

Adaptive evolution of *Saccharomyces cerevisiae* and its application in co-culture with *Saccharomyces kudriavzevii* in the production of fermented *Myrciaria jaboticaba*

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Evolución adaptativa de *Saccharomyces cerevisiae* y su aplicación en co-cultivo con *Saccharomyces kudriavzevii* en la producción de fermentado de *Myrciaria jaboticaba*

Received: 01/31/2021 | Reviewed: 02/07/2021 | Accept: 02/18/2021 | Published: 02/27/2021

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Abstract

The objective of this work was to apply the adaptive evolution technique using the *Saccharomyces cerevisiae* T73 strain to increase its tolerance to ethanol and to evaluate its behavior in co-culture with *Saccharomyces kudriavzevii* CR85 in the production of fermented *Myrciaria jaboticaba*. Fermentations were carried out at 25 °C for 186 hours under agitation of 150 rpm, according to a central. The consumption of sugar, ethanol, glycerol and acetic acid formed during the fermentation process was evaluated. The results showed that there is an improvement in ethanol tolerance in *S. cerevisiae* T73 when submitted to the evolution process. Its use for the production of fermentation of *Myrciaria jaboticaba* in co-culture shows that the highest yield was observed when 0.0372 g.L⁻¹ and 0.0648 g.L⁻¹ of *S. cerevisiae* T73 PE (that underwent evolution) and CR85 respectively. These results differed statistically from the experiments using the original T73 strain. Regarding the production of ethanol in co-culture there is a significant increase when using the evolved T73 strain, showing possible changes in the primary metabolism of the ethanol production process, due to the changes promoted during the adaptive evolution of the T73 strain. The results show the potential of the new strain for the production of fermented with higher concentrations of sugars in the must.

Keywords: Adaptive evolution; Ethanol tolerance; Fermented beverage; *Myrciaria jaboticaba*.

Resumo

O objetivo deste trabalho foi aplicar a técnica de evolução adaptativa utilizando a cepa de *Saccharomyces cerevisiae* T73 para aumentar sua tolerância ao etanol e avaliar seu comportamento em co-cultivo na presença de *Saccharomyces kudriavzevii* CR85 para produzir fermentado de *Myrciaria jaboticaba*. As fermentações foram conduzidas a 25°C por 186 horas sob agitação de 150 rpm, utilizando Delineamento Composto Central. Foi avaliado o consumo de açúcar, etanol, glicerol e ácido acético formado durante o processo de fermentação. Os resultados mostraram que houve melhora na tolerância ao etanol na cepa de *S. cerevisiae* T73 quando submetido ao processo de evolução. Seu na produção de fermentado de *Myrciaria jaboticaba* em co-cultivo mostra que o maior rendimento de etanol foi observado com 0,0372 g.L⁻¹ e 0,0648 g.L⁻¹ de *S. cerevisiae* T73 PE (que passou pela evolução) e CR85, respectivamente. Os resultados diferiram estatisticamente dos experimentos com a cepa T73 original. Em relação à produção de etanol em co-cultura, ocorre um aumento significativo com o uso da cepa T73 PE, evidenciando

possíveis alterações no metabolismo primário de produção do etanol, devido às mudanças promovidas durante a evolução adaptativa da cepa. Os resultados mostram potencial para produção de fermentados com maiores concentrações de açúcares no mosto a partir da nova linhagem evolucionada.

Palavras-chave: Evolução adaptativa; Tolerância etanólica; Bebida fermentada; *Myrciaria jaboticaba*.

Resumen

El objetivo de este trabajo fue aplicar la técnica de evolución adaptativa utilizando la cepa *Saccharomyces cerevisiae* T73 para incrementar su tolerancia al etanol y evaluar su comportamiento en co-cultivo con *Saccharomyces kudriavzevii* CR85 en la producción fermentado de *Myrciaria jaboticaba*. Las fermentaciones se realizaron a 25°C durante 186 horas con agitación de 150 rpm, según un Delineamiento Compuesto Central. Se evaluó el consumo de azúcar y la producción de etanol, glicerol y ácido acético formado durante la fermentación. Los resultados mostraron mejoras en la tolerancia al etanol por parte de la cepas *S. cerevisiae* T73 cuando se somete al proceso de evolución. Su uso para la producción de fermentado de *Myrciaria jaboticaba* en co-cultivo muestra que el mayor rendimiento de etanol se observó con 0.0372 g.L⁻¹ y 0.0648 g.L⁻¹ de *S. cerevisiae* T73 PE (que ha evolucionado) y CR85 respectivamente. Estos resultados difieren estadísticamente de los experimentos que utilizan la cepa original de *S. cerevisiae* T73. Con respecto la producción de etanol en co-cultivo se observa un aumento significativo al utilizar la cepa T73 PE, posiblemente debido los cambios en el metabolismo primario de producción de etanol, promovidos durante la evolución adaptativa de la cepa T73. Los resultados muestran el potencial de la nueva cepa para la producción de fermentados con mayores concentraciones de azúcares en el mosto.

Palabras-clave: Evolución adaptativa; Tolerancia al etanol; Bebida fermentada; *Myrciaria jaboticaba*.

1. Introduction

Jaboticaba is a native fruit from South America belonging to the *Myrtaceae* family. This fruit is ubiquitously distributed in the Brazilian biomes, with the largest quantities seen in the Atlantic Forest region, especially in the Brazilian territory from Pará to Rio Grande do Sul (Batista et al., 2018). The tree is usually small and the flowers and fruits are carried directly on the trunks and branches. Ripe fruits of jaboticaba have a white pulp (composed mainly of sugars) and a dark rind whose promising health effects have attracted the attention of scientists and consumers.

It is a subtropical climate plant and has high nutritional properties with high fiber content, carbohydrates, flavonoids, anthocyanin vitamins, and phenolic compounds, such as ellagic acid, condensed tannins, cyanidin 3-glucoside and delphinidin 3-glucoside are the main bioactive compounds present in jaboticaba (Batista et al., 2014; Plaza et al., 2016). In addition to natural consumption, the fruit can be used to make syrups, wine, liquor, vinegar and jelly (Duarte et al., 2016).

Although difficult to pin down an exact figure, the idea that postharvest losses (PHL) of perishables in Brazil stand at 30-45% has been widely accepted. In spite of the modernization of production systems and the logistics and distribution of perishables in the last decades, postharvest losses continue to be a persistent and relevant problem (Henz, 2017).

There is an abundance of exotic tropical fruits in Brazil with potential for use by the food industry. Different new uses and new methods for processing tropical fruits need to be developed to minimize production losses, generate more profits and promote the sustainable use of biomes, such as the cerrado (Brazilian savannah) and the Amazon forest. One possible use of these fruits is in the production of fruit wines (Duarte et al., 2016).

In addition to the pleasant taste, fruit fermentates can contain several types of antioxidant compounds that are important bioactive compounds (Čakar et al., 2019). Currently, phenolic compounds have received great attention for aggregation as potential agents for the prevention and treatment of many oxidative stress-related diseases.

Fruit fermentation is made using yeasts, which turn fruit sugar into ethyl alcohol, anhydrous carbon dioxide and a number of secondary elements in varying amounts. Due to this, the fermented fruit is considered an elaborate product, differing from manufactured products, characterized by mixtures of various raw materials (Walker & Stewart, 2016).

Saccharomyces cerevisiae is a yeast of great importance in many industries and it has been frequently used to produce food products and beverages (Ferreira et al., 2018).

It is the microbe most used for industrial bioethanol production (Widiastuti et al., 2011). However, in the industrial fermentation processes, *S. cerevisiae* cells are often stressed by a variety of environmental factors, such as high temperature (Chen et al., 2019), hyperosmolarity (Hohmann, 2002), oxidation (Jamieson, 1998), the depletion of nutrients (Dinh et al., 2008), and the accumulation of ethanol (Ding et al., 2009).

Such stresses can affect cell viability and the final ethanol yield, and can even cause cell death (Ma & Liu, 2010). Among these environmental challenges, the ethanol accumulated during cultivation might be the major inhibitor to the completed fermentation. While ethanol is the product from the fermentation, ethanol itself functions as a stressor for the growth of *S. cerevisiae* cells (Izawa et al., 2010).

Several species from the genus *Saccharomyces* are involved in different industrial processes such as, baking, brewing, winemaking or bioethanol production. *Saccharomyces cerevisiae* plays a dominant role as a starter strain in wine fermentations, and dominates during the final stages of the process (Pretorius, 2000). However, natural hybrids between *S. cerevisiae* and *Saccharomyces kudriavzevii* are frequently isolated from cold-climate European countries (Erny et al., 2012; Peris et al., 2012).

We are still quite uncertain about the role of *S. kudriavzevii*'s genomic contribution in natural *S. cerevisiae* × *S. kudriavzevii* hybrids. *S. kudriavzevii* has been isolated from decaying leaves in Japan (Naumov, 2000), from oak bark samples in Portugal (Sampaio & Gonçalves, 2008) and from oak trees in Spain (Lopes et al., 2010), but not in fermentative industrial environments. Due to the lack of information, studies that evaluate the behavior of *S. kudriavzevii* in fermented drinks are of great scientific importance to clarify its potential in the sector.

In this context, the objective of this work was to use the laboratory adaptive evolution technique with the *Saccharomyces cerevisiae* T73 strain to increase its tolerance to ethanol, as well as to evaluate its behavior in co-culture with *Saccharomyces kudriavzevii* in the production of *Myrciaria jaboticaba* fermentate.

2. Methodology

2.1 Yeast strain and inoculum preparation

The microorganisms used in the present work were the *Saccharomyces cerevisiae* T73 and *Saccharomyces kudriavzevii* CR85 strains supplied by the Institute of Agrochemical and Food Technology (IATA), located in the city of Valencia, Spain. Yeasts were maintained in solid GPY medium (5 g.L⁻¹ yeast extract, 5 g.L⁻¹ peptone, 20 g.L⁻¹ glucose, and 20 g.L⁻¹ Agar).

In the inoculum preparation stage, cultures were scraped and placed in enpedorf tubes (1.5 mL) containing 1 mL of liquid GPY medium (without agar addition) and incubated at 30 °C for 24 hours.

2.2 Adaptive evolution of *Saccharomyces cerevisiae* T73 strain and evaluation of ethanol tolerance.

In this step batch fermentations were made using 100 mL glass vials and 50 mL useful volume (Santos, et al., (2020). The culture medium used was sugarcane synthetic must, consisting of 10 g.L⁻¹ glucose, 10 g.L⁻¹ fructose, 55 g.L⁻¹ sucrose, 1.8 g.L⁻¹ malic acid, 8.0 g.L⁻¹ citric acid, 0.75 g.L⁻¹ KH₂PO₄, 0.5 g.L⁻¹ K₂SO₄, 0.25 g.L⁻¹ MgSO₄.7H₂O, 0.155 g.L⁻¹ CaCl₂.2H₂O and 0.2 g.L⁻¹ NaCl. To this solution were also added 38.6 mL of the vitamin solution (composed of 0.05 g.L⁻¹ d-biotin, 1.0 g.L⁻¹ Ca-d-pantothenate, 1.0 g.L⁻¹ nicotinic acid, 25.0 g.L⁻¹ myo-Inositol, 1.0 g.L⁻¹ thiamine hydrochloride, 1.0 g.L⁻¹ Pyridoxal Hydrochloride and 0.2 g.L⁻¹ p-Aminobenzoic Acid), 38.6 mL of amino acid solution (composed of 0.743 g.L⁻¹ Tyrosine, 1.727 g.L⁻¹ Isoleucine, 4.113 g.L⁻¹ Aspartic Acid, 4.4 g.L⁻¹ Glutamic Acid, 1.797 g.L⁻¹ Arginine, 3.367 g.L⁻¹ Leucine, 0.163 g.L⁻¹ l-threonine, 3.183 g.L⁻¹ glycine, 2.93 g.L⁻¹ alanine, 2.843 g.L⁻¹ valine, 0.363 g.L⁻¹ methionine, 1.733 g.L⁻¹ phenylalanine, 2.20 g.L⁻¹ serine, 0.66 g.L⁻¹ histidine and 1.183 g.L⁻¹ lysine) and 10 mL of the trace element solution (composed of 1.5 g.L⁻¹ EDTA,

0.45 g.L⁻¹ ZnSO₄·7H₂O, 0.1 g.L⁻¹ MnCl₂, 0.03 g.L⁻¹ CoCl₂·6H₂O, 0.03 g.L⁻¹ CuSO₄, 0.04 g.L⁻¹ Na₂MoO₄·2H₂O, 0.45 g.L⁻¹ CaCl₂·2H₂O, 0.3 g.L⁻¹ FeSO₄, 0.1 g.L⁻¹ Boric acid and 0.01 g.L⁻¹ KI (Santos, et al., (2020).

Evolutionary fermentations were started with 8 % ethanol in synthetic must and cell concentration of 0.0464 g.L⁻¹, constantly under agitation of 150 rpm at 30 °C until reaching the steady growth phase. With every two repetitions of the fermentations, a new fermentation was started with 0.5 % more ethanol until reaching a total of 13 %.

After each fermentation, a colony was isolated in solid GPY medium with 12 % ethanol, starting with an adjustment to the cell concentration to 0.0464 g.L⁻¹ and collecting a 100 microliter aliquot of a 10⁻² dilution. The plates were incubated at 30 °C for 48 h and the largest colony was used to start the next fermentation.

To evaluate ethanol tolerance the crops were conducted with synthetic must composed of 210 grams of sugar plus 6 % ethanol. We accompanied the cell growth (spectrophotometer at 600 nm) of the *S. cerevisiae* T73 OR (Original) and T73 PE (That went through the evolution process) strains at four different points of evolution (evolution fermentations 0, 3, 7 and 22). For the conversion of absorbance (ABS) results to cell concentration (X in g.L⁻¹) two standard curves were constructed, one for *S. cerevisiae* T73 (X = 0.248.ABS) and one for *S. kudriavzevii* CR85 (X = 0.216.ABS). In mixed crops the mean of the two curves (X = 0.232.ABS) was used, since it did not present significant differences at the 5 % level (t-student test).

Fermentations were carried out in 100 mL glass vials (PHOX) with 50 mL of useable volume and an initial cell concentration of 0.0464 g.L⁻¹ at 30 °C in a shaking chamber at 150 rpm (TECNAL® TE - 4200) for 192 h.

2.3 Application of evolved and non-evolved *Saccharomyces cerevisiae* strains to evaluate kinetic behavior using the natural must of *Myrciaria jaboticaba*.

The production of a fermented beverage based on *Myrciaria jaboticaba* was chosen to evaluate the kinetic parameters of the yeasts, thus assessing the current application mixture.

The jaboticabas of the species *Myrciaria jaboticaba* were harvested at Jabuticabal Farm, located in the municipality of Hidrolândia, Goiás, Brazil. The fruits were selected with regard to the presence of defects or pests and washed with chlorinated water at 100 ppm. To obtain the must, the pulp was extracted by pulping and separated from the shell.

After pulping, the soluble solids content was corrected with sucrose P.A. until it reached 379.62 g.L⁻¹. To prevent bacterial growth, 1.5 % potassium metabisulphite was added to the must.

The fermentations performed at this stage were conducted in 100 mL glass vials with a 50 mL working volume at 25 °C for 186 hours while being agitated at 150 rpm.

Fermentations were performed in duplicates according to central composite design factorial 2² (Box & Hunter, 1987), which indicates a factorial experiment with two control factors, each one tested at levels encoded by - α , -1, 0, 1 and + α . An experimental matrix design was generated using Statistica™ software. This matrix with two designs is shown in Table 1. The selected factors (independent variables) were yeast T73 (Original and Partially Evoked) and CR85 yeast.

Table 1. Actual and coded matrix of the DCCR used for the *Myrciaria jaboticaba* fermentation with strains T73 (OR or PE) and CR85.

Experiments	T73 OR or PE (g.L ⁻¹)	CR85 (g.L ⁻¹)
C1	+1 (0,0635)	-1 (0,009504)
C2	-1 (0,0109)	-1 (0,009504)
C3	+1 (0,0635)	+1 (0,05529)
C4	-1 (0,0109)	+1 (0,05529)
C5	-1,4142 (0)	0 (0,0324)
C6	+1,4142(0,0744)	0 (0,0324)
C7	0 (0,0372)	-1,4142 (0)
C8	0 (0,0372)	+1,4142 (0,0648)
C9	0 (0,0372)	0 (0,0324)
C10	0 (0,0372)	0 (0,0324)
C11	0 (0,0372)	0 (0,0324)

Source: Authors.

Consumable sugar, ethanol, glycerol, acetic acid concentration was adopted as a response variable, which is one that suffers the effects in experiments when stimuli are intentionally introduced by the control factors. Thus, two designs were used to investigate the influence of adaptive evolution applied to the strain T73 OR and T73 PE in co-culture with the CR85 strain.

The consumed sugar, ethanol, glycerol and acetic acid results were analyzed by Statistica 10 software, using the multiple regression technique (p-value <0.1).

Maximum Growth Specific Speeds (μ_{max}) were also evaluated, this being the highest value obtained between Specific Speeds (μ_x) during fermentation), Process Productivity (P_p), Cell Mass Productivity ($P_{p/x}$) and the Fermentative Efficiencies (FE) of pure and co-cultivated crops. Equations 1, 2, 3 and 4 were used, respectively, to determine these parameters.

$$\mu_x = \frac{X_i - X_{(i-1)}}{t_i - t_{(i-1)}} \cdot \frac{1}{\left(\frac{X_i + X_{(i-1)}}{2}\right)} \quad (1)$$

$$P_p = \frac{ET_{tf} - ET_{t0}}{t_f} \quad (2)$$

$$P_{p/x} = \frac{ET_{tf} - ET_{t0}}{t_f} \cdot \frac{1}{X_f} \quad (3)$$

$$EF = \frac{\left(\frac{ET_{tf} - ET_{t0}}{S_{t0} - S_{tf}}\right) \cdot 100}{51,1} \quad (4)$$

X_i (g_{cells}.L⁻¹) is the cell concentration at a given time of fermentation, $X_{(i-1)}$ (g_{cells}.L⁻¹) is the cell concentration at the time before X_i , t_i (hours) the fermentation time in a given process time, $t_{(i-1)}$ (hours) being the fermentation time prior to t_i , ET_{tf} (g_{ethanol}.L⁻¹) ethanol concentration at end of fermentation, ET_{t0} (g_{ethanol}.L⁻¹) the concentration of ethanol at the beginning of the

fermentation, t_f (hours) the fermentation time, X_f ($\text{g}_{\text{cells}}\cdot\text{L}^{-1}$) the cellular concentration at the end of the fermentation, S_{i0} ($\text{g}_{\text{sugars}}\cdot\text{L}^{-1}$) the concentration of sugars at the beginning of the fermentation and S_{if} ($\text{g}_{\text{sugars}}\cdot\text{L}^{-1}$) the concentration of sugars at the end of the fermentation.

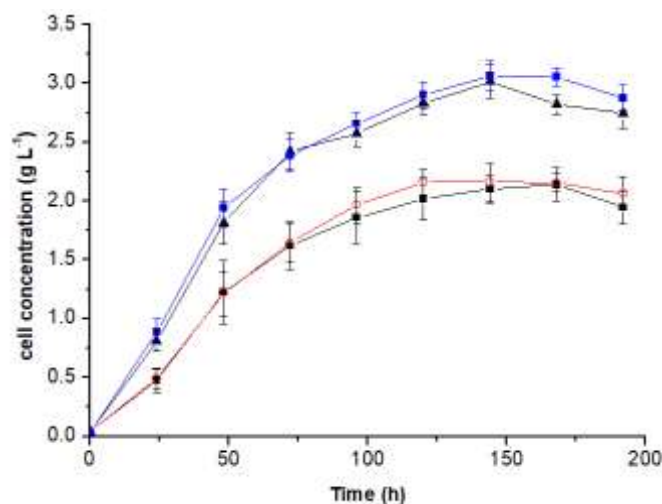
2.4 Analytical Determination

Quantitation of sugars, ethanol, glycerol and acetic acid was performed by High Performance Liquid Chromatography (HPLC). The samples were filtered through 0.22 μm nylon filters and analyzed in duplicates by Thermochromatograph HPLC (Thermo Fisher Scientific, Waltham, MA), with a refractive index and ultraviolet detector at 210 nm. The column used was HyperREZTM XP Carbohydrate H + 8 μm (Thermo Fisher Scientific), protected by HyperREZTM XP CarbohydrateGuard pre-column (Thermo Fisher Scientific). The analysis conditions were: 1.5 mM H_2SO_4 mobile phase, 0.6 $\text{mL}\cdot\text{min}^{-1}$ flow and 50 $^\circ\text{C}$ oven temperature (Silva et al., 2019).

3. Results and Discussion

During the process of adaptive evolution significant improvement was observed at 5 % level (Student's t-test) in relation to ethanol tolerance until the seventh fermentation process. From this moment on the tolerance was no longer statistically significant. Figure 1 shows the results of cell concentration during the fermentation process in a synthetic must with 6 % ethanol in its constitution.

Figure 1. Kinetics curves comparing cell growth in 6% ethanol: X ($\text{g}_{\text{cells}}\cdot\text{L}^{-1}$). Original (\blacksquare); P3 - Third Evolution Fermentation (\circ); P7- Seventh Evolution Fermentation (\blacktriangle); P22 - Twenty-second evolution fermentation (\blacksquare).



Source: Authors.

It is observed that the partially evolved strain, under any of the conditions used, presented higher cell growth compared to the original yeast, the data of which shows a better adaptation of the yeast when it is in the presence of ethanol in the course of fermentation (Figure 1). From 11 % of ethanol in the progressive fermentation processes no further improvement of the yeast was observed regarding the tolerance to ethanol possibly because there hadn't been a sufficient number of fermentations or because it developed some mechanism of stable genetic conservation, preventing its evolution from the seventh fermentation, thus requiring a greater number of fermentations to adapt to more stressful conditions in the presence of ethanol.

Considering that significant differences were observed at the 5 % level (t-student test) in ethanol tolerance between evolution fermentations 3 and 7, a new stage of application and comparison of these strains within a conventional fermentation process began using jabuticaba must as raw material. Jabuticaba stands out for the presence of phenolic compounds, as is the case with anthocyanins, which can act as antioxidants helping in the fight against free radicals.

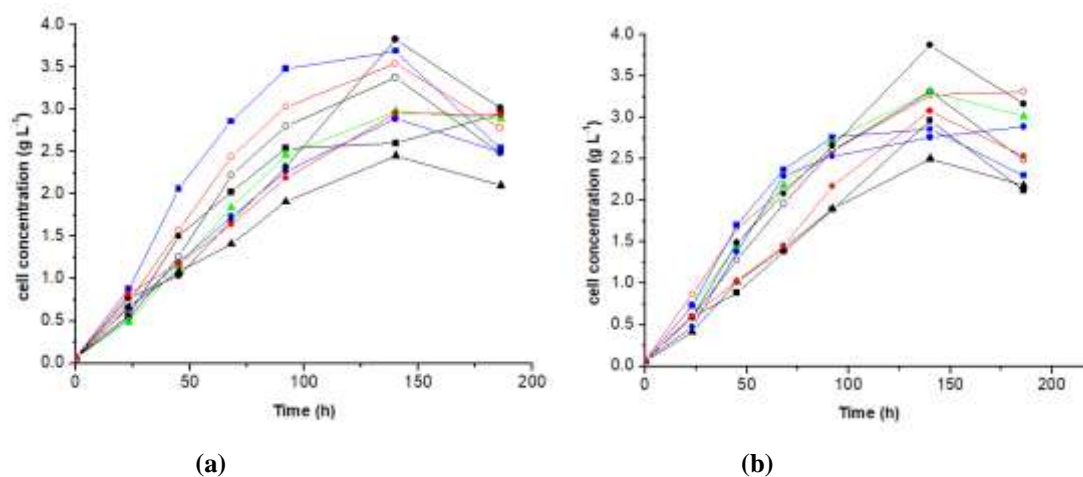
In order to improve the characteristics of the fermented beverage, it was decided to work with a mixture of *Saccharomyces cerevisiae* strains and *Saccharomyces kudriavzevii* CR85. Studies indicate significant improvements in glycerol and ethanol composition, promoting characteristics of greater acceptance in wines (Alonso-del-Real et al., 2017).

In the present work, the jabuticaba must used for the development of a fermented beverage presented a value of $379.62 (\pm 0.2) \text{ g.L}^{-1}$ total sugars and a pH of $3.64 (\pm 0.05)$. Thus, jabuticaba pulp is considered chemically as acidic, which for industrial purposes may present possible delays with regard to microbial changes, prolonging its useful life. According to Costa et al., (2020) the characteristics in terms of soluble solids are high and necessary for products with alcoholic fermentates.

After characterization and adjustment of the sugar content in the must, the fermentation of the beverage from this raw material followed.

The Figure 2 shows growth curves for co-culture experiments and pure cultures using the *Saccharomyces cerevisiae* T73 OR (Figure 2a), *Saccharomyces cerevisiae* T73 PE (Figure 2b) and *Saccharomyces kudriavzevii* CR85 strains. It is observed that in the dispersion figures only 9 of the 11 experiments are presented since the experiments C9 to C11 refer to triplicate of the central point of the experimental design used in the present work and an average of these results was used for better visualization.

Figure 2. Cell concentration in *Myrciaria jaboticaba* fermented (g L^{-1}). T73 OR (a) and T73 PE (b) under the following conditions: C1 (■); C2 (○); C3 (▲); C4 (●); C5 (▲); C6 (■); C7 (●); C8 (○); C9 (●) of the DCCR.



Source: Authors.

Figure 2 illustrates the kinetics of yeast growth during 186 h of fermentation. As with Arroyo-López et al. (2009a), the fermentations showed no lag phase. This phenomenon can be observed when yeasts are in an active growth period due to their pre-adaptation under conditions similar to those used in the fermentation process. Another very significant observation between the experiments of Figures 2 (a) and (b) is the difference between their behaviors, indicating clear changes in metabolism between the original and partially evolved T73 strains.

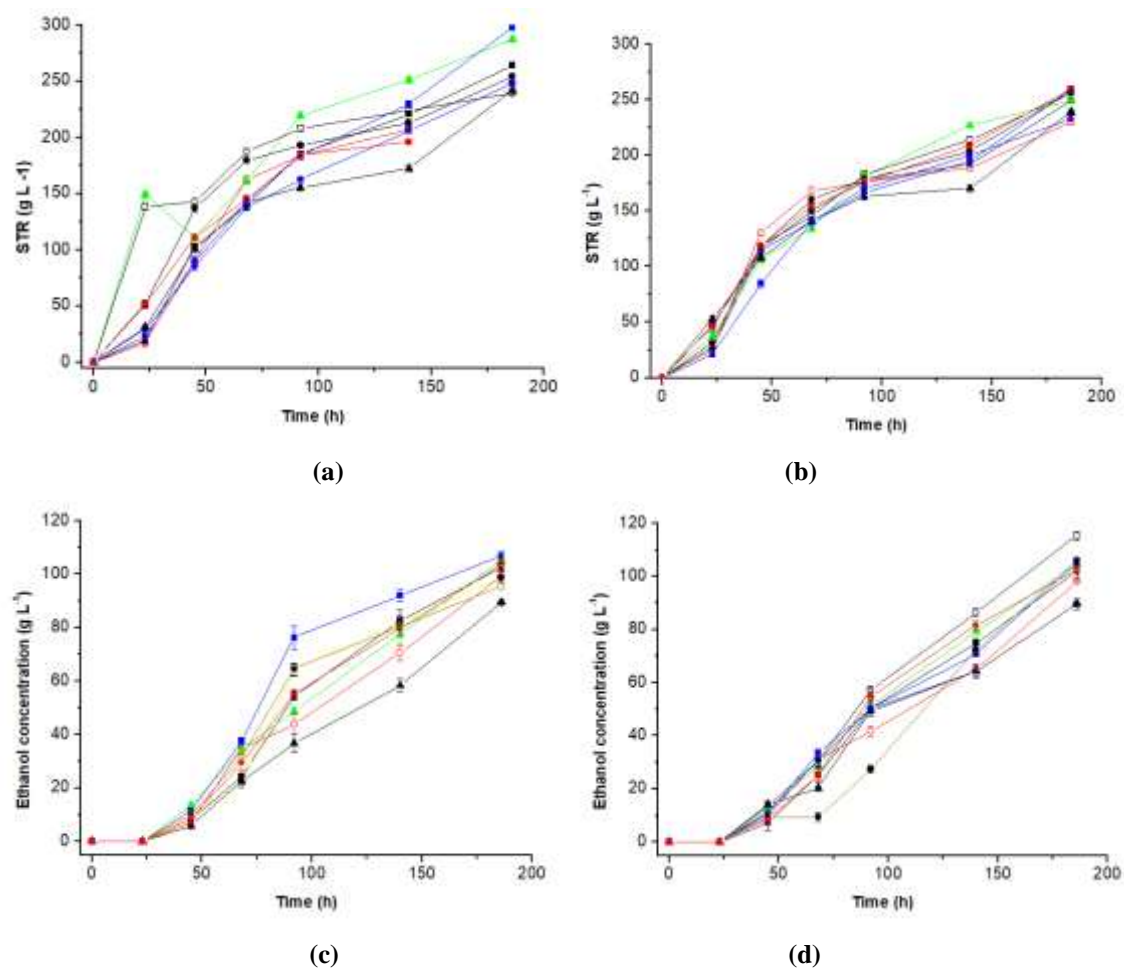
The only condition that shows a slight synergism are with yeasts T73 PE and CR85, with initial cell concentrations of 0.0372 and 0.0648 g.L^{-1} , respectively. This observation is characterized by the increase of mixed crop biomass when compared

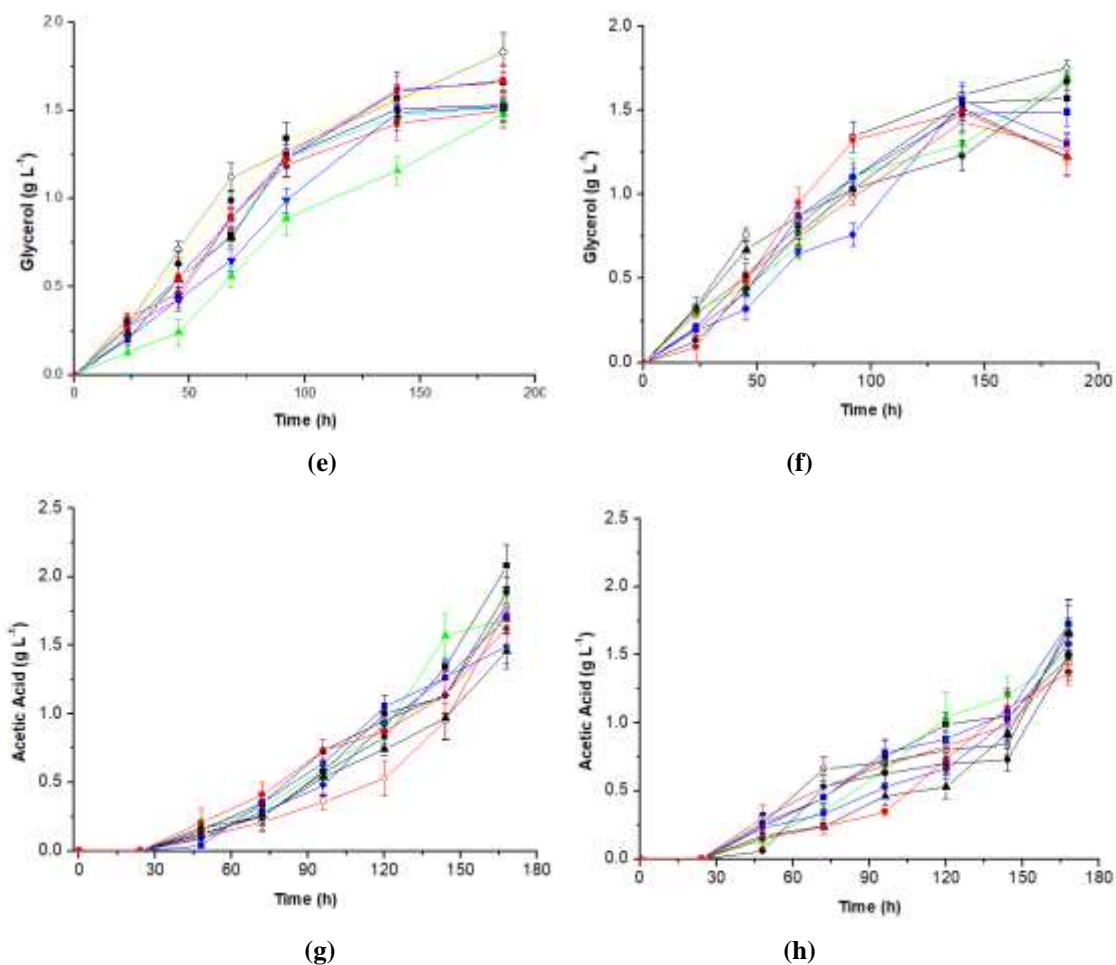
to pure crops. In the experimental conditions with an initial concentration of 0.0635 and 0.0095 g.L⁻¹ of yeast T73 PE and CR85, respectively, as well as using a ratio of 0.0372 : 0.0372 g.L⁻¹, the phenomenon of antagonisms was observed, which corresponds to the decay of biomass when compared to each microorganism that composes it.

In pure experiments using *S. kudriavzeii* CR85, low cell growth was observed, as expected. Henriques et al., (2018) state that the *S. kudriavzeii* CR85 strain typically shows slower growth during fermentation compared to the *S. cerevisiae* T73 strain. According to Henriques et al., (2018) and Gonzalez et al., (2006) the *Saccharomyces kudriavzevii* strain CR85 is only better in comparison to *Saccharomyces cerevisiae* T73 at temperatures below 15°C.

The results of sugar consumption, ethanol production, glycerol and acetic acid from fermentations can be observed in Figure 3.

Figure 3. Results of total sugar consumption (STR), ethanol, glycerol and acetic acid found in DCCR using T73 OR (a, c, e and g) and T73 PE (b, d, f and h) under the conditions C1 (■); C2 (○); C3 (▲); C4 (●); C5 (▲); C6 (■); C7 (●); C8 (○); C9 (●).





Source: Authors.

Observing the sugar consumption in the experiments (Figure 3a and 3b), we verified that the pure cultures with *Saccharomyces kudriavzevii* CR85 demonstrated significantly lower sugar consumption rates ($p < 0.1$) when compared to the experiments that used *Saccharomyces cerevisiae* T73. Such behavior is common since this strain is not a good fermentative when compared to *Saccharomyces cerevisiae*. González et al., (2007) report that *Saccharomyces cerevisiae* has a higher ability to metabolize sugars when compared to *Saccharomyces kudriavzevii*. This behavior was also reported by López-Malo et al., (2013).

When evaluating the kinetic parameters of pure and mixed cultures, there is a significant influence on the different concentration conditions of the strains used at the beginning of the fermentation process. These differences are also observed between the original and partially evolved T73 strains (OR and PE), as shown in Table 2.

Table 2. Results of Maximum Specific Cell Growth Rate (μ_{\max}), Process Productivity (P_P), Cell Mass Productivity ($P_{P/X}$), and Fermentative Efficiency (FE) found in DCCR using *S. cerevisiae* T73 PE, T73 OR and *S. kudriavzevii* CR85 strains.

Exp.	μ_{\max} (h ⁻¹)		P_P (g _{ethanol} .L ⁻¹ .h ⁻¹)		$P_{P/X}$ (g _{ethanol} .g _{cells} ⁻¹ .L ⁻¹ .h ⁻¹)		FE	
	T73 OR	T73 PE	T73 OR	T73 PE	T73 OR	T73 PE	T73 OR	T73 PE
C1	0,068±0,001	0,069±0,002	0,552±0,006	0,548±0,009	0,187±0,012	0,257±0,003	76,11±0,26	80,07±0,50
C2	0,072±0,001	0,074±0,003	0,533±0,009	0,532±0,002	0,191±0,004	0,161±0,009	74,14±0,17	84,33±2,05
C3	0,065±0,006	0,072±0,001	0,566±0,011	0,600±0,007	0,196±0,010	0,199±0,007	71,71±0,72	87,60±0,05
C4	0,070±0,003	0,065±0,005	0,536±0,002	0,563±0,010	0,215±0,012	0,195±0,014	78,64±0,89	88,22±2,19
C5	0,071±0,001	0,069±0,003	0,481±0,007	0,482±0,007	0,229±0,014	0,221±0,012	72,49±0,97	73,41±0,97
C6	0,074±0,004	0,072±0,002	0,574±0,007	0,565±0,009	0,226±0,009	0,245±0,011	70,20±0,12	79,32±0,13
C7	0,072±0,001	0,068±0,002	0,531±0,001	0,571±0,002	0,176±0,005	0,181±0,009	76,04±1,87	80,89±1,45
C8	0,067±0,006	0,069±0,002	0,514±0,005	0,620±0,009	0,207±0,007	0,251±0,029	78,23±0,88	88,08±0,05
C9	0,073±0,003	0,069±0,001	0,557±0,009	0,551±0,005	0,189±0,005	0,217±0,001	78,62±0,47	79,57±1,39

Source: Authors.

There is no clear relationship within fermentation processes when it comes to evaluating the maximum specific cell growth rates, process productivity and productivity by cell mass, but the highest process productivity was found in the mixture which used the T73 PE strain. (An experiment with 0.0372 g.L⁻¹ and 0.0648 g.L⁻¹ of T73 PE and CR85, respectively). This result is statistically different at the 5 % level (t-student test) from the higher yield found in the T73 OR strain experiments (0.0744 g.L⁻¹ and 0.0324 g.L⁻¹ of T73 PE and CR85, respectively). This same behavior is observed for cell mass productivity.

These results may possibly indicate a better adaptation of this strain with the ability to metabolize sugars under conditions of higher concentrations of ethanol in the fermentation process.

Such a statement can be confirmed from the improvement in the fermentative efficiency, because in all the experiments that used the T73 PE strain, the results were superior in relation to the T73 OR strain. This is clear evidence that proves changes in the functioning of its metabolism. The condition that best represents changes in this phenomenon (22.17 % improvement in fermentation efficiency) can be seen among the experiments that used the T73 (OR or PE) and CR85 yeast in the initial cell concentrations of 0.0635 and 0.0553. g.L⁻¹, respectively. Even while presenting superior results, the only condition that did not present a significant difference at 5% level (t-student test) was when the same proportion (0.0372 g.L⁻¹) of both yeasts was used.

With *Saccharomyces* there is usually a direct relationship between sugar consumption and ethanol production, i.e., the higher the sugar consumption, the higher the ethanol production. The aspect that often varies between strains is their Fermentative Efficiency, as observed in the present study when applying the technique and adaptive evolution.

From the ethanol production results, significant values were obtained (p-value <0.1). Using the T7 PE strain, the reduction of the non-significant term, produced a coefficient of determination (R²) of 94.1%, showing a good fit with the experimental data and the model. When using strain T73 OR it is observed that the linear term of the strain CR85 was not significant. Equations 5 and 6 show the reduced models to predict ethanol results under the different conditions studied for T73 OR (X_{1OR}) and T73 PE (X_{1PE}) strains, respectively, with strain CR85 (X₂).

$$Y = 103,81 + 4,49 * X_{1OR} - 1,46 * X_{1OR}^2 - 1,62 * X_2^2 + 3,0494 X_{1OR} X_2 \quad (5)$$

$$Y = 102,96 + 3,97 * X_{1PE} - 2,78 * X_{1PE}^2 + 3,52 * X_2 + 3,97 * X_2^2 \quad (6)$$

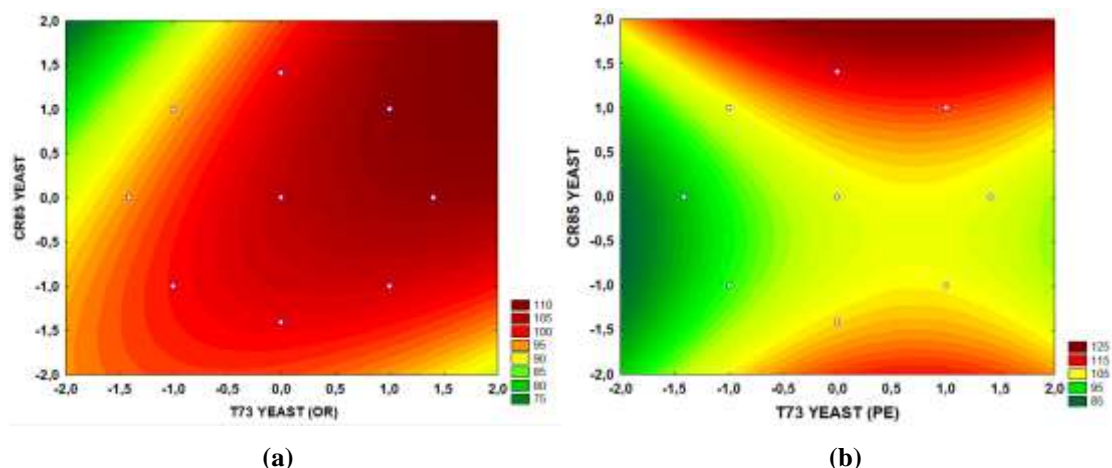
Equation 5 shows that the lowest coefficient represents the quadratic term of the T73 PE strain, showing that ethanol production is influenced by the high concentrations of the T73 and CR85 strains, where they tend to contribute positively to the increase in ethanol production.

Figure 1 (a) and (b) illustrate ethanol production during the fermentative processes in a co-culture of the T73 OR and T73 PE strains, respectively, with the CR85 strain during 186 h of fermentation.

The highest concentrations of ethanol (110.36 g.L^{-1}) in the experiments using T73 OR were found when the highest concentration levels of the co-culture strains were added. In the experiments using T73 PE the highest ethanol concentrations (115.29 g.L^{-1}) were found. Another important aspect is that in relation to ethanol production, the initial T73 PE concentration did not interfere significantly.

In the present work it is observed that the low sugar consumptions in the pure cultures of *S. kudriavzevii* CR85 resulted in a low conversion to ethanol, reaching maximum values of approximately 90 g.L^{-1} . Such results can also be verified in Figure 4.

Figure 4. Contour curves of ethanol production found in the DCCR using *S. cerevisiae* T73 OR (a), T73 PE (b) and *S. kudriavzevii* CR85 strains.



Source: Authors.

Evaluating the production of ethanol (Figure 4), it was observed that the highest concentrations were reached in T73 PE and CR85 mixed cultures, in which 110.36 g.L^{-1} of ethanol in the mixture of 0.0635 (T73 OR) : 0.0552 (CR85) g.L^{-1} of initial biomass and 115.29 g.L^{-1} of ethanol in the mixture of 0.0372 (T73 PE) : 0.0648 (CR85) g.L^{-1} of initial biomass. Evaluating the same experimental conditions in both designs, a significant increase was observed when the T73 PE strain was used, with an increase of 1.18 g.L^{-1} and 19.7 g.L^{-1} of ethanol for the mixture at 0.0635 (T73) : 0.0552 (CR85) g.L^{-1} and 0.0372 (T73) : 0.0648 (CR85) g.L^{-1} of initial biomass, respectively. These results show changes in the primary metabolism of the ethanol production process, due to changes promoted during the adaptive evolution of the T73 strain.

One of the explanations for the change in this behavior may have been due to changes promoted in the transport of sugars through the plasma membrane of cells, since according to Carlsen et al., (1991), during the fermentation process of *S. cerevisiae*, the gradual increase of ethanol (5 to 20 %) in the culture medium acts as a strain inhibiting agent. This inhibition correlates with an increase in the alcohol partition coefficient, suggesting that the initial action of ethanol occurs at hydrophobic sites, possibly in the plasma membrane. As a result, the membrane permeability is altered, as is the sugar transport system.

Another possible explanation for the behavior observed in the T73 PE strain may have been due to some alteration in gene expression, allowing its partial adaptation to ethanol (Fleet & Heard, 2002; Pérez-Torrado et al., 2016). These changes may promote restrictions during energy production due to increased expression of the genes associated with glycolysis and mitochondrial function. Alonso-del-Real et al., (2017) in his research with strains T73 and CR85 noted that the concentration of CR85 did not influence ethanol production. Peris et al., (2016) studying micro-vinification with Tempranillo and macabeo grapes, found that *S. cerevisiae* produces more ethanol than *S. kudriavzevii*.

The sequence of intracellular reactions to ethanol formation, alternative metabolic pathways appear to provide the greatest adaptation of yeast during fermentation. These routes mainly lead to the formation of glycerol (Rosa & Péter, 2005). The literature reports approximately 1 % (w / v) of sugar consumed by yeast is diverted to glycerol synthesis, acids and cell mass formation. Figures 3 (e) and (f) show the kinetic behaviors of glycerol production.

The use of *S. kudriavzevii* CR85 did not make a statistically significant contribution to glycerol production possibly because of jaboticaba must composition. There are no reports in the literature on the use of *S. kudriavzevii* in this type of raw material, and the present work is an initial and potential reference for comparisons in future works.

Peris et al., (2016) in their studies with *Saccharomyces cerevisiae* and *S. kudriavzevii* performed micro-vinification with *Tempranillo* and *macabeo* and obtained practically identical results (1.27 to 1.76 g.L⁻¹ glycerol).

Novo et al., (2014) using grape must observed that evolved strains and ethanol-tolerant mutants, respectively, produced (p<0.5) more glycerol than unevolved and non-mutated strains.

Another secondary product of alcoholic fermentation is acetic acid (Rosa & Péter, 2005). In *Saccharomyces cerevisiae* the additional metabolism of acetic acid is of utmost importance, since this acid is the essential component of yeast fatty acid biosynthesis (Van den Berg & Steensma, 1995).

According to the results of Figure 3 (g) and (h), the highest concentration of acetic acid (2.08 g.L⁻¹) was found for the experiment using initial yeast concentration of 0.0635 and 0.0095 g.L⁻¹ of T73 OR and CR85, respectively. The highest concentration of acetic acid in the experiments with the T73 PE strain (1.73 g.L⁻¹) was found in the mixture of 0.0744 and 0.0324 g.L⁻¹ of T73 PE and CR85, respectively.

Although the lower acetic acid production responses were found in the experiments using partially evolved strains, the results did not differ statistically (p<0.05) from those using the original strain. The values obtained in this study with both strains (T73PE and OR) are very similar to those found by Morales et al., (2015), Contreras et al., (2015), Alonso-del-Real et al., (2017) and Henriques et al., (2018). Novo et al., (2014) In their studies with evolved and non-evolved strains different behavior was observed to the present study, that is, the evolved strains produced the most acetic acid when compared to the unevolved strains.

Another important aspect of the present study is that depending on the initial proportion of yeast in fermentation, different compositions may be presented of this metabolite at the end of the process. The results show that the higher the initial concentration of T73 (OR and PE) and CR85 strains, the greater the production of acetic acid in the fermentation process.

Evaluating the set of experimental results of the *Myrciaria jaboticaba* fermented drink it is observed that the results of concentration of sugars, ethanol, glycerol and acetic acid are within the desired standards for a product of this category. In general, the condition that allowed the most commonly acceptable composition for the *Myrciaria jaboticaba* beverage was that around the mixture of 0.0372 and 0.0648 g.L⁻¹ initial concentration of *S. cerevisiae* T73 PE and *S. kudriavzevii* CR85, as we saw and which was the combination that presented the highest concentrations of ethanol and glycerol and acetic acid contents that do not compromise the quality of the fermented product. These compounds are responsible for good characteristics of fermented beverages and can attribute important sensory aspects for the positive acceptance of this type of product.

4. Conclusion

In the present study there is a large amount of information on the kinetic and metabolic behavior of co-culture between *Saccharomyces cerevisiae* T73 and *S. kudriavzevii* CR85 in *Myrciaria jaboticaba* must. It was possible to observe evident changes in the metabolic behavior of the partially evolved *S. cerevisiae* T73 strain when compared to *S. cerevisiae* T73 which was not submitted to any evolutionary process (original). The main changes are in the aspects related to ethanol tolerance, increased ethanol productivity and improved fermentative yield. The use of co-culture with different proportions of the T73 and CR85 strains shows that their interactions can lead to changes in the composition of the fermented beverage of *Myrciaria jaboticaba*, especially regarding ethanol concentration. The results suggest the continuity of the evolutionary process and genomic sequencing to identify the main changes in *Saccharomyces cerevisiae* T73.

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