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Capability of *Candidas* to Ferment Mixtures of Carbohydrates to Alcohol in Free Cells and Co-Culture

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Abstract: Organic biomass is an attractive feedstock for second generation alcohol production. Wild-type strains of the genus *Candida* showed capabilities different to produce alcohol fermenting a carbohydrates mixture (synthetic medium), individually and in co-culture. Therefore, the main objective of this work was to evaluate the capability of *Candida* wild-type strains isolated from termite gut and rumen liquid, to ferment the most commonly carbohydrates presented in citrus residues, individually and in co-culture to alcohol production. *C. Tropicalis* (LR4) presented higher percentage of carbohydrate consumption (74.20% ± 4.60%), alcohol production (44.53 ± 0.01 gL^{-1}) and maximal alcohol productivity (6.40 ± 0.01 gL^{-1} day) than *C. Glabrata* (T1). Co-culture schemas, CC1 (LR4: 60%; T1: 40%) and CC3 (first LR4 alone and 2 days later T1) presented the highest alcohol production (45.20 ± 1.30 gL^{-1} and 46.80 ± 2.60 gL^{-1}, respectively). Maximal alcohol productivity was obtained with CC2 (LR4: 80%; T1: 20%) and CC3 schemas, 7.70 ± 0.29 gL^{-1} day and 7.80 ± 0.44 gL^{-1} day, respectively. The results suggest the usefulness of these wild-type strains in co-culture as an alternative to alcohol production from carbohydrates mixtures at concentrations commonly found in citrus waste.

Key words: Alcohol production, batch co-culture, carbohydrates mixture, *Candida* wild-type strains.

1. Introduction

The efficient conversion of lignocellulosic biomass into alcohol has become a world priority for producing an environmentally friendly renewable energy [1]. Besides, a sustainable and economically viable bioalcohol process is dependent on the availability of a robust alcohol producing microorganisms, able to ferment all carbohydrates present in the feedstock including pentose carbohydrates as L-arabinose and D-xylose [2]. The microorganisms commonly used for industrial alcohol production have several advantages. However, they exhibit some problems to assimilate pentoses carbohydrates [3]. Moreover, it has been reported that microorganisms that assimilate pentoses show low tolerance to inhibitors and require a small and well-controlled supply of oxygen to enhance alcohol production [3, 4], therefore research of wild microorganisms adapted to specific substrates is still considered and attractive alternative to pentoses fermentation, since the knowledge of the strains behavior could be useful for future strategies and for its improvement. Yeasts of the genus *Candida* sp. have shown the ability to ferment pentoses and hexoses carbohydrates from degradation of hemicellulose and cellulose, individually and in co-culture [5-7]. The co-culture system appears to be an advantageous system over individual cultures because of the potential for synergistic utilization of the metabolic pathways of the strains involved [8, 9]. Co-culture fermentations may result in increased yield, improved control of product qualities, possibility of utilizing cheaper substrates and potential of improving existing processes [8]. These methods have been described to improve the efficiency of lignocellulosic biomass fermentation, having a great impact on the development of biofuels, bioenergy and biobased products [4, 8, 9]. On the other hand, carbohydrates concentrations of agroindustrial residues vary

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depending on the crop used and cultivation conditions. In citrus waste it has been reported around of 44% of fermentable carbohydrates, representing 50%-51% of glucose, 28%-30% of fructose, 9%-16% of galactose, 7%-8% of arabinose, 2%-4% of xylose and 1%-2% of sucrose [10, 11]. México is one of the mayor citric producers and only around the 40% of the fruit is used in the industrial processing. Due to the fact that most of the agricultural waste including citric residues contains lignocellulolytic biomass that can be transformed into fermentable hexoses and pentoses, the main objective of this work was to evaluate the capability of *Candida* wild-type strains isolated from termite gut and rumen liquid, to ferment the most commonl carbohydrates presented in citrus residues, individually and in co-culture to alcohol production.

2. Materials and Methods

2.1 Microorganisms and Synthetic Medium

The two microorganisms used in this study were wild-type strains of *Candida*, *C. tropicalis* (LR4, NRRL Y-50876) isolated from fistulated bovine rumen, and *C. glabrata* (T1, NRRL Y-50877) isolated from termite gut both from Yucatán, México. The strains were isolated and identified according to the methodologies reported in patent applications MX/a/2013/014178 and MX/a/2013/014179 respectively. For maintenance, strains were grown individually on Yeast Peptone Dextrose (YPD) medium with 20% (vv^{-1}) of glycerol and kept at -20 °C until use. Carbohydrates mixture medium was used in the following proportions: glucose 51%, fructose 30%, galactose 8%, arabinose 7%, sucrose 2% and xylose 2% to obtain a 100 gL^{-1} total carbohydrates concentration in the minimal yeast nitrogen base medium [12].

2.2 Inoculum Preparation and Individual Fermentation Conditions

Pre-inoculum was prepared by adding 10 mL of YPD medium containing previously activated glycerol preserved cells, to a 250 mL flask with 100 mL of YPD and grown at 35 °C and 200 rpm for 10 h. Inoculum was prepared from an aliquot of the pre-inoculum containing 20×10^6 cells mL^{-1}, YPD medium was eliminated by centrifugation at 4 °C and 5,300 rpm for 30 min and the cells were washed several times with 0.85% (wv^{-1}) saline water. The pellet free of YPD medium was suspended in 25 mL of fermentation medium and inoculated into 500 mL Erlenmeyer flask containing 250 mL of the same medium previously adjusted to pH 4.5 (HCl 50% vv^{-1}). Carbohydrate mixture consumption kinetics were performed with the strains individually at inoculum concentration of 20×10^6 cells mL^{-1} and incubated during 10.5 days at 35 °C without agitation for *C. tropicalis* strain and 200 rpm for *C. glabrata* strain [13].

2.3 Co-cultures Schemas and Fermentation Conditions

Co-cultures schemas were prepared as follows: first schema (CC1) was performed with inoculums concentration of 60% of *C. tropicalis* and 40% of *C. glabrata* to reach a final concentration of 20×10^6 cells mL^{-1}, both strains were added at the beginning of the fermentation and incubated at 35 °C without agitation. Second schema (CC2) was inoculated with a concentration of 20% of LR4 and 80% of T1 strain inoculated at the fermentation start and incubated at 35 °C at 200 rpm. The third one (CC3) was inoculated with LR4 strain at concentration of 20×10^6 cells mL^{-1} at the beginning of the fermentation and sequentially (2 days later), a similar concentration of 20×10^6 cells mL^{-1} of T1 strain was added, incubated was performed at 35 °C without agitation the 2 first days then agitation was initiated at 200 rpm. Carbohydrates mixture medium (seen in previous section) was used to obtain a 200 gL^{-1} total carbohydrates concentration in the minimal yeast nitrogen base medium [12].

The pellet of each strain free of YPD medium was

suspended in 25 mL of fermentation medium and inoculated into 500 mL Erlenmeyer flask containing 250 mL of the same medium previously adjusted to pH 4.5 (HCl 50% vv^{-1}). In all fermentations, samples were collected each 12 h, a milliliter was used for microbial growth determination, the rest was centrifuged for 20 min at 5,300 rpm and 4 °C and the supernatant was stored at -20 °C for further analysis.

2.4 Analytical Methods

Total microbial population was determined by direct microscopic counting method and dry weight. Due to the differences in shape and size of the strains, total population of each one of the microorganisms, in co-culture schemas was possible to determine. Carbohydrates consumption was monitored by high-performance liquid chromatography (HPLC). A system (Scientific Thermo Finnigan Surveyor) with a Surveyor Plus LC Pump, auto sampler and RI Detector was used. The separation was performed using a Phenomenex Rezex RPM-Monosaccharide Pb^{+2} (8%) column of 300 mm × 7.8 mm. The column and detector temperature were 80 °C and 42 °C respectively with a flow of 0.5 mL min^{-1}. Samples were filtered before injection using PTFE acrodisc filters (Millipore Millex). Concentrations were determined from standard curves prepared by mixing analytical grade carbohydrates standards (Sigma). Alcohol determination was performed by the potassium dichromate method described by Bohringer (1964) [14]. Fermentation samples were distilled using a microdestillator with a vigreux column. Alcohol yield $Y_{P/S}$ was calculated by dividing the maximum alcohol production (gL^{-1}) by the carbohydrate consumption (gL^{-1}). Fermentation efficiency (E) was calculated by dividing the experimental $Y_{P/S}$ by theoretical $Y_{P/S}$ (0.51 gg^{-1}) multiplied by 100. Maximal productivity (P_{max} gL^{-1}) was calculated by dividing the maximum alcohol production by the time (h) at which it obtained. Carbohydrate consumption and alcohol production rates were estimated by the Gompertz model using the non-linear regression program (Origin Pro 8 SRo), according to the expression $y(t) = a^{[-b^{(-kt)}]}$ where: $y(t)$ is the carbohydrate consumption or alcohol production at time (t) ; a is the maximum carbohydrate consumption (%) or alcohol production (gL^{-1}) at $t \to \infty$; b is a constant related to the initial conditions when $t = 0$, k is the carbohydrate consumption or alcohol production rate constant (h^{-1}) [15].

2.5 Statistical Analysis

Carbohydrate consumption, alcohol production, kinetic parameters related, microbial growth, alcohol yield, efficiency and maximal alcohol productivity results, were analyzed by multiple comparisons of means by Turkey Kramer's test using the statistical software Statgraphics Centurion XV.I (Statpoint, Inc.).

3. Results and Discussion

3.1 Individual Culture Fermentation

To evaluate the consumption capability of *C. tropicalis* and *C. glabrata* on a carbohydrate mixture, strains were grown individually in a medium containing glucose, sucrose, fructose, galactose, xylose and arabinose at concentrations that simulates citrus residues medium according to the literature [10, 11]. The consumption profiles expressed in consumption percentage of each carbohydrate presented in the medium during 10.5 days of fermentation by *C. tropicalis* are presented in Fig. 1A. The results indicated that this strain was able to assimilate more than 65% of each hexose concentration and at least 40% of each pentose concentration. After three days of fermentation, glucose, fructose and galactose, presented consumption values of 57%, 37% and 54%, respectively, which represented the 48% of total carbohydrate consumed. After 7 days glucose and fructose reached a constant

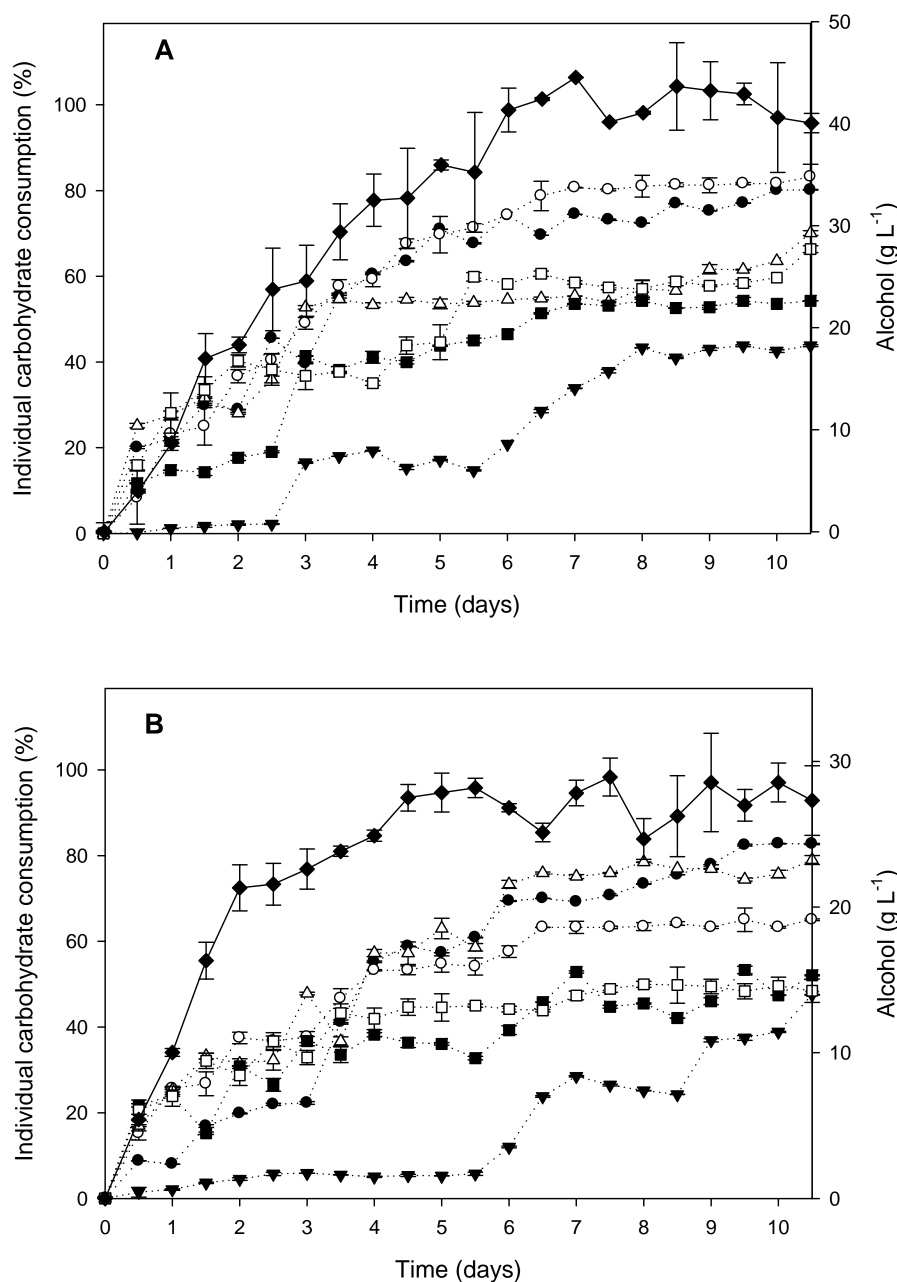

Fig. 1 Individual carbohydrate consumption expressed in percentage and alcohol production (gL^{-1}) obtained by *C. tropicalis* (A) and *C. glabrata* (B) wild-type strains in a carbohydrate mixture as carbon source. Glucose (○); Galactose (△); Fructose (□); Sucrose (●); Xylose (▼); Arabinose (■); Alcohol (◆).

consumption (80.70% ± 0.09% and 58.50% ± 0.41%, respectively). Galactose presented small consumption increments to reach its maximal (70.10% ± 0.01%) after 10 days as well as sucrose, which was consumed gradually during the whole fermentation until a maximal of 80.10% ± 0.02%. With respect to pentoses, xylose consumption initiated after 2.5 days of fermentation with values around 16%, which remains constant until day 5.5, and then the consumption gradually increased to reach the highest value of 43.30% ± 0.03% after 8 days and remains constant until fermentation ended. Arabinose was slightly consumed from day one to day 2.5 with a value of 19%, later it increased rapidly to obtain 41% of consumption at 3

fermentation days, a slow increment was observed to attain 54.30% ± 0.38% at day 8 to remain constant until the end of the fermentation.

Consumption rates of each carbohydrate by *C. tropicalis* are shown in Table 1, the results indicated that glucose was the fastest hexose consumed (0.023 gh^{-1}), followed by sucrose and galactose and the fastest pentose consumed was arabinose (0.024 gh^{-1}). Overall, after 10.5 days of fermentation, a remainder of 26% of the total carbohydrate concentration presented in the medium was observed, nevertheless a high initial carbohydrate concentration was used. The results showed that *C. tropicalis* strain did not present glucose catabolic repression to other hexoses evaluated, however in the case of pentoses, xylose might present catabolic repression due to the fact that its consumption started after the 58% of glucose was consumed (Fig. 1A). De Bari et al. [16] observed that the glucose and xylose consumption occurred sequentially by *S. stipitis*, nonetheless xylose uptake significantly increased only when the glucose concentration decreased; Govindaswamy et al. [17] reported that glucose utilization precedes xylose utilization in mixed carbohydrate fermentation, they suggested that inhibition of xylose fermentation is likely due to competition for the yeast carbohydrate transporter system, since glucose is transported across the cell by a yeast hexose transporter system that is believed to be the same for xylose transportation [16]. Fonseca et al. [18], have reported the consumption of arabinose for biomass formation with *C. arabinofermentans* inmedium without agitation. Although Schimer-Michel et al. [19], have reported that arabinose is metabolized only after depletion of glucose and xylose, the results obtained herein suggest that the strain *C. tropicalis* is able to consume arabinose at the same time that xylose is consumed; nevertheless, deeper arabinose metabolization studies are needed.

Carbohydrate consumption profiles by the strain *C. glabrata* in the carbohydrate mixture medium are shown in Fig. 1B. *Candida glabrata* strain was capable to assimilate around 60% of total carbohydrate presented in the medium during the whole fermentation. Before four days, glucose, fructose and galactose showed consumption values of 47%, 43% and 37% respectively, which represented around 40% of the total carbohydrate presented in the medium, this value was slightly lower than the obtained with *C. tropicalis* at the same time. Fructose, glucose and galactose reached its maximal carbohydrate consumption around day 8 with values of 50%, 64% and 78% respectively, and sucrose (83%) after 10 days. Fructose as is shown in Table 1 presented the highest consumption rate among substrates (0.031 gh^{-1}) twice that obtained with *C. tropicalis*, furthermore it presented significant differences ($p < 0.05$) to the rest of the substrates except

Table 1 Kinetic parameters of maximal carbohydrate consumption (%) and total substrate consumption rates of each carbohydrate presented in the mixture medium of the strains *C. tropicalis* and *C. glabrata* individually.

	Concentration (gL^{-1})	*C. tropicalis*		*C. glabrata*	
		TSC* (%)	μ_s** (gh^{-1})	TSC* (%)	μ_s* (gh^{-1})
Glucose	51	83.2 ± 0.10^a	0.023^a	65.0 ± 0.31^c	$0.022^{a,b}$
Fructose	30	66.3 ± 0.41^d	0.015^a	48.5 ± 0.78^e	0.031^a
Galactose	8	70.1 ± 0.01^c	0.022^a	78.9 ± 0.12^b	0.019^b
Sucrose	2	80.1 ± 0.01^b	0.022^a	82.7 ± 0.05^a	0.019^b
Arabinose	7	54.3 ± 0.11^e	0.024^a	52.1 ± 0.33^d	0.014^b
Xylose	2	43.8 ± 0.06^f	0.018^a	47.5 ± 0.22^e	0.013^b

*TSC: Total substrate consumption at 10.5 days of fermentation.

**μ_s: Total substrate consumption rates determined by adjustments to the Gompertz model ($R^2 \geq 0.95$) of carbohydrate consumption time curse values, SDs of μ_s were $\leq$20% from two independent experiments.

Values in a column with the same letter were not significantly different at $p < 0.05$ determined by multiple comparisons of means by Tukey Kramer'stest.

Table 2 Kinetic parameters of maximal carbohydrate consumption (%) and total substrate consumption rates of each carbohydrate presented in the mixture medium of the co-culture schemas.

	CC1***		CC2***		CC3***	
	TSC* (%)	μ_s** (gh^{-1})	TSC* (%)	μ_s** (gh^{-1})	TSC* (%)	μ_s** (gh^{-1})
Glucose	82.3 ± 0.36^a	0.020^{b,c}	77.4 ± 0.95^c	0.041^b	95.2 ± 0.36^a	0.030^a
Fructose	62.8 ± 2.78^c	0.042^{a,b}	68.2 ± 0.12^d	0.079^a	77.8 ± 0.01^c	0.019^b
Galactose	86.0 ± 0.50^a	0.017^{b,c}	83.9 ± 0.46^b	0.022^{c,d}	83.0 ± 0.11^b	0.019^b
Sucrose	74.1 ± 0.02^b	0.023^b	92.1 ± 0.10^a	0.015^{c,d}	83.0 ± 0.51^b	0.023^{a,b}
Arabinose	51.6 ± 0.88^d	0.021^{b,c}	58.3 ± 0.15^e	0.028^{b,c}	57.7 ± 0.62^d	0.017^b
Xylose	44.9 ± 0.52^e	0.012^c	47.4 ± 0.15^f	0.012^e	51.3 ± 0.15^e	0.023^{a,b}

*TSC: Total substrate consumption at 10.5 days of fermentation.

**μ_s: Total substrate consumption rates determined by adjustments to the Gompertz model ($R^2 \geq 0.95$) of carbohydrate consumption time curse values, SDs of μ_s were $\leq$20% from two independent experiments.

***CC1: 60% of LR4 strain and 40% of T1 strain, CC2: 20% of T1 strain and 80% of LR4 strain and CC3: LR4 at 100% at 0 h of fermentation, and T1 at 100% after 48 h.

Values in a column with the same letter were not significantly different at $p < 0.05$ determined by multiple comparisons of means by Tukey Kramer'stest.

glucose. As regards to pentoses, xylose started with a slow consumption within first 2 days (5%), then it remained constant until day 5.5 and increased gradually to reach around 48% of consumption after 10 days (Fig. 1B). Although *C. glabrata* presented lower xylose consumption rate (0.013 gh^{-1}) than *C. tropicalis* (0.018 gh^{-1}), a better consumption was performed by *C. glabrata* strain in co-culture with higher values of maximal carbohydrate consumption (Table 2). Arabinose showed half of its total consumption after 2 days of fermentation then a gradual consumption was observed to reach its maximal value of 53% at 9.5 days. Other studies (Schimer-Michelet al. [19]) reported that arabinose started to be metabolized in a later phase, when both glucose and xylose were exhausted, similar metabolic profile has been observed for other *Candida* sp. Bettiga et al. [2] reported parallel consumption of arabinose and xylose in a mixture containing glucose; nevertheless, it was obtained with a modified *S. cerevisiae* strain. Alcohol production of each strain during fermentation is also shown in Fig. 1.

Maximum alcohol production was observed at day 7 for *C. tropicalis* strain (44.50 ± 0.04 gL^{-1}) and at day 7.5 for *C. glabrata* strain (28.92 ± 1.30 gL^{-1}), in both cases most of the carbohydrates stopped their consumption. The alcohol production with *C. tropicalis* strain was 35% higher than the alcohol concentration obtained by *C. glabrata*, which can be explained by the lower consumption of glucose and fructose observed with *C. glabrata* and by the influence of the agitation that favors *C. glabrata* microbial growth, and also oxygen dissolution in the medium, reducing alcohol production. Nevertheless, the ability of alcohol production by *C. glabrata* has been reported with similar characteristics with those of *S.cerevisiae* that showed increment in alcohol concentration under limited oxygen conditions [7]. In both strains alcohol production started after 0.5 days of fermentation, production rate was higher with *C. glabrata* (0.040 ± 0.01 gh^{-1}), while a higher production of 44.5 ± 0.04 gL^{-1} was obtained with *C. tropicalis* strain, as well as a higher yield (0.32 ± 0.01 gg^{-1}) and maximal alcohol productivity (6.40 ± 0.01 $gL^{-1}d^{-1}$) (Table 3). Nonetheless, glucose and fructose are the mayor carbohydrates presented in citrus waste [10, 11], a complete and efficient conversion of hexoses and pentoses carbohydrates presented in the lignocellulosic hydrolysates is a prerequisite for maximizing the profitability of an industrial process for bioethanol production [9].

Cell growth obtained by dry weight of the strains *C.*

tropicalis and *C. glabrata* is shown in Fig. 2. Maximal growth was observed for *C. glabrata* at 3.5 days of fermentation around 2.60 ± 0.24 gL^{-1} of dry weight, this value is higher than the maximal value obtained by the strain *C. tropicalis* (1.93 ± 0.13 gL^{-1}) after 3 days of fermentation. Growth rate and duplication time obtained by *C. glabrata* were 0.086 ± 0.01 h^{-1} and 4.59 ± 1.39 h respectively. These values were also higher than the obtained for *C. tropicalis* strain (0.060 ± 0.01 h^{-1} and 2.54 ± 1.10 h) in Fig. 2. The results suggest that *C. glabrata* could use the carbon source to produce biomass and microbial maintenance, instead of metabolites production that is reflected in the lower alcohol concentration.

3.2 Co-culture Fermentation

Due to the fact that *C. tropicalis* strain showed the best alcohol production from hexoses evaluated and *C. glabrata* showed higher pentoses consumption, and in order to provide the simultaneous conversion of mixed carbohydrates, to increase the substrate consumption and improve alcohol production, different co-culture schemas were evaluated. Fig. 3A shows the carbohydrate consumption percentages and alcohol production of co-culture schema CC1. As in the culture medium of single strains, the fermentation was performed during 10.5 days, after this time around 74% of the total carbohydrates presented in the medium was consumed with at least 62% of each hexose, these values are higher than the obtained with *C. glabrata* strain alone and similar to the values obtained by *C. tropicalis*. After 3.5 days glucose, fructose and galactose presented consumption values of 58%, 54% and 49% respectively, which represented around 50% of total carbohydrates consumed in the medium, this value is higher than the obtained by both strains in individual cultures at the same fermentation day. Glucose after day 1.5 presented a gradual consumption of 22% to the obtained 82% at day 10.5, the same behavior was observed for fructose that reached 63% and sucrose that presented 74% at the end of the fermentation. Galactose increased its consumption to reach constant consumption at day 7 with around 85%. Individual carbohydrate analysis showed that total glucose consumption was similar in co-culture CC1 as the obtained by strain *C. tropicalis* and higher than the obtained by strain *C. glabrata* the consumption alone, fructose consumption was 3% and 12% higher than *C. tropicalis* and *C. glabrata* respectively. Galactose consumption was also higher in this schema than with the strains alone, around 10% for *C. tropicalis* and 5% for *C. glabrata*, nevertheless

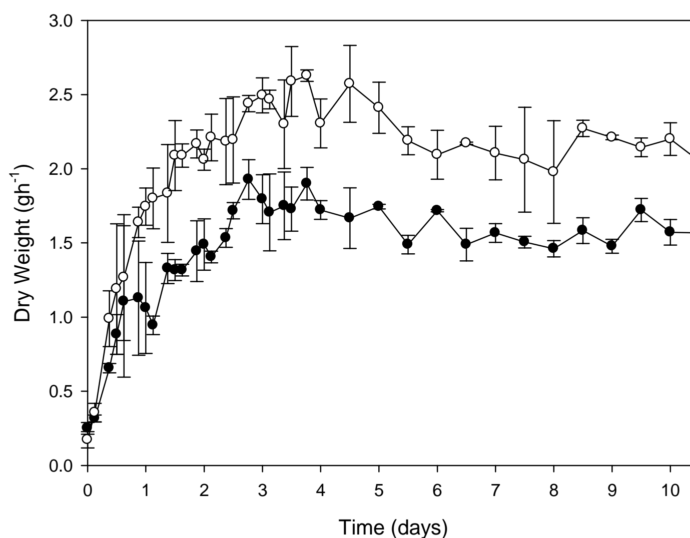

Fig. 2 Cellular growth in dry weight (gh^{-1}) of wild-type strains *C. tropicalis*—LR4 (●) and *C. glabrata*—T1 (○) in sugar mixture as carbon source.

sucrose was less consumed, around 20% lower than obtained in individual cultures. In relation to the xylose consumption started after 2 days of fermentation and presented a similar consumption to strain *C. glabrata* (6%) to increase gradually to 45%, value situated in between C. *tropicalis* and *C. glabrata* consumptions. Arabinose presented a similar behavior than in individual cultures with a consumption of 52%. As regards alcohol production, the maximal concentration was obtained at 8 days of fermentation with 45.20 ± 1.30 gL^{-1}, this value is similar to the obtained with *C. tropicalis* strain but higher than the obtained by *C. glabrata* alone, with a yield ($Y_{p/s}$) of 0.34 ± 0.01 and maximal alcohol productivity of 5.70 ± 0.16 $gL^{-1}d^{-1}$ (Table 3). The results obtained are in concordance with the reported by De Bari et al. [16], who observed faster carbohydrate consumption in a co-culture by *S. stipitis* and *S. cerevisiae* than in single cultures, nonetheless they presented lower yields than the obtained here in similar concentrations (0.28 ± 0.02 gg^{-1}). Fig. 3B showed the behavior of the growth of cells in co-cultures of *C. glabrata* and *C. tropicalis* strains inoculated simultaneously in CC1. Initially the culture started with (15.50 ± 0.70) × 10^6 cell mL^{-1} (60%) of *C. tropicalis* and (9.50 ± 2.12) × 10^6 cell mL^{-1} (40%) of *C. glabrata*, where from the 5 days presented a more important cell growth. The growth curves show that between 5.5 and 7.5 days with decreasing population of *C. glabrata*, increases the population *C. tropicalis.* Maximal growth was observed for *C. glabrata* at 10 days of fermentation around (66 ± 4) × 10^6 cell mL^{-1} of co-culture, this value is higher than the maximal value obtained by the strain *C. tropicalis* ((33 ± 9) × 10^6 cell mL^{-1})after 5.5 days of fermentation. Although *C. tropicalis* was inoculated in higher proportion, the cellular growth did not remain constant as shown with low population during the remainder of the fermentation. Despite growth conditions were set up to favor *C. tropicalis* growth; the variation on the microorganism cellular concentration may be due to the differences on the strains growth rate and duplication time of individual strains as discussed before, higher for *C. glabrata* than *C. tropicalis*. The absence of agitation in the medium did not affect *C. glabrata* growth and even though a reduction of the concentration of *C. tropicalis* in the medium was observed, the alcohol production remained higher than in single *C. glabrata* medium.

Fig. 3C shows the carbohydrate consumption behavior and the alcohol production obtained in the co-culture schema CC2 though 10.5 days of fermentation. A maximal consumption of 74% of total carbohydrate was observed, where 5% of consumption was pentoses contribution. After 3.5 days of fermentation, consumption of 74%, 54% and 49% of glucose, fructose and galactose was achieved, which represented around 60% of the total carbohydrate presented in the medium. The values obtained at this time were higher than the obtained by the strains evaluated individually and in CC1 schema. At day 0.5 a 21% of glucose was consumed to reach the steady stateat day 5 (76%), with the highest glucose consumption rate obtained among the schemas evaluated (0.041 h^{-1}), fructose and galactose presented their maximal consumption percentages after 6 days of fermentation with values of 68% and 83% respectively. The higher consumption rate among the carbohydrates evaluated in CC2 schema was obtained with fructose (0.079 h^{-1}) (Table 2). Sucrose was consumed gradually until 92% after day 9, the consumption rate of this carbohydrate was smaller than the rest of the schemas (0.015 h^{-1}). Pentoses behavior showed that xylose was consumed in a very slow way until day 6, then consumption incremented gradually to 47% similar value obtained with strain *C. glabrata* evaluated individually. As regards arabinose, it started to be consumed gradually to reach a maximal value after day 10 of 58% with consumption rate of (0.028 h^{-1}), these values were the highest obtained for this carbohydrate among the schemas evaluated. Alcohol production showed a maximal

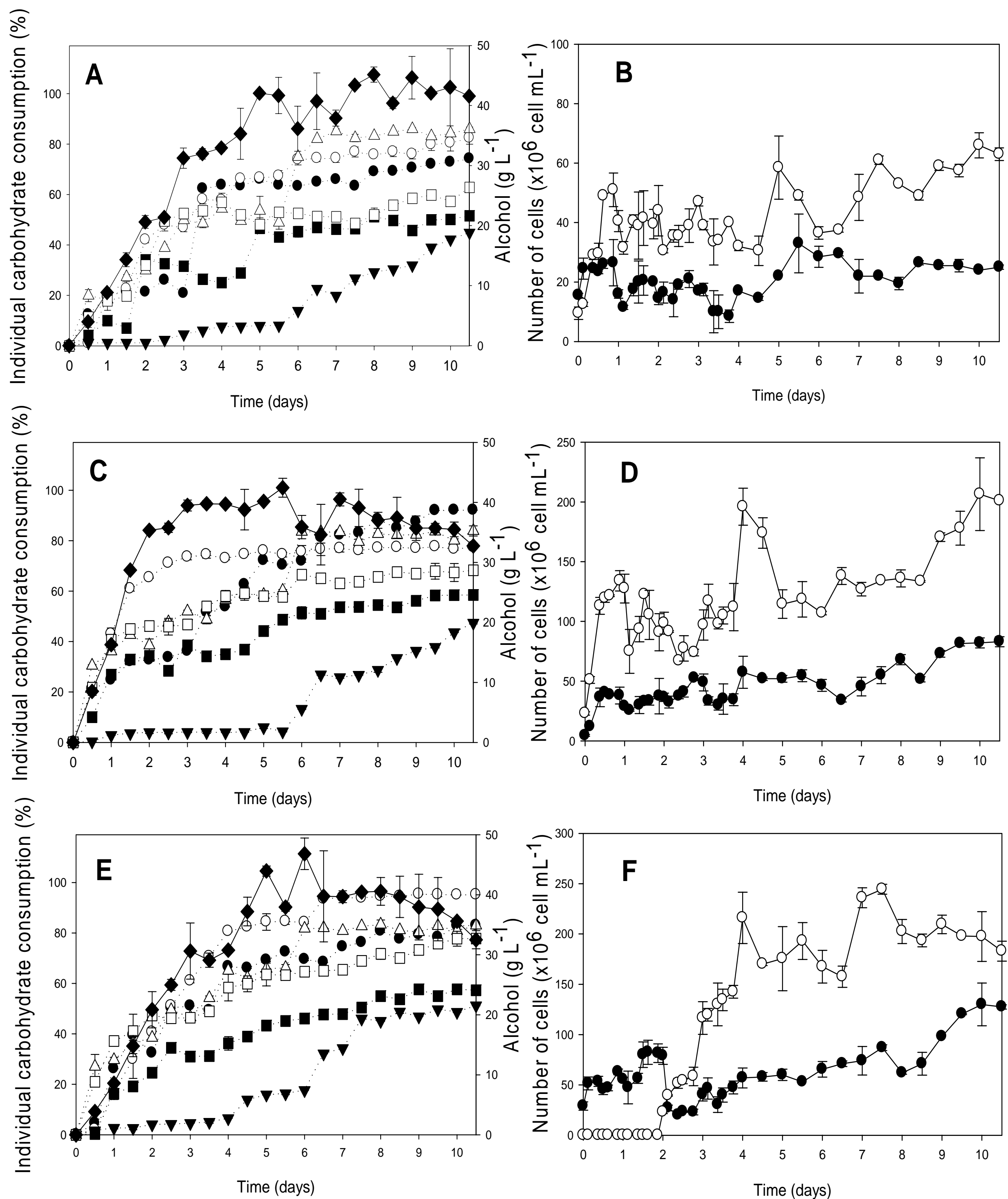

Fig. 3 Individual carbohydrate consumption expressed in percentage and alcohol production (gL^{-1}) in co-cultures of carbohydrate mixture as carbon source of (A) CC1, (C) CC2 and (E) CC3; Glucose (○); Galactose (△); Fructose (□); Sucrose (●); Xylose (▼); Arabinose (■); Alcohol (◆). Number of cells in co-cultures ($\times 10^6$ cell mL^{-1}) (B) CC1, (D) CC2 and (F) CC3; *C. tropicalis* LR4 (●) and *C. glabrata* T1 (○).

Table 3 Kinetic parameters of alcohol production obtained by *Candida* wild strains and co-cultures schemas in carbohydrates mixture.

System schema	TSC* (%)	Alcohol (gL^{-1})	Yp/s (gg^{-1})	μ_p** (gh^{-1})	Maximal alcohol production		Maximal productivity ($gL^{-1}d^{-1}$)
					t (d)	SC*** (%)	
C. tropicalis	74.2 ± 4.60[b]	44.5 ± 0.04[a]	0.32 ± 0.01[a,b]	0.025 ± 0.00[b]	7	69.1 ± 0.70[a]	6.4 ± 0.01[b]
C. glabrata	60.3 ± 4.40[c]	28.9 ± 1.30[b]	0.26 ± 0.01[c]	0.040 ± 0.01[a,b]	7.5	58.1 ± 1.80[b]	3.9 ± 0.17[d]
CC1****	73.1 ± 4.70[b]	45.2 ± 1.30[a]	0.34 ± 0.01[a]	0.029 ± 0.00[a,b]	8	66.4 ± 5.10[a,b]	5.7 ± 0.16[c]
CC2****	73.5 ± 5.10[b]	42.4 ± 1.60[a]	0.31 ± 0.01[a,b]	0.042 ± 0.01[a]	5.5	65.0 ± 1.90[a,b]	7.7 ± 0.29[a]
CC3****	85.2 ± 2.00[a]	46.8 ± 2.60[a]	0.30 ± 0.01[b]	0.037 ± 0.01[a,b]	6	73.7 ± 1.80[a]	7.8 ± 0.44[a]

*TSC: Total substrate consumption at 10.5 days of fermentation.

**μ_p: alcohol production rates determined by adjustments to the Gompertz model ($R^2 \geq 0.95$) of production values obtained during time curses.

***SC: Substrate consumption in the day that was obtained maximum alcohol production.

****CC1: 60% of LR4 strain and 40% of T1 strain, CC2: 20% of T1 strain and 80% of LR4 strain and CC3: LR4 at 100% at 0 h of fermentation, and T1 at 100% after 48 h.

Values in a column with the same letter were not significantly different at $p < 0.05$ determined by multiple comparisons of means by Tukey Kramer's test.

concentration of 42.44 ± 1.60 gL^{-1} at 5.5 days, and it showed a reduction on the fermentation time to the maximal alcohol production that is reflected on the production rate (0.042 ± 0.01 gL^{-1}). High alcohol productivity among the system tested was also observed (7.70 ± 0.29 $gL^{-1}d^{-1}$) (Table 3). Compared to the literature, the values of productivity are lower than the obtained by Fu et al. [9] in a co-culture with *Z. mobilis* and *S. stipitis* and De Bari et al. [16] and in co-cultures of *S. stipitis* and *S. cerevisiae*, both in a medium in a mixture of glucose and xylose, nonetheless they used a yeast well adapted to alcohol production and simple medium added only with glucose and xylose was used.

The behavior of the growth of cells during fermentation of the microorganisms presented in the system CC2 is shown in Fig. 3D, initially the culture started with (6.00 ± 0.70) × 10^6 cell mL^{-1} (20%) of *C. tropicalis* and (22.00 ± 1.41) × 10^6 cell mL^{-1} (80%) of *C. glabrata*, results indicated that *C. glabrata* maintained a higher cell growth throughout the fermentation and an increase in the population with 4 days of fermentation, a slight variation in *C. tropicalis* was observed, where the cellular growth average in the fermentation obtained at 10.5 days was (44.41 ± 14.64) × 10^6 cell mL^{-1}. Although *C. glabrata* was inoculated in higher proportion, the cellular growth did remain constant as shown with high population during the remainder of the fermentation. Maximal growth was observed for *C. glabrata* at 10 days of fermentation around (206.5 ± 30.41) × 10^6 cell mL^{-1} of co-culture, this value is higher than the maximal value obtained by the strain *C. tropicalis* ((81.5 ± 2.12) × 10^6 cell mL^{-1}) after 9.5 days of fermentation.

In order to increase total carbohydrates consumption and alcohol productivity, a sequentially co-culture schema was proposed (CC3) Fig.3E shows carbohydrate consumption behavior during the 10.5 days of fermentation. A maximal consumption of 85.2% of total carbohydrate was observed, where 5% of the total carbohydrates consumed was from pentoses contribution. After 3.5 days of fermentation, consumption of 71%, 49% and 54% of glucose, fructose and galactose was achieved, which represented around 60% of the total carbohydrates presented in the medium, those results are similar to the obtained in schema CC2 and higher than schema CC1. A gradual increase of glucose consumption was observed to reach a maximal consumption of 95% with a rate of (0.030 h^{-1}) (Table 2). Fructose and galactose presented consumption of 45% at day 3, then a gradual increment was observed to reach values around 78% and 83% respectively, with consumption rates of 0.019 h^{-1} in both cases. Sucrose was

consumed moderately to reach 80% after day 8, the consumption rate of this carbohydrate was similar to the obtained in schema CC2 (0.023 h^{-1}). Pentoses behavior showed that xylose was consumed slowly until day 4.5 that may be due to consumption of 82% of glucose and the absence of *C. glabrata* strain within day 2. Then the consumption incremented gradually to 51% with a rate of 0.023 h^{-1}, these were the highest values obtained among the system tested. Arabinose started to be consumed gradually to reach a maximal value after day 9 of 58% similar to the maximal consumption of CC2 schema, nevertheless consumption rate was lower for CC3 than CC2 schema (0.017 h^{-1}) (Table 2). The results obtained in this study are in concordance with the reported by Guanet al. [20] who evaluated the carbohydrate mixture consumption with non-recombinant strains for alcohol production in a sequential schema of *C. shehatae* and *S. cerevisiae* or *B. bruxellensis*. The sequential fermentation evaluated showed a better total carbohydrate consumption of 85% (Table 3), value higher than the obtained among the schemas evaluated and with the strains alone, significant differences were also observed. Alcohol production showed a maximal concentration of 46.81 ± 2.60 gL^{-1} at day 6, which corresponds to the time when the carbohydrate presented a maximal consumption. Later the metabolite decreases to around 35 gL^{-1} at day 10.5. The alcohol production obtained with this schema was also the highest obtained among the schemas tested. Production rate (0.037 ± 0.007 h^{-1}) was lower than the obtained with CC2. However, the maximal alcohol productivity at the time with maximum alcohol production was the highest obtained among the systems (7.8 ± 0.44 $gL^{-1}d^{-1}$) (Table 3). As the results reported by de Bari et al. [16] simultaneous consumption of carbohydrates ensured higher productivities in the co-cultures schemas evaluated. The addition of *C. tropicalis* strain at the beginning of the fermentation favored hexoses consumption and alcohol production, then *C. glabrata* strain addition incremented pentoses consumption and as a consequence improved alcohol production. Fig. 3F showed the cellular concentration behavior in the sequential culture. *C. tropicalis* showed a typical growth during the first 2 days of fermentation, after addition of strain *C. glabrata* the total cellular concentration incremented and as a consequence the cellular percentage of each strain was affected. The growth curves show that with increasing population of *C. glabrata*, decreases the population *C. tropicalis* and an inhibitory effect is observed when inoculating the *C. glabrata.* Maximal growth was observed for *C. glabrata* at 7.5 days of fermentation around (244.5 ± 4.95) × 10^6 cell mL^{-1} of co-culture, this value is higher than the maximal value obtained by the strain *C. tropicalis* ((130.0 ± 21.21) ×10^6 cell mL^{-1}) after 10 days of fermentation. The low yield obtained in alcohol production and consumption of carbohydrates may be due to the initially high concentration of carbohydrates (200 gL^{-1}) used in synthetic mixture in the co-culture fermentation. This high concentration is used looking to get a higher concentration of alcohol. Similar concentrations of glucose were obtained in sugar cane bagasse and grape must [21, 22]. In grape must processing it was reported a consumption of carbohydrates with *S. cerevisiae* UCD522 of 69.54% and *C. sake* CBS5093 of 44.69%. A sequential co-culture using wild strains of *Candida* can improve the rates of consumption (Table 2).The results suggest the usefulness of this co-culture as an alternative to alcohol production from carbohydrates mixtures at high concentrations.

4. Conclusions

Two wild-type *Candida* strains individually and in co-culture fermentation schemas the most common carbohydrates presented in citrus residues, one as a good alcohol producer (*C. tropicalis*) and the other as a pentose consumer (*C. glabrata*). Culture individual and co-culture schemas evaluated improved high percentage carbohydrates consumption and alcohol

productivity. In the sequential co-culture schema (CC3), the complementation of the strains metabolisms improved to be a high conversion of mixed carbohydrates (i.e. xylose) and as a consequence, increased substrate utilization and alcohol productivity. Further research is needed to obtain the optimal operating conditions and ferment citrus waste for alcohol production.

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