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# DIFFERENTIAL MALIC ACID DEGRADATION BY INDIGENOUS AND COMMERCIAL *SACCHAROMYCES CEREVISIAE* WINE STRAINS

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## Introduction

**Saccharomyces cerevisiae** *is mankind's oldest domesticated organism and the world's premier commercial microorganism for biotechnological applications, being wine production a reference issue.*
*Malic acid contributes to the acidic taste of wine and also serves as a substrate for contaminating lactic acid bacteria that can cause wine spoilage after bottling. It is therefore essential to remove excess malic acid from the wine to ensure its physical, biochemical and microbial stability [1,2].*

*The ability of a yeast strain to degrade extracellular malic acid is dependent on the transport and the efficacy of the intracellular malic enzyme [3,4]. Previous studies have shown that* **S. cerevisiae** *can import malic acid and other dicarboxylic acids only via simple diffusion and is therefore unable to effectively degrade or utilize extracellular malic acid. However, the S. cerevisiae malic enzyme has a very low substrate affinity (Km=50 mM) which further contributes to the inefficient degradation of malic acid by* **S. cerevisiae** *[5].*

*The aim of the present study was (i) to screen a collection of 294 indigenous* **S.cerevisiae** *strains selected from the Vinho Verde Region regarding ethanol tolerance, capacity to utilize acetic and malic acid as well as $H_2S$ production, (ii) to evaluate differential malic acid degradation patterns in synthetic wine musts among three selected isolates in comparison to commercial yeast strains and (iii) to elucidate the activity of enzymes involved in malic acid metabolism.*

## Materials and Methods

*Phenotypic characterization*
*The ability to sustain growth on media containing ethanol and acetic acid was tested on YNB medium (Difco) containing glucose (2.0%, w/v), acetic acid (0.25%, v/v) and ethanol (10.0%, v/v), adjusted to pH 4.0. The capacity to utilize malic or acetic acid was investigated on YP medium, pH 4.0, containing methyl orange (0.005%, w/v) and acetic acid (0.25%, v/v) or malic acid (0.5%, w/v). Hydrogen sulphide production was tested on Biggy agar .*

*Growth conditions*
*Fermentations were carried out using a previously described synthetic culture medium (MS) that partially simulates the composition of a standard grape juice [6]. All the strains were inoculated at 1x10⁶ cells/ml into 500 ml flasks with synthetic grape must and fermentations were carried out at 20ºC, with constant shaking (120 rpm) and sealed with fermentation caps.*

*Glucose determination*
*The determination of glucose concentration was performed by a enzymatic/colorimetric method (GOD/POD).*

*Malic acid determination*
*The concentration of malic acid were determined enzymatically with specialized kit (BOEHRINGER MANNHEIN/R-BIOPHARM Roche Biochemicals, Germany) according to the manufacturer's instructions.*

*Enzymatic assays*
*Cells were collected at several times (22,46,68,138h of growth) and cellular extracts obtained. Protein quantification was performed by Bradford method. Enzymatic assays were performed adapting protocols provided by SIGMA-ALDRICH.*

*Three enzymes with key-role in malic acid metabolism were chosen.*

**Malic enzyme (mitochondria)**

L-malate + β-NADP → Piruvate + $CO_2$ + β-NADPH

**Fumarase (cytosol)**

L-malate → Fumarate + $H_2O$

**Malate dehydrogenase (2 mitochondrial + 1 cytosol)**

L-malate + β-NAD → Oxaloacetate + β-NADH + $H^+$

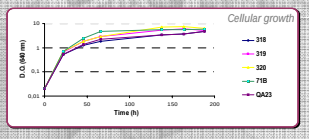
# RESULTS

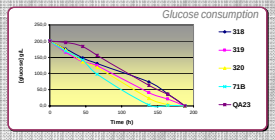
## 1 Physiological characterization of *S. cerevisiae* strains

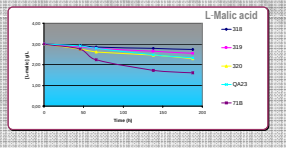
| Ethanol resistance | Malic acid utilization | Acetic acid utilization | $H_2S$ production | Number of isolates |
|---|---|---|---|---|
| - | - | - | + | 4 |
| - | - | - | ++ | 2 |
| - | - | + | - | 2 |
| - | - | + | + | 20 |
| - | - | + | ++ | 10 |
| - | + | - | + | 12 |
| - | + | - | ++ | 6 |
| - | + | + | - | 6 |
| - | + | + | + | 4 |
| - | + | + | ++ | 1 |
| + | - | - | + | 6 |
| + | - | - | ++ | 5 |
| + | - | + | + | 48 |
| + | - | + | ++ | 6 |
| + | + | - | + | 2 |
| + | + | - | ++ | 20 |
| + | + | + | - | 3 |
| + | + | + | + | 1 |
| + | + | + | ++ | 1 |
| ++ | - | - | + | 10 |
| ++ | - | - | ++ | 5 |
| ++ | - | + | + | 6 |
| ++ | - | + | ++ | 1 |
| ++ | + | - | + | 7 |
| ++ | + | - | ++ | 3 |
| ++ | + | + | + | 1 |
| ++ | + | + | ++ | 6 |

- *A remarkable phenotypical diversity was observed. The most frequently occurring metabolic profiles (red cells) was associated to*
  - *low (-) or good (+) ethanol tolerance in the presence of acetic acid;*
  - *intermediate (+) or good (++) capability to utilize acetic acid;*
  - *moderate (+) $H_2S$ production;*
  - *no capacity to utilize malic acid.*
- *Only 35 strains were able to utilize malic acid, being two of them very efficient concerning malic acid metabolism.*
- *The 9 strains marked by a red square are preferential candidates for further studies, the strains marked by a dotted red square are of secondary importance.*
- *9 of the 168 strains exhibit interesting phenotypical traits that eventually can have a positive impact as future "specialist strains" for the Vinho Verde Region.*

## 2 Growth in a synthetic must culture medium containing glucose (20 %, w/v) and D,L- malic acid (0.6%, w/v)



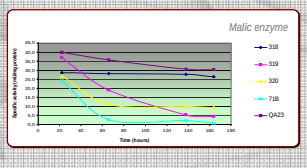




*The growth profiles of indigenous strains (318, 319, and 320) in a synthetic must medium were very similar to the ones observed for the commercial strains QA23 and 71B.*

*71B consumed glucose more rapidly (5 days) followed by strain 320 (7 days). Strains 318,319 and QA23 needed more time to deplete glucose from the medium (8 days).*

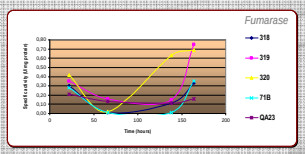
**The commercial strain 71B degraded 46%, whereas 320 and QA23 24% and 23%,respectively. Strains 318 and 319 only showed 8% and 15% malic acid degradation, respectively.**

## 3 Enzymatic assays

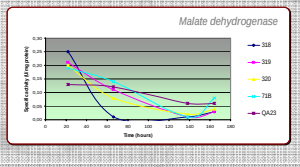



*After 22h of growth malic enzyme activity decreased in strains 319, 320 and 71B, whereas strains 318 and QA23 slightly decreased or maintained the activity of malic enzyme.*

*Considering that strain 71B consumed most malic acid in the above mentioned synthetic must medium, it seems that consumption of this organic acid is not necessarily/exclusively linked to the activity of the malic enzyme.*




*After 22h of growth the enzyme displays loss of activity (catabolic repression) that is recovered after glucose depletion. Strains 319 and 320 showed twice the fumarase activity of the other strains.*




*Malate dehydrogenase activity decreases during glucose consumption . Strain 318 showed the most pronounced decrease, that may explain the low malic acid degradation (8%).*

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## Conclusions

*Exploring the biodiversity of indigenous fermentative strains, using simple selection criteria is the basis for further studies that provide deeper insight in the genetic variability. As the use of genetically modified yeasts in winemaking is a highly controversial topic, we consider that the systematic exploration of a wine regions' biodiversity is an important contribution towards the selection and understanding of strains carrying specific enological traits. Such studies are an essential complement to the existing knowledge about genetically modified strains.*

*The most efficient malic acid degrading strain (commercial strain 71B) did not show a higher activity of enzymes involved in malic acid consumption compared to other* **S. cerevisiae** *strains. The absence of correlations between malic acid consumption and enzyme activity indicates that other factors may be responsible for the use of this organic acid. Our data also show significant differences between fumarase and malic enzyme activities among indigenous and commercial S. cerevisiae strains.*

## Acknowledgements

*This study was financially supported by the programs POCTI/ BIO/38106/2001, POCI 2010 (FEDER/FCT, POCTI/AGR/56102/2004), and AGRO (ENOSAFE, Nº 762).*