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ETHANOL PRODUCTION FROM *Dekkera bruxellensis* IN SYNTHETIC MEDIA WITH PENTOSE

Carolina B. Codato[1,*], Cristina Martini[1],
Sandra R. Ceccato-Antonini[1] and Reinaldo G. Bastos[1]

[1]Universidade Federal de São Carlos, Centro de Ciências Agrárias, Dept. Tecnologia Agroindustrial e Socio-Economia Rural, Rodovia Anhanguera, km 174, Araras 13600-970, Brazil

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Abstract – Ethanol is obtained in Brazil from the fermentation of sugarcane, molasses or a mixture of these. Alternatively, it can also be obtained from products composed of cellulose and hemicellulose, called "second generation ethanol – 2G". The yeast *Saccharomyces cerevisiae*, commonly applied in industrial ethanol production, is not efficient in the conversion of pentoses, which is present in high amounts in lignocellulosic materials. This study aimed to evaluate the ability of a yeast strain of *Dekkera bruxellensis* in producing ethanol from synthetic media, containing xylose or arabinose, xylose and glucose as the sole carbon sources. The results indicated that *D. bruxellensis* was capable of producing ethanol from xylose and arabinose, with ethanol concentration similar for both carbon sources, 1.9 g L^{-1}. For the fermentations performed with xylose and glucose, there was an increase in the concentration of ethanol to 5.9 g L^{-1}, lower than the standard yeast *Pichia stipitis* (9.3 g L^{-1}), but with similar maximum yield in ethanol (0.9 g g TOC^{-1}). This proves that the yeast *D. bruxellensis* produced lower amounts of ethanol when compared with *P. stipitis*, but showed that is capable of fermenting xylose and can be a promising alternative for ethanol conversion from hydrolysates containing glucose and xylose as carbon source.

Keywords: Dekkera bruxellensis, xylose, arabinose, ethanol.

INTRODUCTION

The increase in the demand for ethanol, stimulated by the increase of the proportion of ethanol in the mixture with gasoline, flex-fuel vehicles, and the constant global concerns regarding environmental issues, are incentivizing the industry sector to expand production to meet future demands for fuel (Tomalsquim, 2012). On the other hand, the increase of ethanol production triggers some concerns about possible competition between sugar cane plantations for agricultural lands, which negatively affects more diverse agriculture production. An alternative for this issue comes by way of second-generation ethanol production, which is a process from lignocellulosic residues that demands the transformation of cellulose and hemicellulose into their monomers (glucose and xylose) followed by their conversion into ethanol by microorganisms.

The yeast *Saccharomyces cerevisiae* is able to rapidly ferment glucose and fructose into ethanol, with high tolerance to the obtained product and to temperature variations, which are desirable features for a strain with industrial use (Andrietta et al., 2007).This yeast has been indicated for the fermentation of hexoses present in lignocellulosic hydrolysates due to its ability to produce ethanol and to its tolerance to the inhibitors present (Hahn-Hägerdal et al., 1991). However, it is not able to ferment sugars such as xylose, arabinose and others. These sugars make up the substrate of interest in lignocellulosic biomass, so that research for yeasts able to ferment them is necessary to increase ethanol production (Rossi and Andrietta, 2009;

**Corresponding author*: Carolina B. Codato. E-mail: codato.carolina@gmail.com

Ceccato-Antonini et al., 2017).

In this way, a viable production of ethanol from biomass implicates the use of microorganisms that are able to ferment not only glucose but all sugars present in the lignocellulosic hydrolysates, such as D-xylose, L-arabinose, D-cellobiose, D-galactose and D-mannose with high yield and productivity (van Maris et al., 2006; Hahn-Hägerdal et al., 2007; Bettiga et al., 2008; Fukuda et al., 2009; Martini et al., 2016).

Many yeasts ferment xylose directly into ethanol but production is limited. Only a few species can effectively produce ethanol from xylose, such as *Pachysolen tannophilus, Candida shehatae* and *Pichia stipitis* (Nigam et al., 1985). Toivola et al. (1984) carried out a systematic screening of strains from around 200 yeast species and identified *P. stipitis* as one of the species with the highest yield and productivity of ethanol from xylose. *Dekkera* yeast strains were studied to explore their potential to produce ethanol from renewable sources in conditions adequate for industrial processes, such as with limited oxygen and low pH, revealing their high potential in industrial processes of this nature (Galafassi et al., 2011). In this context, studies about the cultivation of *Dekkera bruxellensis* in synthetic media became necessary to evaluate its fermentative capacity from media containing pentose and/or glucose, since studies in this area proved that, even with low productivity, this strain is able to reach high yields of conversion of pentoses into ethanol.

MATERIALS AND METHODS

Microorganisms and culture media

The yeast *D. bruxellensis* (CCA155-CCT7784) from the culture collection of the Laboratory of Agricultural and Molecular Microbiology (Laboratório de Microbiologia Agrícola e Molecular – LAMAM), UFSCar, Campus Araras, was used in the experiment. In addition, *P. stipitis* (NRRL Y-7124) was kindly supplied by Prof. Dr. Inês Conceição Roberto (Escola de Engenharia de Lorena/ Universidade de São Paulo - EEL/USP) and tested for comparative purposes. The strains were maintained on YPD (10 g L^{-1} yeast extract; 20 g L^{-1} glucose; 20 g L^{-1} peptone; 20 g L^{-1} agar; for liquid medium agar was not included) slants at 4°C with constant transfers to new medium.

Fermentative trials were performed with strains of *D. bruxellensis* and *P. stipitis* with and without pentose recycling, in synthetic media containing xylose, arabinose or xylose + glucose as carbon sources. Concentrations from 20 to 100 g L^{-1} xylose and arabinose, and a mixture of 40 g L^{-1} xylose + 10 g L^{-1} glucose were tested with synthetic fermentation media: monobasic potassium phosphate (5.0 g L^{-1}), potassium chloride (1.0 g L^{-1}), ammonium chloride (1.5 g L^{-1}), yeast extract (6.0 g L^{-1}), magnesium sulphate heptahydrate (1.0 g L^{-1}), pentose source (arabinose or xylose) with variable concentration and glucose (10 g L^{-1}) whenever necessary.

Fermentation with pentose recycling in synthetic fermentation medium

Inoculum preparation

The inoculum was obtained from the cultivation of *D. bruxellensis* in YPD liquid culture media. The inoculum was placed in an agitator at 30°C and at 160 rpm for 24 hours. After this period, it was re-inoculated in a saline solution (8.5 g L^{-1} NaCl) for cell washing. Following this, it was adapted in YNB medium (6.7 g L^{-1}) containing 10 g L^{-1} pentose source and then placed in an agitator at 30°C and 160 rpm for 24 hours. Subsequently, the cells were centrifuged and re-inoculated in YNB medium (6.7 g L^{-1}) containing 20 g L^{-1} pentose source, following the above parameters. After this period, the inoculum was centrifuged and washed in saline solution (8.5 g L^{-1} NaCl) and a proportion of 10 g of wet mass L^{-1} of fermentation medium was used.

Fermentation

Fermentation was carried out in increasing concentrations of arabinose and xylose (20, 40, 60, 80 and 100 g L^{-1}) as sole carbon sources. The pH was 5.0, temperature 30°C, cell concentration 10 g L^{-1} fermentation medium, final volume 20 mL and agitated at 150 rpm. The concentration increase was performed by centrifugation of the cells, thereby renewing the culture medium. Total organic carbon and pH were measured daily and after each cycle the samples were distilled for the determination of ethanol content.

Fermentations without pentose recycling in synthetic fermentation medium

Inoculum preparation

The inoculum was obtained from the yeasts (*D. bruxellensis* or *P. stipitis*) tested in flasks containing liquid culture medium (YPD) in an agitator at 30°C and 160 rpm for 48 hours. After this period the cells were centrifuged and adapted in YNB medium (6.7 g L^{-1}) containing 20 g L^{-1} of pentose source in the same conditions described in the fermentation with pentose recycling in a synthetic medium.

Fermentation

The fermentation was carried out at pH 5, temperature 30°C, cell concentration 10 g L^{-1}, final volume of 200 mL, and agitation at 150 rpm. The fermentation medium consisted of a mixture of 40 g L^{-1} xylose + 10 g L^{-1} glucose prepared as above described. Total organic carbon and pH testing were performed every 12 h. Samples were distilled and the ethanol content was measured.

Analysis

Analysis of Total Organic Carbon (TOC) was performed with the TOC - LCPN Shimadzu® equipment. The analysis is based in the organic material oxidation of the sample at 720°C in the presence of an oxidizing atmosphere (synthetic air) catalyzed by alumina and platinum, transforming the material into CO_2 and a mixture of gases including the nitrogen compounds. The TOC measurement is made by an infrared (NDIR) gas analyzer with a 4.0 μg L^{-1} detection limit that detects absorbance of CO_2 at a specific wavelength in the infrared spectrum.

After each fermentative cycle (recycled fermentation of pentose) and every 12 hours (fermentation without recycle) samples were centrifuged and, in the supernatant, pH was determined using a digital pH-meter; the alcohol content (g L^{-1}) was evaluated in the hydroalcoholic solution using a DMA-45 Anton Paar® densitometer after the sample distillation. The alcohol yield ($Y_{p/s}$) was calculated based on the alcohol content of the fermented medium and the consumption of the TOC (g alcohol g TOC^{-1}), and the maximum alcohol yield ($Y_{p/s}$ max) was calculated from the maximum ethanol produced by consumption of the TOC. Alcohol productivity (g L^{-1} h^{-1}) was calculated based on the alcohol content at the end of each fermentative cycle or in relation to the maximum production at a particular time.

RESULTS AND DISCUSSION

Fermentation with pentose recycling in synthetic fermentation medium

In all conditions carbon consumption was observed, being most notable for 20 g L^{-1} of arabinose and xylose (Fig. 1A e B), with 85 and 69% of consumption, respectively. The highest yield ($Y_{p/s}$) 0.34 g g^{-1} was obtained at 80 g L^{-1} of arabinose concentration (Tab. 1) in 120 hours, following by 0.28 g g^{-1} at 60 g L^{-1} of xylose concentration, requiring 144 hours. According to the literature, species such as *Scheffersomyces shehatae*, *Pachysolen tannophilus* and *Scheffersomyces stipitis* are capable of achieving high ethanol yields from xylose on the order of 0.38, 0.36 and 0.49 g g^{-1}, respectively (Cheng et al., 2008; Antunes et al., 2014; Yu et al., 2015). According to Galafassi et al. (2011), the yeast *D. bruxellensis* in conventional batch cultivations with synthetic medium under oxygen limiting conditions showed a yield of 0.44 g g^{-1}. The condition with increased ethanol production, 1.9 g L^{-1}, occurred in 60 g L^{-1} xylose medium. In this condition, the volumetric productivity (Qp) of ethanol was the highest (0.013 g L^{-1} h^{-1}). It is a low value compared with strains indicated as able to achieve high ethanol yields from xylose. As described in Cheng et al. (2008), the yeast *Pachysolen tannophilus* in sugarcane bagasse hydrolysate achieved 0.73 g L^{-1} h^{-1}. However, other studies reported 0.03 g L^{-1} h^{-1} with *Schizosaccharomyces pombe* in semi-synthetic medium (Silva, 2015) and 0.07 g L^{-1} h^{-1} with *Pichia stipitis* in supplemented hydrolyzed sugarcane bagasse (Ferreira et al., 2011).

When 80 g L^{-1} of xylose was added, there was low ethanol production, with inhibition observed at 100 g L^{-1}. TOC values indicated variations at all concentrations, with more pronounced substrate consumption at 100 g L^{-1}, corroborating a slower metabolism. According to studies by Zhu et al. (2007), xylose assimilation is considered slower when compared to glucose, and has its consumption strongly influenced by its concentration. In a study set up by Roberto et al. (1991) with *P. stipitis,* fermentation inhibition was registered at initial concentrations of xylose between 76 g L^{-1} and 99 g L^{-1}.

The pH values did not change considerably until the concentration of 60 g L^{-1} for arabinose (Fig. 1D). At 100 g L^{-1} there was moderate acidification and also the highest consumption of arabinose. In comparison to xylose, it presented slightly lower levels of ethanol. pH values of around 2 are reported in the literature as being able to limit *Dekkera* growth in YEPD medium (Bassi et al., 2013). According to Freer et al. (2003), *D. bruxellensis* is able to produce high amounts of acetic acid from glucose in a well-mixed medium, even when presenting a slow metabolism. In addition, these yeasts can adapt to environments with nutrient limitation and high ethanol concentrations, being able to also use the latter as a carbon source (Suárez et al., 2007).

For fermentation in which xylose was the only carbon source (Fig. 1A and C), a higher ethanol production was observed at 60 g L^{-1}, which was also the condition that presented the greatest decrease in pH. According to Ciani and Ferraro (1997), *Dekkera* yeasts in semi-anaerobic conditions produce a mixture of ethanol and acetic acid due to a positive Custer Effect that is caused when the fermentative process is stimulated by aeration.

Fermentations without pentose recycling in synthetic fermentation medium

The fermentation showed maximum levels of ethanol between 24 and 36 hours, for *D. bruxellensis* and *P. stipitis* with 5.92 g L^{-1} and 9.32 g L^{-1} respectively (Fig. 2A and B). Considering the theoretical ethanol yield from glucose (0.511 g g^{-1}), tests would allow a maximum ethanol production from glucose of 5.11 g L^{-1} ethanol because the fermentation medium contained 10 g L^{-1} of glucose. Therefore, from the maximum concentration found, 5.92 g L^{-1}, we can say that the yeast *D. bruxellensis* was able to produce ethanol from xylose, since the values found are beyond the theoretical production from glucose. According to these results, the productivity value in 36 hours was higher for *P. stipitis* with 0.26 g L^{-1} h^{-1}, against 0.16 g L^{-1} h^{-1} for the strain of *D. bruxellensis*. This result proves a slower metabolism of *D. bruxellensis*, which gradually consumed the TOC concentration from 12 hours. Furthermore, at 24 hours *P. stipitis* had reduced 31% of the total carbon present

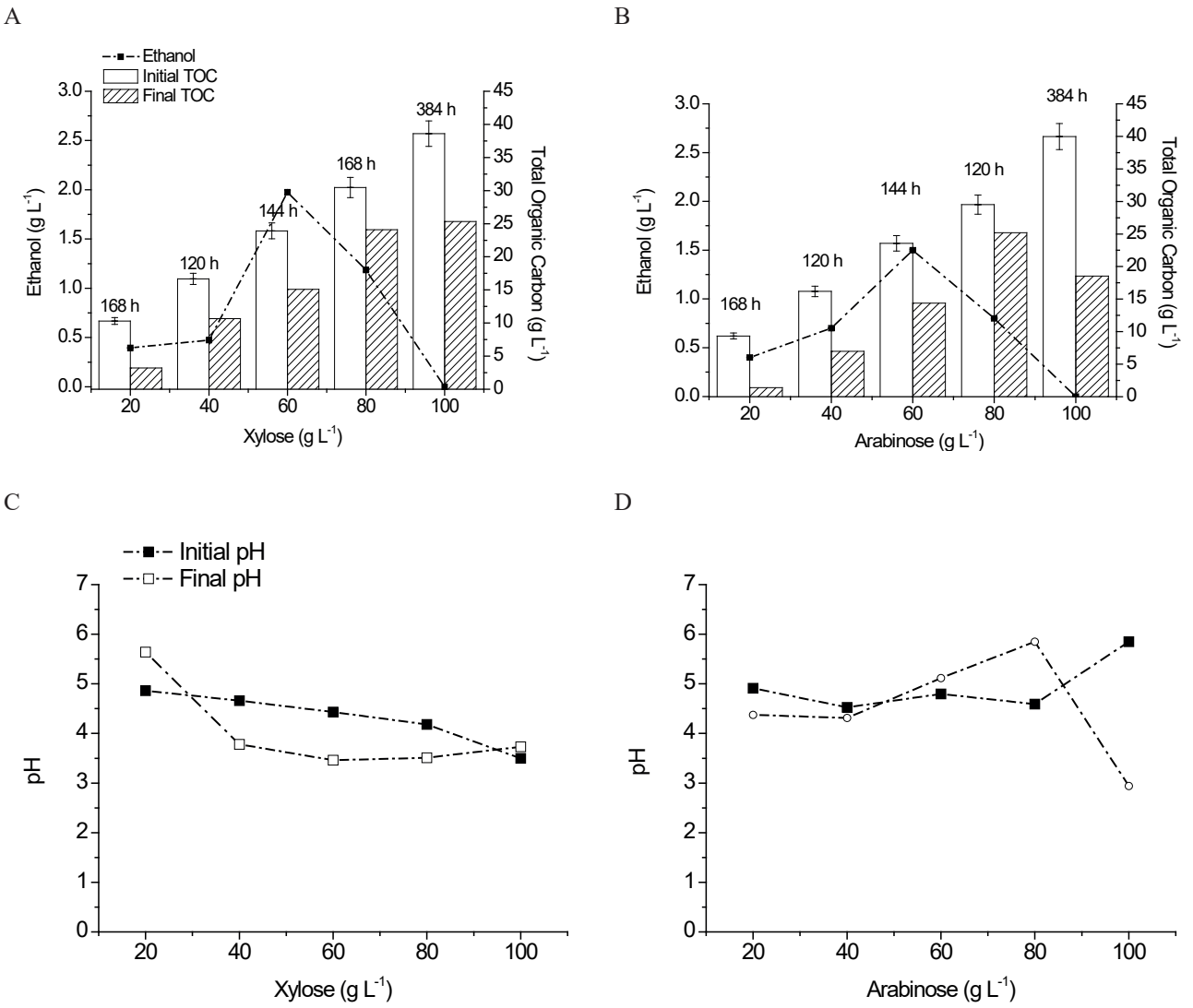

Figure 1. Total organic carbon (TOC), ethanol and pH for the fermentation by *D. bruxellensis* made with 20 mL of synthetic fermentation medium containing xylose (A and C) or arabinose (B and D) as the sole carbon sources, in a recycled fermentation with increased pentose concentration, in 50 mL flasks with a pH of 5.0, 10 g L^{-1} inoculum, 150 rpm and 30°C. Time expressed in hours for each cycle (A and B).

Table 1. Consumption of total organic carbon (TOC), ethanol yield based in TOC (Yp/s) and volumetric productivity of ethanol (Qp) for each concentration of xylose (Xy) and arabinose (Ar) in recycled fermentation medium with increased pentose concentration by *D. bruxellensis.*

Parameter	Pentose concentration (g L^{-1})									
	20		40		60		80		100	
	Xy	Ar	Xy	Ar	Xy	Ar	Xy	Ar	Xy	Ar
TOC consumption (%)	69	85	36	57	37	39	21	15	34	54
Yp/s (g g^{-1})	0.08	0.07	0.12	0.10	0.29	0.21	0.27	0.34	0	0
Qp (g L^{-1} h^{-1})	0.002	0.002	0.004	0.006	0.014	0.010	0.010	0.007	0	0

in the medium, suggesting the potential for producing ethanol from xylose.

According to du Preez et al. (1986), on medium containing pentoses and hexoses, the hexose will be consumed first and, after being exhausted, the pentose will be used. Tests carried out by Galafassi et al. (2011) showed productivity of 0.135 g L^{-1} h^{-1} using synthetic softwood hydrolysate without furfural with the yeast *Brettanomyces naardenensis*, a similar value to that found with *D. bruxellensis*. The same authors revealed that, due to the presence of glucose in the feeds, the ethanol yield in the fermentative process was higher with xylose,

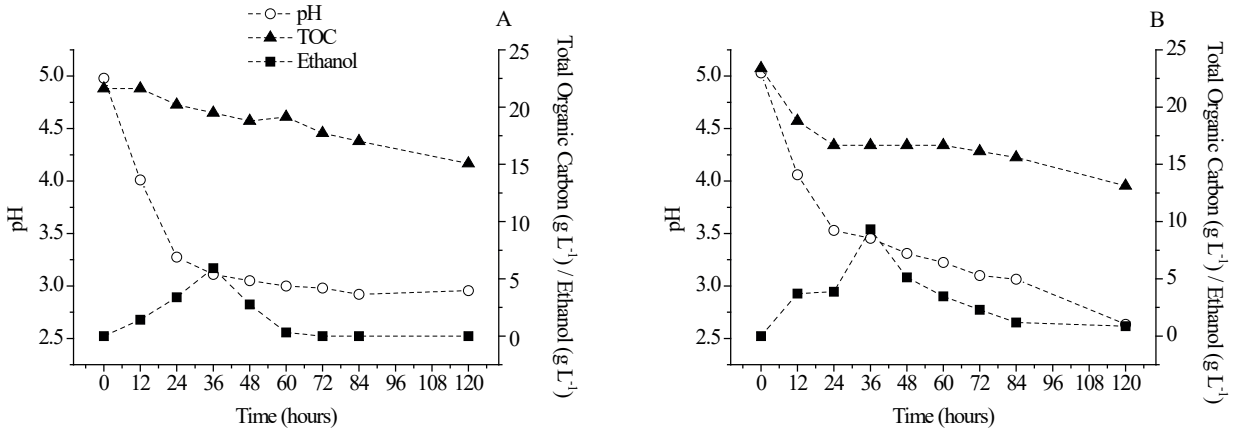

Figure 2. Total organic carbon (TOC), pH and ethanol for the fermentation by *D. bruxellensis* (A) and *P. stipitis* (B), of synthetic fermentation medium containing 40 g L^{-1} of xylose and 10 g L^{-1} glucose as the sole carbon sources, pH 5.0, 10 g L^{-1} inoculum, 150 rpm and 30°C.

which influenced the decision of adding a low glucose concentration (10 g L^{-1}) to the fermentation medium. In addition, increasing the feed rate higher yield levels and productivity were obtained. This fact can indicate that xylose fermentation under oxygen limitation causes a redox unbalance that can probably be associated with the use of a different co-factor by two enzymes (xylose reductase and xylose dehydrogenase) that are initially involved in xylose metabolism (Galafassi et al., 2011). Study carried out by Meneghin et al. (2013) indicates that aeration is an important parameter in the cultivation of *D. bruxellensis* in a batch culture during the alcoholic fermentation process. According to Schneider and Jeffries (1989) and Rizzi et al. (1989), the aeration rate determines the amount of carbon used by the cell for cell growth or ethanol formation, with an ideal oxygenation level existing that leads to a higher yield and productivity of ethanol, with a low biomass yield. The oxygenation in xylose-fermented yeasts is necessary for maximum ethanol production and, even then, it reaches low levels when compared to the production by *S. cerevisiae* from glucose.

After 36 hours, there was a decrease in ethanol concentration for the two strains. As it was batch fermentation, it is possible that ethanol had been consumed as a carbon source as variations in TOC were not considerable, unlike the ethanol concentrations. According to the literature, the ethanol yield obtained by fermenting pentose yeast in culture medium with xylose is mainly limited by two factors: concurrent ethanol use in the presence of high concentrations of xylose and the formation of xylitol and others by-products (Duarte, 1990). According to Kiipper (2009), ethanol consumption occurs because the reducing sugar concentration decreases rapidly during the first 24 hours and starts to decrease gradually until 120 final hours of the experiment, when ethanol is used as the carbon source. Furthermore, when the xylose concentration reaches a certain level and oxygenation increases, some microorganisms used preferably ethanol as a carbon source (Maleszka and Schneider, 1982).

Acidification of the culture medium occurred for the two species; however, in the early hours it was more pronounced for *D. bruxellensis*. According to Freer et al. (2003), this species produces a significant amount of acetic acid from glucose in shaken culture. The authors have shown that the temperature may also influence the formation of acetic acid, which is greater at 30°C, with ethanol or glucose as carbon source. In fact, according to Blomqvist et al. (2010), *D. bruxellensis* was able to utilize ethanol as a carbon source for the production of acetic acid.

The maximum yield to ethanol was similar for both tested strains (Tab. 2), around 71% of the theoretical stoichiometry for carbon to ethanol yield. Although with a lower productivity (0.16 g L^{-1} h^{-1}), the yeast *D. bruxellensis* may be an alternative to ethanol conversion from hydrolysates containing glucose and xylose as carbon source.

CONCLUSIONS

The results suggest that *D. bruxellensis* yeast produced low amounts of ethanol in synthetic media containing pentose, with yields around 0.34 and 0.28 g g^{-1} for arabinose and xylose, respectively. However, the condition with higher ethanol production, 1.9 g L^{-1}, and increased volumetric productivity, 0.013 g L^{-1} h^{-1}, occurred in medium with 60 g L^{-1} xylose. In culture medium containing xylose

Table 2. Maximum ethanol yield based in TOC ($Y_{p/s}$) and volumetric productivity of ethanol (Qp) during *D. bruxellensis* and *P. stipitis* fermentation of a synthetic medium contain xylose and glucose as the sole carbon source.

Yeast	$Y_{P/S}$ max ($g_{ethanol}$ g_{TOC}^{-1})	Qp ($g_{ethanol}$ L^{-1} h^{-1})
D. bruxellensis	0.90	0.16
P. stipitis	0.91	0.26

and glucose (4:1) the ethanol production was around 6 g L^{-1} ethanol compared to 9.48 g L^{-1} of the standard *P. stipitis* yeast; however, the maximum ethanol yield was similar for both strains tested (0.9 g g^{-1}). Considering the theoretical ethanol yield from glucose, the yeast *D. bruxellensis* was also able to produce ethanol from xylose, since the values found were beyond the theoretical production from glucose. Therefore, the yeast was revealed to be able to ferment xylose in a synthetic medium, in ideal conditions, revealing its ability to produce ethanol from hydrolysates containing pentose as carbon sources.

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