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Metabolism of *Schizosaccharomyces pombe* under reduced osmotic stress conditions afforded by fed-batch alcoholic fermentation of white grape must

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ABSTRACT

Strains of *Schizosaccharomyces pombe* are being increasingly investigated with regards to their grape winemaking potential either in combination with the typical production yeast, *Saccharomyces cerevisiae,* or in monoseptic fermentations. Their ethanol tolerance and ability to degrade L-malic acid is oenologically convenient but contrasts with the comparatively high acetic acid and acetaldehyde formation potential which is considered undesirable, especially in white winemaking. The purpose of this work was to investigate the performance of a selected *S. pombe* strain in monoseptic femerntations of white grape must. Traditional batch fermentations were compared with an innovative and automated fed-batch fermentation technique were sugar concentrations are kept low during fermentations to decrease sugar induced osmotic stress. Because of its known effect on growth and ethanol tolerance, the effect of Mg was also tested. While Mg supplementation was not shown to significantly influence residual values of sugars, ethanol, glycerol, organic acids and acetaldehyde, the application of the fed-batch technique led to a fundamental change in yeast physiology. While glycerol values were only slightly reduced, the fed-batch approach allowed obtaining wines devoid of acetic acid whose levels were considerable in wines produced by the traditional batch technique (0.6 g/L). The work demonstrates that the acetic acid metabolism of *S. pombe* is associated to sugar induced osmotic stress such as for *S. cerevisiae*, too, and may be controlled by application of suitable fermentation techniques for winemaking.

1. Introduction

Alcoholic fermentation (AF) is a complex biochemical process based on the microbial conversion of sugars to ethanol, carbon dioxide and fermentation by-products, such as glycerol, organic acids and carbonyls (Pretorius, Van der Westhuizen, & Augustyn, 1999). In winemaking, *Saccharomyces cerevisiae* is typically considered the main responsible for AF, whereas non-*Saccharomyces* yeasts have usually been considered as 'spoilage' yeasts because of their association with products presenting organoleptic defects (Ciani, Comitini, Mannazzu, & Domizio, 2010; Contreras et al., 2014; Fleet, Lafon-Lafourcade, & Ribereau-Gayon, 1984). The excessive production of acetic acid, acetaldehyde, and ethyl acetate is among the potential undesirable metabolic activities of non-*Saccharomyces* wine yeasts (Ciani et al., 2010).

However, growing anecdotal and experimental evidence obtained over the last decade suggests that the winemaking potential of non-*Saccharomyces* yeast has been undervalued and that selected strains may play a relevant role with regards to aroma complexity and final wine quality (Ciani et al., 2010; Ciani & Maccarelli, 1998; Padilla, Gil, & Manzanares, 2016). *Schizosaccharomyces pombe* is of particular interest. Unlike most other non-*Saccharomyces*, *S. pombe* can ferment grape musts to dryness because of its high alcohol and SO_2 tolerance (Koukou, Tsoukatos, & Drainas, 1990; Queiroz & Pareilleux, 1990). In addition to its fermentation performance, it has several interesting metabolic capabilities, too. Notably, *S. pombe* strains have been shown to convert L-malic acid into ethanol and CO_2, to reduce gluconic acid (Peinado, Maestre, Mauricio, & Moreno, 2009), to express urease (Benito et al., 2013; Benito et al., 2016; Silva et al., 2003; Taillandier, Gilis, & Strehaiano, 1995) and to produce significant amounts of cell wall polysaccharides (Domizio, Liu, Bisson, & Barile, 2017). Over the last years, several studies considering the nutrition, growth and metabolism of *S. pombe* (Hoffman, Wood, & Fantes, 2015; Petersen & Russell, 2016) have been presented. However, fundamental aspects of the metabolism of *S. pombe* such as its response to osmotic stress remain unknown. Recently, it was shown that reducing sugar induced osmotic stress during alcoholic fermentation by application of a fed-batch approach led to fundamental changes in the metabolism of *S. cerevisiae* that may be favourable for wine quality (Frohman & Mira de Orduña, 2013).

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Notably, the formation of acetic acid was greatly reduced.

The aim of the current work was to investigate the metabolic response of *S. pombe* under reduced osmotic stress conditions attained by an automated fed-batch technique (Frohman & Mira de Orduña, 2018) allowing alcoholic fermentation at constant and reduced sugar levels. The kinetics of sugar, ethanol and several secondary metabolites were documented. In addition, the effect of medium magnesium supplementation was considered based on previous works showing that Mg plays a crucial role in metabolism (Walker, Birch-Andersen, Hamburger, & Kramhoft, 1982), cell division (Wolf, Trapani, & Cittadini, 2008) and may reduce ethanol toxicity associated stress (Chun-Keng, Feng-Wu, & Li-Jia, 2003; Dombek & Ingram, 1986). The *S. pombe* strain used was selected in a previous study based on its ethanol resistance, fermentation performance and L-malic acid degradation capacity, as well as its resistance towards the dehydration conditions applied for starter preparation (Roca-Domènech et al., 2016).

2. Materials and methods

2.1. Medium, microorganism and culture conditions

The same flash-pasteurized Riesling must from the Palatinate region of Germany was used for all batch and fed-batch fermentations. Its sugar concentration was 191.9 g/L combined glucose and fructose, the pH was 3.37, the titratable acidity was 4.72 g/L expressed as tartaric acid and the yeast assimilable nitrogen (YAN) concentration was 150 mg/L N. For nitrogen yeast nutrition, the must was supplemented with 0.3 g/L of a complex supplement (Uvavital D, Eaton, Germany) and 0.2 g/L of laboratory grade $(NH_4)_2HPO_4$ (ACS, Sigma Aldrich, EU). Mg supplemented must was produced by increasing the native Mg concentration of 45 mg/L with $MgSO_4$ to reach 167 mg/L Mg.

S. pombe strain CECT11197 was obtained from the culture collection of the Department of Biochemistry and Biotechnology, University Rovira i Virgili, Tarragona, Spain and was pre-grown under aerobic conditions in YEPD (Yeast Extract Peptone Dextrose Medium, Fisher Scientific) in shaker flasks (150 rpm) for 40 h at 25 °C to obtain a yeast starter for inoculations. Batch fermentations were conducted by adding this yeast starter to the entire amount of must in 5 L glass bottles (Schott Duran, Germany) sealed with suitable air locks to allow for fermentation gas release and to prevent air ingress. These fermentations were inoculated to a titer of 1×10^7 cells/mL as determined by a Neubauer counting chamber with respect to the total must volume.

Fed-batch treatments were conducted according to Frohman and Mira de Orduña (2018). Briefly, a Fourier-Transform Near-Infrared (FT-NIR) Spectrophotometer (MPA, Bruker, Germany) equipped with a suitable relfectance *in-situ* probe (IN271F, Bruker Optics Germany) was used to quantify the concentrations of sugars and ethanol in real-time based on previously established prediction models using the equipment's chemometric software (OPUS, Bruker, Germany). Sugar concentration data was then used to automatically control a peristaltic pump (Reglo ICC Digital Peristaltic Pump, 3-channels, Ismatec, WA) that delivered the fresh Riesling must (kept at 0 °C) to the fermentation at such rates as to keep sugar concentrations stable at approximately 65 g/L during fermentations. Fed-batch fermentations had a starting volume of 68 mL consisting of 45 mL of yeast starter and 23 mL of flash-pasteurized Riesling must to obtain an initial sugar concentration of approximately 65 g/L. Inoculation occurred at a titer of 1×10^7 cells/mL as determined by a Neubauer counting chamber with respect to the total final must volume to be delivered (1.8 L). During the initial phase of fed-batch fermentations (up to a volume of 500 mL), the head space of fed-batch incubations was flushed with CO_2 in order to prevent oxidation. All fermentations were carried out at 22 ± 1 °C.

2.2. Sample taking, analytical methods and statistical analysis

For chemical analyses, the biomass was removed by centrifugation (5 min, 15,000*g*) and the supernatant stored frozen (−20 °C) until analysis. YAN was measured by quantifying amino nitrogen according to the NOPA method (Dukes & Butzke, 1998) and inorganic nitrogen by means of an enzymatic method for ammonia (Megazyme, Ireland). Sugars, ethanol, glycerol, acetic acid, lactic acid and malic acid were analysed with a HPLC system (Agilent 1260 Infinity, EU) equipped with a 64225A degasser, a G1310B isocratic pump, a GT329B autosampler, a G1316A column oven, a G1314F UV detector and a refractive index detector (RI-102, Shodex). Data acquisition and analysis were performed with the instrument software provided (Agilent OpenLab CDS Chemstation v.A02.09). The mobile phase consisted of 0.65 mM H_2SO_4 and was filtered prior to utilization (0.22 µm, nylon, Millipore, Ireland). 500 µL of sample was mixed with 4.5 mL of the mobile phase and cleaned up using a commercial solid phase extraction cartridge loaded with 200 mg sorbent (Waters Oasis HLB 6 cc). 20 µL sample was then injected and separated at 80 °C on a PS-DVB phase (Aminex HPX-87H, 300 × 7.8 mm, Bio-Rad) at a flow rate of 0.5 mL/min. Sugars, ethanol and acids were quantified by refractive index while acetic acid was quantified by UV spectroscopy at 210 nm. Acetaldehyde was measured enzymatically with a commercial test kit (Megazyme, UK). External calibration standards were used to validate all analyses.

All fermentations were carried out in triplicate and data representation and rate fittings were carried out using OriginLab Origin v7.0. Results were statistically analysed using the XLSTAT 19.5 statistical software package.

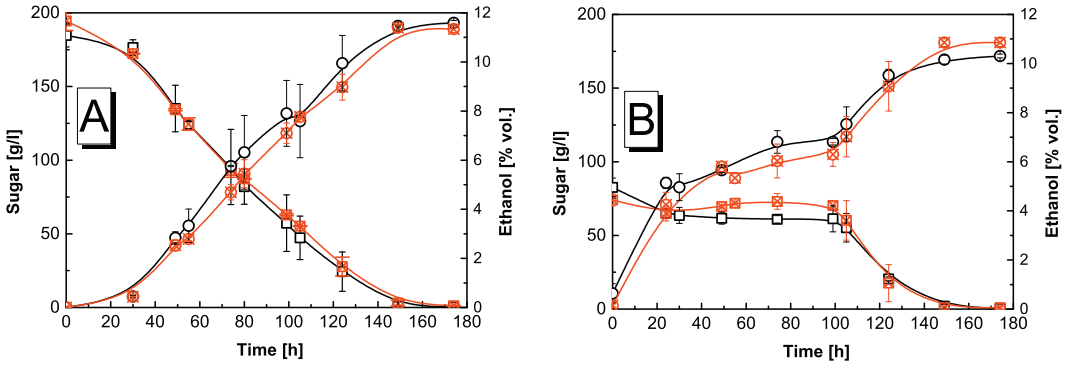

Fig. 1. Consumption of sugar (□) and production of ethanol (○) during traditional batch (A) and fed-batch fermentations (B). Fermentations with Mg supplementation represented by red symbols with crosses (sugar: ⊠; ethanol: ⊗). Average values ± SD shown (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

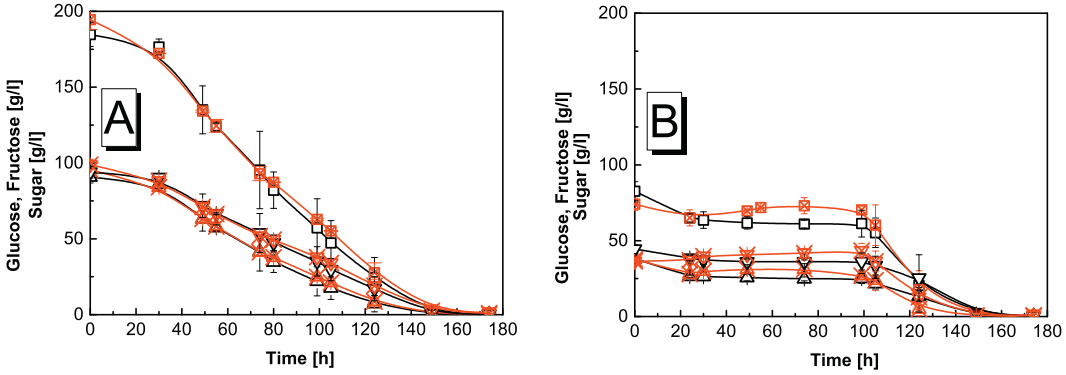

Fig. 2. Time course of total sugars (glucose + fructose, □), glucose (Δ) and fructose (∇) during traditional batch (A) and fed-batch fermentations (B). Fermentations with Mg supplementation represented by red symbols with crosses (total sugars: ⊠; glucose: ⋇; fructose: ⋇). Average values ± SD shown (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Sugars and ethanol

Fig. 1 shows the time course of sugar and ethanol concentrations in traditional batch (A) and fed-batch (B) fermentations. Traditional batch fermentations without and with Mg-supplementation reached dryness (< 5 g/L of sugar) after 149 h. In fed-batch fermentations, sugars were kept constant at approximately 65 g/L until all must was consumed and during this period (100 h), 6–7% (v/v) of ethanol were reached. After conclusion of the must-feeding phase, the residual sugar was also completely degraded after 149 h irrespective of Mg supplementation. All fermentations displayed a slight glucophilic sugar degradation behaviour that was not significantly influenced by the type of fermentation or Mg supplementation (Fig. 2). There was no statistically significant difference between the residual sugar concentrations of any of the treatments (Table 1). In spite of this, significant differences were found with regards to the final ethanol concentrations. Wines produced with the fed-batch technique had 0.55–1.3% (v/v) less ethanol (Table 1).

3.2. Malic acid degradation

From an initial must concentration of 2.6 g/L, malic acid was rapidly degraded (< 60 h) in traditional batch fermentations (Fig. 3). In fed-batch incubations, malic acid concentrations never exceeded 0.6 g/L and remained below the LOD for most of the duration of incubations. The higher initial malic acid concentration in the fed-batch treatment with added Mg was attributable to differences in the process control (must feeding rate). Except for this slight difference, Mg supplementation had no effect on the time course of malic or lactic acid levels, which stayed relatively constant throughout all fermentations. There was a slight trend towards higher final lactic acid concentrations in wines produced by the fed-batch technique, which was not statistically relevant (Fig. 3).

3.3. Sugar induced osmotic stress response related metabolites

Fig. 4 shows the kinetics of several sugar induced osmotic stress associated fermentation by-products in traditional batch and fed-batch fermentations. Supplementation with Mg led to slightly different kinetics of glycerol and acetic acid in some incubations. However, the effects were not consistent and Mg supplmentation had no statistically significant effect on the final values (Table 1) of any of the metabolites. In contrast, significant differences were observed with regards to the kinetics and the final values between traditional batch and fed-batch fermentations. In batch fermentations, glycerol levels increased rapidly up to 70–80 h of fermentation time and then stayed constant. In fed-batch incubations, glycerol formation already levelled off after 30 h and final glycerol concentrations were approximately 20% lower (Table 1). The differences were striking with regards to acetic acid kinetics and final values (Fig. 4, Table 1). A strong initial production of acetic acid in traditional batch fermentations transitioned to a slower production phase after approximately 90 h resulting in a mean final concentration of 600 mg/L acetic acid across all batch incubations. In contrast, acetic acid never exceeded 220 mg/L in fed-batch incubations (Fig. 4) and no acetic acid was detectable in any of the fed-batch incubations after 25 h of incubation time (Table 1). The course of acetaldehyde concentrations followed typical kinetics with an initial increase followed by a partial reuptake (Jackowetz, Dierschke, & Mira de Orduña, 2011). Peak and final acetaldehyde concentrations were similar across all incubations (Fig. 4). There was a trend towards lower residual acetaldehyde concentrations (40% less) in wines produced by the fed-batch technique, which was not statistically significant (Table 1).

4. Discussion

Shizosaccharomyces pombe is a grape must fermentation associated non-*Saccharomyces* yeast with significant potential for winemaking (Benito et al., 2016). *S. pombe* strains have been shown to possess the ability to completely convert grape sugars to alcohol, and to degrade malic acid (Dharmadhikari & Wilker, 1998), which typically has to be

Table 1
Final concentrations of various fermentation parameters. Values displayed are means of triplicate experiments ± SD. Different superscript letters indicate statistical significance between averages at $p < 0.05$.

	Total Sugar (g/L)	Ethanol (% vol.)	Glycerol (g/L)	Acetic ac. (g/L)	Acetaldehyde (mg/L)	Malic ac. (g/L)	Lactic ac. (g/L)
Batch	0.81 ± 0.08^{a}	11.47 ± 0.10^{a}	11.96 ± 1.11^{a}	0.55 ± 0.07^{a}	67.54 ± 26.45^{a}	n.d.	0.12 ± 0.00^{a}
Batch + Mg	3.04 ± 1.85^{a}	11.40 ± 0.13^{ab}	11.64 ± 0.05^{ab}	0.66 ± 0.04^{a}	50.52 ± 2.60^{a}	n.d.	0.12 ± 0.00^{a}
Fed-Batch	1.94 ± 1.33^{a}	10.16 ± 0.18^{c}	9.52 ± 0.90^{ab}	n.d.	35.95 ± 1.32^{a}	n.d.	0.17 ± 0.02^{a}
Fed-Batch + Mg	0.96 ± 0.79^{a}	10.85 ± 0.16^{b}	8.83 ± 0.49^{b}	n.d.	39.94 ± 2.5^{a}	n.d.	0.16 ± 0.01^{a}

n.d.: not detected.

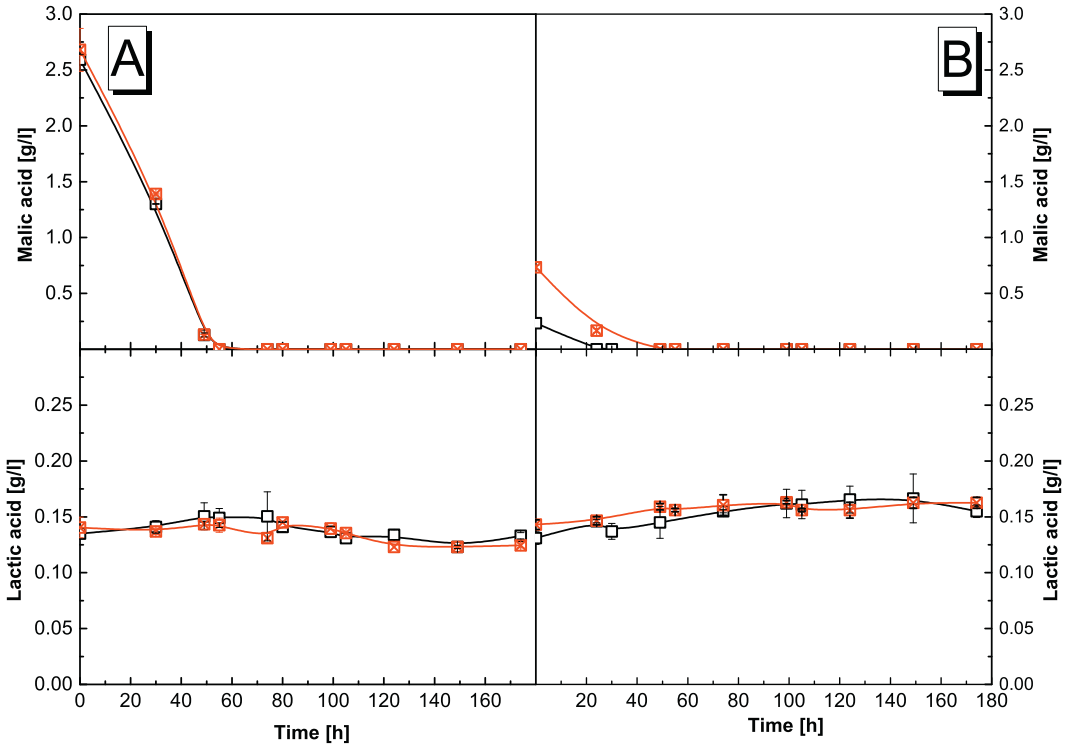

Fig. 3. Time course of malic and lactic acid during traditional batch (A) and fed-batch fermentations (B). Fermentations with Mg supplementation represented by symbols with crosses. Average values ± SD shown (n = 3).

achieved during a malolactic fermentation (Sumby, Grbin, & Jiranek, 2014). In addition, its production of aldehyde compounds may be of interest for red wine colour development (Morata et al., 2012; Mylona et al., 2016).

In this work, its utilization as sole production organism was studied in monoseptic white grape must fermentations in traditional batch incubations, as well as fed-batch fermentations that have been shown to improve fermentation performance of *S. cerevisiae* and reduce the production of undesirable fermentation by-products (Frohman & Mira de Orduña, 2013). In addition, grape must supplementation with Mg was investigated. In *S. cerevisiae*, magnesium has been shown to act as a growth enhancer and to increase ethanol tolerance (Walker et al., 1982). Mg supplementation has also been associated with improved growth and ethanol tolerance in *S. pombe* (Hu, Bai, & An, 2003). However, in this study, Mg supplementation was not found to have a consistent effect on the time course or final values of any of the metabolites studied. Although the natural Mg concentration of the grape must used (45 mg/L Mg) was relatively low with regards to typical must Mg levels reported in the literature (50–200 mg/L Mg, Margalit, 2016), it is possible that it was still too high to cause a Mg deficiency. Since specific chelation for Mg sequestration is difficult, the effect of Mg may be better studied in synthetic grape must fermentations.

Overall, the differences caused by the fermentation mode were notable. While sugar degradation was complete in all treatments, final alcohol concentrations were slightly reduced in wines produced by the fed-batch technique. In studies with *S. cerevisiae*, fed-batch incubations did not lead to ethanol reductions compared with the traditional batch approach (Frohman & Mira de Orduña, 2013). As much as an ethanol reduction would be desirable considering the climate change associated increase in wine ethanol concentrations and its implications (Mira de Orduña, 2010), it is suggested that the results obtained in this study were an artefact of the technique applied rather than a physiological response to lower sugar concentrations. Specifically, the CO_2 flushing applied to prevent oxidation in the small initial fed-batch fermentation volume is expected to have caused ethanol stripping.

Unlike malolactic fermentation (MLF) by lactic acid bacteria (Lonvaud-Funel, 1999), malic acid degradation by *S. pombe* does not lead to lactic acid formation, which can lead to lactic notes from formation of ethyl lactate after esterification. Rather, malic acid metabolism by *S. pombe* leads to formation of ethanol and CO_2 (Benito et al., 2016). In both fermentation treatments, malic acid was degraded below the detection threshold of the method applied. Because of the high initial cell density present in fed-batch fermentations, must was essentially depleted from malic acid upon introduction to fed-batch fermentations. Lactic acid concentrations remained almost constant in all treatments, confirming the significant potential of *S. pombe* for biological deacidification especially in high acidity musts.

The polyol glycerol is an alcoholic fermentation by-product and plays significant roles in yeast physiology such as maintaining cellular redox homeostasis and functioning as an osmoprotectant (Michnick, Roustan, Remize, & Barre, 1997; Scanes, Hohmann, & Priori, 1998). Consequently, under high sugar induced osmotic pressure, glycerol formation is increased and correlates with formation of acetic acid in *S. cerevisiae* (Pigeau & Inglis, 2007). A recent study investigating the alcoholic fermentation of *S. cerevisiae* showed that the reduced osmotic stress afforded by a fed-batch approach led to a 45% reduction in final glycerol and a 80% reduction in final acetic acid levels as compared with the traditional batch approach (Frohman & Mira de Orduña, 2013). In traditional batch fermentations, the *S. pombe* strain CECT11197 used in this study yielded acetic acid concentrations (0.55 g/L) that were similar to those reported by Mylona et al. (2016) for another *S. pombe* strain (0.51 g/L for strains 7VA) but significantly lower than those reported by Miljić, Puškaš, Vučurović, and Muzalevski (2017) and Mylona et al. (2016), for strains 2139 and 938 which exceeded 1 g/L of acetic acid. Conversely, Mylona et al. (2016) and especially Du Plessis et al. (2017) reported much lower levels for *S. pombe* strains than ranged between 0.07 g/L (expressed as volatile acidity) and 0.35 g/L acetic acid. In this study, the fed-batch approach allowed obtaining wines devoid of acetic acid while glycerol levels still remained higher than those repoted by Mylona et al. (2016).

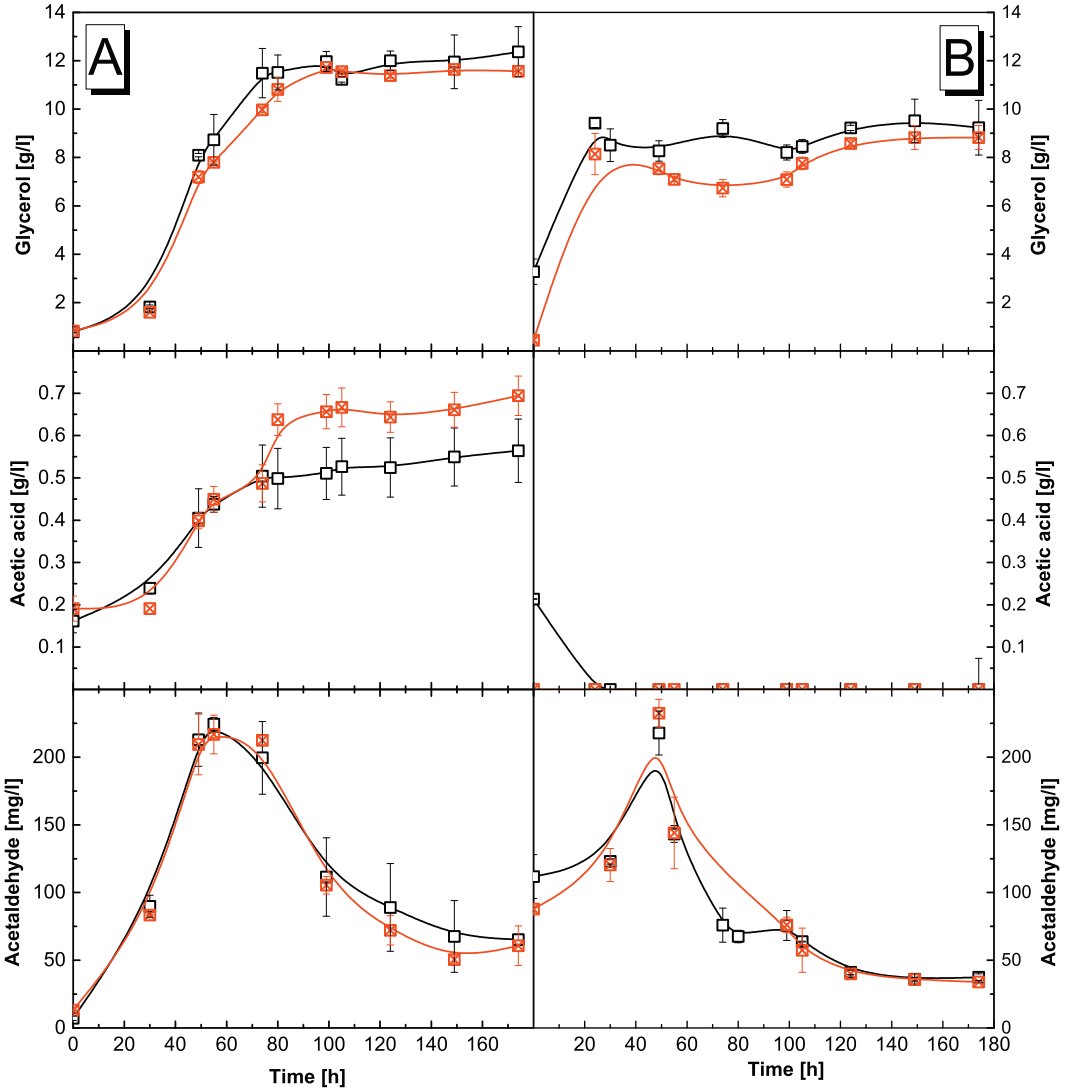

Fig. 4. Time course of sugar induced osmotic stress associated metabolites glycerol, acetic acid and acetaldehyde during traditional batch (A) and fed-batch fermentations (B). Fermentations with Mg supplementation represented by symbols with crosses. Average values ± SD shown (n = 3).

Accordingly, application of the fed-batch technique may allow to greatly reduce volatile acidity even with strains that produce significant amounts of acetic acid in traditional batch culture, while maintaining relatively high levels of glycerol, which is associated with positive sensory notes in wine (Lubbers, Verret, & Voulley, 2001; Noble & Bursick, 1984).

Acetaldehyde is the major carbonyl compound formed during alcoholic fermentation and has implications for sensory qualities and stability of wines as well as for the use of the wine preservative SO_2 (Jackowetz & Mira de Orduña, 2013). Compared with other wine associated yeast, *S. pombe* produced relatively large amounts of acetaldehyde in resting cell and grape must incubations (Li & Mira de Orduña, 2011, 2017). While this metabolic property may be interesting in red wine fermentations where acetaldehyde contributes to the stabilization of red wine colours (Benito, Calderón, & Benito, 2017; Mylona et al., 2016), in white wines it is considered undesirable since acetaldehyde will bind signifcant quantities of SO_2 (Jackowetz et al., 2011), a wine conservative whose levels are sought to be limited. In this study, there was a trend towards lower acetaldehyde levels in fed-batch treatments. In fact, the residual acetaldehyde concentrations of 40 mg/L in wines produced by the fed-batch technique in this study were congruent with the average concentrations measured in 127 commercial white wines in a recent study (Jackowetz & Mira de Orduña, 2013). Hence, in spite of the higher known acetaldehyde production potential of *S. pombe* strains, application of *S. pombe* strain CECT11197 with the fed-batch technique allowed obtaining wines with residual acetaldehyde levels comparable to current commercial standards. The acetaldehyde residues obtained with the *S. pombe* strains selected by Mylona et al. (2016) were even lower in batch fermenations. Accordingly, application of these superior strains with a fed-batch technique may allow to further reduce acetaldehyde levels.

5. Conclusions

The current work demonstrated the suitability of a selected *Shizosaccharomyces pombe* strain for monoseptic fermentations of white grape juice. Its ability to completely convert grape sugars to alcohol and its strong malic acid degradation capacity may be advantageous for a number of vinification challenges including acidic white musts. Applying a novel fed-batch technique, it was possible to avoid formation of acetic acid. The application of suitable strains reported elsewhere with the innovative fed-batch fermentation approach may provide for an efficient utilization of *S. pombe* in white winemaking.

Acknowledgements

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