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RESEARCH ARTICLE

CARBOHYDRATE ASSIMILATION PROFILE OF TWO WILD STRAINS OF GENUS *CANDIDA* IN A MIXTURE OF HEXOSES AND PENTOSES BY ALCOHOL PRODUCTION.

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Abstract

Bioethanol is an alternative energy source used as fossil fuel complement. It can be obtained from microbial fermentation of sugars. Efficient fermentations require microorganisms capable of use all the carbohydrates present in raw materials but most of them have different preferences for its assimilation. Mixed cultures are an alternative to perform carbohydrate fermentations due the synergistic combination of different metabolic capacities. In this work the carbohydrate assimilation profile of two *Candida glabrata* strains (code T1 and LR2) isolated from termite gut and bovine ruminal fluid, respectively, was studied in single and mixed cultures on sugar mixtures. A minimal medium based on nitrogen with 120 g/l of carbohydrates (in proportions similar to those found in citric residues) was inoculated with 2.0 x 10^7 cell/ml. In mixed cultures (code MC1 and MC2), strains were inoculated at proportion 50:50 with the same inoculum concentration. Fermentations were made during ten days at 35°C (by T1 single culture and MC1) and 40°C (by LR2 single culture and MC2) at 200 rpm. LR2 had higher cellular population and biomass and T1 had better growth rate and doubling time. All the cultures assimilate all sugars. MC1 had the best assimilation of glucose (94.81±0.12%), fructose (83.83±0.60%) and arabinose (62.12±0.42%). T1 single culture had the best assimilation of galactose (52.64±0.71%) and xylose (56.75±0.66%). T1 and MC1 had the best ethanol production (24.28 ±0.78 g/l and 24.32 ±1.00 g/l, respectively). MC2 had the highest maximum productivity (6.98 ± 0.73 g/l·day) and alcohol production rate (0.128±0.004 g/lh).

Introduction:-

Increasing interest in the production of bio-fuel due to the increased demand of energy and fuels, depletion of fossil fuels, concerns about energy security and environmental pollution has led to an interest in the development renewable and sustainable energy sources. Bioethanol is an alternative energy source that can be used as a substitute

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of fossil fuels (Balat and Balat, 2009; Gupta and Verma, 2015; Karmee, 2016). Ethanol is produced in the alcoholic fermentation by microorganisms in which the carbohydrates of a raw material are used by strains to produce alcohol. Vegetable biomass is a raw material of increasing interest to produce ethanol due to the abundancy of the lignocellulosic biomass and the abundance of residues of vegetable origin. The lignocellulosic biomass contain sugars (hexoses and pentoses) like glucose, galactose, xylose and arabinose (Gupta y Verma, 2015; Achinas and Euverink, 2016). Efficient fermentations need strains able to use all the sugars. Unfortunately, many microorganisms can´t use all the pentoses and hexoses or show differential and preferential capacities of consumption for the different sugars. *Saccharomyces cerevisiae* is one of most used microorganisms in fermentation process because of its high levels of ethanol production. This yeast is able to use hexoses but it can´t use efficiently pentoses like xylose and arabinose that are main sugars in the lignocellulosic biomass. Other microorganisms are able to use hexoses and pentoses, like *Pachysolem tannophilus, Scheffersomyces stipitis, Kluyveromyces marxianus* and *Candida shehatae*, but requires strict conditions like control in the aeration level, substrate concentrations, nutritional factors, etc. Conversion of pentoses to ethanol in yeast occurs through a serie of biochemical redox reactions that conducts to the intermediate D-xylulose, which proceeds to the pentose phosphate pathway and later to the glycolysis pathway and alcohol production through phosphorylation to D-xylulose-5-phospate by xylulose kinase (XK). *S. cerevisiae* doesn't have some of the enzymes of the catabolism of pentoses or have copies with low activity, like xylose reductase (XR) and xylitol dehydrogenase (XHD) (Girio et al., 2010; Chandel et al., 2011; Tesfaw and Assefa, 2014). Despite the physiologic capacity of pentose-fermenting microorganisms, normally they present preferences for others sugars like the glucose over pentoses. In sugar mixtures the assimilation capacity of a certain strain can change in function of its preference of carbohydrates. This leads to an incomplete and inefficient fermentation of non-glucose sugars and low productivities. Pentose-fermenting yeasts have a retard in consumption of pentoses in glucose/pentoses mixtures and they present a diauxic growth. Also, other yeasts like *S. cerevisiae* present different assimilation of hexoses in mixtures. This strain can use glucose, fructose and sucrose, but in presence of a mixture of these sugars the fermentation usually doesn´t occurs completely. The yeasts consume fructose when glucose is finished. At this point, ethanol concentration and others inhibitors are high and poison the yeast, leading to an incomplete utilization of the fructose. Also, fructose has catabolite repression by glucose (Jasman et al., 2012). There are different ways to overcome the problem of incomplete utilization of the carbohydrates. Some microorganisms can co-ferment carbohydrates like cellobiose and xylose (*Lipomyces starkeyi*, *Cryptococcus curvatus*) or glucose and xylose (*Trichosporum cutaneum*) simultaneously (Zhang et al., 2015). The use of strategies of metabolic or genetic engineering is another way to improve the carbohydrate assimilation and the co-fermentation of different sugars. Another strategy is the use of mixed cultures of strains with different metabolic capabilities for carbohydrate assimilation due the synergic effect of combination of metabolism of two or more strains, and additionally perform the ethanol production. Additional advantages of mixed cultures over single cultures are: compounds and growth factor produced for microorganisms in the mixture can benefit or complement each other and exclude unwanted microorganisms and mixed cultures enable the utilization cheap substrates (Bader et al., 2010; Jasman et al., 2013; Gutiérrez-Rivera et al., 2015; Jahnke et al., 2016). With all above, it's important to know the assimilation profile of carbohydrates in order to do better fermentation strategies and better designs of mixed cultures.

One advantage of the use of mixed cultures is to avoid the use of patented microorganisms due the possibility of use wild strains. The search of microorganisms capable of utilize cellulosic and hemicellulosic carbohydrates materials is important due to the biotechnological potential to use lignocellulosic biomass to produce biofuels like bioethanol. Natural niches like the gut of xylophage insects and the rumen are potential sources of novel microorganisms and enzymes with cellulolytic and hemicellulolytic activity. The termite gut is a niche in which microorganisms like bacteria, yeast and protozoa live in symbiosis and many of them use cellulose and hemicellulose of the wood biomass (Prillinger et al., 1996; Schäfer et al., 1996; Brune, 2014). On the other hand, rumen is a part of the digestive system with an anoxic environment with a complex ecosystem that contains bacteria and fungi that live in symbiosis and contain a complex enzymatic machinery that allow them the hydrolysis and fermentation of cellulosic and hemicellulosic carbohydrates (Christopherson and Suen, 2013; Zorec et al., 2014).

Candida is a genus of yeasts wich some of the species are pentose-fermenting strains (e.g., *C. shehatae*, *C. guillermondii* and *C. tropicalis*) and they are used in fermentation of lignocellulosic feedstocks and mixture of sugars to produce ethanol, alone or in mixed cultures (Acourene and Ammouche, 2012; Hickert et al., 2013; Thongdumyu et al., 2014; Hermansyah et al., 2016; Sopandi and Wardah, 2017). *Candida* genus comprises over 150 species ubiquitous around the world. Some species are commensals and/or pathogens for humans and grow as yeast or as filamentous type growth (hyphae or pseudohyphae) (Brunke and Hube, 2013; Rodrigues et al., 2014). *C.*

glabrata is an asexual facultative aerobic and haploid yeast that generally grows as blastoconidia cells. This yeast has been used for the production of metabolites like pyruvate and surfactants (Brunke and Hube, 2013; Rodrigues et al., 2014; Li et al., 2016; Lima et al., 2017). In this work the carbohydrate assimilation and fermentation capacities of two strains of *C. glabrata* isolated from termite stomach (named T1) and ruminal bovine fluid (named LR2) were studied in a sugar mixture of glucose, fructose, galactose, arabinose and xylose in single and mixed cultures in order to determine its assimilation profiles alone and together for these carbohydrates during the production of ethanol.

Materials and Methods:-

Strains and Media:-

The strain *C. glabrata* LR2 was isolated from bovine ruminal fluid and *C. glabrata* T1 (NRRL Y-50877) was isolated from termite stomach. Yeast strains were propagated in 100 ml of YPD broth containing 10 g/l yeast extract (BD, United States of America), 20 g/l peptone from casein (Fluka Analytical, Switzerland) and 20 g/l dextrose (Meyer, Mexico). The fermentation media was prepared according to a minimal medium yeast nitrogen base (Atlas, 2010) as follows: 5 g/l $(NH_4)_2SO_4$ (Sigma, United States of America); 1 g/l KH_2PO_4 (Karal, Mexico); 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l $CaCl_2$, 0.4 mg/l $ZnSO_4 \cdot 7H_2O$, 0.4 mg/l $MnSO_4 \cdot H_2O$, 0.04 mg/l $CuSO_4 \cdot 5H_2O$ (J.T. Baker, United States of America); 0.1 g/l NaCl (CTR, Mexico); 1 mg/l KI, 0.5 mg/l H_3BO_3, 0.2 mg/l $Na_2MoO_4 \cdot 2H_2O$ (Fermont, Mexico); 0.2 mg/l $FeCl_3$ (Sigma-Aldrich, United States of America) and a mixture of carbohydrates as carbon source with a total concentration of 120 g/l in a proportion similar to those found in citrus residues (Wilkins et al., 2005; Boluda-Aguilar and López-Gómez, 2013): 51% glucose, 2% xylose (Sigma-Aldrich, United States of America); 30% fructose (Sigma, United States of America); 8% galactose, 7% arabinose (Fluka Analytical, Switzerland); 2% sucrose (BD, United States of America). The pH of all the medias was adjusted at 4.5 with concentrated HCl.

Inoculum Preparation:-

A flask containing 100 ml of YPD medium was inoculated with 3 ml of YPD-glycerol (60:40 v:v)-conservated strains with a concentration of 1.0 x 10^9 cells/ml of the strain *C. glabrata* T1 or LR2 and then incubated at 35°C with constant agitation on an orbital shaker at 200 rpm for 6 h until reaching the log phase (monitored by cell growth). A sample of each flask was taken at 6 h to obtain and estimate the number of cells using a Neubauer chamber, reaching approximately 4.0 x 10^8 cells/ml. The culture from each flask was divided into Falcon tubes of 50 ml and centrifuged at 4700 rpm with a temperature of 4° C for 20 min. Cell pellets were washed two times with sterile NaCl 0.85% and resuspended in the minimal medium with carbohydrates.

Fermentation in synthetic media with mixture of Carbohydrates:-

Fermentations were carried out in minimal medium yeast nitrogen base with a mixture of carbohydrates (hexoses and pentoses) as carbon source. An aliquot of each cell resuspension in yeast nitrogen base medium with carbohydrates was used for the inoculation of sterile flasks containing synthetic medium. There were a total of four cultures: a single culture of *C. glabrata* T1, a single culture of *C. glabrata* LR2, and two mixed cultures, namely MC1 and MC2 where the only difference between them was the incubation temperature. Each culture had an initial population of 2.0 x 10^7 cells/ml in 300 ml of synthetic medium. In the mixed cultures, both strains were inoculated at a proportion of 50:50 simultaneously (1.0 x 10^7 cells/ml of each strain). The individual culture of *C. glabrata* T1 and the mixed culture MC1 were incubated at 35° C. The individual culture of LR2 and the mixed culture MC2 were inoculated a 40° C. All the cultures were in constant agitation on a rotary shaker at 200 rpm during 240 h. All experiments were performed in duplicate. Samples (15 ml) were taken at different times and were used for monitoring the cellular growth and determination of biomass (dry weight), carbohydrates and ethanol.

Growth Determination:-

To determine the growth of the cultures, 15 ml of sample was taken of each flask at 0, 16, 24, 40, 48, 64, 72, 88, 96, 120, 144, 168, 192, 216 and 240 h and 0.5 ml of the sample was utilized for the estimation of the cellular population by direct counting cell under the microscope in a Neubauer chamber. The other 14.5 ml were used for determination of biomass as dry weight. The sample was centrifuged at 4700 rpm and 4° C for 20 min. Cell pellet was washed two times with sterile distillated water and dried over an aluminum tray in an oven at 80° C to constant weight. The supernatant was stored at -20° C until carbohydrates and ethanol analysis.

Carbohydrates Determination:-

Concentration of the sugars (glucose, fructose, galactose, arabinose and xylose) was determined by Ultra Performance Liquid Chromatography (UPLC) coupled to mass spectrometer (MS). Supernatants were diluted in a

range from 200 fold to 2 fold with ultrapure water, according to the carbohydrate concentrations expected, and filtered through Phenex-PTFE (polytetrafluoroethylene) membrane syringe filters (0.2 µm pore size and 15 mm diameter, Phenomenex, United States of America). The analysis was made with an Acquity UPLC H-Class system equipped with a Xevo TQ-S MS detector (Waters, United States of America) using a column Acquity UPLC BEHAmide (150 mm x 3.0 mm i.d., 1.7 µm particle size, Waters, United States of America). The temperature of the column was 35° C and the samples (0.2 ml in duplicate) were injected in the system and eluted at a flow rate of 0.2 ml/min during 25 min. A gradient elution was carried out with a mixture of solvent A (acetonitrile: water 30:70 with 0.1% NH_4OH) and B (acetonitrile: water 80:20 with 0.1% NH_4OH) as follows: 100% (B) at 0 min, 20:80 (A:B) at 8 min, 25:75 (A:B) at 12 min, 40:60 (A:B) at 18 min, finally 100% (B) from 18.01 min to 25 min. The carbohydrates were identified by comparison to retention times and molecular weight of standards of the six sugars (Sigma-Aldrich, United States of America) that were mixed and quantified with a standard curve performed in triplicate with this mixture.

Ethanol Determination:-

The ethanol was determined by the spectrophotometric method of potassium dichromate according to Estrada-Martinez R. (2013). Briefly, 5 ml of supernatant was added to 5 ml of distilled water and the mixed was distillated until recover 5 ml. After, 2 ml of the solution of potassium dichromate (added with 323 ml of sulfuric acid and 33.768 g of potassium dichromate to distillated water and dilute into a 1000 ml volumetric flask) was added to 1 ml of the distillate and putted in repose during 10 min. Five ml of distillated water was added and the absorbance was read at 585 nm. The concentration of the alcohol was quantified with a standard curve of ethanol (Sigma-Aldrich, United States of America) generated in duplicate.

Statistical analysis and kinetic parameters Calculation:-

The parameters cellular population, dry weight, grow rate and maximum growth rate, duplication time, rate of substrate consumption, ethanol production, maximum productivity and rate of ethanol production were calculated as follows:

Cellular population was estimated by direct counting cell under the microscope in a Neubauer chamber and them it was calculated on base of the equation (01):

$$Cellular\ population\left(\frac{cells}{ml}\right) = \left(\frac{Number\ of\ counted\ cells}{Number\ of\ counted\ squares\ of\ the\ chamber}\right)(D)(F) \quad (01)$$

Where D is the dilution factor and F is the chamber factor (0.25 x 10^6).

Dry weight (biomass) was calculated on base of the equation (02):

$$Dry\ weight\ \left(\frac{g}{l}\right) = \left(\frac{Final\ weight - initial\ weigth}{Sample\ volume}\right) \quad (02)$$

Where the sample volume is expressed in liters.

The growth rate (µ) was calculated on base of the equation (03):

$$\mu\ (h^{-1}) = \frac{dx}{dt} \quad (03)$$

Where x is the cell concentration in terms of dry cell weight per volume (g/l) and dx/dt is the differential expression of the biomass production respect to the time (h) calculated in each point of the growth kinetic, and the maximum value was the μ_{max}

The doubling time (Td) was calculated on base of the equation (04):

$$Td\ (h) = \frac{Ln(2)}{\mu_{max}} \quad (04)$$

Rate of substrate consumption (rs) of each carbohydrate was calculated on base of the equation (05):

$$rs\ \left(\frac{g}{lh}\right) = -\frac{dS}{dt} \quad (05)$$

Where S is the substrate (sugar) concentration (g/l) and dS/dt is the differential expression of the substrate consumption respect to the time (h) calculated in each point of the kinetic. The maximum value is the rate of consumption reported in this work for each sugar.

Rate of ethanol production (rp) was calculated on base of the equation (06):

$$rp\left(\frac{g}{lh}\right) = \frac{dP}{dt} \quad (06)$$

Where P is the product (ethanol) concentration (g/l) and dP/dt is the differential expression of the production of alcohol respect to the time (h) calculated in each point of the kinetic. The maximum value is the rate of production reported in this work.

Maximum productivity (P_{max}) was calculated on base of the equation (07):

$$P_{max}\left(\frac{g}{l\ day}\right) = \frac{Ethanol\ production}{time\ of\ maximun\ production} \quad (07)$$

Where the time is expressed in days and the ethanol production in g/l.

Analysis of variance was carried out by an ANOVA test to determine significant differences between the samples. The statistical level of significance was set at $P \leq 0.05$ with two replicates (n=2). The data were performed using the software Statgraphics Centurion XVI.

Results and Discussion:-

Kinetic parameters of growth of single Cultures:-

The yeast *C. glabrata* T1 and LR2 were isolated from termite stomach and bovine ruminal fluid, respectively. Its optimal conditions for fermentation were previously determined at 35° C and 200 rpm for T1 and 40° C and 200 rpm for LR2 (Estrada-Martinez R., 2013). It was expected that strains T1 and LR2 had a physiologic capacity to utilize cellulose and hemicellulose sugars (hexoses and pentoses). In this work, these strains were incubated in a sugar mixture of hexoses and pentoses in order to determine their assimilation profiles and the possible future application of them in the fermentation of citrus residues. The growth kinetic parameters of T1 and LR2 in the synthetic medium are shown in Table 1. According to this, the strain *C. glabrata* LR2 has slightly higher cellular population and maximum biomass compared with the strain T1. In the other side, the strain T1 presented the major growth rate and the least time of doubling time. However, these differences between the yeasts were not statistically significant.

Table 1:- Kinetic parameters of growth by individual cultures of strains *C. glabrata* (T1) and *C. glabrata* (LR2) during ten days of fermentation on synthetic medium with a sugar mixture (51% glucose, 30% fructose, 8% galactose, 7% arabinose, 2% xylose and 2% sucrose).

PARAMETER	STRAIN	
	C. glabrata* T1	***C. glabrata* LR2****
Cellular population (10^6 célls/ml)	214 ± 14.14[a]	248 ± 12.37[a]
Biomass (g/l)	1.84 ± 0.13[a]	1.92 ± 0.04[a]
μ_{max} (h^{-1})	0.093 ± 0.007[a]	0.076 ± 0.005[a]
Td (hours)	7.50 ± 0.57[a]	9.12 ± 0.64[a]

a, b: different letters in the same line show significant differences between strains ($p<0.05$).
**C. glabrata* T1 at 35° C and 200 rpm
** *C. glabrata* LR2 at 40° C and 200 rpm

Kinetic of the fermentation of single Cultures:-

The consumption of carbohydrates, the alcohol production and the yeasts growth are shown in Fig. 1. Although both strains have been identified as *C. glabrata*, they showed differences between them. Fig. 1 shows that T1 presented slightly higher ethanol production compared with LR2 (24.28±0.78 g/l compared with 21.36±1.41 g/l), in contrast, this strain reached the maximum production more quickly than T1 (3.7 days for LR2 compared with 6 days for T1). Both strains were able to assimilate glucose, fructose, galactose, arabinose and xylose in the minimal medium and produced ethanol. Glucose was the mainly sugar consumed, followed by the fructose, which present a retard of 16 h in its consumption until 30% of glucose was consumed. The galactose also had a retard of 16 h and the xylose of 48 h in the assimilation by the strain T1 when more than 70% of glucose was consumed, while LR2 strain consumed the galactose since the beginning and the consumption of xylose had a delay of 24 h when more than 50% of glucose was consumed. Both strains consumed arabinose by first. Sucrose was not detected. As can be seen in Fig. 1, strain T1 had a rapid growth in the first 16 h and then had a slowly growth until 96 h. After this point started to decrease until 144 h, when ethanol reached the maximum production. After this point, alcohol started the assimilation by T1 in parallel with the increase of the cellular population. Strain LR2 started ethanol consumption when reached the

maximum alcohol production (88 h). LR2 had a rapid growth in the first 24 h, after this point, the growth stabilized until 72 h and then the cellular population slowly increased until the end of the fermentation (10 days) in parallel with the ethanol and sugar consumption. Zili et al. (2015) with a culture of a strain of *C. glabrata* in a rich YP medium with glucose or trehalose produced ethanol and cell growth during the fermentation of the sugars, and after the sugars was exhausted, the ethanol was used as a carbon source. It´s reported that *C. glabrata* it´s a yeast that can assimilate glucose and trehalose but not galactose or sucrose due to the lost genes related with the metabolism of these carbohydrates like invertase and genes of the Leloir pathway (Bolotin-Fukuhara and Fairhead, 2014; Zili et al., 2015). Sucrose consumption requires a first hydrolysis with extracellular or intracellular invertases that disrupts it in its constituent monosaccharides glucose and fructose (Gargel et al., 2014; Marques et al., 2016). With respect to galactose, its reported that many genes (*GAL1, GAL10, GAL7, GAL3, GAL80, GAL4*) of the Leloir galactose metabolisms pathway in yeast were reportedly lost in *C. glabrata* in a reductive evolution process suggested due to the commensal and pathogenic lifestyle (Hittinger et al., 2004). Surprisingly, in this work both strains were able to assimilate the galactose. Further investigation is necessary to verify the assimilation of galactose and sucrose in T1 and LR2. Preez et al. (1986) found that *C. shehatae* used glucose, mannose, galactose, xylose, arabinose and cellobiose in a synthetic media with 20 g/l of individual sugars and obtained a μ_{max} of 0.14, 0.20, 0.14, 0.14, 0.04 and 0.04 h^{-1}, respectively during the fermentation of the respective sugars with a utilization of substrate near to 100%, except for cellobiose, which utilization was about 75%. In the same media, *Pichia stipitis* used glucose, mannose, galactose, xylose, arabinose, rhamnose and cellobiose and obtained a μ_{max} of 0.23, 0.18, 0.20, 0.22, 0.14, 0.16 and 0.21 h^{-1}, with a utilization of near of 100%. These results, except for the growth rate in arabinose and cellobiose by *C. shehatae*, were higher than those obtained in this work despite the strains growth in a sugar mixture. Preez et al. also found in sugar mixtures with 10 g/l of each carbohydrate (glucose, mannose, galactose, xylose and the disaccharide cellobiose) a μ_{max} of 0.20 h^{-1} and 0.22 h^{-1} for *C. shehatae* and *P. stipitis*, respectively, while the ethanol production was 13.4 g/l after near 40 h of fermentation and 18.6 g/l after near 50 h of fermentation, respectively. Both strains preferred glucose and observed a retard in the fermentation of mannose, galactose, xylose and cellobiose (cellobiose was not consumed by *C. shehatae*). Mannose, galactose and xylose were consumed by *C. shehahate* after the depletion of glucose while only xylose and cellobiose were consumed by *Pichia stipitis* after the depletion of glucose. In this study, the growth rate of both strains was lower. Strains T1 and LR2 preferred glucose and the consumption of fructose, galactose and xylose by T1 presented a retard, while LR2 presented a retard in the assimilation of the fructose and xylose (see Fig. 1). Both strains started the assimilation of sugars before the depletion of the glucose. The results of ethanol production were higher for T1 and LR2. More recently, Jasman et al. (2012) studied the fermentation of mixed sugars of glucose, fructose and sucrose by strains of *S. cerevisiae*. They found that the residual concentration of fructose was higher than the residual concentration of glucose in most of the strains, and the yeast had preference to consume glucose than fructose, similar to the results showed by strains T1 and LR2 in this work. Laplace et al. (1991) found that the fermentative behavior of xylose by *C. shehatae* and *Pichia stipits* depends on the oxygen transference rate (OTR). They obtain a μ_{max} at 0.24 h^{-1} and 0.30 h^{-1} and consumed 100% of xylose (50 g/l) with a OTR of 3.90 $mol \cdot l^{-1} \cdot h^{-1}$ at 50 and 57 h of fermentation by *C. shehatae* and *P.* stipitis, respectively. Sugar consumption and growth rate decreased in parallel to the reduction of aeration. In anaerobic conditions they obtain a μ_{max} of 0.07 h^{-1} and 0.09 h^{-1} with 15.6% and 13.5% of xylose consumed on 72 h of fermentation by *C. shehatae a*nd *P. stipis*, respectively. The ethanol and xylitol also were affected by the level of aeration. Ethanol yield tends to reach a maximum in high levels of aeration while xylitol yield decreased at these conditions. It´s known that oxygen supplementation is one factor that affect the xylose metabolism and the flux of the carbon to ethanol production or biomass production (Dussan et al., 2016). In this study it wasn't aeration in the cultures of T1 and LR2. Considering the previous information, in conditions of controlled aeration some of the carbohydrates, like the xylose, could have a better consumption and fermentation by strains T1 and LR2. Further investigation is necessary in order to determine the conditions of aeration for improve the fermentation of the strains.

The search of thermotolerant pentose-fermenting strains is important to reduce the costs of operation and cooling systems, especially in tropical countries. Tanimura et al. (2012) screened natural yeast in different sources to search thermotolerant xylose assimilation microorganisms and found a strain of *C. shehatae* capable of use xylose and glucose for growth and ethanol production at 35-39° C. The best temperature for ethanol production was 37° C with approximately 6 g/l and 2.5 g/l of ethanol for glucose and xylose fermentation, respectively in synthetic. In contrast, at 39° C the capability of production decreased to 1 g/l and 0.5 g/l, respectively. Additionally, this strain was capable of assimilate galactose, sucrose, maltose, trehalose and others sugars, but not arabinose. The strain LR2 had the optimal fermentation at 40° C. T1 and, specially, LR2, are potential thermotolerant strains. Also, the capacity to

use pentoses like arabinose and xylose, or sugars like galactose make them interesting for the fermentation of lignocellulosic residues at high temperatures.

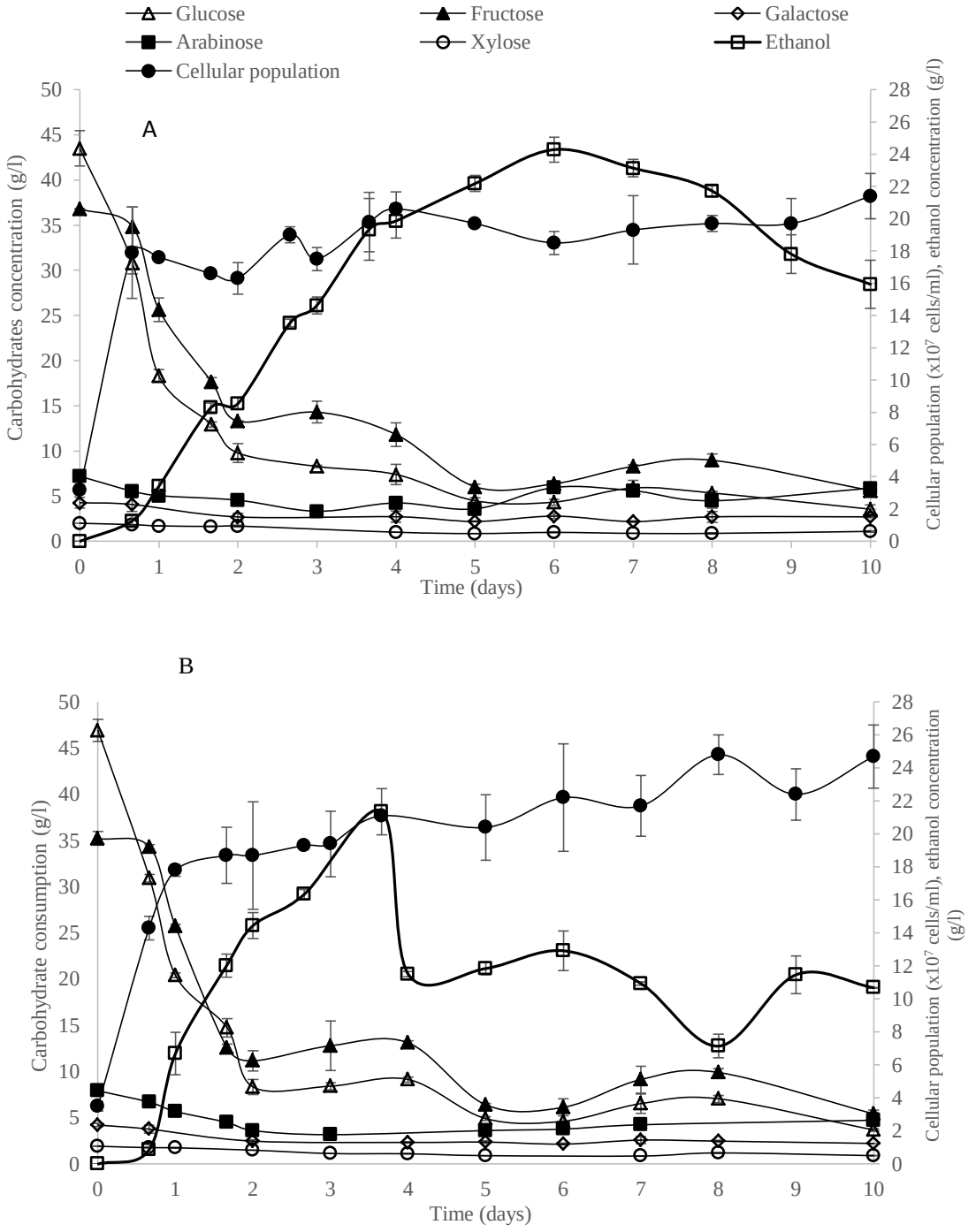

Fig. 1:- Carbohydrate consumption and ethanol production during 10 days of fermentation on synthetic medium with a sugar mixture at 120 g/l of carbohydrates (51% glucose, 30% fructose, 8% galactose, 7% arabinose, 2% xylose and 2% sucrose) by the strain: A) *C. glabrata* (T1) at 35° C and 200 rpm, B) *C. glabrata* (LR2) at 40°C and 200 rpm.

Assimilation percentages and rates of carbohydrate consumption: single and mixed cultures:-

The percentage of consumption of sugars by both strains in single culture and by the mixed cultures MC1 and MC2 is shown in Fig. 2. The Fig. 3 shows the rate of substrate consumption by all the cultures. As can be seen in Fig. 2, both mixed cultures were capable of assimilate glucose, fructose, galactose, arabinose and xylose. Glucose was the most consumed sugar for all the cultures, followed by fructose at the day of maximum ethanol production. T1 from higher to lower assimilation percentage glucose, fructose, xylose, arabinose and galactose with similar preference of galactose, xylose and arabinose. While for the strain LR2 it was: glucose, fructose, arabinose, galactose and xylose with similar assimilation for galactose and xylose and for fructose and arabinose. For mixed culture MC1 the preference was (in order) glucose, fructose, arabinose, xylose and galactose while by culture MC2 was glucose, fructose, arabinose, galactose and xylose with similar assimilation of fructose, galactose and arabinose. Between cultures, MC1 had the best assimilation of glucose, fructose and arabinose with significant differences. Also, MC1 had a slightly higher consumption of galactose and a better xylose assimilation in comparison with LR2 single culture and with the mixed culture MC2, with significant differences. T1 had the best assimilation for xylose and galactose between cultures with significant differences. LR2 had better consumption of glucose, fructose arabinose and xylose in comparison with culture MC2, and also had similar consumption for galactose. In MC2, the xylose consumption was remarkably lower in comparison with the other three cultures at the 40 h, after this point, the culture continued the carbohydrate assimilation until reach similar percentages to the other cultures (results not shown).

As can be seen in Fig. 3, glucose had the maximum rate of consumption compared with other sugars. Glucose presented the best assimilation and rate of consumption, and as pointed out it was the preferred sugar by all the cultures. The consumption rates of glucose, galactose and arabinose was similar between the four cultures, without significant differences. The rate of assimilation of fructose by MC1 was the lowest and also it had the highest rate of consumption of xylose. MC2 had the lowest rate of assimilation of xylose. The consumption of xylose was lower in comparison with other sugars.

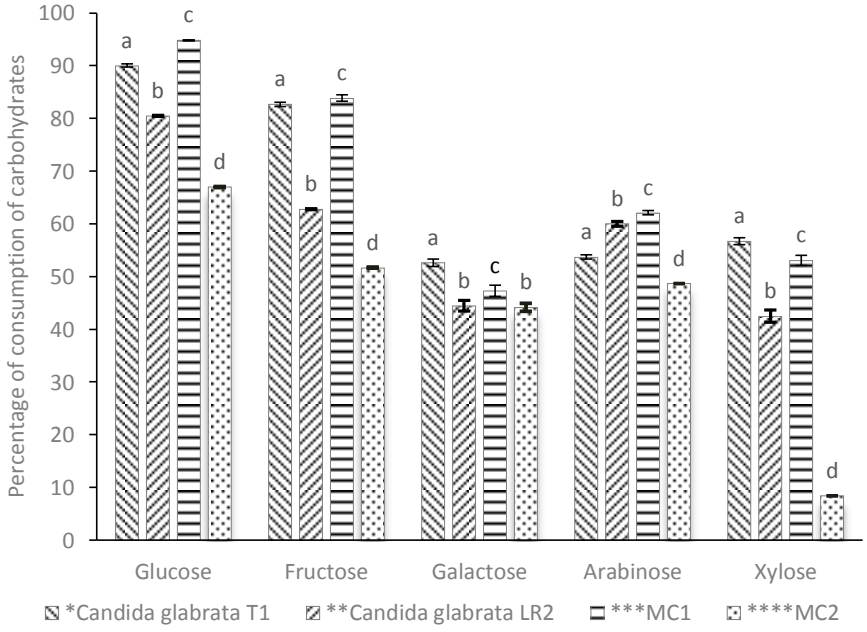

Fig. 2:- Assimilation percentages of the carbohydrates at the maximum production time of ethanol by single and mixed cultures of *C. glabrata* (T1) and *C. glabrata* (LR2) during the fermentation of a synthetic medium with a sugar mixture at 120 g/l of carbohydrates (51% glucose, 30% fructose, 8% galactose, 7% arabinose, 2% xylose and 2% sucrose).

a, b, c, d: different letters in the same sugar show significant differences between cultures ($p<0.05$).

**C. glabrata* T1 at 35° C and 200 rpm

** *C. glabrata* LR2 at 40° C and 200 rpm
***MC1: mixed culture *C. glabrata* T1 and *C. glabrata* LR2 at radio 50:50 with a total cellular population of 20 x 10^6 cells/ml inoculated simultaneously at 35° C and 200 rpm.
****MC2: mixed culture *C. glabrata* T1 and *C. glabrata* LR2 at radio 50:50 with a total cellular population of 20 x 10^6 cells/ml inoculated simultaneously at 40° C and 200 rpm.

Jeffries and Sreenath (1988) found that in cultures of *C. shehatae* the rate of xylose uptake in mediums of xylose with increasing concentrations of glucose supplements decreased more strongly than the rate of glucose uptake in mediums of glucose with increasing concentrations of xylose supplements. Thus, glucose was more effective for the repression of the xylose assimilation. The rates of consumption were higher than the obtained in this work, because those were obtained in cultures growing in rich medium instead of minimal medium. Most microorganisms have preference for glucose over other sugars and present carbon catabolite repression, expression of genes of enzymes of sugar metabolic pathways and transporters, and/or competence with sugar transporters. Also, expression and activity of the metabolic genes and sugar transporters is influenced by the sugars concentration, so at non-optimal concentrations, the sugar assimilation is reduced or repressed. This is resolved by genetic or metabolic engineering by modified the sugar metabolic pathways or by mixed cultures with microorganisms with different and complementary metabolic capacities (Demir and Kurnaz, 2006; Leandro et al., 2009; Seiboth and Metz, 2011; Marques et al., 2016).

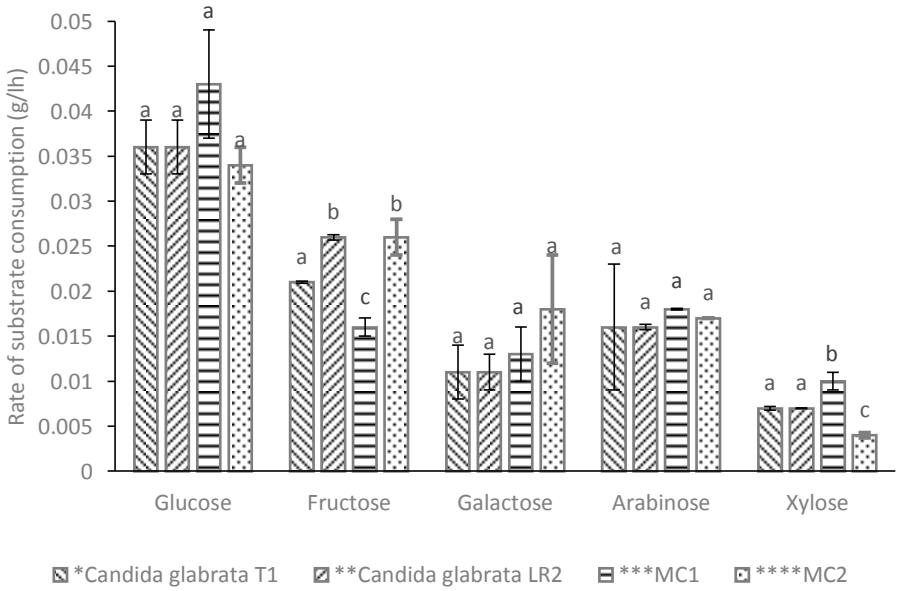

Fig. 3:- Rate of carbohydrate consumption by single and mixed cultures of *C. glabrata* (T1) and *C. glabrata* (LR2) during the fermentation of a synthetic medium with a sugar mixture at 120 g/l of carbohydrates (51% glucose, 30% fructose, 8% galactose, 7% arabinose, 2% xylose and 2% sucrose).
a, b, c: different letters in the same sugar show significant differences between cultures ($p<0.05$).
**C. glabrata* T1 at 35° C and 200 rpm
** *C. glabrata* LR2 at 40° C and 200 rpm
***MC1: mixed culture *C. glabrata* T1 and *C. glabrata* LR2 at radio 50:50 with a total cellular population of 20 x 10^6 cells/ml inoculated simultaneously at 35° C and 200 rpm.
****MC2: mixed culture *C. glabrata* T1 and *C. glabrata* LR2 at radio 50:50 with a total cellular population of 20 x 10^6 cells/ml inoculated simultaneously at 40° C and 200 rpm.

Production parameters of ethanol by single and mixed Cultures:-
Table 2 shows the parameters of ethanol production. MC1 and T1 had the higher ethanol production and slightly higher production than the single culture of LR2, with no significate difference between the three cultures. These cultures had a higher alcohol production in comparison with culture MC2. On the other side, MC2 produced ethanol with the major production rate and reach the maximum production in less time, i.e. 40 h. Additionally, MC2 had a

significantly higher maximum productivity in comparison with MC1 and T1, and slightly higher than LR2 single culture. MC1 had the lowest maximum productivity of all cultures, followed by T1, without significant difference between these two. Moreover, MC1 had a significant better production rate of ethanol compared with T1 and similar with LR2. Maybe this reflects the combination of metabolism of different strains in a mixed culture. MC1 had a higher production rate, similar to LR2, but a smaller maximum productivity, reaching the maximum production at 7 days, similar to T1. This possibly is consequence of the growth parameters (Table 1). MC1 was incubated at the optimal ethanol production conditions of T1 (35° C and 200 rpm), which had a better growth rate and doubling time at these conditions than LR2 at optimal conditions (40° C and 200 rpm) (μ_{max}: 0.093 and 0.076h^{-1}, Td: 7.50 and 9.12 for T1 and LR2, respectively, Table 1). Because of its higher μ_{max}, T1 grew faster and dominated the fermentation over the time. In consequence, the ethanol production was similar to the alcohol production of T1. The high rate of ethanol production of MC1 probably was consequence of an initial co-fermentation of carbohydrates by both strains that results in a quickly initial production of ethanol. MC1 also had better assimilation of glucose, fructose and arabinose, as mentioned above (Fig. 3), probably because both yeasts contribute to consume the carbohydrates. It´s remarkably the better utilization of arabinose by MC1 in comparison with T1, and the similarity with the assimilation of the sugar by LR2, which presented a preference for this carbohydrate. Moreover, the consumption of xylose by MC1 was higher in comparison with the assimilation by LR2. The efficiency in the consumption of pentoses is important during the production of alcohol by carbohydrates fermentation because the difficult of its metabolism (repressed by glucose), especially in sugar mixtures.

In similar way to MC1, the results obtained with MC2 reflects the combination of the metabolism of both strains. The lower ethanol production is related with the growth parameter (Table 1). T1 had better growth rate and doubling in comparison with LR2 (at optimal conditions). MC2 was incubated at the optimal fermentation conditions of LR2. Perhaps at this last condition (40|° C, 200rpm) T1 had highly parameters of growth in comparison with LR2; at the same time LR2 produced more ethanol. This would establish a competition between alcohol production by LR2 and the growth of T1 in the mixed culture, and as a consequence, the alcohol production is amortized.

Hickert et al. (2013) used a single culture of *C. shehatae* and a mixed culture of *S. cerevisiae* with *C. shehatae* for ethanol production by a synthetic medium of glucose (20 g/l), xylose (20 g/l) and arabinose (10 g/l). They observed that the single culture was capable of consume the three sugars. Glucose was the preferred sugar consumed and the assimilation of pentoses was retarded; the arabinose assimilation was dramatically retarted until glucose and xylose were exhausted. The production of alcohol was about 23 g/l at 100 h, approximately. In the mixed culture, the arabinose was not consumed; but they obtained a production of approximately 13 g/l in about 50 h. In comparison, in this study the ethanol production of LR2 (21.36 ±1.41 in 88 h) was a similar to the alcohol production of *C. shehatae* single culture. In contrast, LR2 was capable to consume the glucose and arabinose since the beginning of the fermentation. Moreover, MC2 had an ethanol production (11.59 ±1.21 at 40 h) similar to the obtained by these authors with the mixed culture (*C. shehatae* and *S. cerevisiae*), in contrast with T1, LR2 and MC1, which had higher alcohol production. The mixed cultures could be improve with a sequential inoculation. Guan et al. (2013) produced ethanol by a mixed culture of *S. cerevisiae* or *Brettanomyces bruxellensis* both with *C. shehatae* in a sugar mixture of 50 g/l of glucose, 40 g/l of xylose and 50 g/l of cellobiose with a first inoculation of *C. shehatae* for the consumption of glucose and xylose. After 48.5 h, they inoculated the ethanol-tolerant yeast of *S. cerevisiae* that fermented glucose from hydrolyzed cellobiose or a strain of *B. bruxellensis*, that fermented the cellobiose. With the second strain there was an increment of ethanol production from approximately 33.8 g/l to 57.0 g/l. Similar strategy was adopted by Gutiérrez-Rivera et al. (2015) with a mixed culture of *S. stipitis* and *S. cerevisiae*. They evaluated the fermentation in a hydrolysate of sugarcane bagasse supplemented with molasses with simultaneous inoculation of both strains at different inoculum ratios resulting in similar ethanol productions in all the mixed cultures, apparently indicating that the strain of *S. stipitis* had a predominance in the cultures due to the competition between Crabtree-positive yeast (like *S. cerevisiae*) and Crabtree-negative yeast (like *S. stipitis*) during the fermentation of glucose under aerobic conditions. They established a strategy of sequential inoculation, first with *S. stipitis* (which can use xylose and it´s less tolerant to ethanol than *S. cerevisiae*) and after 42 h with *S. cerevisiae*. The ethanol production was enhanced from approximately 29 g/l in simultaneous inoculation to 53.80 g/l in sequential inoculation. So, a strategy of sequential inoculation could improve the ethanol production and the utilization of the sugars in mixed cultures of the strains T1 and LR2. The evolution of the population of both strains and its interaction in the mixed culture also must be investigated to improve the fermentation.

Table 2:- Production parameters of alcohol by single and mixed cultures of *C. glabrata* (T1) and *C. glabrata* (LR2) during ten days of fermentation on synthetic medium with a sugar mixture (51% glucose, 30% fructose, 8% galactose, 7% arabinose, 2% xylose and 2% sucrose).

PARAMETER	CULTURE			
	C. glabrata* T1	***C. glabrata* LR2****	**MC1*****	**MC2********
Ethanol (g/l)	24.28 ±0.78[a]	21.36 ±1.41[a]	24.32 ±1.00[a]	11.59 ±1.21[b]
P_{max} (g/l day)	4.05 ± 0.13[a]	5.84 ± 0.38[b]	3.47 ± 0.14[a]	6.98 ± 0.73[b]
rp (g/lh)	0.053±0.001[a]	0.079 ± 0.008[b]	0.082±0.004[b]	0.128±0.004[c]
Day of max. production	6.0	3.7	7.0	1.7

a, b, c: different letters in the same line show significant differences between cultures ($p<0.05$).
**C. glabrata* T1 at 35° C and 200 rpm
** *C. glabrata* LR2 at 40° C and 200 rpm
***MC1: mixed culture *C. glabrata* T1 and *C. glabrata* LR2 at radio 50:50 with a total cellular population of 20 x 10^6 cells/ml inoculated simultaneously at 35° C and 200 rpm.
****MC2: mixed culture *C. glabrata* T1 and *C. glabrata* LR2 at radio 50:50 with a total cellular population of 20 x 10^6 cells/ml inoculated simultaneously at 40° C and 200 rpm.

Conclusion:-

The assimilation profiles of two strains of *C. glabrata* were studied in single and in two mixed cultures during the fermentation of a carbohydrate mixture of glucose, fructose, galactose, arabinose and xylose in synthetic media. It was found that in single cultures, the strain T1 had higher μ_{max} and lower doubling time but the strain LR2 had higher biomass and cellular population. The cultures were capable to consume all the sugars in the mixture, The assimilation profiles during the ethanol production for both strains in single and mixed cultures were different. All the cultures preferred glucose and fructose, but T1 single culture had similar preference by galactose, arabinose and xylose. LR2 and MC1 preferred arabinose over galactose and xylose and MC2 preferred galactose and arabinose over xylose. Between all the cultures, MC1 had the best consumption of glucose, fructose and arabinose and T1 had the best assimilation of galactose and xylose. Despite both strains were combined in mixed cultures, ethanol production wasn´t improved. T1 single culture and MC1 had slightly higher production compared with LR2 single culture but this strain produced ethanol more quickly. MC2 had the lowest alcohol production, the lowest sugar consumption, and the best production rate. Since both strains were inoculated simultaneously in mixed cultures, probably there was a competition due to the differences in growth parameters. The consumption of carbohydrates and alcohol production could be improved with a strategy of mixed cultures with sequential inoculation. The knowledge of the assimilation profiles of both strains in single and mixed cultures will help to search possible use of them in the fermentation of citrus residues.

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