

Yeasts with Fermentative Potential Associated with Fruits of Camu-Camu (*Myrciaria Dubia*, Kunth) from North of Brazilian Amazon

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Abstract

Purpose

Considering the high biotechnological potential of yeasts associated to edible fruits, a screening for these microbes able to alcoholic fermentation was performed in ripe fruits of camu-camu.

Methods

Fruits of camu-camu (*Myrciaria dubia*, Kunth) were collected in north of Brazilian Amazon, in floodplain of Cauamé river. Yeasts were isolated, and fermentation capability was evaluated using Durham tubes. Quantitative assays were performed to calculate ethanol yield (g.g^{-1}), specific growth rate (h^{-1}) and ethanol productivity ($\text{g.L}^{-1}.\text{h}^{-1}$). Taxonomic identification was performed by ribosomal genes nucleotides sequences analysis by alignment using BLASTn.

Results

A total of fifteen yeasts colonies were isolated, and eight of them presented the ability to ferment glucose to ethanol. Six of them were identified as three different species: *Candida orthopsilosis*, *Pichia kudriavzevii* and *Meyerozyma caribbica*. When cultured in broth containing 180 g.L^{-1} of glucose, *M. caribbica* reaches 91.7 percent of the maximum theoretical ethanol concentration (84.4 g.L^{-1}), presenting ethanol yield and productivity of 0.4688 g.g^{-1} and $0.781 \text{ g.L}^{-1}.\text{h}^{-1}$, respectively.

Conclusions

The endophytic microbiota of camu-camu includes *C. orthopsilosis*, *P. kudriavzevii* and *M. caribbica*. This paper is a rare report of *C. orthopsilosis* with endophytic habit, because most of the references indicate it as human pathogenic. Besides this, *M. caribbica* is a promising fermenter for alcoholic beverages, due to its osmotolerance and high ethanol yield. This is the first paper reporting endophytic yeasts associated with fruits of *Myrciaria dubia*.

1. Background

Yeasts are microbes from Fungi kingdom that present asexual reproduction by fission or budding, and with sexual reproductive structure not presenting fruiting bodies (Kurtzman et al. 2011). Currently there are more than 2500 described species and recognized at specialized literature (Azhar et al. 2017) but, despite of this number, there are only about 80 species used to bioprocesses in laboratory scale and a few more than twelve used in industrial processes (Türker 2014).

Amazon is the greatest tropical rainforest of the world, containing about 220 edible fruits species (Carvalho 2012), including camu-camu (*Myrciaria dubia*, Myrtaceae, Myrtales, Magnoliopsida, Magnoliophyta, Plantae). Fruits are potentially the natural habitat to a great variety of microbes because their abundance of sugars and water, being favorable to proliferation of yeasts, mainly those able to

perform alcoholic fermentation. Despite of all this potential, researches about yeasts communities associated to Amazonian fruits are scarce, some of those dating more than two decades ago (Ganter et al. 2017).

Myrciaria dubia has attracted a lot of attention because of its remarkable quantity of bioactive compounds (Azevedo et al. 2019). Specially because of antioxidant activity, their fruits have been used for functional food production, including an artisanal beer with high ascorbic acid concentration (Pimentel et al. 2019). Besides that, there are not specific studies published about the microbiota associated with their leaves, roots or fruits. The elucidation about yeasts associated to this plant may result in new strains with applicability as to biofuel as to food's industry.

In this context, as for taxonomic purposes as for biotechnological applications, sampling efforts are a necessary approach. The aim of this work was to isolate and identify yeasts associated with fruits of *Myrciaria dubia* able to perform alcoholic fermentation, evaluating some kinetic parameters along the fermentative process.

2. Materials And Methods

2.1. Collect and Isolation

Ripe fruits of *M. dubia* were collected manually from the bushes (Cauamé river floodplain, 2°51'54.79"N, 60°39'44.19"W), and maintained under refrigeration ($4\text{ }^{\circ}\text{C} \pm 2$) until their processing (about 2 hours). The fruits were washed in water to remove macro-particles, immersed in sodium hypochlorite solution (1% v/v) for 1 minute, ethanol 70% for 1 minute and, finally, washed in sterilized distilled water for 2 minutes. After superficial decontamination, fruits were stored in a sterilized vessel at room temperature until presenting aroma resembling bread or alcoholic beverage, about 7 days (samples processed in the first day after collected did not present colonies growth). Then, their peels were removed and the mesocarps were mashed in sterilized distilled water (1:10 w/v).

Aliquots of 100 μL were spread in plates containing GYMP Agar (glucose 10 g.L^{-1} , yeast extract 3 g.L^{-1} , malt extract 3 g.L^{-1} , peptone 5 g.L^{-1} , Agar 20 g.L^{-1} , pH 5.0), incubated at $28\text{ }^{\circ}\text{C}$ for until 48 hours. Colonies presenting typical yeast's morphology were isolated and stored in sterilized mineral oil.

2.2. Fermentation assays

A qualitative fermentation test was performed, evaluating the capability of the isolates to ferment glucose, D-xylose, sucrose and maltose. A loopful of each isolate was inoculated in tubes containing liquid media composed of the respective sugar (40 g.L^{-1}) and yeast extract (10 g.L^{-1}). The tubes were incubated for until 14 days at $28\text{ }^{\circ}\text{C}$, evaluated each 24 hours to identify fermentation, evidenced by gas retention into Durham tubes (Barnett et al. 2000). The assays were performed in triplicate.

The isolates with positive results in the qualitative tests were used in a quantitative test, besides that evaluating their osmotolerance. The selected isolates were inoculated in 250 mL Erlenmeyer flasks containing 100 mL of broth (pH 5,0) composed of glucose (180 g.L⁻¹) and yeast extract (10 g.L⁻¹), incubated at 28 °C, 120 rpm for until 120 hours. Fermentation was monitored each 12 hours by measuring the mass of CO₂ released. Total inoculum was about 5 g.L⁻¹ (dry weight) and kinetic parameters were evaluated according to further description.

2.3. Taxonomic Identification

Yeasts with fermentative capability were identified using the nucleotides sequences of rDNA genes and Internal Transcribed Spacer (ITS) region. The genomic DNA was extracted according to previous description (Rosa et al. 2009), and amplified by PCR techniques using primers NL1 and NL4 (Lachance et al. 2003). The amplicons were used to sequencing reaction using BigDye kit (Applied Biosystems®), and nucleotides sequences were obtained using a 3500 automatic genetic analyzer (Applied Biosystems®). The sequences were compared to the GenBank database by alignment, using the on-line tool BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.4. Kinetic parameters and Analytical methods

Ethanol concentration ([EtOH], g.L⁻¹) along fermentation was determined by stoichiometric calculation, as previous description (Dijck et al. 2000). Specific growth rate (μ_{MAX} , h⁻¹) was calculated by the equation " $\ln[EtOH]_n/[EtOH]_i = \mu_{MAX} * t \pm b$ ", being $[EtOH]_n/[EtOH]_i$ the relation between current and initial ethanol concentration along *log* phase, and "t" is time in hours, according to previous description (Duarte et al. 2008).

Ethanol yield (Y_{EtOH} , g.g⁻¹) was calculated by the relation between mass of ethanol produced and mass of glucose consumed. Ethanol productivity (Q_{EtOH} , g.L⁻¹.h⁻¹) was calculated by the relation between final ethanol concentration and total time of fermentation before the stationary phase. Total reducing sugars concentration ([TRS], g.L⁻¹) was determined using Dinitrosalicylic acid method (DNS). Aliquots of 20 μ L of the samples (diluted in distilled water, 1:10 v/v) were mixed to 180 μ L of distilled water and 300 μ L of DNS solution. After 5 minutes of boiling, were added 1500 μ L of distilled water and absorbance was determined at 540 nm. The [TRS] was calculated using the formula "[TRS] = (ABS + 0.070)/0.015" (R² = 0.990).

3. Results

3.1. Isolation and Fermentative Capability

A total of 15 colonies were isolated, being identified with a numeric code preceded by "CC" (due to camu-camu, the vernacular form in Portuguese to *Myrciaria dubia*). More than a half was able to ferment

glucose, and some were able to ferment sucrose. No colonies presented capability to ferment D-xylose and maltose. Complete results are presented at Table 1.

Table 1
Results of fermentative capability evaluation for yeasts isolated from *Myrciaria dubia*.

Isolate	Sugars			
	Glucose	Sucrose	Maltose	D-Xylose
CC001	++	-	-	-
CC002	++	-	-	-
CC003	+++	-	-	-
CC004	+	+	-	-
CC005	+	+	-	-
CC006	+++	-	-	-
CC007	+++	-	-	-
CC008	-	-	-	-
CC009	-	-	-	-
CC010	-	-	-	-
CC011	-	-	-	-
CC012	-	-	-	-
CC013	-	-	-	-
CC014	-	-	-	-
CC015	+	+++	-	-

Subtitle: (+++) positive results in 48 hours; (++) positive results in 72 hours; (+) positive results in 96 or more hours; (-) no fermentation evidence along 14 days.

3.2. Taxonomic identification

A total of six isolates were identified and the obtained sequences were deposited in NCBI database under accession number PRJNA645409. The isolates were identified according to Table 2.

Table 2
Results of fermentative capability evaluation for yeasts isolated from *Myrciaria dubia*.

Isolate	Specie	High similarity	Strains related
CC001	<i>Pichia kudriavzevii</i>	100%	ATCC 34135
CC002	<i>Pichia kudriavzevii</i>	100%	ATCC 34135
CC005	<i>Candida orthopsilosis</i>	99,57%	CBS 11337
CC015	<i>Candida orthopsilosis</i>	99,57%	CBS 11337
CC003	<i>Meyerozyma caribbica</i>	99,81%	CBS 9966
CC006	<i>Meyerozyma caribbica</i>	99,81%	CBS 9966

3.3. Kinetic parameters and Analytical methods

In assays with initial glucose concentration of 180 g.L^{-1} , *Pichia kudriavzevii* CC001 and *Candida orthopsilosis* CC015 presented final ethanol concentration of 39.94 and 34.57 g.L^{-1} , respectively. These results correspond respectively to 43.4 and 37.5 percent of theoretical maximum, for this reason considered not promising to fermentation and not evaluated for other kinetic parameters.

Meyerozyma caribbica CC003 presented final ethanol concentration of 84.4 g.L^{-1} , corresponding to 91.7 percent of the theoretical maximum. The final [TRS] indicated full consumption of glucose, meaning that this isolate is osmotolerant. The Y_{EtOH} was 0.4688 g.g^{-1} , $Q_{\text{EtOH}} = 0.781 \text{ g.L}^{-1}.\text{h}^{-1}$ and $\mu_{\text{MAX}} = 0.025 \text{ h}^{-1}$. Complete kinetic curve of ethanol production by *M. caribbica* CC003 is presented at Fig. 1.

4. Discussion

The occurrence of *P. kudriavzevii* was reported associated with blossom and ripped fruits of apple, pear and plum in southwest Slovakia (Vadkertiová et al. 2012). Its association with natural fermentation of ripped pulp fruits was described when microbes associated with *Ziziphus mauritiana* were screened (Niyanga et al. 2007). Robs et al. (1989) reported its occurrence associated with rotten fruits in a pineapple plantation in Rio de Janeiro, Brazil. Furthermore, unpublished data of Rosa reports the occurrence of this yeast in rotten fruits of *Byrsonima* sp. (Ganter et al. 2017), a common fruiting plant around the collecting site of this work.

P. kudriavzevii is also reported in association with natural fermentation of cereal dough in West Africa (Houngbédji et al. 2018), in Chinese liquor Daqu (Xu et al., 2019) and component of the terroir of North Patagonian winemaking (Mónaco et al. 2016). Together, these facts indicate that *P. kudriavzevii* is a typical endophytic yeast associated with fruits, including *Myrciaria dubia*, and safe for human feeding.

Most of the reports about *C. orthopsilosis* in scientific databases are related to human health problems. This species is closely related to *C. parapsilosis*, commonly described as a commensal in human skin

and pathogenic yeast, capable to develop human tissue invasion and damage (Gácsér et al. 2007). Scarce reports describe this yeast associated to fruits of *Opuntia stricta* (Kurtzman et al. 2011) and tomatoes (Robl et al. 2014).

Its occurrence was described in natural fermentation of cotton seeds and rice beverage produced by Brazilian Amerindians (Ramos et al. 2011). Probably, the difference between endophytic and pathogenic lineages of *C. orthopsilosis* must be evaluated using other molecular markers. Because of the possibility of being a pathogenic isolate, its uses in industrial processes should be avoided until confirmative analysis for taxonomic identification and safe use.

Firstly, *Meyerozyma caribbica* was described as *Pichia caribbica*, distinguished from *P. guilliermondii* (Vaughan-Martini et al. 2005). The type-strain was isolated from sugar cane in Cuba, what explains its name “*caribbica*”. Further analysis repositioned it in a new genus, currently named *Meyerozyma* (Kurtzman and Suzuki, 2003).

Its occurrence was reported in association with rhizosphere in high salinity soil in South Korea (Kim et al. 2015), in corn-derived starch granules at Illinois, USA (Kurtzman et al. 2011) and associated with different insects of the order Diptera (De-Marco et al. 2018). *M. caribbica* was also reported as a prevalent species in the natural wet fermentation of coffee fruits and beans in Brazil (Evangelista et al. 2015), and in *Mangifera indica* fruits in Mexico (Bautista-Rosales et al. 2011). These facts indicate that this species is worldwide distributed and presents a cosmopolitan habit.

The biotechnological applications of *M. caribbica* include its use as oleaginous yeast for biodiesel production (Chebbia et al. 2019) and as biological control agent against *Colletotrichum gloeosporioides* in fruits of *Mangifera indica* (Aguirre-Güitrón et al. 2019). The yeast powder presented effectiveness even after 6 months of storage. *M. caribbica* did not induce any animal toxicity or obvious cytotoxic activity (Ocampo-Suarez et al. 2017). It was used in a mixed inoculum with *Saccharomyces cerevisiae* to produce cachaça (sugar cane spirit) because of its ability to produce ethyl acetate and other compounds resembling of the fruit’s aroma (Amorim et al. 2016). Together with its natural occurrence associated with fruits, it indicates that *M. caribbica* is safe to the food industry.

The results of qualitative fermentation tests corroborate as predicted by literature, with most of the yeasts belonging to ascomycetous groups and able to ferment glucose (Ganter et al. 2017). Final ethanol concentration obtained by *P. kudriavzevii* CC001 is similar to observed for different wild-type strains isolated by Chamnipa et al. (2018). The low yield observed can be explained by the relatively low incubation temperature, as the highest yields were obtained when temperatures over 40 °C were used (Dhaliwal et al. 2011; Pongcharoen et al. 2018). This possibility must be re-evaluated in a further research Project.

Ethanol production by *C. orthopsilosis* was similar to the obtained by Chamnipa et al. (2018) when using a wild-type strain. Low yield in alcoholic fermentation seems to be a common feature for this species.

The percentage of the maximum theoretical observed to *M. caribbica* CC003 (91.7 %) is greater than the 84.9 percent obtained by Sukpipat et al. (2016) when using a strain of *M. caribbica* for fermenting glucose to ethanol. The values of Y_{EtOH} here observed were similar to the obtained when *S. cerevisiae* and *P. kudriavzevii* were used to produce ethanol using glucose as substrate (Phong et al. 2019), and when *M. caribbica* was used to produce a new sweet sorghum distilled beverage (Lopes et al. 2019). Furthermore, Y_{EtOH} by CC003 is more than four times greater than the obtained when a wild-type strain of *M. caribbica*, isolated from Vietnam, was used to ferment glucose to ethanol (Tolieng et al. 2018).

Its ability to grow in medium with elevated glucose concentration and also presenting high ethanol yield indicates osmotolerance, being this strain suitable to fermentation of high original gravity brewing wort (Dragone et al. 2007). These results indicate that *M. caribbica* CC003 is a promising fermenter, including for food as well as alcoholic beverage industries.

The values of Q_{EtOH} and μ_{MAX} were lower than all the compared references, indicating slow speed of fermentation probably due to relatively small initial inoculum. Temperature and initial inoculum are factors that need to be adjusted in further works.

5. Conclusions

Endophytic microbiota of *Myrciaria dubia* includes *Pichia kudriavzevii*, *Candida orthopsilosis* and *Meyerozyma caribbica*.

This paper is a rare report of endophytic occurrence of *C. orthopsilosis*, as most of the references indicate this species as human pathogenic. There are scarce papers reporting the natural occurrence associated with fruits and other environmental samples. Other molecular markers must be evaluated to establish the difference between safe and pathogenic lineages of this species.

Besides having high ethanol yield, *Meyerozyma caribbica* CC003 also presents tolerance to elevated amounts of glucose and ethanol. It is a promising fermenter for alcoholic beverage production.

The subsequent efforts will be employed to produce an alcoholic beverage using *M. caribbica* CC003, evaluating its chemistry profile, nutritional properties and organoleptics characteristics.

Declarations

Ethics approval: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing Interests: The authors declare that they have competing interests.

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Author's contributions: ITSRM performed isolation, fermentation assays and calculated kinetic parameters. VAS worked on demands of molecular biology and nucleotides sequencing. GRD worked on manuscript writing and proof-reading. SAF acted as a research's mentor and provided financial support. MJSV acted as laboratory coordinator, research's mentor and provided financial support.

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Figures

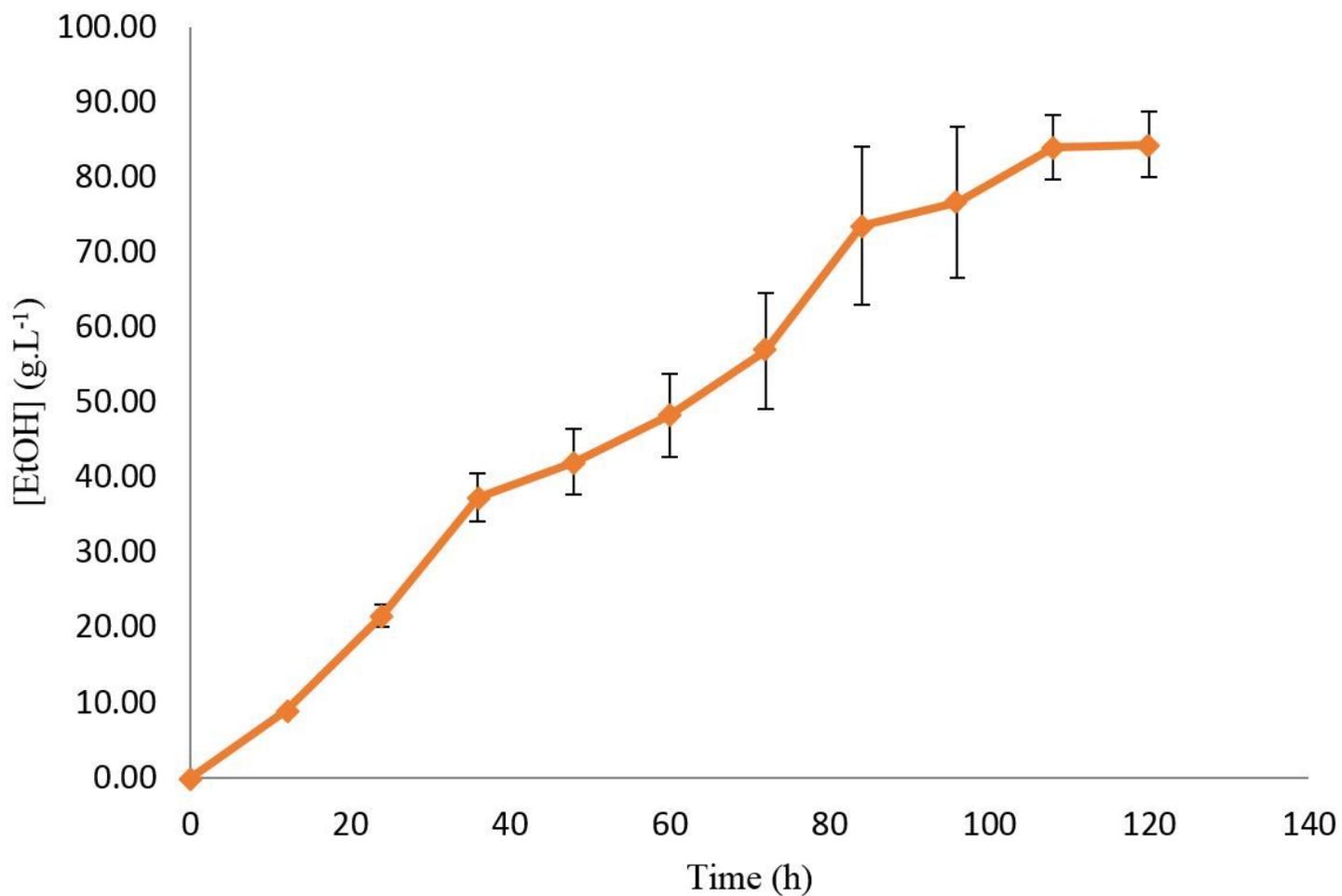


Figure 1

Ethanol production by *Meyerozyma caribbica* CC003 along 120 hours of fermentation. Stationary phase was observed after 108 hours. Error bars indicate standard deviation.