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The isolation of pentose-assimilating yeasts and their xylose fermentation potential



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ABSTRACT

For the implementation of cellulosic ethanol technology, the maximum use of lignocellulosic materials is important to increase efficiency and to reduce costs. In this context, appropriate use of the pentose released by hemicellulose hydrolysis could improve the economic viability of this process. Since the *Saccharomyces cerevisiae* is unable to ferment the pentose, the search for pentose-fermenting microorganisms could be an alternative. In this work, the isolation of yeast strains from decaying vegetal materials, flowers, fruits and insects and their application for assimilation and alcoholic fermentation of xylose were carried out. From a total of 30 isolated strains, 12 were able to assimilate 30 g L⁻¹ of xylose in 120 h. The strain *Candida tropicalis* S4 produced 6 g L⁻¹ of ethanol from 56 g L⁻¹ of xylose, while the strain *C. tropicalis* E2 produced 22 g L⁻¹ of xylitol. The strains *Candida oleophila* G10.1 and *Metschnikowia koreensis* G18 consumed significant amount of xylose in aerobic cultivation releasing non-identified metabolites. The different materials in environment were source for pentose-assimilating yeast with variable metabolic profile.

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Introduction

Bioethanol or first generation ethanol in Brazil is obtained by fermenting glucose from sugar cane juice and molasses (a residue from sugar making) using *Saccharomyces cerevisiae*. While this technology is a consolidated industrial process,

production of second generation ethanol remains a challenge. One of the bottlenecks is the utilization of pentoses released by hydrolysis of hemicellulose that correspond to around 30% of the lignocellulosic feedstock. Yeasts able to convert xylose into ethanol have been described like as *Scheffersomyces* (*Pichia*) *stipitis*, *S. (Candida) shehatae* and *Pachysolen tannophilus*.^{1,2} However the yield has not reached a satisfactory level for industrial

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applications. On the other hand, pentose-assimilating yeasts can be used for obtainment of high aggregate value products. The pentose-assimilating capacity of yeasts *Pseudozyma antarctica* PYCC 5048^T, *P. aphidis* PYCC 5535^T and *P. rugulosa* PYCC 5537^T and production of a glycolipid biosurfactant was demonstrate³ as well as the production of xylitol and arabitol by *Debaryomyces hansenii* from pentose.⁴

A number of recent studies have been focused on the genetic engineering of *S. cerevisiae*, aimed at making it able to produce ethanol from glucose and xylose.⁵ Nevertheless, the yield achieved resembles the xylose-fermenting species, the xylose utilization is slow and occurs only after glucose exhaustion.^{3,4,6,7} The search for genetically modified strains is the focus of several studies. Thus, the study of microorganisms pentose-fermenting and isolation of genes involved in pentose fermentation can afford support for these studies.

Another research tendency is the use of pentose to produce alternative compounds like organic acids,^{8–12} xylitol,¹³ lipids for biodiesel production,^{14–16} isobutanol¹⁷ and hydrogen¹⁸ all using mainly yeast and bacteria. Besides compounds already cited, new alternative products with high aggregate value, are still appearing, increasing the options for the utilization of these sugars in biorefineries system.¹⁹

Considering the importance of the use of pentoses from lignocellulosic materials, the present work aimed at isolating yeast strains with the ability to assimilate and/or ferment xylose for future use for obtainment of products with biotechnological interest. Thus, this study contributes by increasing the information on xylose-fermenting strains and offers genomes for future studies of the metabolism of microorganisms.

Materials and methods

Isolation and identification of strains

The strains were isolated from fruits, flowers, insects and grape residues. About 0.5 g of material was suspended in 4 mL of YEPX medium (1% of yeast extract, 2% of peptone and of 2% xylose) and incubated at 30 °C. A loop of the homogenized culture was then streaked on the solid YEPX medium in Petri dishes to isolate the colonies. The strain identification was made using sequences of the D1/D2 domains of the rDNA.²⁰

Xylose assimilation assay

To evaluate the potential of xylose assimilation, the strains were pre-cultivated in a YEP medium (1% yeast extract, 2% peptone and 2% glucose) and the biomass was centrifuged, washed twice with sterile distilled water and a suspension of them used as inoculum at 1 g of dry biomass per liter of culture medium composed of KH₂PO₄ (2.0 g L⁻¹), (NH₄)₂SO₄ (2.0 g L⁻¹), MgSO₄ · 7H₂O (1.0 g L⁻¹), urea (0.3 g L⁻¹), CaCl₂ (0.3 g L⁻¹) and xylose (30.0 g L⁻¹). The incubation was carried out at 30 °C and 150 rpm. The assays were done in duplicate and with three repetitions.

Ethanol production assay

The alcoholic fermentation was assayed in 125 mL Erlenmeyer flasks adapted for alcoholic fermentation, closed with a valve containing sodium metabisulfite solution at 1 g L⁻¹ to ensure that no oxygen got, and containing 60 mL of medium described above but with yeast extract at 10 g L⁻¹ and xylose at 100.0 g L⁻¹. The incubation was at 30 °C. The assays were done in duplicate with three repetitions.

Analytical methods

To evaluate the dry cell mass, samples of fermented medium were centrifuged at 10,000 × g, by 15 min, the supernatant was discarded and the precipitated cells were dried at 60 °C until they reached constant weight.

The reducing sugar concentration was determined using the dinitrosalicylic (DNS) acid method, based on Miller (1959).²¹

The ethanol concentration was measured by a gas chromatograph (HP 5890) with an FFAP capillary column (polyethylene glycol – 30 mm × 0.22 mm × 0.3 μm) and a flame ionization detector, one split/splitless injector. Nitrogen was utilized as a carrier gas at 30 mL min⁻¹. Temperatures at injector and detector were 250 °C.

The xylitol quantification was performed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). All samples were filtered (0.22 μm membrane) and injected (20 μL) in HPAEC-PAD System (ICS, Dionex Corporation, USA) equipped with automatic sampler AS40. The form of wave pattern used was the standard quadruple with the following potential pulse and durations: E1 = 0.10 V (t1 = 0.40 s); E2 = -2.00 V (t2 = 0.02 s); E3 = 0.60 V (t3 = 0.01 s); E4 = -0.10 V (t4 = 0.06 s). An Isocratic run was performed with 10 mM NaOH at a flow rate of 1 mL min⁻¹ and 35 °C.

Results

Isolation, identification of yeasts and evaluation of xylose assimilation

A total of 30 strains were isolated and able to grow on the liquid YEPX medium, but, among those, four were not able to grow in liquid medium with xylose as the sole carbon source (Table 1). In aerobic cultivation, nine strains consumed all the xylose present in the medium (30 g L⁻¹) in 120 h.

The strain *Pichia guilliermondii* G1.2 was the most efficient in the xylose consumption (maximum at 48 h) while strains *Candida tropicalis* FP, *C. tropicalis* S4 and *P. guilliermondii* G4.2 exhausted the sugar in 72 h.

Species or genera isolated in this work, such as *Aureobasidium pullulans*, *Issatchenkia terricola*, *I. orientalis*, *Metschnikowia bicuspidata*, *M. chrysoperlae*, *M. zobellii*, *P. guilliermondii*, *P. stipitis*, *Rhodotorula minuta* and *R. mucilaginosa* are mentioned in the literature as being able to produce ethanol from xylose.^{22–29} The genera *Hanseniaspora* and the specie *I. terricola* are glucose fermenting, but there is contradicting information about

Table 1 – Yeasts isolated and xylose consumption during aerobic cultivation using a basal medium and xylose (30 g L⁻¹) as the sole carbon source.

Isolated strains	Samples	Xylose consumption		Maximum biomass production		Yield (biomass/xylose) (g g ⁻¹)
		(g L ⁻¹)	Time (h)	(g L ⁻¹)	Time (h)	
<i>Aureobasidium pullulans</i> G19	Flor (Nyctaginaceae)	9.9	120	9.7	120	0.9
<i>Candida tropicalis</i> E2	Grape residue	30.0	120	8.0	120	0.2
<i>C. tropicalis</i> FP	Anthill	30.0	72	7.2	120	0.2
<i>C. tropicalis</i> S2	Grape leaf	10.0	120	3.3	96	0.3
<i>C. tropicalis</i> S3	Grape	10.0	120	3.1	120	0.3
<i>C. tropicalis</i> S4	Grape leaf	30.0	72	7.3	120	0.2
<i>C. tropicalis</i> T	Wine must	20.1	120	7.1	120	0.3
<i>C. oleophila</i> G10.1	Leaf (Asteraceae)	30.0	96	8.2	120	0.3
<i>Hanseniaspora guilliermondii</i> G9.1	Leaf (Asteraceae)	7.6	120	2.6	96	0.3
<i>Hanseniaspora</i> sp. G1.3	Fruit (Anarcadiaceae)	19.4	120	3.7	72	0.2
<i>Hanseniaspora</i> sp. G11.1	Fruit (Rutaceae)	7.6	120	3.5	120	0.5
<i>Hanseniaspora</i> sp. G13	Fruit (Rutaceae)	10.4	120	2.7	72	0.2
<i>Hanseniaspora</i> sp. G14	Flower (Myrtaceae)	9.9	120	2.0	120	0.2
<i>Hanseniaspora</i> sp. G15	Fruit (Malpighiaceae)	3.5	120	2.2	96	0.6
<i>Hanseniaspora</i> sp. G17	Flower (Rubiaceae)	5.4	120	2.8	48	0.5
<i>Hanseniaspora</i> sp. G2	Flower (Amaryllidaceae)	10.2	120	5.6	48	0.5
<i>Hanseniaspora</i> sp. G4.1	Flower (Nyctaginaceae)	23.0	120	8.2	120	0.3
<i>Hanseniaspora</i> sp. G7.1	Fruit (Myrtaceae)	23.9	120	8.1	120	0.3
<i>Issatchenkia terricola</i> G20	Fruit (Myrtaceae)	5.1	120	5.0	96	0.9
<i>Metschnikowia korensis</i> G18	Flower (Amaryllidaceae)	30.0	120	6.7	96	0.2
<i>Pichia guilliermondii</i> G1.2	Fruit (Anarcadiaceae)	30.0	48	10.8	120	0.3
<i>P. guilliermondii</i> G4.2	Fruit (Rosaceae)	30.0	72	10.6	120	0.3
<i>P. guilliermondii</i> B1	Wine must	nd ^a	–	nd	–	–
<i>P. guilliermondii</i> B2	Grape	nd	–	nd	–	–
<i>P. kluyveri</i> G1.1	Fruit (Anarcadiaceae)	20.5	120	7.2	120	0.3
<i>Rhodotorula mucilaginosa</i> G7.2	Flower (Euphorbiaceae)	29.4	120	11.5	120	0.4
<i>R. mucilaginosa</i> G11.2	Flower (Plantaginaceae)	17.3	120	6.7	120	0.4
<i>Rhodotorula</i> sp. G10.2	Flower (Plantaginaceae)	27.8	120	8.1	120	0.3
<i>Yarrowia lipolitica</i> CO1	Grape	nd	–	nd	–	–
<i>Yarrowia lipolitica</i> CO2	Grape	nd	–	nd	–	–

Identification based on sequences of the D1/D2 domains of the rDNA (at least 98% similarity, with reference to homologous sequences from GenBank).

^a Not quantified, the strains were not able to grow with xylose as the sole carbon source.

the xylose assimilation by these yeasts.^{22,25} The most efficient xylose-assimilating yeasts were chosen to continue the experiments and used for fermentation for ethanol production in subsequent assays.

Ethanol production by yeasts

The ethanol production was evaluated for 30 strains and four of them were able to produce ethanol from xylose (from 3 to 6 g L⁻¹) (data not shown). The three strains of *C. tropicalis* showed different profiles of consumption of xylose and ethanol production. The highest ethanol production was observed with *C. tropicalis* S4 with 6 g L⁻¹ of ethanol and whose xylose assimilation was 56 g L⁻¹ resulting in a yield of 0.1 g g⁻¹, following by *C. tropicalis* FP that produced 4.6 g L⁻¹ of ethanol from 84 g L⁻¹ of xylose (yield of 0.05 g g⁻¹).

The strains *Rhodotorula* sp. G10.2 and *C. tropicalis* E2 showed similar production of ethanol (around 3 g L⁻¹) to each other but *Rhodotorula* sp. G10.2 consumed 73 g L⁻¹ of xylose with yield of 0.04 g g⁻¹ and *C. tropicalis* E2 consumed 53 g L⁻¹ with yield of 0.05 g g⁻¹ (Fig. 1A).

Fig. 1B shows the xylose assimilation and biomass production by strains that not were able to produce of ethanol. *P. guilliermondii* G4.2, *C. oleophila* G10.1 and *M. korensis* G18 showed yields of biomass of 0.7 g g⁻¹ meanwhile *P. guilliermondii* G1.2 and *R. mucilaginosa* G7.2 had yields of 1.1 up to 1.3 g g⁻¹, suggesting that *P. guilliermondii* G4.2, *C. oleophila* G10.1 and *M. korensis* G18 produced not identified metabolite.

Since *C. tropicalis* has been reported as a xylitol-producing species³⁰ and taking into account its consumption of xylose, the xylitol quantification in the fermentation media was carried out (Table 2).

The strain *C. tropicalis* E2 reached a production of 22 g L⁻¹ of xylitol from 100 g L⁻¹ of xylose (yield of 0.2 g g⁻¹) while S4 produced 15.5 g L⁻¹ of xylitol (0.15 g g⁻¹) and *C. tropicalis* FP produced 4.5 g L⁻¹ of xylitol (0.04 g g⁻¹). The comparison between xylitol and ethanol fermentative parameters shows that the strain *C. tropicalis* E2 exhibits more potential for xylitol production while *C. tropicalis* FP produced similar concentrations of both alcohols but with low yield. In the culture medium of strain *C. tropicalis* S4 a higher concentration of xylitol than ethanol was measured.

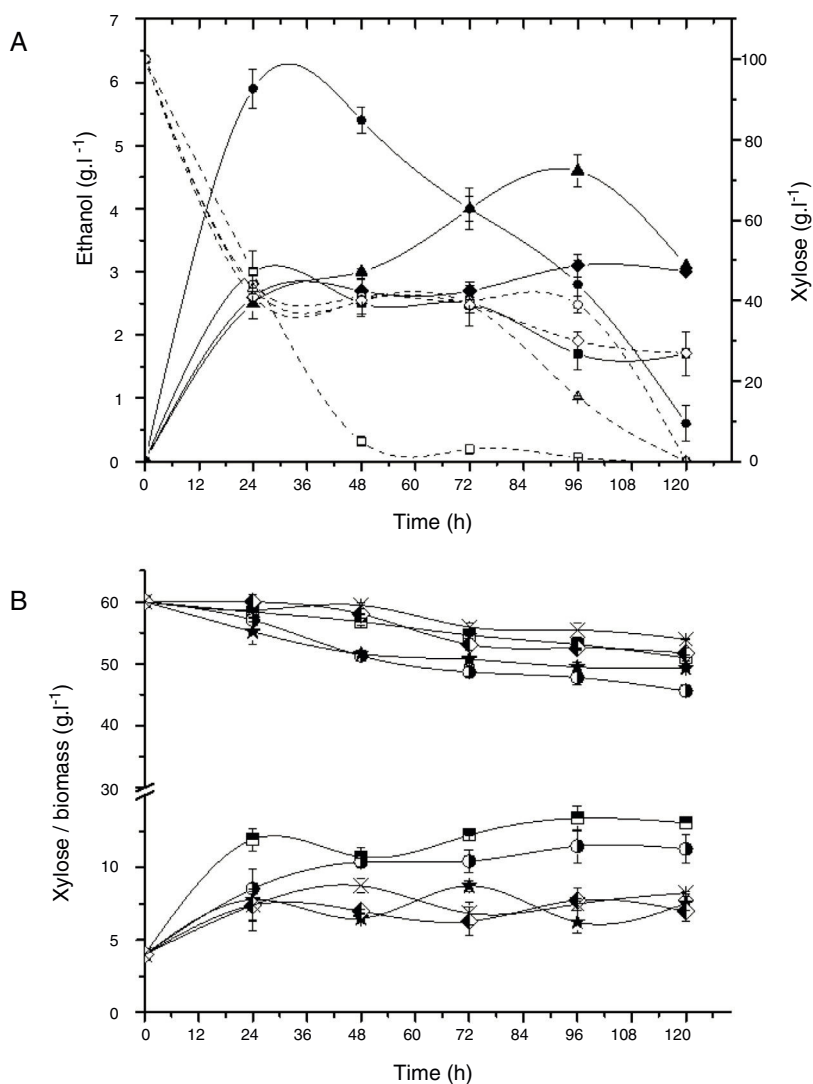


Fig. 1 – Alcoholic fermentation assay in a basal medium with xylose (100 g L⁻¹) and yeast extract (10 g L⁻¹). (A) Ethanol production and xylose consumption by *C. tropicalis* E2 (squares), *C. tropicalis* S4 (circles), *C. tropicalis* FP (triangle), and *Rhodotorula* sp. G10.2 (diamond); Open symbols: xylose; Full symbols: ethanol. (B) Consumption of xylose and biomass by *P. guilliermondii* G1.2 (squares half up) and G4.2 (circle half right), *C. oleophila* G10.1 (star), *M. koreensis* G18 (diamond half left), *R. mucilaginosa* G7.2 (cross).

Table 2 – Xylitol productivity by *C. tropicalis* FP strain. Fermentation realized in a basal medium with xylose (100 g L⁻¹) and yeast extract (10 g L⁻¹) at 30 °C in 120 h.

Parameters evaluated	Xylitol			Ethanol		
	E2	S4	FP	E2	S4	FP
Sugar consumed (g L ⁻¹)	100	100	100	100	100	100
Production (g L ⁻¹)	22	15.5	4.5	3.0	5.9	4.6
Yield – Y _{p/s} (g g ⁻¹)	0.22	0.15	0.04	0.03	0.06	0.04
Volumetric productivity – Q _p (g L ⁻¹ h ⁻¹)	0.18	0.12	0.03	0.12	0.24	0.05
Conversion efficiency – η (%) ^a	24	16	4	6	12	9

^a Theoretical yield for xylitol production defined at 0.9 g g⁻¹³⁰ and theoretical yield for ethanol production defined at 0.51 g g⁻¹³¹

A decreasing in the ethanol concentration during the fermentative route was observed (Fig. 1A), probably because the yeast can assimilate ethanol and, in this case, the ethanol determined in the medium is, perhaps, not real.

Discussion

In general, the yeasts strains presented xylose assimilation but low ethanol production. *C. tropicalis* S4 produced the highest amount of ethanol (6 g L^{-1}), with a yield of 0.1 g g^{-1} . This strain showed good sugar consumption and the species is not among the most promising yeasts for ethanol production. In fact, the literature shows that the most promising species for ethanol production are *Scheffersomyces stipitis*, *S. shehatae* and *Pachysolen tannophilus*, while *C. tropicalis* is related to xylitol production.

The strains *C. oleophila* G10.1, *M. koreensis* G18 and *P. guilliermondii* G4.2 showed smaller yields of biomass production than *P. guilliermondii* G1.2 and *R. mucilaginosa* G7.2. This sugar consumption with minor growth suggests the production of metabolites which were not ethanol or xylitol. Thus, these strains need further investigation about alternative compounds like organic acids.

P. guilliermondii is frequently cited for production of xylitol and/or ethanol, but *C. oleophila* and *M. koreensis* have not been reported in the literature for the fermentation of hydrolysate lignocellulosic material. *C. oleophila* has been reported as an agent of biologic control due to its ability to suppress the growth of some fungi that cause fruit spoilage like *Botrytis cinerea* and *Penicillium digitatum*.^{32,33} *M. koreensis* was described more recently²⁷ and was reported to be a carbonyl reductase³⁴ and an L-talitol producer.³⁵

The specie *A. pullulans*, a yeast-like, is able to assimilate glucose and xylose but is reported as unable to ferment glucose. This feature makes this yeast interesting in a possible co-cultivation with *S. cerevisiae*. Furthermore, it is reported as being producer of high xylanase activity^{22,28} allowing the hydrolysis of derived from hemicellulose in the production of ethanol from lignocellulosic material.

The strain *C. tropicalis* S4 showed slower xylose assimilation but consumed all the sugar of the medium in 120 h and produced the most ethanol. Furthermore, the decrease in the ethanol in the fermentation media over the course of time indicates that, perhaps, it is able to assimilate the ethanol, which would justify the slow xylose utilization.³⁶

Under the conditions tested, *C. tropicalis* FP consumed all the xylose. Nevertheless, it presented a low yield for both ethanol and xylitol. Also, during the fermentation process, the strain did not present a large increase in the biomass (maximum biomass was 7 g L^{-1}), that suggests the presence of other coproducts. Meanwhile, *C. tropicalis* E2 presented high xylose assimilation with a higher yield for xylitol than for ethanol, showing its promise for research into xylitol production.

The amount of xylitol produced by *C. tropicalis* strains shows the importance of the individual study of the characteristics of each strain, since differences may occur in the production of compounds, although belonging to same species. While the strain E2 produced a small quantity of

ethanol and a larger quantity of xylitol, the strain FP produced the same quantity of both compounds.

The ethanol and xylitol production is strongly influenced by the fermentation conditions such as pH, temperature, oxygen and nutrients which were not studied in this work. Maybe productivity could be increased in different fermentation conditions, mainly controlling the oxygen supply.

The missing of ethanol production from xylose by the most of yeasts is marked by a redox imbalance that occurs between cofactors NAD and NADP during the formation of xylitol and xylulose. The xylose reductase enzyme reduces D-xylose to xylitol using NADPH, whereas xylitol dehydrogenase requires NAD⁺ mainly, which hampers the recycling of cofactors. This imbalance causes xylitol accumulation in the medium and the pathway does not proceed to produce ethanol. The recycling of NADP⁺ can be relieved through respiratory chain. However, depending on the oxygen level, cell growth may occur instead of ethanol production.^{37,38}

Xylitol is industrially interesting for its applicability as a sweetener for diabetics and is utilized in the food, pharmaceutical, oral health and cosmetics industries. In addition, its production from several lignocellulosic materials, such as corn cob and cotton stalk, has been tested.^{30,39,40} Furthermore, to increase the xylitol yield from lignocellulosic materials, some studies looked at strategies such as hydrolysate detoxification or yeast adaptation, cell immobilization and varying the process conditions.^{41–46}

In conclusion, the assimilating yeasts are common in the environment but the understanding of the absorption and assimilation of the sugar is needed so that biotechnological utilization of this pathway is possible. Our results showed the importance of the individual study of each strain, since they have different characteristics concerning response to external factors, even if the strains belong to the same species. The study of the production of alternative compounds to ethanol is important because the application of these strains in industrial processes has not been well studied.

Conflicts of interest

The authors declare no conflicts of interest.

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