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# KCl/KOH supplementation improves acetic acid tolerance and ethanol production in a thermotolerant strain of *Kluyveromyces marxianus* isolated from henequen (*Agave fourcroydes*)

# La suplementación con KCl/KOH mejora la tolerancia al ácido acético y la producción de etanol en una cepa termotolerante de *Kluyveromyces marxianus* aislada de henequén (*Agave fourcroydes*)

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## Abstract

*Kluyveromyces marxianus* exhibits a high growth rate and is a promising non-conventional yeast for different biotechnological applications. One of these applications is the production of bioethanol from lignocellulosic hydrolysates at high temperature. Here, a new thermotolerant strain of *K. marxianus* (Kmx24) was isolated from a cooked henequen core. Its tolerance to acetic acid, one of the most common fermentation inhibitors present in lignocellulosic hydrolysates, was examined. Growth and ethanol production from glucose by Kmx24 were inhibited at acetic acid concentrations > 1.5 g/L. This sensitivity to acetic acid was alleviated by supplementation of the culture medium with KCl/KOH (40/10 mM) both at 30 and 42ºC, although the observed effect was not so marked at 42ºC. Increased cell viability and cell wall integrity were observed on addition of KCl/KOH in the presence of acetic acid. It is suggested that improved acetic acid tolerance is due to an increase in potassium uptake allowing an efficient proton efflux, increased membrane integrity and strengthened membrane electrochemical potential. The link between KCl/KOH supplementation and cell wall integrity needs further study. Temperature stress caused a decrease in the cell wall integrity which was not relieved by KCl/KOH addition.

*Keywords*: *Kluyveromyces marxianus*, bioethanol, acetic acid tolerance, potassium, cell wall.

## Resumen

*Kluyveromyces marxianus* tiene una alta velocidad de crecimiento y es una levadura no convencional prometedora para diferentes aplicaciones biotecnológicas. Una de estas aplicaciones es la producción de bioetanol a partir de hidrolizados lignocelulósicos a alta temperatura. Aquí, una nueva cepa termotolerante de *K. marxianus* (Kmx24) fue aislada de una piña de henequén cocida. Se examinó su tolerancia al ácido acético, uno de los inhibidores de fermentación más comunes presente en hidrolizados lignocelulósicos. El crecimiento y la producción de etanol a partir de glucosa por la cepa Kmx24 se inhibieron a concentraciones de ácido acético > 1.5 g/L. Esta sensibilidad al ácido acético disminuyó al suplementar el medio de cultivo con KCl/KOH (40/10 mM) tanto a 30 como a 42°C, aunque el efecto observado no fue tan pronunciado a 42°C. Se observó una mayor viabilidad celular e integridad de la pared celular tras la adición de KCl/KOH en presencia de ácido acético. Se sugiere que la mejoría en la tolerancia al ácido acético se debe a un aumento en la entrada de potasio que permite eficientar el flujo de protones hacia el exterior, una mayor integridad de la membrana y un reforzamiento del potencial electroquímico de membrana. El entendimiento de la relación entre la suplementación de KCl/KOH y la integridad de la pared celular necesita más estudios. El estrés por temperatura provocó una disminución en la integridad de la pared celular que no se alivió con la adición de KCl/KOH.

*Palabras clave*: *Kluyveromyces marxianus*, bioetanol, tolerancia a ácido acético, potasio, pared celular.

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# 1 Introduction

The non-conventional yeast *Kluyveromyces marxianus* has several characteristics of interest for industrial applications, such as a high growth rate, thermotolerance, a broad spectrum of carbon source utilization, including lactose and inulin, and the capability of producing ethanol, flavor and fragrance molecules as well as enzymes and heterologous proteins (Fonseca *et al*., 2008; Lane and Morrissey 2010; Morrissey *et al*., 2015; Varela *et al*., 2017; Nurcholis *et al*., 2019; Cordero-Soto *et al*., 2021). The physiological and metabolic diversity of *K. marxianus* make this yeast a promising cell factory for biorefinery applications using lignocellulosic biomass residues or dairy industry lactose-rich effluents as feedstocks for different applications (Lane *et al*., 2011; Radecka *et al*., 2015; Dasgupta *et al*., 2017; Leandro *et al*., 2020; Do *et al*., 2019).

Due to its thermotolerance, *K. marxianus* has also been considered a promising yeast for the production of ethanol by simultaneous saccharification and fermentation (SSF) (Ballesteros *et al*., 1991; Ballesteros *et al*., 1993; Olofsson *et al*., 2008; Suryawati *et al*., 2008; Park *et al*., 2015; Choudhary *et al*., 2016). In SSF, fermentation is carried out at higher temperatures (>40ºC), which are closer to the optimal temperature of the cellulase enzymes that simultaneously hydrolyze the cellulose fraction of the pretreated biomass. Therefore, the use of thermotolerant microorganisms like *K. marxianus* is required for fermentation. In the SSF configuration, investment costs are lower than when hydrolysis and fermentation are performed separately and the inhibition of cellulases by their end-product (glucose) is reduced due to the simultaneous consumption of the released sugar by the fermenting microorganism (Olofsson *et al*., 2008). An obstacle to the use of lignocellulosic biomass as a feedstock is the presence of fermentation inhibitors that can diminish cell growth and fermentation performance (Du *et al*., 2010). Inhibitors can be removed but this increases production costs and may lead to sugar loss (Kumar *et al*., 2020). Acetic acid is one of the most abundant fermentation inhibitors present in lignocellulosic biomass hydrolysates (Almeida *et al*., 2007). It is therefore important that the fermentative microorganisms which grow in lignocellulosic hydrolysates have a certain tolerance to acetic acid.

The toxic effects of acetic acid and tolerance mechanisms have been extensively described in *Saccharomyces cerevisiae* (Mira *et al*., 2010a; Piper, 2011; Giannattasio *et al*., 2013; Palma *et al*., 2018; Guaragnella and Bettiga, 2021). Briefly, when the extracellular pH is lower than the pKa of acetic acid (4.7), the undissociated form prevails and, unlike acetate, can enter the cells by simple diffusion through the plasma membrane. During its passage, the acid alters the structure and organization of membrane proteins and lipids, leading to loss of membrane integrity. Once inside the cell, where the pH is close to neutral values, the acid dissociates and liberates protons that acidify the cytosol. Intracellular pH is recovered by pumping the protons out of the cell at the expense of ATP, with the consequent reduction or complete arrest of growth and fermentation capabilities. The acetate anion accumulated inside the cell decreases the activity of important glycolytic enzymes, increases the turgor pressure, induces the accumulation of reactive oxygen species (ROS) that damage proteins and DNA, and can even lead to apoptosis.

The tolerance of *S. cerevisiae* to acetic acid has been improved by medium composition design and strain improvement. For example, the addition of metal ions ($Zn^{2+}$, $Mg^{2+}$, and $Ca^{2+}$) or potassium in the culture medium resulted in enhanced tolerance to acetic acid (Mira *et al*., 2010a; Ismail *et al*., 2014; Xu *et al*., 2019). *S. cerevisiae* adaptation and tolerance to acetic acid involves the expression of around 650 genes associated with multiple cellular processes such as transcription, pH homeostasis, carbohydrate metabolism, biogenesis of vacuole, ribosome, mitochondria and cell wall, and the sensing, signaling and uptake of various nutrients such as potassium, iron, glucose and amino acids (Mira *et al*., 2010b). Despite the polygenic nature of acetic acid tolerance in *S. cerevisiae*, the overexpression or deletion of individual genes has been used as a strategy to improve its acetic tolerance, as well as strategies based on laboratory evolutionary adaptation and genome shuffling (Guaragnella and Bettiga, 2021). Individual genes have included those encoding the yeast aquaglyceroporin, acetate transporter, plasma membrane and vacuolar $H^+$ ATPases, transcription factors and activators, and acetyl-CoA synthetase, among others. Recently, a laboratory evolution approach allowed the identification of a high-affinity potassium transporter as another target for rational strain engineering (Xu *et al*., 2019).

Unlike *S. cerevisiae*, the stress physiology of *K. marxianus* has not been studied in detail. An early

study showed that the mitogen-activated protein kinase Hog1 gene may be required for adaptation of *K. marxianus* to acetic acid and to osmotic and oxidative stress (Qian *et al*., 2011). In this work, we report that supplementation of the culture medium with potassium ions greatly enhanced acetic acid tolerance of a thermotolerant strain of *K. marxianus* newly isolated from henequen (*Agave fourcroydes* Lem.), thus improving ethanol production in the presence of this weak acid. The possible mechanisms underlying the improved tolerance phenotype are discussed.

## 2 Materials and methods

### 2.1 Yeast strains and general culture media

The *K. marxianus* Kmx24 strain was isolated using standard methodologies from a cooked henequen core processed at the GeMBio Laboratory, Centro de Investigación Científica de Yucatán (CICY) in Merida, Yucatan, as described by Pérez Brito *et al*. (2007). The *K. marxianus* strains CBS 6556 and CBS 745 were obtained from the ARS culture collection (NRRL) under accession numbers Y-7571 and Y-1598, respectively. According to the information provided by the NRRL, these strains were isolated from pozol (Chiapas) and rotting sisal leaf (country of origin not indicated), respectively. *S. cerevisiae* S288C was kindly donated by Luis Caspeta (Instituto de Biotecnología, UNAM) and *S. cerevisiae* ATCC 965813 was purchased from the American Type Culture Collection (ATCC). The culture medium used for maintenance and inocula preparation was YPD broth containing 20 g/L bacteriological peptone, 10 g/L yeast extract, and 20 g/L glucose. 15 g/L of bacteriological agar were added for YPD agar preparation. The strains cultured in YPD broth were stored and maintained in 25 % (v/v) glycerol at -80°C.

### 2.2 Identification and thermotolerance of *K. marxianus Kmx24*

Yeast DNA was extracted and isolated from 3 mL of an overnight culture in YPD broth, using the ZR Fungal/bacterial DNA Miniprep kit (Zymo Research). For identification, the rRNA internal transcribed spacer (ITS) region was amplified and sequenced using the ITS1 and ITS4 primers described by White *et al*. (1990). The 25-$\mu$L PCR mixtures contained 25 ng of template DNA, 1x Standard Taq reaction buffer (New England Biolabs), 0.2 mM of each dNTP, 0.5 mM of each primer and 1.25 units of Taq DNA polymerase. DNA amplification was performed under the following conditions: 5 min of initial denaturation at 95ºC; 35 cycles of 1 min at 94ºC, 2 min at 55.5ºC and 2 min at 72ºC, followed by one cycle of final extension (10 min at 72ºC). After purification with the DNA Clean and Concentrator kit (Zymo Research), the PCR product was sent to Macrogen (Korea) for sequencing with the primers ITS1 and ITS4. The sequences thus obtained were compared with those deposited in GenBank using the Blast program (Altschul *et al*., 1990) for identification. Thermotolerance of the Kmx24 strain was assessed by spotting liquid cultures onto solid medium using strains CBS 6556, CBS 745 and S288C as controls. The strains were grown overnight at 30ºC to the stationary phase in YPD broth, adjusted to an optical density at 600 nm ($OD_{600}$) of 0.2 using YPD broth and spotted (2.5 $\mu$L) onto YPD agar plates which were incubated at 30, 37, 42, 45 and 48ºC for 48 hours (Mukherjee *et al*., 2014).

### 2.3 Fermentation conditions

The fermentation medium contained 5 g/L yeast extract, 2 g/L $NH_4Cl$, 1 g/L $KH_2PO_4$, 0.3 g/L $MgSO_4{\cdot}7H_2O$, and 50 g/L glucose. The pH of this medium was adjusted to 5.5 with 2 N NaOH in all experiments. When the medium was supplemented with acetic acid, the pH was adjusted to 5.5 after the addition of acetic acid. This fermentation medium was not limited in nitrogen, phosphorus, potassium or magnesium (see supplementary information SI1). When indicated, KCl and KOH were added from concentrated stock solutions at final concentrations of 40 mM KCl and 10 mM KOH, as described by Lam *et al*. (2014). Inocula were prepared in YPD broth from one colony inoculated in 5 mL of medium and grown to an early stationary phase at 30ºC and 200 rpm for 16 hours. The fermentations were carried out in 125 mL Erlenmeyer flasks sealed with phenolic screw caps and containing 62.5 mL of fermentation medium at 30ºC or 42ºC, with an agitation of 150 rpm. The flasks were inoculated with an initial $OD_{600}$ of 0.25. Cell growth was followed by measuring the $OD_{600}$ using a BioPhotometer Plus (Eppendorf) instrument. When $OD_{600}$ readings higher than 1 were reached, the cultures were diluted in order to obtain readings lower than 1. Experiments were performed in duplicate and error bars were placed to represent the SD of the results.

### *2.4 Lyticase sensitivity assay*

The lyticase sensitivity assay was performed following the protocol described by Cunha *et al*. (2018) and using the *Arthrobacter luteus* lyticase (Sigma-Aldrich) which is a pure enzyme with $\beta$-1,3-glucan hydrolase activity that degrades yeast cell walls and is used for cell wall integrity determination (Ovalle *et al*., 1998). In this test, the $OD_{600}$ of cell suspensions treated with lyticase decreases as the cell wall lyses. The assays were performed after growth, using samples taken at 24 hours of fermentation. The $OD_{600}$ was measured at 30-min intervals to monitor cell lysis.

### *2.5 Analytical methods*

Glucose, acetic acid, glycerol and ethanol were quantified by high-performance liquid chromatography (HPLC) using a Varian ProStar 210 chromatograph equipped with a Knauer-IR refractive index detector. A Bio-Rad Aminex HPX-87H column operated at 65°C with 0.005 M $H_2SO_4$ as mobile phase (0.6 mL/min) was used for the separation as described by Rentería-Martínez *et al*. (2021). Cell viability was determined after 24 hours of culture. For this, serial dilutions up to $10^{-6}$ were plated onto YPD medium in duplicate. The plates were incubated at 30°C for 4-5 days until growth was observed and colony forming units (CFU) were counted.

## 3 Results

### *3.1 Identification and thermotolerance evaluation*

The Kmx24 strain used in this study was identified as *K. marxianus* by sequencing of the ITS region. The corresponding nucleotide sequence was deposited in GenBank under accession number MW193124. The Blast search tool showed that the ITS sequence of Kmx24 was 99.9% identical to that of *K. marxianus* strains and isolates of different origins and to the type strain *K. marxianus* CBS 712. The effect of elevated temperatures on *K. marxianus* Kmx24 was tested by examining the growth of this strain at 30, 37, 42, 45 and 48ºC in YPD agar plates (Figure 1). *S. cerevisiae* ATCC 96581 and S288C and *K. marxianus* CBS 6556 and CBS 745 were used as references of non-thermotolerant and thermotolerant yeasts, respectively. As expected, the *S. cerevisiae* strains could not grow at 42, 45 and 48ºC while *K. marxianus* Kmx24, CBS 6556 and CBS 745 were clearly thermotolerant and able to grow at up to 45ºC. Kmx24 and CBS 6556 showed better growth at 42 and 45ºC than CBS 745. However, the three *K. marxianus* strains, Kmx24, CBS 6556 and CBS 745, failed to grow at 48ºC. This temperature limit had already been reported by Lane *et al*. 2011 for *K. marxianus* CBS 745.

### *3.2 Effect of KCl/KOH supplementation on growth in the presence of acetic acid*

Mira *et al*. (2010a) showed that supplementation of the culture medium with KCl (10-20 mM) enhanced the tolerance of *S. cerevisiae* to acetic acid. Xu *et al*. (2019) recently showed that extracellular KCl supplementation (1-100 mM) increased the tolerance of *S. cerevisiae* to propionic acid and other organic acids such as acetic acid. Lam *et al*. (2014) had previously shown that increasing extracellular $K^+$ and pH through supplementation of the medium with KCl/KOH (40/10 mM) enhanced the tolerance of *S. cerevisiae* to ethanol by reinforcing the plasma membrane integrity through strengthening the opposing potassium and proton electrochemical gradients. As plasma membrane is also the first target of acetic acid toxicity, the effect of KCl/KOH supplementation was tested on *K. marxianus* Kmx24 fermenting glucose to ethanol in the presence of acetic acid. For this, the Kmx24 strain was cultured in the presence of 0, 1.5, 3, 4.5 and 6 g/L of acetic acid at 30 and 42ºC, with and without KCl/KOH (40/10 mM) supplementation. 42ºC was chosen because it is the SSF temperature for *K. marxianus* (Ballesteros *et al*., 2004).

Acetic acid severely impaired growth at 30 and 42ºC (Figure 2A and 2B, empty symbols). Lower cell growth was observed in the presence of 1.5 g/L of acetic acid compared to the condition without acetic acid. No growth was detected in the presence of 3, 4.5 and 6 g/L of acetic acid at 30 and 42ºC without KCl/KOH. Supplementation with KCl/KOH (Figure 2A and 2B, filled symbols) improved growth in the presence of 1.5 g/L of acetic acid and allowed growth with 3, 4.5 and 6 g/L of acetic acid at both temperatures. The maximal biomass production was reached in the absence of acetic acid (Table 1). On the other hand, in the absence of acetic acid, lower $OD_{600}$ was reached at 42ºC than at 30ºC (Table 1 and Figure 2A and 2B, 0 g/L of acetic acid). KCl/KOH addition did not substantially increase biomass at 42ºC (Table 1 and Figure 2B, 0 g/L of acetic acid, empty versus filled

Table 1. $OD_{600}$ values after 48 hours of culture in the presence of different concentrations of acetic acid, with and without KCl/KOH supplementation.

| Acetic acid (g/L) | $OD_{600}$ at 48 hours | | | |
|---|---|---|---|---|
| | 30ºC | | 42ºC | |
| | w/o KCl/KOH | w/ KCl/KOH | w/o KCl/KOH | w/ KCl/KOH |
| **0** | 16.47 | 21.32 | 13.88 | 15.23 |
| **1.5** | 10.92 | 17.13 | 8.23 | 9.63 |
| **3** | 0.28 | 14.32 | 0.18 | 7.36 |
| **4.5** | 0.14 | 14.88 | 0.12 | 7.83 |
| **6** | 0.13 | 12.06 | 0.11 | 6.96 |

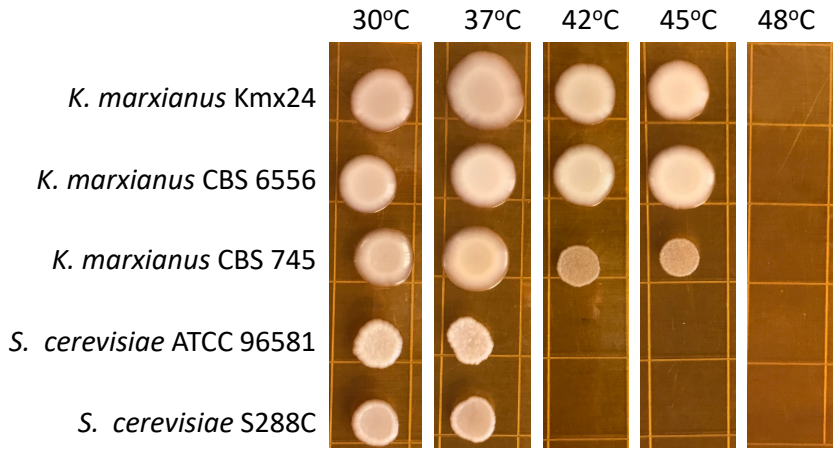


Fig. 1. Growth ability of Kmx24 on YPD agar at increasing temperatures.

symbols). The detrimental effect of temperature could not be relieved by KCl/KOH. At 30ºC the maximal biomass production was observed after 48 h. This value decreased from 21.3 to 12.1 between 0 and 6 g/L of acetic acid in the cultures with KCl/KOH (Figure 2A and Table 1). Growth was not affected in the first 10 h of cultures under all acetic acid concentrations tested. At 42ºC the maximal biomass decreased from 15.2 to 7.0 between 0 and 6 g/L of acetic acid in the presence of KCl/KOH (Figure 2B and Table 1). In this case, a slowdown of growth was apparent at 4.5 and 6 g/L of acetic acid during the first hours of cultivation. The addition of KCl/KOH was therefore not so efficient at restoring growth in the presence of high concentrations of acetic acid combined with high temperature.

The pH was monitored throughout the cultivations (Figure 3). In the cultures without acetic acid at 30ºC and 42ºC and without KCl/KOH supplementation (Figure 3A and 3B, 0 g/L of acetic acid, empty symbols), three phases were observed: the pH remained constant at pH 5.5 during the first 10 hours of culture, then it dropped to around 4.5 after 24 hours of culture and, finally, it remained almost stable during the following hours. This profile is consistent with the decrease of pH typically observed during fermentation without pH control. The decrease in the pH is due to the assimilation of ammonium into biomass that leads to the concomitant release of protons, and to the production of organic acids (Castrillo *et al*., 1995; Akin *et al*., 2008). The buffering capacity of the medium was probably exceeded after 10 hours of growth. When the medium was supplemented with KCl/KOH in the absence of acetic acid at 30ºC and 42ºC, a similar trend was observed, although the decrease of pH was slight (Figure 3A and 3B, 0 g/L of acetic acid, filled symbols). It was clear that the medium did not get buffered after the addition of KCl/KOH. In the cultures with 1.5 g/L of acetic acid, similar pH profiles were obtained at both temperatures

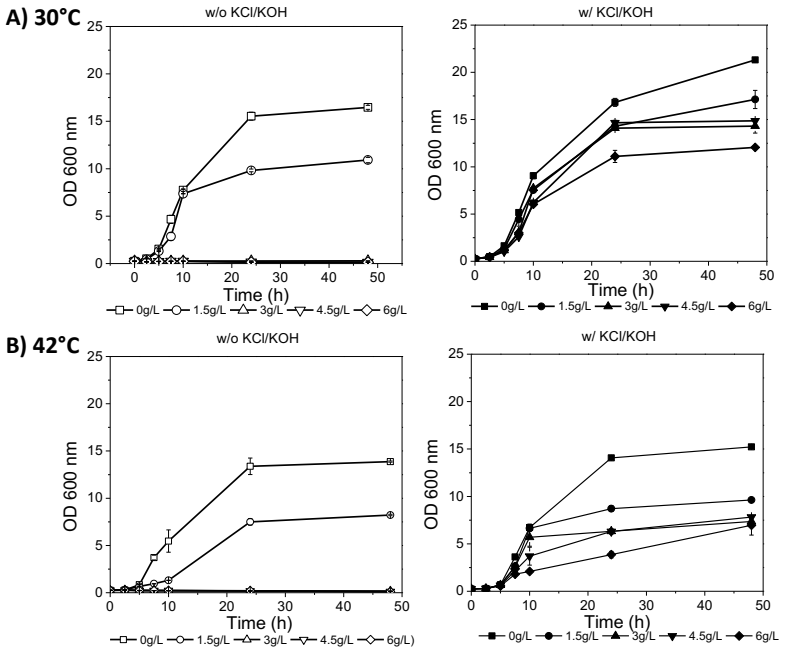


Fig. 2. Influence of acetic acid on yeast growth with or without KCl/KOH supplementation. Kmx24 was cultivated at A) 30°C or B) 42°C in fermentation medium containing the indicated concentrations of acetic acid without (empty symbols) or with (filled symbols) KCl/KOH (40 mM).

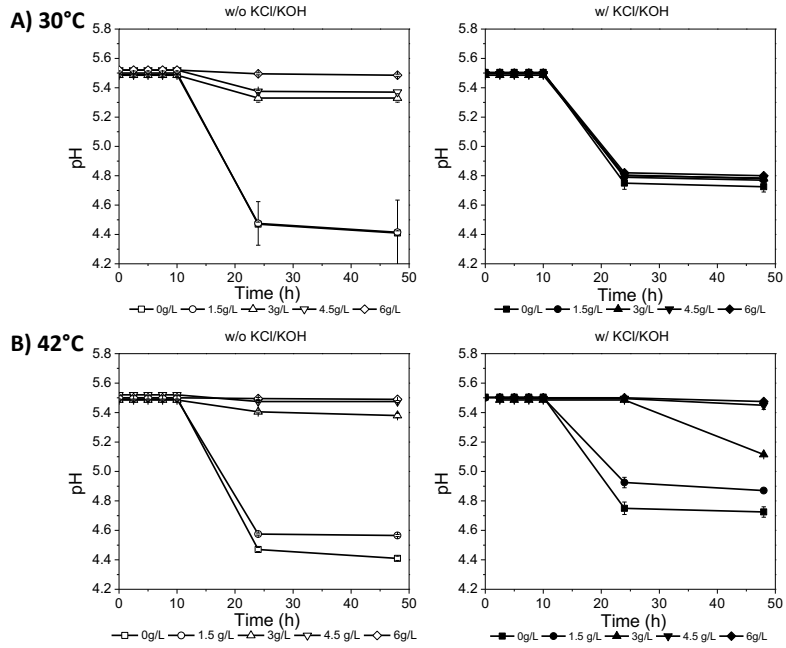


Fig. 3. Evolution of the pH during the growth of Kmx24 with or without KCl/KOH supplementation. Kmx24 was cultivated at A) 30°C or B) 42°C in fermentation medium containing the indicated concentrations of acetic acid without (empty symbols) or with (filled symbols) KCl/KOH (40 mM).

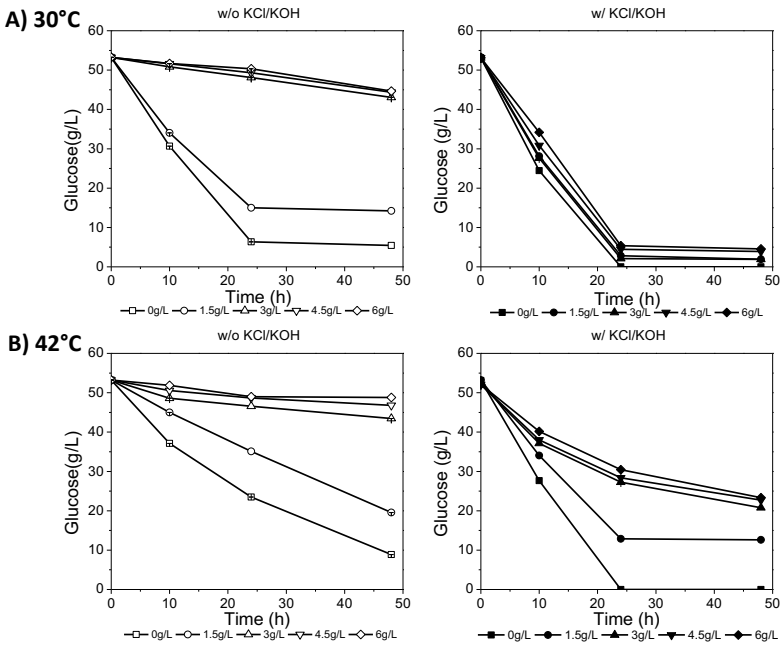


Fig. 4. Glucose profiles of Kmx24 grown in fermentation medium with or without KCl/KOH supplementation. Kmx24 was cultivated at A) 30°C or B) 42°C in the presence of the indicated concentrations of acetic acid without (empty symbols) or with (filled symbols) KCl/KOH (40 mM).

Table 2. Glucose consumption, ethanol production and yield of *K. marxianus*, after 48 hour in the presence of different concentrations of acetic acid, with and without KCl/KOH supplementation.

| Acetic acid (g/L) | Glucose (g/L) | | | | Ethanol (g/L) | | | | $Y_{E/G}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 30ºC | | 42ºC | | 30ºC | | 42ºC | | 30ºC | | 42ºC | |
| | w/o KCl/KOH | w/ KCl/KOH | w/o KCl/KOH | w/ KCl/KOH | w/o KCl/KOH | w/ KCl/KOH | w/o KCl/KOH | w/ KCl/KOH | w/o KCl/KOH | w/ KCl/KOH | w/o KCl/KOH | w/ KCl/KOH |
| **0** | 5.44 | 0.00 | 8.89 | 0.00 | 19.73 | 24.18 | 17.00 | 20.64 | 0.41 | 0.46 | 0.38 | 0.39 |
| **1.5** | 14.22 | 1.94 | 19.56 | 12.61 | 14.73 | 22.45 | 11.45 | 12.09 | 0.38 | 0.44 | 0.34 | 0.35 |
| **3** | 43.06 | 1.89 | 43.44 | 20.78 | 1.55 | 21.64 | 1.45 | 9.91 | 0.15 | 0.42 | 0.15 | 0.32 |
| **4.5** | 44.38 | 3.89 | 46.78 | 22.72 | 1.27 | 20.73 | 0.91 | 9.09 | 0.14 | 0.42 | 0.14 | 0.31 |
| **6** | 44.67 | 4.56 | 48.78 | 23.33 | 1.09 | 19.00 | 0.36 | 8.55 | 0.13 | 0.39 | 0.08 | 0.30 |

with and without the addition of KCl/KOH (Figure 3A and 3B, 1.5 g/L of acetic acid, filled symbols). The growth improvement observed with KCl/KOH supplementation at this acetic acid concentration was therefore not due to medium buffering. Under acetic conditions at which no growth had been observed (3, 4.5 and 6 g/L of acetic acid), different pH profiles were obtained with and without KCl/KOH. Without supplementation the pH remained constant, which is consistent with the absence of growth (Figure 3A and 3B, 3, 4.5 and 6 g/L of acetic acid, empty symbols). When KCl/KOH was added to the medium, the three-phase pH profile was restored in the presence of 3-6 g/L of acetic acid at 30ºC and 4.5 g/L of acetic acid at 42ºC (Figure 3A and 3B, filled symbols). This is consistent with the growth restoration observed in these conditions. In the presence of 4.5 and 6 g/L of acetic acid at 42ºC (Figure 3B, filled versus empty symbols) a slight pH drop was registered when the medium was supplemented with KCl/KOH, which is consistent with the slower growth and lower biomass production under these conditions (Table 1).

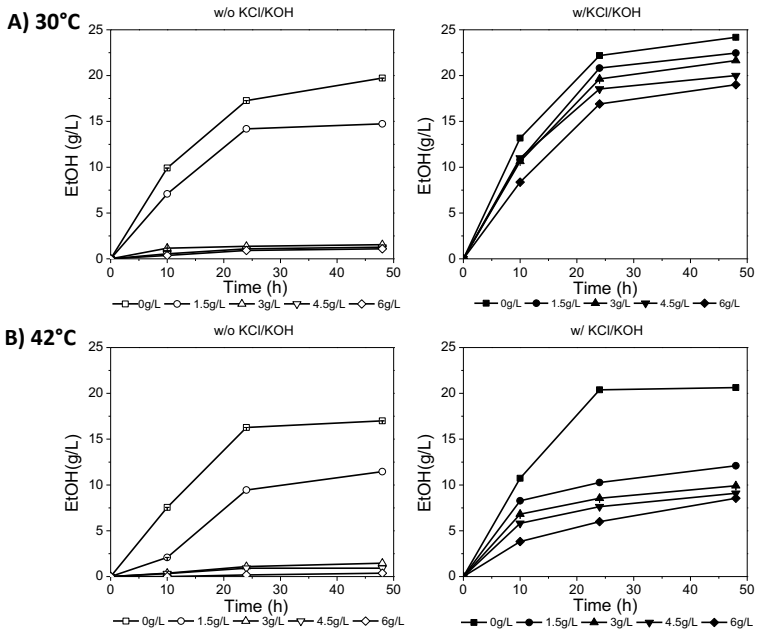


Fig. 5. Ethanol profiles of Kmx24 grown in fermentation medium with or without KCl/KOH supplementation. Kmx24 was cultivated at A) 30°C or B) 42°C in the presence of the indicated concentrations of acetic acid without (empty symbols) or with (filled symbols) KCl/KOH (40 mM).

### *3.3 Effect of KCl/KOH supplementation on glucose consumption and metabolite production in the presence of acetic acid*

Figures 4 and 5 show the glucose and ethanol profiles in the presence of acetic acid under the different conditions tested. Table 2 shows ethanol titers and yields after 48 h of culture. The glucose consumption and ethanol production profiles were consistent with those obtained for growth at 30 and 42ºC. In the presence of 1.5 g/L of acetic acid, lower glucose consumption and ethanol production were observed than in the condition without acetic acid. No or incipient glucose consumption and ethanol production were observed in the presence of 3, 4.5 or 6 g/L of acetic acid (Figure 4A, 4B, 5A and 5B, empty symbols). Supplementation of the medium with KCl/KOH (Figure 4A, 4B, 5A and 5B, filled symbols) improved glucose consumption and ethanol production at 1.5 g/L of acetic acid. The cultures grown with 3, 4.5 and 6 g/L of acetic acid were able to consume glucose and to produce ethanol at both temperatures. At 30ºC the glucose was almost completely consumed at all acetic acid concentrations tested when the medium was supplemented with KCl/KOH. The highest ethanol yield (0.46 g/g or 90% of the theoretical) was however obtained in the absence of acetic acid in the cultures supplemented with KCl/KOH, in contrast to 0.41 g/g (80.4% of the theoretical) without supplementation. In the presence of 1.5 to 6 g/L of acetic acid and KCl/KOH, the ethanol yields were between 0.44 to 0.39 g/g (86 to 76% of the theoretical), respectively. At 42ºC the glucose was not completely consumed even when KCl/KOH was added to the medium, and ethanol yields were lower than at 30ºC. In the absence of acetic acid, the yield was 0.39 g/g (76% of the theoretical) with KCl/KOH. For concentrations of acetic acid between 1.5 and 6 g/L the yield was 0.31 g/g (61% of the theoretical). As observed for growth, KCl/KOH supplementation was not sufficient to restore ethanol production in the presence of acetic acid at 42ºC.

The concentration of acetic acid and glycerol present in the culture medium under the different conditions tested are presented in Figure 6. In the absence of externally added acetic acid and KCl/KOH supplementation, some acetic acid was produced as a fermentation by-product both at 30 and 42ºC (Figure 6A and 6B, left panels with empty symbols and solid lines). Less acetic acid was produced at 42ºC than at 30ºC (1.5 versus 3.5 g/L at 48 h).

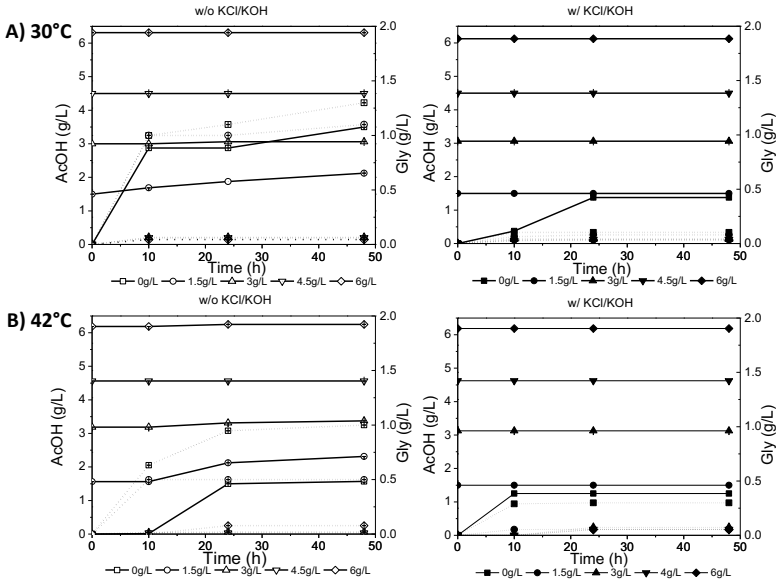


Fig. 6. Acetic acid and glycerol profiles of Kmx24 grown in fermentation medium with or without KCl/KOH supplementation. Kmx24 was cultivated at A) 30°C or B) 42°C in the presence of the indicated concentrations of acetic acid without (empty symbols) or with (filled symbols) KCl/KOH (40 mM). Solid lines for acetic acid (AcOH) and dashed lines for glycerol (Gly).

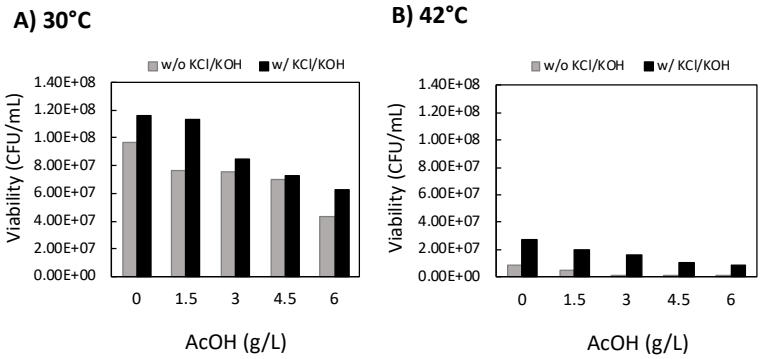


Fig. 7. Viable cells of Kmx24 after 24 hours of growth with or without KCl/KOH supplementation. Kmx24 was cultivated at A) 30°C or B) 42°C in the presence of the indicated concentrations of acetic acid without (grey bars) or with (black bars) KCl/KOH (40 mM).

At 1.5 g/L of externally added acetic acid, some extra acetic acid was produced (approximately 0.5 and 1 g/L at 30 and 42ºC at 48 h, respectively). The concentration of acetic acid remained stable in the presence of 3, 4.5 and 6 g/L of externally added acetic acid, which is consistent with no growth. When KCl/KOH was added to the medium (Figure 6A and 6B, right panels with filled symbols and solid lines), less acetic acid was produced in the absence of externally added acetic acid, both at 30 and 42ºC (between 1 and 1.5 g/L, respectively, at 48 h). The concentration of acetic acid remained constant in the cultures grown at 30 and 42ºC in the presence of 1.5-6 g/L of externally added acetic acid with KCl/KOH supplementation, meaning that acetic acid was not consumed nor produced during growth. Some glycerol was produced in the cultures grown without KCl/KOH supplementation, both at 30 and 42ºC (less than 1 g/L), but when the medium was supplemented with KCl/KOH, glycerol production was very low (Figure 6A and 6B, left and right panels, dashed lines).

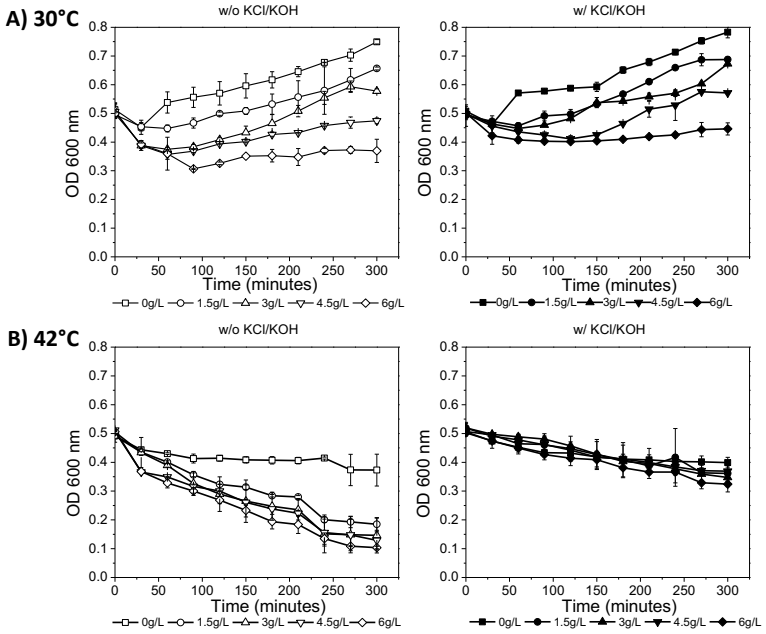


Fig. 8. Profiles of lyticase effect on the cell wall of Kmx24 after growth with or without KCl/KOH supplementation. Kmx24 was cultivated at A) 30°C or B) 42°C in the presence of the indicated concentrations of acetic acid without (empty symbols) or with (filled symbols) KCl/KOH (40 mM).

### *3.4 Effect of KCl/KOH supplementation on cell viability and cell wall integrity*

To test if cell viability was also improved with KCl/KOH, viable cell counts were evaluated after 24 h of culture by the count plate method (Figure 7). In the absence of KCl/KOH (grey bars) and without acetic acid, cell viability was much lower at 42ºC (Figure 7B) than at 30ºC (Figure 7A), which is consistent with the lowest growth, glucose consumption and ethanol production observed at this temperature. When acetic acid was added to the medium, cell viability decreased at increasing acetic acid concentrations, both at 30 and 42ºC. With KCl/KOH supplementation (black bars), cell viability was significantly improved at all acetic acid concentrations and both temperatures. However, the improvement at 42ºC was much less marked than at 30ºC, even in the absence of acetic acid. KCl/KOH addition was therefore not so effective at improving cell viability at 42ºC, which is consistent with the growth, glucose consumption and ethanol production data.

It has been shown that *S. cerevisiae* cells exposed to acetic acid become fragile, with a thinner cell wall (Dong *et al*., 2017). To test if the better performance of *K. marxianus* in the presence of KCl/KOH could be due to increased cell wall robustness, a lyticase sensitivity test was performed (Cunha *et al*. 2018). In the absence of KCl/KOH, *K. marxianus* grown at 42ºC without acetic acid was more sensitive to lyticase than cells at 30ºC (Figure 8A and 8B, left panels with empty symbols). Supplementation with KCl/KOH did not increase the lyticase resistance or sensitivity patterns of cells grown at 30 and 42ºC in the absence of acetic acid (Figure 8A and 8B, right panels with filled symbols). So, growth at elevated temperature affected the cell wall integrity of this strain and KCl/KOH addition did not improve or restore cell wall robustness. In the presence of 1.5-6 g/L of acetic acid without KCl/KOH, cells at 30ºC were also initially sensitive to lyticase but recovered during the course of the assay (Figure 8A, left panel with empty symbols). When the medium was supplemented with KCl/KOH (Figure 8A, right panel with filled symbols), the $OD_{600}$ decrease was less marked in the first minutes of the assay, indicating that the cells were initially less sensitive to lyticase. At 42ºC, cells in the presence of acetic acid and without KCl/KOH were sensitive to lyticase from the first acetic acid concentration tested (Figure 8B, left panel with empty symbols), indicating that the presence of increasing concentrations of acetic acid at 42ºC exacerbated cell

wall damage. Unlike what was observed at 30ºC, cells did not recover during the course of the lyticase assay. With KCl/KOH supplementation, the lyticase sensitivity of cells cultivated in the presence of acetic acid at 42ºC was reduced and became similar to that of cells grown without acetic acid (Figure 8B, right panel with filled symbols). So, KCl/KOH addition improved cell wall robustness in the presence of acetic acid but not at elevated temperature.

## 4 Discussion

Henequen is the common name of *Agave fourcroydes*, an agave species used for fiber production in the Yucatan peninsula (Mexico). Henequen has been domesticated by the Maya since Prehispanic times and traditionally used as a source of textile fibers, food and fermented beverages, medicine and construction material (Colunga-Garcíamarín and May-Pat, 1998). Different strains of *K. marxianus* have been retrieved from agave juice and alcoholic beverages produced from different agave species in Mexico (Lappe-Oliveras *et al*., 2008, Verdugo-Valdez *et al*., 2011). A *K. marxianus* strain (UFS-Y2791) has also been reported from *Agave americana* juice in South Africa (Schabort *et al*., 2016). *Agave americana* is native to Mexico and Texas but has been distributed worldwide, while henequen is only present in Mexico. Using a comparative genomic study of fourteen *K. marxianus* strains from diverse environments, including only one strain from agave (*K. marxianus* UFS-Y2791 from South Africa), Ortiz-Merino *et al*. (2018) have proposed that agave strains may represent a divergent group with new genetic diversity compared to strains from dairy environments and miscellaneous non-dairy strains. So, the Kmx24 strain and other *K. marxianus* isolates from agave substrates in Mexico constitute a new source of diversity within this yeast species.

Thermotolerance is an important characteristic for yeasts in high-temperature processes as the SSF of lignocellulosic biomass hydrolysates, to reduce media cooling costs in industrial bioprocesses, minimize unwanted bacterial contamination and allow stable fermentation processes in tropical regions (Abdel-Banat *et al*., 2010). Thermotolerance is a characteristic of *K. marxianus*, although not all *K. marxianus* strains are equally tolerant to high temperatures (Lane *et al*., 2011). The Kmx24 strain could grow at up to 45ºC in a solid medium. This temperature limit had already been reported for *K. marxianus* by Lane *et al*. (2011), who found that only two of the thirteen strains they tested showed robust growth at up to 48ºC in a solid medium. These differences in thermotolerance were apparently not related to the origin of the isolates, dairy or non-dairy. Thermotolerance is not frequent in yeasts. Lehnen *et al*. (2019) have found that no conserved metabolic traits have been found among thermotolerant yeast species and that *K. marxianus* is highly adaptive to temperature changes and presents a variety of different strain-dependent phenotypes.

Concerning acetic acid tolerance, strain Kmx24 could not grow and produce ethanol at concentrations of acetic acid >1.5 g/L at both temperatures tested. This strain was more sensitive to acetic acid at 42 than at 30ºC. The sensitivity of *K. marxianus* to acetic acid at high temperature (42ºC) has already been reported (Rugthaworn *et al*., 2014). The strain used, *K. marxianus* TISTR 5925, had been isolated from a fruit market in Thailand. As *K. marxianus* Kmx24, strain TISTR 5925 was more sensitive to acetic acid at 42 than at 30°C with a half maximal inhibitory concentration ($IC_{50}$) for growth of 1.8 g/L at 42°C. However, unlike strain Kmx24, *K. marxianus* TISTR 5925 could still grow at concentrations above 1.5 g/L at 30ºC without reaching the $IC_{50}$. On the other hand, Oliva *et al*. (2003) reported that *K. marxianus* CECT 10875 was not affected by the presence of 0-10 g/L of acetic acid and that growth and ethanol production were still 53% and 85% of the control at 10 g/L of acetic acid. This strain was therefore more tolerant to acetic acid than Kmx24 and TISTR 5925. *K. marxianus* CECT 10875 had been obtained by thermal treatment of a *K. marxianus* strain of unknown origin (Ballesteros *et al*., 1993). Strain CECT 10875 may have gained more tolerance to acetic acid and other fermentation inhibitors during the thermal treatment. Acetic acid tolerance is therefore variable among *K. marxianus* strains. The physiological, metabolic and genetic diversity among *K. marxianus* strains has been highlighted in different studies (Rocha *et al*., 2011; Lane *et al*., 2011; Fasoli *et al*., 2016). Considerable intraspecies diversity has also been found for acetic acid tolerance in *S. cerevisiae* (Haitani *et al*., 2012).

The presence of acetic acid at moderate concentrations >1.5 g/L (pH 5.5) impaired growth, glucose consumption and ethanol production in Kmx24 at 30 and 42ºC. The inhibition of growth observed in the presence of acetic acid is consistent with what has been reported for *S. cerevisiae*. ATP is diverted from anabolic processes (growth) to expel the protons that accumulate in the cytoplasm due to the dissociation of acetic acid, leading to increased lag

times, decreased growth rate and reduction in biomass yield (Pampulha and Loureiro-Dias, 2000). The anions accumulated in the cytoplasm are translocated across the plasma membrane to the external medium through energy-demanding proton antiporters (Fernandes *et al*., 2005). Before these detoxification mechanisms can actively take place, *S. cerevisiae* exhibits a more or less extended latency phase (up to 48 hours) during which cells adapt and modify their cellular processes to put into operation detoxification mechanisms such as those described above and restart growth (Mira *et al*., 2010a; Giannattasio *et al*., 2013). Higher concentrations of acetic acid cause complete growth inhibition and even apoptosis (Giannatasio *et al*., 2013). Unlike *S. cerevisiae*, the adaptive response and tolerance to acetic acid of *K. marxianus* have not been extensively studied. Here, the viable counts showed that a significant number of viable cells of *K. marxianus* Kmx24 were present after 24 hours of exposure to acetic acid >1.5 g/L at 30ºC even though no growth, glucose consumption and ethanol production occurred. This shows that acetic acid was not lethal at the concentrations tested and that some cells persisted in a viable but non-growing state. At 42ºC with and without acetic acid, the fraction of cells that remained viable was lower than at 30ºC, showing that elevated temperature affected cell viability to a greater extent than acetic acid.

The toxic effect of acetic acid on growth, glucose consumption and ethanol production was significantly counteracted by the addition of KCl/KOH to the culture medium at both temperatures tested. This effect was less pronounced at 42 than at 30ºC. According to the pH profiles during growth in the media to which KCl/KOH was added, the observed effect was not due to buffering of the medium. So, it was rather the presence of extra potassium ions that allowed growth and ethanol production. This is the first report in which potassium ion supplementation has been associated with increased tolerance to acetic acid in *K. marxianus*. In the medium containing acetic acid and KCl/KOH, growth and ethanol production were observed without an extended lag phase and the number of viable cells increased. The strain growing in the presence of acetic acid did not apparently consume or accumulate acetic acid inside the cell, as shown by the constant acetic acid profiles during growth. This may be explained by a repression effect of glucose on the uptake and metabolism of other carbon sources, as observed in *S. cerevisiae* growing on glucose in a fermentation medium supplemented with acetic acid (Cunha *et al*., 2018). These results are consistent with previous studies in which supplementation of the medium with KCl improved the tolerance of *S. cerevisiae* to several organic acids including acetic acid (Mira *et al*., 2010b; Xu *et al*., 2019). Other studies have shown that *S. cerevisiae* and *Zygosaccharomyces bailii*, a food spoilage yeast, accumulated intracellular potassium during long term adaptation to benzoic acid, a weak acid used as a preservative in the food industry (Macpherson *et al*., 2005). Besides, Mira *et al*. (2010b) and Xu *et al*. (2019) have shown that TRK1, the gene encoding the high affinity potassium transporter of *S. cerevisiae*, is the genetic determinant of improved acetic acid tolerance. Deletion of this gene increased the sensitivity of *S. cerevisiae* to acetic acid, whereas a higher expression level of TRK1 or the expression of evolved TRK1 mutants with improved uptake of potassium increased tolerance to acetic acid. This indicated that potassium uptake was definitively involved in the improved-tolerance phenotype.

Xu *et al*. (2019) have proposed two hypotheses to explain this improved phenotype in *S. cerevisiae*. First, increased potassium uptake might promote a more efficient export of protons to decrease intracellular acidification. Indeed, in the plasma membrane, the import of potassium ions through TRK1 provides the major return current for proton export by the ATP-dependent PMA1 proton efflux pump (Yenush *et al*. 2005). Additionally, the activity of PMA1 is positively regulated not only in response to intracellular pH decrease but also to increased potassium uptake (Yenush *et al*., 2005; Ariño *et al*., 2010). So, an increase in potassium uptake leads to a more efficient export of protons. Besides intracellular acidification, plasma membrane potential dissipation is another negative effect of weak acids on cells. As the undissociated forms of weak acids cross the plasma membrane by simple diffusion, they affect lipid organization and membrane-embedded proteins leading to loss of membrane integrity and dissipation of the membrane electrochemical potential which is essential for nutrient transport (Mira *et al*., 2010a). The second hypothesis proposed by Xu *et al*. (2019) is that increased potassium transport may improve tolerance to weak acids by strengthening the transmembrane proton and potassium gradients, leading to the stabilization of the plasma membrane potential. This hypothesis is consistent with the findings of Lam *et al*. (2014), which found that KCl/KOH supplementation heightened the overall population viability and boosted ethanol and other high chain alcohol tolerance in *S. cerevisiae* by strengthening the electrical membrane potential,

which is essential to support the transport of nutrients for cell growth. Proton antiporters belonging to the DHA1 family of MFS secondary transporters have been shown to be involved in the extrusion of acetate in *S. cerevisiae* (dos Santos *et al*., 2014). As these transporters rely on a membrane-potential-based mechanism for transport, strengthening of the membrane electrochemical potential may in addition contribute to the efficient extrusion of acetate by $H^+$ antiporters. As potassium homeostasis is crucial for all organisms and potassium transport is functionally and evolutionarily conserved in yeasts, fungi and other organisms, similar mechanisms probably explain the increased acetic acid tolerance of *K. marxianus* and *S. cerevisiae* on supplementation with potassium salts.

Another mechanism reported for increased tolerance and adaptation to acetic acid in *S. cerevisiae* is cell wall reconfiguration to limit the entry or re-entry of acetic acid into the cell (Palma *et al*., 2018; Ribeiro *et al*., 2021). The increased resistance of cells to lyticase digestion in cultures with added KCl/KOH indicated they were less susceptible to this enzyme. Lyticase mainly hydrolyse $\beta$1,3-glucans in yeasts cell walls. This means that the cells contained a higher proportion of $\beta$1,3-glucans in their cell walls or that these glucans were less accessible to lyticase, indicating in both cases a remodeling of the cell wall in the cultures supplemented with KCl/KOH. A recent study showed that the increase in cell wall stiffness of *S. cerevisiae* cells adapted to acetic acid is due to a change in the molecular architecture of the cell wall involving glucan elongation and cross-link to chitin (Ribeiro *et al*., 2021). Concerning cell wall remodeling in cultures supplemented with potassium, no similar results have been reported and this subject should be further studied. Finally, growth, viable counts, glucose consumption and ethanol production were lower at 42 than at 30ºC, even when the medium was supplemented with potassium and in the absence of acetic acid. Heat stress not only affects cellular membranes but also diverse proteins and the cytoskeleton (Auesukaree, 2017). Temperature stress response is multifaceted in yeast and involves several mechanisms such as heat shock response, change in membrane composition and modification of carbohydrates fluxes (Morano *et al*., 2012). During growth at 42ºC, *K. marxianus* Kmx24 was more sensitive to lyticase than at 30ºC, indicating that the cell wall had been remodeled at this temperature. As mentioned above, no conserved metabolic traits have been found in thermotolerant yeasts. Cell wall structure may also be an interesting target for further studies on thermotolerance.

## Conclusions

Increasing extracellular potassium concentration enhanced the tolerance of a thermotolerant strain of *K. marxianus* to acetic acid. This is the first report of the positive effect of potassium supplementation on acetic acid tolerance and ethanol production in *K. marxianus*. The potassium effect was however less marked at elevated temperature, which means that other mechanisms are involved in response to high temperature stress. It is suggested that cell wall architecture remodeling may be involved in increased tolerance to acetic acid and high temperature stress response in *K. marxianus*. Further studies are needed to understand the link between potassium homeostasis and cell wall architecture as well as the cellular response of *K. marxianus* to elevated temperature and its thermotolerance mechanisms.

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