metabolites were measured at the end of fermentation (day 11 for EC1118 and day 13 for EC1118 *mks1*Δ). Once again, the mutation caused a significant increase in glycerol (Fig. 2C), with a lower acetic acid level (Fig. 2D). The initial fermentations with the C9 strain did not allow a standardised ethanol measurement. In this case however, EC1118 *mks1*Δ produced a significant reduction in the final ethanol level, with a reduction of 1 g/100 ml, which was a 9% less than that produced by parental strain EC1118 (Fig. 2E). The yields per gram of consumed reducing sugars are indicated in Supplementary Table 2. Therefore, a diversion in the glycolytic flux from ethanol to glycerol was apparently caused by this gene deletion. As this is a very interesting phenotype with a biotechnological potential, the same CRISPR-Cas9 construct was used to delete the existing copies of the *MKS1* gene in three other commercial strains: T773, 71B and M2. Fermentations were carried out as previously described in red grape juice (Supplementary Figure S1). These strains displayed differences in the fermentation speed measured by reducing sugar consumption (Supplementary Figure S1A). Although the *MKS1* mutants always showed slower fermentation, they all reached completion. Once more, the final ethanol production significantly reduced

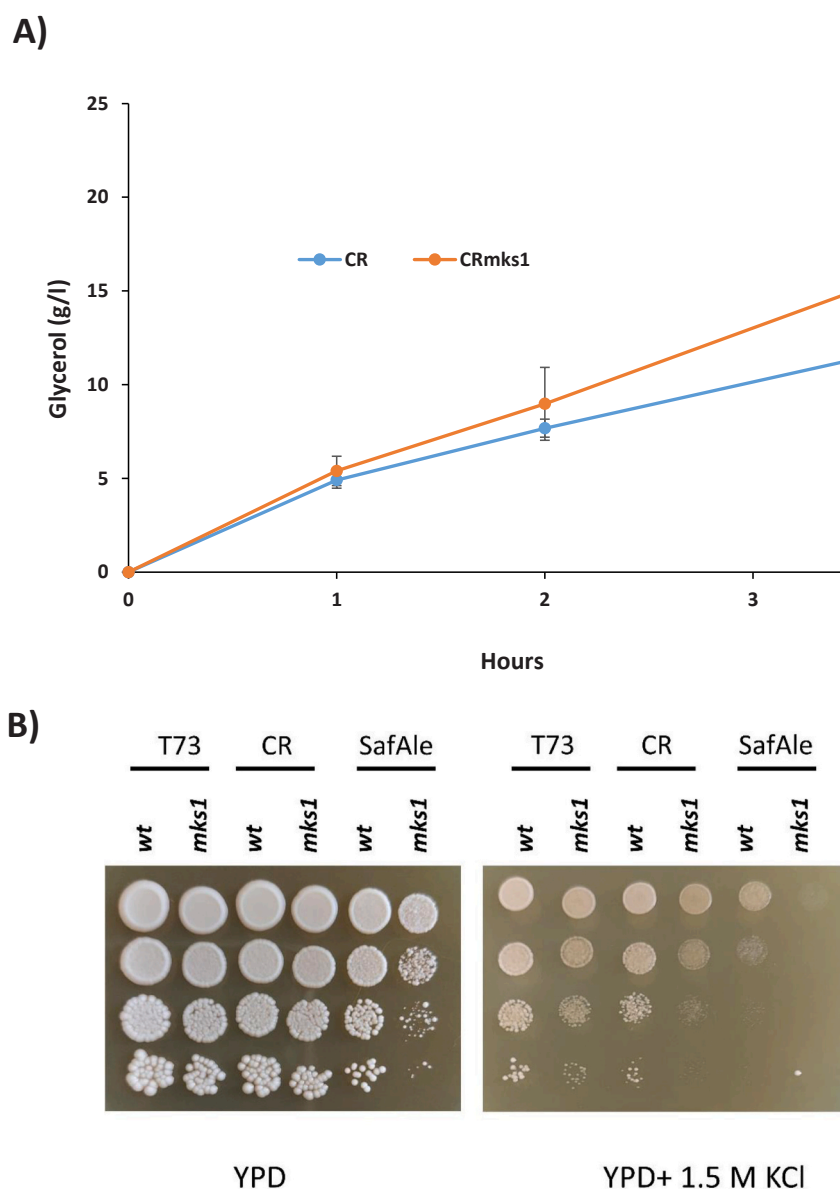


Fig. 5. *MKS1* increases glycerol during baking. A) Glycerol production in a liquid dough medium by Cinta Roja and its *mks1Δ* derivative. The shown values are the means of three replicates. Error bars represent standard deviation. B) Hyperosmotic tolerance on plates with 1.5 M KCl of the deletion mutants of *MKS1* in wine (T73), baking (CR) and brewing (SafAle) yeasts.

(Supplementary Figure S1B) by 9% for the T73 and 71B genetic backgrounds, and by 10% for M2. For glycerol (Supplementary Figure S1C), a rise in the metabolite concentration was observed. Acetic acid (Supplementary Figure S1D) decreased in all cases, particularly in the M2 genetic background. Therefore, the role of *MKS1* is consistent in all the tested genetic backgrounds and is compatible with glycerol overproduction at the expense of ethanol yields.

In order to test the role of oxygen in the production of those metabolites of oenological interest, a simple approach was applied using Erlenmeyer flasks and comparing static fermentation under the shaking condition, where oxygenation was increased and respiratory metabolism was allowed (Fig. 3). The M2 strain and its *MKS1* null mutant derivative were employed as an example given that it was the strain with a more marked ethanol reduction. For the shaking condition, reducing sugars were metabolised very quickly compared to the non-shaking condition (Fig. 3A). Once more, the mutant strain showed delayed sugars consumption (Fig. 3A). As expected, the full aerobic condition led to lower ethanol levels, but once again, the mutant strain displayed

decreased ethanol production compared to the wild-type strain (Fig. 3B). Similar glycerol levels were produced by parental strain M2 regardless of shaking and, once more, the *MKS1* deletion mutant increased this product under this condition, but to a lesser extent under the shaking condition (Fig. 3C), perhaps due to the diversion of reducing equivalents to mitochondrial functions. Aeration caused high acetic acid production for wild-type strain M2 (Fig. 3D). However, this was reduced in M2 *mks1*, and even dropped to lower levels for this mutant under static conditions and resulted in greater overall reduction (> 5-fold). Retrograde Response controlled D-lactic acid production and this aspect was also measured (Fig. 3E). In this case, shaking decreased strongly lactic acid for the wild-type strain. Under both conditions however, *MKS1* gene deletion led to an increase in this organic acid, but that phenotype was not a common feature in all the genetic backgrounds. For instance, it was seen under static conditions in the 71B strain, but not in the T73 strain (data not shown).

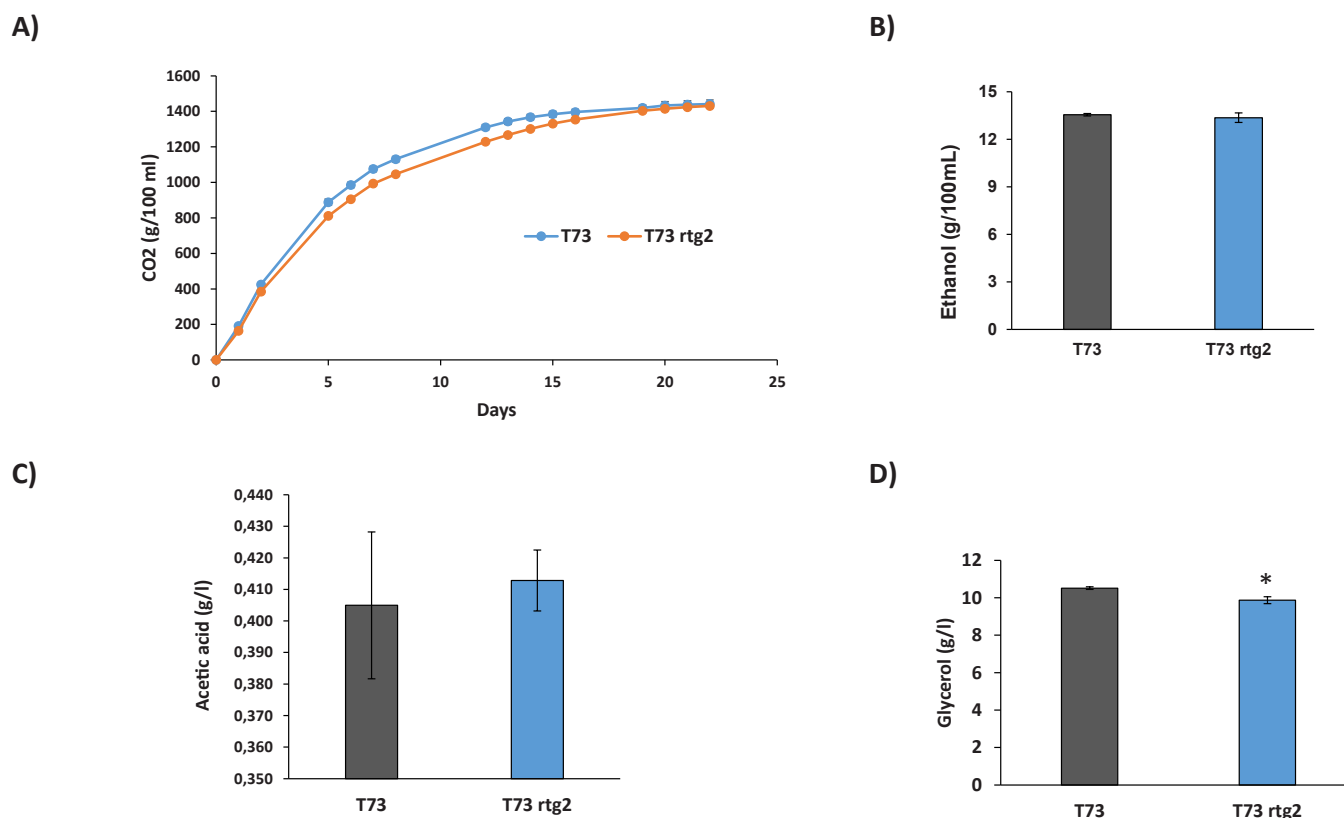


Fig. 6. *RTG2* deletion has not big impact on wine fermentation. Strains T73 and T73 *rtg2*Δ were grown in red grape juice. A) Fermentation progress measured as weight loss. B) Ethanol production at the end of fermentation. C) Acetic acid production. D) Glycerol production. The shown values are the means of three replicates. Error bars represent standard deviation (* $p \leq 0.05$, two-tailed t-test).

3.3. *MKS1* role in brewing and baking yeasts

As *MKS1* deletion has an impact on relevant metabolites during winemaking, it may affect the performance of other yeasts when manufacturing other fermented foods. To test this notion, *MKS1* was deleted from brewing yeast, Safale US-05 (Fig. 4). To test its influence during malt fermentation, the small vials used for standardised vinifications (see Fig. 1) were used, and fermentation progress was followed by weight loss measurements (Fig. 4A). *MKS1* deletion caused a delay in the start of brewing and its overall profile was quite different. The mutant strain did not reach the same levels as the parental strain. The ethanol measurement taken on day 8 (Fig. 4B) indicated that the mutant strain actually produced less ethanol than the parental strain. As growth was slower in the mutant, those levels could reflect that fermentation had not completely finished. Glycerol (Fig. 4C) and acetic acid (Fig. 4D) were also measured. Glycerol markedly increased, which also occurred during winemaking. So it would seem that there was also (at least) a partial diversion from the glycolytic flux from ethanol to glycerol. Acetic acid also reduced, which also happened during grape juice fermentation seeing that the impact of *MKS1* on metabolism seemed to be similar regardless of the employed genetic background and fermentation substrate.

MKS1 gene deletion in baking strain Cinta Roja was tested in media called Liquid Dough, which mimics sweet dough (Panadero et al., 2005). This involves easy inoculation and sample collection. As breadmaking does not involve long times and complete sugars exhaustion is not expected, samples were taken after inoculation over a 4-hour period and glycerol was measured as a marker of the *MKS1* deletion impact on fermentation (Fig. 5A). As expected, glycerol accumulated in the medium as fermentation progressed. The increase in the *mks1*Δ strain was bigger, which indicates that this mutation would also increase glycerol

under a laboratory-scale baking condition. Glycerol is produced to keep the redox balance, but is also a compatible osmolyte that prevents hyperosmotic stress, a critical feature in the high-osmolarity environment of dough. The *MKS1* deletion in all the three industrial strains was tested during a spot test run on plates with high osmolarity (1.5 M KCl, Fig. 5B). In wine strain T73, *MKS1* deletion did not improve stress tolerance, and colonies were even slightly smaller under stress. This negative effect became even more evident in baking strain Cinta Roja. *MKS1* deletion was more deleterious in brewing strain SafAle by causing a growth defect even under non-stressful conditions. In any case, *MKS1* did not lead to any relative improvement under hyperosmotic shock. Therefore, RR activation by *MKS1* deletion does not improve osmotic stress tolerance under standard laboratory conditions.

3.4. Role of *RTG2* in wine yeasts

Given the relevant yeast performance shown by *MKS1*, the deletion of the activator of the RR pathway *RTG2* was performed in wine yeast. Fermentation was followed in microvinifications like those depicted in Figs. 1 and 4 by measuring weight loss (Fig. 6A). Fermentation was slightly delayed in the mutant strain, but reached completion (no reducing sugars were detected at the end time; data not shown). Therefore, *RTG2* deletion did not exhibit the opposite effect of *MKS1* deletion in fermentation speed terms, which indicates that balanced RR is required for optimal yeast performance. There were no significant variations in either the final ethanol (Fig. 6B) or the acetic acid levels (Fig. 6C). There was a very small, albeit significant, decrease in glycerol production (Fig. 6D), which could be expected for an *MKS1* antagonist, but not to the extent that *MKS1* had accomplished. Therefore, *Mks1* may have a wider range of targets than *Rtg2*, and *RTG2* deletion is not useful for influencing the production of metabolites of oenological interest.

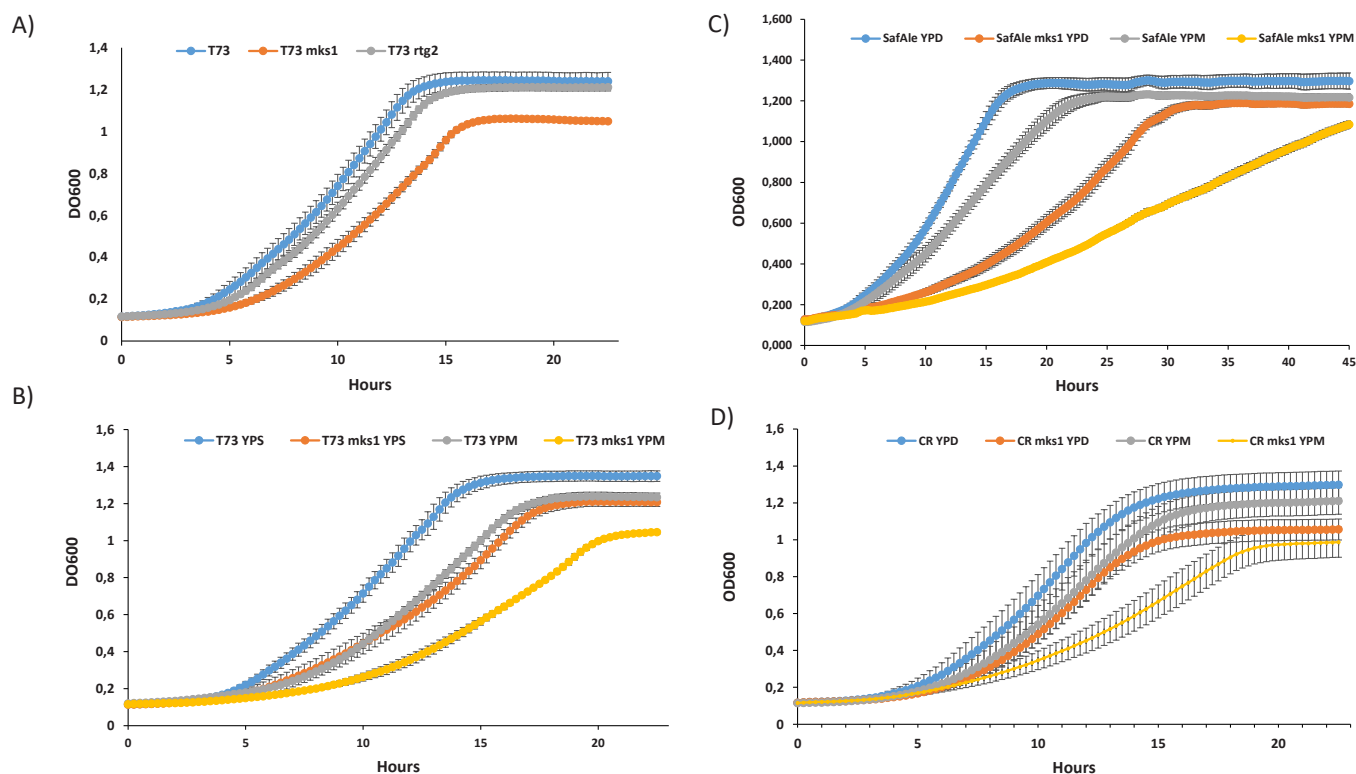


Fig. 7. *MKS1* impacts growth in several carbon sources. Growth curves in a 96-well microplate reader were obtained using rich media with different carbon sources: glucose (YPD), sucrose (YPS) and maltose (YPM). A) Growth curves of wine yeast T73 and mutants T73 *mks1*Δ and T73 *rtg2*Δ in YPD. B) Growth of strains T73 and T73 *mks1*Δ in YPS and YPM. C) Growth of the *MKS1* deletion in brewing yeast SafAle US-05 in YPD and YPM. D) Same as C for the CintaRoja baking strain. The shown values are the means of three replicates. Error bars represent standard deviation.

3.5. *MKS1* deletion impairs growth in a variety of carbon sources

As the *MKS1* deletion mutant had low fermentative power in several industrial media and reduced cell proliferation during grape juice fermentation, growth curves were performed in rich media with different carbon sources. The aim of this experiment was to determine if this effect was due to the harsh conditions of industrial growth media or to an intrinsic condition of *MKS1* deletion regarding specific carbon source assimilation. Firstly, standard rich media YPD, which contain glucose as a sole carbon source, were used to test the deletions of genes *MKS1* and *RTG2* in wine strain T73 (Fig. 7A). The *rtg2*Δ strain exhibited delayed growth, but eventually reached a similar saturation optical density (OD) as the parental strain. However, the *MKS1* deletion strain did not reach the saturation level and its growth speed was clearly slower. Therefore, *Mks1* is required not only in the exponential phase, but to also enter the stationary phase. The role of *MKS1* was tested in two disaccharides of industrial relevance: sucrose, YPS (the main carbon source of molasses, in which yeasts biomass is obtained); maltose, YPM (the main carbon source in brewing and baking) (Fig. 7B). Maltose is a worse carbon source than sucrose for this wine yeast, which is not surprising considering that the commercial wine yeast biomass is grown in molasses rich in sucrose. In both cases, the pattern caused by *MKS1* deletion was similar: slower growth speed and lower maximum cell density for the mutant strain. Therefore, intrinsic growth deficit occurred in the *mks1*Δ strain regardless of the carbon source. Once again, *RTG2* deletion had a minor impact on these growth media (data not shown). The *MKS1* deletion in brewing yeast SafAle was tested in two carbon sources: YPD and YPM (Fig. 7C). Maltose is relevant in brewing and the parental strain is best adapted to grow in that medium than wine yeast. However as expected, glucose was the optimal carbon source. Once more, *MKS1* mutation slowed down growth speed and lowered OD at saturation in both media. So the effect of *MKS1* on growth

seemed to be independent of the genetic background. Similar results were obtained for baking strain Cinta Roja under the same conditions (Fig. 7D).

3.6. *MKS1* deletion impacts amino acid biosynthesis

In order to prove the effect of *MKS1* deletion on amino acid metabolism, chemical inhibitors were used. Retrograde response increases the synthesis of α-ketoglutarate, which is channelled to glutamate and lysine biosynthesis. For this reason, *MKS1* was originally dubbed as *LYS80*. Its deletion increases tolerance to a lysine-toxic analogue, 2-aminoethylcysteine (Feller et al., 1997). The mutations in *MKS1* and *RTG2* in the wine strain were tested during a spot analysis in the presence of this compound (Fig. 8a). The phenotype was compatible with Retrograde Response regulation, the *MKS1* mutant was more tolerant to the compound and *RTG2* was more sensitive. The same pattern for *MKS1* deletion was seen in the SafAle brewing genetic background (Fig. 8B) and in baking strain Cinta Roja (not shown). Therefore, the up-regulated synthesis of this mutant was a conserved feature across all the tested industrial yeasts. To test if this was a specific effect on some biosynthetic pathways and not a complete increase in amino acid biosynthesis, other toxic amino acid analogues were used (Fig. 8A). 3-aminotriazole inhibits His3 and histidine biosynthesis. Ethionine is a toxic analogue of methionine and canavanine is one of arginine. *MKS1* mutation does not increase tolerance to these compounds, nor does *RTG2* deletion significantly decrease it (as *RTG2* deletion *per se* has a growth defect on minimal medium, it may target other pathways). Sulfometuron methyl inhibits the synthesis of branched amino acids valine and isoleucine in mitochondria; in this case, there is a growth defect in the *MKS1* deletion mutant, but *RTG2* deletion has a negligible effect. The toxic effect on *MKS1* was slightly relieved by adding valine, and more so by the valine+isoleucine combination (isoleucine alone had a negligible effect).

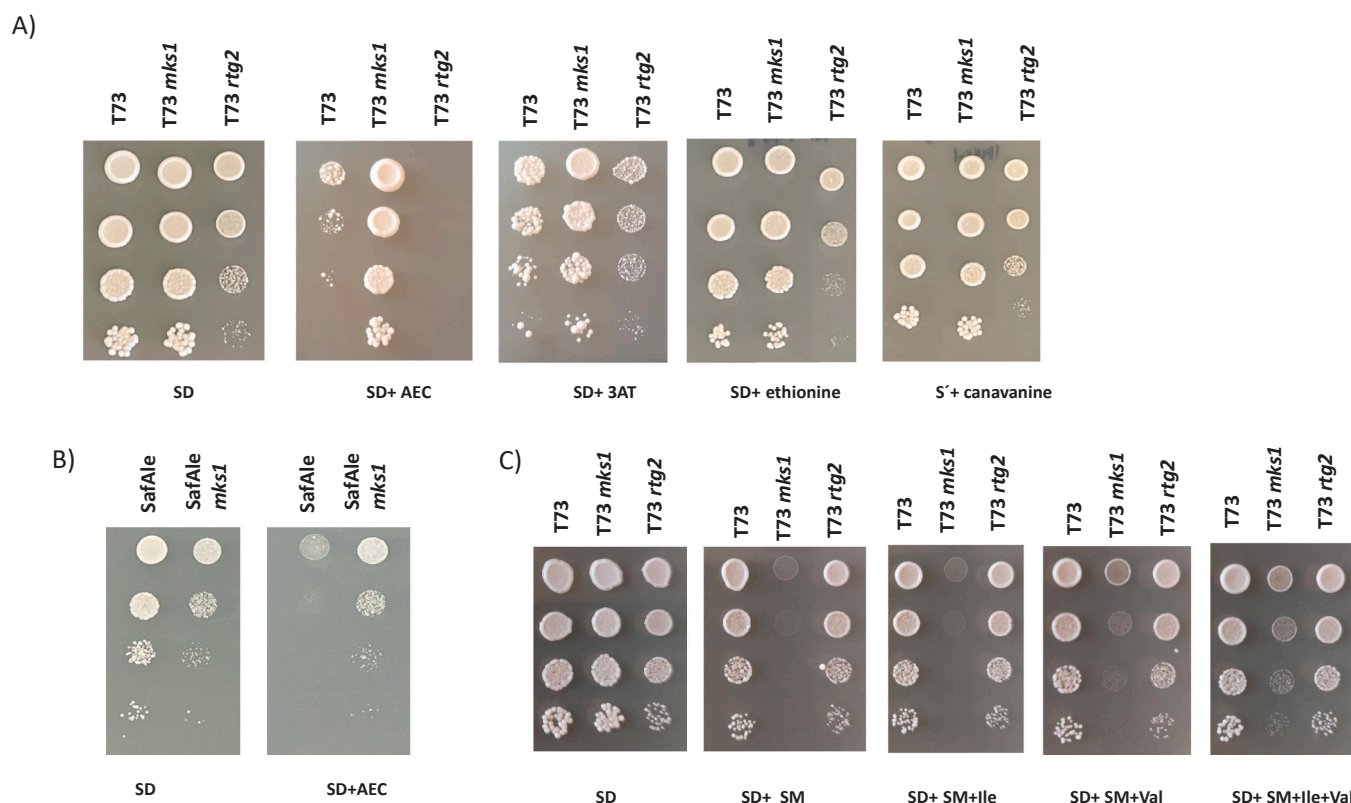


Fig. 8. *Mks1* plays a role in the biosynthesis of a subset of amino acids. Spot analysis of minimal medium plates using inhibitors of amino acid biosynthesis. A) Wine yeast T73 and mutants T73 *mks1*Δ and T73 *rtg2*Δ were tested against aminoethylcysteine (AEC), 3-aminotriazole (3AT), ethionine and canavanine. B) SafAle with *MKS1* deletion was tested for AEC tolerance. C) Wine yeast T73 and mutants T73 *mks1*Δ and T73 *rtg2*Δ were grown in the presence of sulfometuron methyl (SM) alone or in the presence of branched amino acid valine (Val) or isoleucine (Ile).

Thus, as expected, it targeted branch amino acids, perhaps by diverting precursors in mitochondria to other amino acids. Therefore, *MKS1* deletion impacts specific branches of amino acid metabolism and has no global effect on nitrogen metabolism.

4. Discussion

Nutrients are the guiding force that lies behind the biotechnological processes carried out by microorganisms. A balanced growth medium guarantees the correct performance of the yeast *Saccharomyces cerevisiae* during its industrial tasks. The plant-derived substrates used for food production tend to be rich in carbon sources (mainly in the form of monosaccharides and disaccharides), but are poorer in nitrogen sources. The industrial processes that aim to achieve complete carbon source consumption, i.e. winemaking, depend more on the presence of enough ammonium and amino acids to fulfil yeasts' requirements. This is why an exogenous nitrogen source sometimes needs to be added to grape juice. Despite helping fermentation progress, this addition can, however, cause an alteration to the aroma profile, which largely depends on amino acid metabolism (Gobert et al., 2019). Beet and cane molasses used for biomass propagation also require the addition of inorganic nitrogen to compensate for excess sucrose (Pérez-Torrado et al., 2015). Regulation of both metabolism and growth is achieved with nutrient signalling pathways (Conrad et al., 2014). One of them is the Retrograde Response pathway, which channels carbon backbones from glycolysis to amino acid synthesis (Jazwinski, 2013), and so it lies at the core of the carbon/nitrogen relation. In this study, we focused on RR repressor, *Mks1*. Its deletion slowed down wine fermentation speed in several genetic backgrounds, and in both red and white grape juices, and also caused a growth defect in different carbon sources in brewing and baking strains. The impact on the fermentative performance of *MKS1*

deletion has already been detected while screening a wine yeast deletion collection (Peter et al., 2018). So deregulation of the pathway *per se* is bad for yeast performance, probably because of resource misallocation. The mutation of RR activator *RTG2*, which should theoretically have the opposite effect, also impaired growth and fermentation, albeit to a lesser extent. Hence in this case, deregulation could be worse than lack of activation. The biosynthesis of amino acids is a very costly endeavour that has to be tightly regulated to avoid wasting energy. The fact that *MKS1* deletion is sensitive to branched amino acid synthesis inhibitor sulfometuron methyl (Fig. 8) may indicate that channelling resources to α -ketoglutarate-derived amino acids, like glutamic acid or lysine, may cause deficits in other nitrogen metabolism branches with a possible deleterious effect.

The most interesting result of our study was that *MKS1* deletion altered carbon metabolism, and led to an increase in glycerol and a decrease in ethanol during winemaking and under brewing conditions. Glycerol overproduction at the expense of the glycolytic flux is a desired trait to obtain low-alcohol wines, but the overexpression of glycerol-producing enzymes brings about an undesirable increase in volatile acidity, which is likely due to redox-balance issues (Zhao et al., 2015) (Remize et al., 1999). Increasing the oxygen content to induce a shift to a more respirofermentative metabolism is one way of lowering ethanol at the end of fermentation, but increased acetic acid production also has a downside (Fig. 3; Tronchoni et al., 2022). During this deletion, mutant acetic acid did not increase. In fact its levels lowered, which reveals a very high ethanol reduction potential with no organoleptic negative effects, such as increased volatile acidity. *MKS1* deletion in industrial yeasts fits the expected phenotypic trait of tolerance to lysine-toxic analogue 2-aminoethylcysteine (Fig. 7). This is based on the fact that lysine is synthesised from α -ketoglutarate, and RR increases the synthesis of this precursor. RR also increases lactate dehydrogenase *DLD3*

(da Cunha et al., 2015; Jazwinski, 2013), and lactate indeed grows in the mutant (Fig. 3). Regarding acetic acid reduction, we can only speculate in accordance with the transcriptomic analysis of *MKS1* deletion under laboratory conditions (Dilova et al., 2002). α -ketoglutarate comes from the condensation of oxaloacetate and acetyl-CoA by citrate synthase, and both mitochondrial and peroxisomal isoenzymes are controlled by RR (Dilova et al., 2002; Shamji et al., 2000), which may explain the reduction in acetate if acetyl-CoA is drained from the cellular pool. The fact that the reduction was proportionally larger under aeration conditions (Fig. 3) may indicate that mitochondrial contribution could be more significant in this case. However, that acetyl-CoA depletion in the cytosol could affect other processes, such as lipid biosynthesis, by impairing growth. Ethanol reduction can be explained if its glycolytic precursor, pyruvate, is channelled to produce other metabolites (i.e. amino acids). Oxaloacetate comes from pyruvate in different pathways, including pyruvate carboxylase *PYC1*, another RR target (Komeili et al., 2000). Any depletion from pyruvate will decrease ethanol and reducing equivalents could be channelled to glycerol production. There is no transcriptomic evidence that RR controls either glycerol production or transport. Therefore, RR activation fits all the phenotypic traits seen in different assays and backgrounds during the fermentation of wine, beer and dough. RR down-regulation by the deletion of its activator *RTG2* should bring about an opposite effect, and does so mildly in one aspect, glycerol decreases, but with no consistent impact on ethanol. *MKS1* deletion has a well-known impact on canonical RR targets, but also has a marked signature in lysine biosynthesis (Dilova et al., 2002), while *RTG2* deletion does not show any control of this branch (Epstein et al., 2001). Therefore, *MKS1* and *RTG2* may have a different subset of targets despite both controlling RR during α -ketoglutarate production. *RTG2* deletion mutant sensitivity to 2-aminoethylcysteine (Fig. 8A) may be due to decreased lysine production by reducing its precursor.

5. Conclusion

In conclusion, the manipulation of different Retrograde Response aspects may influence carbon metabolism in industrial yeasts. *MKS1* deletion, which activates the Retrograde Response pathway, is a suitable way to produce low-alcohol beverages without increasing volatile acidity through growing glycerol production. RR de-regulation causes fermentation speed problems that must be taken into account before transferring this technology to industry.

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Ethical statement

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CRediT authorship contribution statement

Víctor Garrigós: Writing – review & editing, Investigation, Formal analysis. **Beatriz Vallejo:** Investigation, Formal analysis. **Agustín Aranda:** Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Philippe Marullo:** Writing – review & editing, Supervision, Funding acquisition. **Emilia Matallana:** Writing – review & editing, Supervision, Funding acquisition. **Cecilia Picazo:** Investigation, Formal analysis. **Emilien Peltier:** Software, Formal analysis. **Esperanza Mollá-Martí:** Investigation, Formal analysis.

Declaration of Competing Interest

Philippe Marullo is working for the Biolaftoff Company.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2024.05.007.

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