RESEARCH ARTICLE

Effect of cytochrome bc₁ complex inhibition during fermentation and growth of Scheffersomyces stipitis using glucose, xylose or arabinose as carbon sources

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One sentence summary: Respiratory and fermentative metabolism of S. stipitis can be differentiated using glucose, fructose or arabinose, whereas respiratory chain inhibition may be used to improve ethanol yields.

ABSTRACT

Scheffersomyces stipitis shows a high capacity to ferment xylose, with a strong oxygen dependence to allow NAD⁺ regeneration. However, without oxygen regeneration of NADH occurs by other metabolic pathways like alcoholic fermentation. There are few reports about inhibitors of mitochondrial respiration and their effects on growth and fermentation. This work aimed to explore the effect of cytochrome bc₁ complex inhibition by antimycin A (AA), on growth and fermentation of S. stipitis using glucose, xylose and arabinose as carbon sources, at three agitation levels (0, 125 and 250 rpm). It was possible to discriminate between respiratory and fermentative metabolism in these different conditions using xylose or arabinose. Despite the inhibition of mitochondrial respiration, the glycolytic flux was active because S. stipitis metabolized glucose or xylose to produce ATP; on 0.5 M glucose the cells yielded 17–33 g L⁻¹ ethanol. However, more complex results were obtained on xylose, which depended upon agitation conditions where ethanol production without agitation increased up to 11 g L⁻¹. Inhibition of respiratory chain in S. stipitis could therefore be a good strategy to improve ethanol yields.

Keywords: Scheffersomyces stipitis; respiratory chain; antimycin A; fermentation; ethanol

INTRODUCTION

The main polymers of lignocellulosic biomass are cellulose and hemicelluloses that comprise about 70% of the total dry biomass weight (Zabed et al. 2017). Glucose represents the main hexose sugar, whereas D-xylose and L- arabinose are the major pentose sugars in lignocellulosic biomass (Subtil and Boles 2011;
MATERIALS AND METHODS

Yeast strain

All experiments were conducted using S. stipitis (NRRL Y-1154), kept at −70°C in an ultrafreezer (Thermo Scientific, MA, USA). The yeast was activated twice using a medium containing 10 g L⁻¹ yeast extract (Sigma-Aldrich, MO, USA), 20 g L⁻¹ peptone (BD, MD, USA), and 20 g L⁻¹ glucose (Sigma-Aldrich), for 24 h at 30°C, and 150 rpm.

Growth kinetics and specific growth rate (μ)

Five μL of the activated yeast were mixed with 145 μL of a medium containing 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone in each well of a honeycomb plate (Growth curves, Picstown, NJ, USA) and incubated at 30°C for 48 h in a Bioscreen C MBR (Growth curves) (Madrigal-Perez et al. 2016). Three different shaking levels were used (intense, medium and null) and the absorbance at 600 nm was taken every 30 min. The carbon sources used were glucose, xylose (Sigma-Aldrich) or arabinose (bioWorld, OH, USA) with three different concentrations (0.5, 0.05 and 0.005 M). These carbon sources are the main hexose and pentoses found in lignocellulosic biomass (Subtil and Boles 2011; Chen and Fu 2016). Growth kinetic parameters were calculated using equation 1:

\[ X_f = X_o e^{\mu t} \]  

(1)

Where \( X_o \) is the initial cells population, \( X_f \) is the final cells population, \( \mu \) is the specific growth rate and \( t \) is the time (h).

To calculate doubling time (dt) equation 2 was used:

\[ dt = \ln 2 / \mu \]  

(2)

Respiratory inhibition kinetics

Antimycin A (AA; Sigma-Aldrich) was used to inhibit the mitochondrial complex III (Nicholls and Ferguson 2013). For these experiments, 5 μL of pre-inoculum were added to 139 μL of YP medium (containing the carbon source under evaluation) supplemented with 6 μL of AA (0.2 mM). Absolute ethanol (Sigma-Aldrich), a non-fermentable carbon source, was used as respiratory growth control (Stahl et al. 2004) at two levels (v/v⁻¹): 0.1% and 1.0%. Some reports mention that S. stipitis is not inhibited when initial concentration is between 20 to 30 g L⁻¹ of ethanol, but the effect of inhibition depends on the presence of oxygen and carbon source (Jeppsson, Alexander and Hahn-Hagerdal 1995; Liang et al. 2014).

Quantification of bioenergetic variables

The bioenergetic variables measured were in situ mitochondrial respiration, and the extracellular acidification rate (ECAR). A base value of specific growth rate was determined using media containing 1% ethanol, 0.2 mM AA and 250 rpm, where growth was minimal (0.011 h⁻¹). Treatments were selected to calculate the bioenergetic variables, when growth rate was higher than this base value, and are shown in Table 1.

Quantification of in situ mitochondrial respiration

In situ mitochondrial respiration was evaluated according to Madrigal-Perez et al. (2016). Briefly, the oxygen consumption was...
The slope of a plot of pH (milliunits) versus time (s) was used to measure polarographically at 28 °C in a Clark detector (YSI, model 5300, Yellow Spring, OH, USA). These assays were conducted using 125 mg of wet cells harvested at mid-logarithmic growth phase (OD<sub>600</sub>~0.6). Cells were resuspended in 5 mL of 10 mM 2-N-(morpholinol) ethane sulphonic acid (MES-TEA; Sigma–Aldrich) buffer, adjusted to pH 6.0 with triethanolamine (Sigma–Aldrich) in a closed chamber with constant stirring. The basal respiration was determined after adding the oxidizable substrate, which in this case was glucose or xylose, both at final concentration of 0.05 M or 0.5 M, and oxygen consumption was monitored for 3 min. The maximal respiratory capacity was analyzed by adding 10 μM of the uncoupling agent cyanide 3–chlorophenylhydrazone (CCCP). Non-mitochondrial oxygen consumption was discriminated by adding 10 μM AA at the end of each determination. Oxygen uptake was expressed in nano atoms oxygen min<sup>−1</sup> mg cells<sup>−1</sup>.

### Measurement of glycolytic flux using extracellular acidification rate (ECAR)

The ECAR was measured according to Olivares-Marin et al. (2018) in conditions where specific growth rate was higher than the base value determined using 1% ethanol with AA, and 250 rpm was used to determine the growth inhibition of respiratory metabolism. Since ethanol is only metabolized by mitochondrial respiration, it was used as positive control of respiratory growth. Additionally, three agitation conditions without AA and 250 rpm, 125 rpm, and 0 rpm (Fig. S2, Supporting Information) were used except that 0.4 mL of AA (0.2 mM) was added. For experiments without AA, medium containing 10 g L<sup>−1</sup> yeast extract and 20 g L<sup>−1</sup> peptone (9.7 mL) was inoculated with 0.3 mL of activated yeast in a 50 mL flask and incubated at 30 °C for 48 h. In case of experiments with AA, same conditions as above were used except that 0.4 mL of AA (0.2 mM) was added. For both conditions arabinose was discarded due to growth inhibition. Biomass was measured gravimetrically using cells dried at 60 °C for 24 h, and consumed sugar was quantified by the dinitrosalicylic acid (DNS) method (Wood et al. 2012). Ethanol was measured using an HPLC 1220 Infinity (Agilent Technologies, CA, USA) fitted with a refractive index detector, and a MetaCarb 600 H plus column (300 × 7.8 mm) (Agilent Technologies) at 55 °C. The mobile phase was sulfuric acid (0.05 M) at a flow rate of 0.35 mL/min and running time of 35 min. The oven temperature was 75 °C and detector temperature was 55 °C with a sample injection of 20 μL. Ethanol and biomass were calculated and reported as g L<sup>−1</sup> of medium.

### Statistical analysis

Results are presented as mean ± standard error of the mean (SE). One-way ANOVA followed by Tukey’s test were used to analyze the results of specific growth rate, maximal and basal respiratory capacity, and glycolytic flux by ECAR, and biomass and ethanol yields. Statistical analyses were performed using GraphPad Prism v.5 software (GraphPad Software, La Jolla, CA, USA).

## RESULTS AND DISCUSSION

### Respiratory inhibition kinetics

Antimycin A was used initially to discriminate the use of ETC in the metabolism of xylose. Glucose was used as control because under static conditions (no agitation) it induces a fully fermentative metabolism in S. stipitis; whereas under agitation a respiratory metabolism is established. Since ethanol is only metabolized by mitochondrial respiration, it was used as positive control of respiratory growth. Additionally, three agitation conditions were tested 0, 125 and 250 rpm. As expected, under growing conditions without AA and 250 rpm, S. stipitis was able to grow in ethanol (Fig. 1A), but was unable to grow when AA was supplemented (Fig. 1B). This confirmed that AA is able to inhibit respiratory growth in S. stipitis, and the calculated μ (0.011 h<sup>−1</sup>) from this treatment was used as reference to discriminate the growth by respiratory metabolism. Regarding glucose, S. stipitis grew at the three agitation rates tested without AA: 250 rpm (Fig. 1C), 125 rpm (Fig. S1, Supporting Information), and 0 rpm (Fig. S2, Supporting Information). When AA was added, growth decrease was observed in all treatments (250 rpm, Fig. 1D; 125 rpm, Fig. S3; 0 rpm, Fig. S4, Supporting Information), but growth inhibition was not observed. Interestingly, S. stipitis barely showed any growth for any xylose concentration when AA was supplemented (Fig. 1F) compared to the treatment without AA (Fig. 1E) at 250 rpm. A similar pattern was detected at 125 and 0 rpm (Fig. S5 and S6, Supporting Information) when compared to conditions with added AA (Fig. S7 and S8, Supporting Information).

### Table 1. Assays selected to test S. stipitis growth in shake-flasks.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Concentration (M)</th>
<th>Agitation rate (rpm)</th>
<th>Inhibitor&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>Glucose</td>
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<td>Xylose</td>
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<sup>a</sup> – without AA

## References

- Granados-Arvizu et al. (2018)
Information). These data indicate that S. stipitis needs a functional ETC to grow in xylose. In case of arabinose, S. stipitis was unable to grow when AA was supplemented (Fig. 1H) compared to the treatment without AA (Fig. 1G) at 250 rpm. When agitation was reduced, the growth of S. stipitis significantly (\( P > 0.05 \)) decreased even without AA (Fig. S9 and S10, Supporting Information). However, when AA was added growth was totally inhibited (Fig. S11 and S12, Supporting Information).

Therefore, these data suggest that S. stipitis needs a completely functional ETC to metabolize arabinose. The effect of respiratory inhibition on specific growth rate using glucose as carbon source is shown in Fig 2A. Under inhibition conditions,
growth rates were lower in all treatments compared to non-inhibited cultures, especially at 0.005 M of glucose where growth was completely inhibited (Fig. 2A). Additionally, it was found that S. stipitis is able to grow at high glucose concentrations (Fig. 2A). When 5 μM of AA was used in Kluyveromyces lactis, the specific growth rate was 0.15 h⁻¹ (Merico et al. 2009), which is higher than the values observed in this study, using 0.11 M glucose and limited oxygen conditions. On the other hand, the inhibition of ETC using xylose as carbon source showed a significant (P < 0.05) effect on specific growth rate at all growth conditions (Fig. 3A), and μ values were below 0.05 h⁻¹. Additionally, arabinose was tested because it is one of the major five-carbon sugars present in biomass hydrolysates (Subtil and Boles 2011). Complete growth inhibition was observed when ETC was blocked using arabinose as carbon source in all treatments (Fig. 4A). Therefore, these data indicate that arabinose metabolism in S. stipitis is fully respiratory. Even without AA, S. stipitis in the presence of arabinose did not growth without agitation (Fig. 4B),
Figure 3. Effect of AA in energetic metabolism of S. stipitis using xylose as carbon source, in cultures at 30°C for 48 h. (A), Specific growth rate (µ) at 250, 125 and 0 rpm with and without AA; results represent mean ± SE from three technical replicates. (B), Comparison of in situ mitochondrial basal respiration; results represent mean ± SE from six independent experiments. (C), Comparison of in situ mitochondrial maximal respiration. Results represent mean ± SE from six independent experiments. (D), Comparison of glycolytic flux by ECAR. Results represent mean ± SE from six independent experiments. (E), Ethanol and (F), Biomass yields, of shake-flask cultures. Results represent mean ± SE from three technical replicates.

showing a high dependence of oxygen to metabolize this five-carbon sugar.

**Effect of glucose, xylose and antimycin A supplementation on the mitochondrial respiration at different agitation rates**

To corroborate the utilization of the ETC for xylose metabolism, the mitochondrial respiration was measured. The lowest concentration of xylose and the null agitation were discarded, due to lack of growth. As expected, it was found that AA inhibits basal respiration (Fig. 3B) and maximal respiratory capacity (Fig. 3C) of S. stipitis, and it was confirmed that at 125 and 250 rpm without AA higher oxygen consumption was observed in xylose metabolism, especially at low concentration. These data support that in xylose metabolism the function of the ETC is essential to support S. stipitis growth. Additionally, it was decided to further investigate the role of the ETC in glucose metabolism
considering that without agitation the AA has a negative role in growth. It was found that basal respiration and maximal respiratory capacity depend on agitation independently of the concentration of supplemented glucose. Interestingly, without agitation and inhibitor, cells still maintain oxygen consumption in both basal respiration (Fig. 2B) and maximal respiration capacity (Fig. 2C). These results explain the importance of ETC in glucose metabolism of S. stipitis without agitation.

**Glycolytic flux of S. stipitis in the presence of xylose, glucose and/or antimycin A under different agitation rates**

Based on the results from inhibition kinetics (Fig. 1a–h; Fig. S1–S12, Supporting Information), six conditions for glucose and four for xylose were selected, because the μ values of these treatments were above respiratory metabolism limit (0.011 h⁻¹). Interestingly, in all growth conditions tested for glucose (Fig. 2D) and xylose (Fig. 3D), AA decreased the glycolytic flux. However, S. stipitis still maintains growth in the presence of glucose or xylose, which may be explained by ATP generation at substrate level by glycolysis pathway that is confirmed by mitochondrial respiration inhibition and the ECAR assay. Additionally, it was observed that glycolytic flux in all growth conditions tested was higher at low concentrations of carbon source. This could be possible because the low-affinity transport system of S. stipitis is used at high carbon source concentrations, whereas the high-affinity system is expressed when carbon source is low (Agbogbo and Coward-Kelly 2008). Thus, high expression of low affinity transport system may be useful to increase ethanol production at high glucose concentration, but further studies are needed to confirm this. This pattern was also observed in the ECAR assay even in conditions where mitochondrial respiration was inhibited.

**Shake-flask cultures**

To evaluate the effect of ETC inhibition on ethanol and biomass production using xylose or glucose as carbon sources, fermentation in shake-flasks was conducted. The inhibition of ETC resulted in a significant (P > 0.05) increase in ethanol production at all glucose treatments tested (Fig. 2E). Using 0.5 M glucose, the inhibition of cytochrome bc₁ of S. stipitis increased ethanol yields from 17 to 33 g L⁻¹, depending on agitation level. Additionally, when 0.05 M glucose was used ethanol yield increased from 2 to 5.7 g L⁻¹, only at high agitation rate. In the case of biomass using glucose as carbon source, S. stipitis showed a significant (P < 0.05) growth reduction at all conditions with ETC inhibition in the range of 0.4 to 2.5 g L⁻¹, in comparison to treatments without AA (Fig. 2F). Growth decreased similarly at both concentrations (0.5 M and 0.05 M) tested. When 0.5 M xylose was used as carbon source, unlike glucose, ethanol production decreased almost 8 g L⁻¹ at 125 rpm but increased to 11 g L⁻¹ without agitation (Fig. 3E). These results are similar to those reported by Acevedo, Conejeros and Aroca (2017), who tested some respiratory chain inhibitors in S. stipitis using xylose at limited oxygen supply conditions. They observed decreased ethanol yields in the range of 25%-50% depending on the inhibitor used, but complex I inhibition with rotenone increased the yield up to 13%. Lee et al. (2000) reported that the addition of rotenone to S. stipitis during the exponential growth phase increased 1.1 times the ethanol concentration, whereas in this work AA addition increased ethanol production 12.2 times at 0.5 M xylose without agitation (Fig. 3E). These results are similar to those reported by Lighthi, Prior and Preez du (1988) reported that AA strongly stimulated ethanol production in P. tannophilus but not in S. stipitis. For biomass production with xylose as carbon source, ETC inhibition caused a significant (P < 0.05) decrease in all treatments. However, treatments without agitation barely showed biomass production (Fig. 3F). The use of arabinose was discarded as carbon source because the calculated μ values were below the respiratory metabolism limit (0.01 h⁻¹), when AA was incorporated (Fig. 4A). Arabinose fermentations treatments conducted without AA showed a strong dependence of μ on agitation and concentration (Fig. 4A), while biomass production required both high concentration and agitation (Fig. 4B). Nevertheless, it must be mentioned that ethanol was not detected in any treatment (data not shown). In consequence, arabinose may be considered as a non-fermentable carbon source by S. stipitis, in agreement with a report of Shi et al. (2000). These results confirm that S. stipitis requires a functional ETC to metabolize xylose and arabinose, whereas this effect was not observed for glucose. Acevedo, Conejeros and Aroca (2017) mentioned that...
respiratory inhibition could be an additional strategy combined with aeration control to improve ethanol yields from \textit{S. stipitis} using xylose. Nonetheless, it is necessary to understand the exerted mechanisms when different ETC inhibitors are used, in addition to oxygen limitation to produce high ethanol yields from \textit{S. stipitis} fermentation processes. In conclusion, the inhibition of cytochrome bc_{1} complex proved to be a good strategy to improve ethanol yields from \textit{S. stipitis}. However, before going into practical trials many factors have yet to be investigated, such as the identification of low cost inhibitors, optimization of fermentation with aeration using these ETC inhibitors as well as the role of AOX systems when these inhibitors are used.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSyr online.

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**Conflict of interest.** None declared.

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