Ethanol production from sugarcane bagasse hydrolysate using newly adapted strains of *Meyerozyma guilliermondi* CBA-524 and *Pichia kudriavzevii* CBA-519

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Abstract

New strains of the yeasts *Meyerozyma guilliermondi* CBA-524 and *Pichia kudriavzevii* CBA-519, capable of growing on sugarcane bagasse hydrolysate, have a great potential for second-generation bioethanol processes. *Meyerozyma guilliermondi* CBA-524, achieved a maximum ethanol concentration of 3.01g/L corresponding to a yield of 0.26 g ethanol per g of sugar and a productivity of 0.125 g/l/h, after 24 h of cultivation. *Pichia kudriavzevii* CBA-519 exhibited a prolonged fermentation time on bagasse hydrolysate resulting in an ethanol yield of 0.26 g/g and a productivity of 0.015 g/l/h after 192 h of fermentation. The ethanol yield and the volumetric productivity using the adapted *P. stipitis* CBS6054 standard were 0.34 g/g and 0.083 g/l/h, respectively, after 48 h of cultivation. Furthermore, the newly adapted *M. guilliermondi* CBA-524 demonstrated significantly enhanced tolerance to sugarcane bagasse hydrolysate, exhibiting a maximum in ethanol concentration after 24 h of fermentation. More adapted yeasts and improved fermentation conditions for this yeast can be of great importance for the increased production of ethanol using sugarcane bagasse hydrolysate.

Keywords: Hydrolysate, Bioethanol, *Meyerozyma guilliermondi, Pichia kudriavzevii.*

Introduction

The technology developed in Brazil for the production of ethanol from sugarcane stands among the most efficient and well-established worldwide. Lignocellulosic byproducts or other cheaper substrates could be used as a complement, or as an alternative, to ethanol, increasing its competitiveness. Second generation ethanol, i.e., produced from cellulosic and hemicellulosic biomass, represents an attractive sustainable energy source that can greatly contribute to the implementation of a clean and environmentally beneficial energy matrix.[1] Lignocellulosic byproducts represent an abundant biomass and a potential renewable source of energy for the production of ethanol via microbial fermentation, commonly including processes of pretreatment, enzymatic hydrolysis, and pentose and hexose fermentation. Hydrolysis with dilute sulfuric acid used in hydrolysis breaks down hemicelluloses, releasing products that can then be used by the yeasts. The hemicellulosic hydrolysate, after concentration by vacuum evaporation and detoxification, showed 30.89 g/l xylose along with other by products (0.32 g/l glucose, 2.31 g/l arabinose, and 1.26 g/l acetic acid),[2] that can be fermented to ethanol.[3] The most studied xylose-fermenting yeasts, that are capable of converting xylose to ethanol, are *Kluyveromyces marxianus*,[4] *Candida boidinii*,[1] *C. guilliermondii*,[5] *C. tropicalis*,[6] *Pichia anomala*,[7] *P. caribbica*,[3] *P. kudriavzevii*[8] and *P. stipites*.[9,10,11] Saccharification techniques need to be applied to lignocellulosic material, so that fermentable sugars can be released in the solution and then fermented by microorganisms. Weak acids, furan derivatives, and phenols, all resulting from the pretreatment degradation process, inhibit the growth of microorganisms, thereby affecting the fermentation efficiency.[12]

Pichia stipitis is considered the most promising and naturally occurring microorganism for the fermentation of hemicellulosic hydrolysates since it generally produces the highest ethanol yield from xylose,[13] and also producing ethanol from hemicellulosic detoxified and non-detoxified hydrolysates.[14] Among the yeasts, *P. stipitis* exhibits the greatest potential for industrial application because this species produces a high ethanol yield,[14,15] does not require additional vitamins during fermentation,[16] and is able to ferment a wider range of sugars, including cellobiose.[9] The efficiency of ethanol fermentation depends on many factors, but especially the strains of yeasts and the available feedstock. Thus, the present study aimed at obtaining a fermenting strain efficient in the production of ethanol from the hemicellulosic hydrolysate of sugarcane bagasse.

Materials and Method

Microorganisms and maintenance: *Meyerozyma guilliermondi* CBA-524 and *Pichia kudriavzevii* CBA-519 used in this study were obtained from the culture collection of Centro de Biotecnologia da Amazônia – (CBA), Brazil. These yeasts were evaluated regarding their ability of using hemicellulosic hydrolysate as a carbon source by Cassa-Barbosa et al.[17] The cells were maintained on YM agar plates at 4ºC and transferred monthly. YM agar consisted of 10.0 g/l of glucose, 3.0 g/l of yeast extract, 3.0 g/l of malt extract, 5.0 g/l of

peptone, and 20.0 g/l of agar. The pH was maintained at 5.0 and the media were sterilized by autoclaving at 121ºC for 15 min. Yeast cultures were transferred into Petri plates containing YM synthetic medium and incubated for 24 h at 30ºC.

Preparation of lignocellulosic hydrolysate: Considerable literature is available on the optimization of different factors affecting dilute acid pretreatment of hydrolysates such as acid concentration, acid-to-substrate ratio, and pretreatment conditions (i.e., temperature and time).[6] Thus, pretreatment conditions were not optimized in the present study, and the pretreatment process was based on results of previous studies. The sugarcane bagasse was dried in a stove and cut into small pieces. The acid solution was prepared with H_2SO_4 1%, 250 mL for each 50 g sugarcane bagasse, and left to rest for 24 hours. The material was autoclaved at 121ºC for 40 minutes, and then pressed to separate the liquid fraction. The resulting broth was neutralized with $Ca(OH)_2$ until adjusting the pH to 5.0. The medium was filtered using filter paper under vacuum.

For hydrolysate medium, were added: citric acid and salt solution 40ml/l, yeast extract 1.25 g/l, KH_2PO_4 1.1 g/l and 500 ml/l of lignocellulosic hydrolysate. The dinitrosalicylic acid method (DNS) described by Miller [18] was used for determining reducing sugars.

Adaptation and yeast growth: The yeasts were previously inoculated in tubes and kept for 12 hours in 10 mL of 50% hydrolysate medium. The yeasts were inoculated in Petri dishes with Sabouraud agar medium, each one in triplicates, for colony counting. Yeast counting was done at each 12 h, for 96 h. The culture of *Meyerozyma guilliermondi* CBA-524, *Pichia kudriavzevii* CBA-519 and *Pichia stipitis CBS6054* was aseptically transferred to sterilized 50 mL tubes containing 10 mL hydrolysate medium broth, and were incubated at 30ºC for 48 h and 150 rpm in an incubator shaker (Innova 4000, New Brunswick Scientific, USA), for the cells to acclimate to the physiological conditions of the culture medium. After incubation of the inoculum on a rotary shaker the cultures were transferred to the fermenter.

Ethanol production: Fermentation was carried out in a 5 L jar of New Brunswick fermentors (Bioflo 110), containing 3L of the hydrolysate medium at 30ºC, agitation of 100 rpm and pH of 5.0. The fractions were collected every 24 hours. *Pichia stipitis* CBS 6054 was used as a control in all the tests.

Analytical methods: Ethanol and monosaccharides (glucose and xylose) concentrations were determined by HPLC (RID -10A Shimadzu and Rezex RPM-Monosaccharide Pb + New column 300x7.8 mm) equipped with a refractive index detector. The samples were filtered through a membrane of 0.22 microns and injected in a volume of 0.6 ml / min with an isocratic water system. The duration of the run was 30 min. Sugar standards (D-glucose and D-xylose) were injected and their retention times were compared to the peaks of the samples. The sugar content of the samples was quantified from calibration curves after different dilutions were injected in the same isocratic water system.

Results and Discussion

Sugar yield after hydrolysis: Temperature, pH, aeration, agitation, initial concentrations of cells and xylose, and nutrient supplementation of the fermentation medium, among other factors, affect xylose fermentation.[19] Since the pretreatment step significantly influences the efficiency of biomass conversion into ethanol, a substantial research effort has been dedicated to improve lignocellulosic material digestibility[20] that precedes enzymatic saccharification.

Depending on the method used in the hydrolysis of lignocellulosic materials, will be the concentration of sugar in the culture medium. We used 50% (v/v) of hydrolysate obtained from the fermentation medium, with a final concentration of 11.53g/l. Chandel et al.[21] obtained 30.29g/l of sugar through acid hydrolysis of sugarcane bagasse, however, we have obtained 23.06g/l. The release of fermentation inhibitors is an important obstacle to the process of lignocellulosic material hydrolysis, given that they negatively affect sugar bioconversion into desired products.[12] Additionally, inhibitory compounds may act synergistically, further compromising microbial hydrolysate fermentation efficiency. To reduce the acid inhibitory action during this process, the option usually lies in adjusting the hydrolysate pH, thereby significantly reducing toxicity.[14,3,4] The broth was neutralized with $Ca(OH)_2$ until the pH was at 5.0. The fermentation of hydrolysate hemicellulosic medium was at a pH of 5.0 and 30ºC temperature, as recommended to *P. stipites*[22,23,24,11,7,25] (22Kumar et al., 2009, 23Wan et al., 2012, 24Scordia et al., 2012, 11Biswas et al., 2013, 7Zha et al., 2013, 25Dasgupta et al., 2013). The detoxification of hemicellulosic acid hydrolysate by boiling and overliming generally results in better fermentability of the hydrolysate. Volatile compounds, such as furfural and phenols are stripped by boiling, while over liming with $Ca(OH)_2$ removes and/or reduces the concentration of other acid components, e.g. acetic and tannic acid.[22] Although CBA-519 had an ethanol concentration of up to 3.00 g/l, with an ethanol yield of 0.26 g/g after 192 h, and exhibited prolonged fermentation time for the hydrolysate, it also had an extended lag phase, suggesting a presence of inhibitory compounds in the hydrolysate that could have caused stress to the non-adapted cells, retarding cell growth and fermentation capacity (Table 1). The adapted strain seems to have acclimatized to the specific stresses presented by the hydrolysate, perhaps including enhanced tolerance to inhibitory compounds during the extended cultivation

period. Microbial adaptation under similar circumstances may be ascribed, according to some workers, to synthesis of novel enzymes or cofactors that increase sugar metabolism efficiency and reduce inhibitory effects.[16]

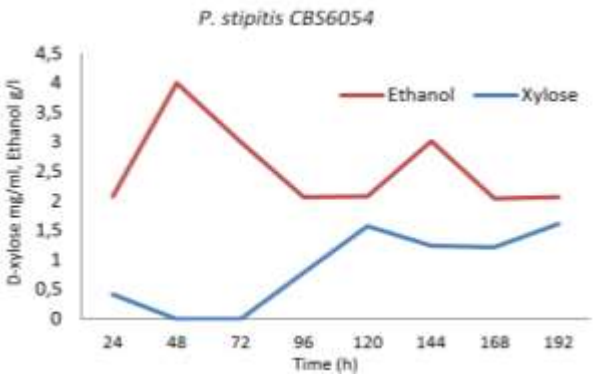
Table 1: Effect of simultaneous saccharifification and fermentation in the concentration of diferent sugars and the yield of ethanol using diferent yeasts

	P stipitis CBS6054			*P kudriavzevii CBA-519*			*M guilliermondii CBA-524*		
Time h	**D-xylose mg/ml**	**D-glucose mg/ml**	**Ethanol g/l**	**D-xylose mg/ml**	**D-glucose mg/ml**	**Ethanol g/l**	**D-xylose mg/ml**	**D-glucose mg/ml**	**Ethanol g/l**
24	0.41	ND	2.08	0.75	5.37	ND	1.48	ND	**3.01**
48	ND	ND	**4.00**	0.71	5.25	ND	1.46	ND	2.07
72	ND	ND	3.00	1.39	3.55	ND	1.44	ND	2.07
96	0.78	ND	2.06	2.45	9.08	ND	3.27	ND	3.05
120	1.57	ND	2.08	2.29	7.74	ND	0.63	ND	1.09
144	1.24	ND	3.01	1.23	5.07	ND	5.09	ND	2.02
168	1.22	ND	2.04	1.14	3.39	ND	2.25	ND	2.06
192	1.61	ND	2.06	0	0.52	**3.05**	-	-	-
216	-	-	-	0	0	2.05	-	-	-
240	-	-	-	0	0	2.09	-	-	-

ND not detected.
- not available

Ethanol production from hydrolysate: The first readings of ethanol were made using the alcoholmeter method, that verifies alcohol production through $Ca(OH)_2$, demonstrating the yeasts' potential for ethanol production from sugarcane bagasse. *Pichia stipitis* had three peaks of 5% at 120, 168 and 192 hours of fermentation, decreasing until zero in the next count. *Pichia kudriavzevii* maintained the alcohol for all the collect made, the peak was 5% in 6 days consecutive, what shows that more days would be better for ethanol produced from lignocellulosic biomass. *Meyerozyma guilliermondii* begun with 4% production, in the next 48 hours it showed the same other yeasts peak 5%, but at the 192 hours the production had zero alcohol.

The fermentations were monitored through measuring D-glucose, D-xylose, and ethanol concentrations of samples taken over fixed intervals, as shown in Table 1 and Fig. 1. Ethanol production was observed in both fermentations as shown in Fig. 1, and performance and yield obtained are shown in Table 2. A typical fermentation profile of giant reed biomass using CBS6054, after 48h, gives a maximum ethanol production of 8.20g/l, a yield of 0.33g/g and a productivity of 0.17g/l/h.[24] The main parameters for the fermentation comparison of *P. stipitis* CBS6054 on the different hydrolysates are ethanol yield and productivity. The ethanol yield obtained in the present work was 0.34 g/g for CBS6054, which is comparable to that reported by Biswas et al.;[11] 0.38g/g, and, Wan et al.[23] 0.42 g/g for CBS6054. In this study for *P. stipitis* CBS6054, ethanol accumulated to a final volumetric concentration of 4.0 g/L and a product yield of 0.34 g of ethanol per g of sugar after 48 h. For *M. guilliermondii* CBA-524 the maximum ethanol concentration was of 3.01g/L with yield of 0.26 g of ethanol per g of sugar after 24 h of cultivation. Results show that in the first 24 h of cultivation with *M. guilliermondii* CBA-524 the maximum accumulation of ethanol was comparable to that obtained by *P. stipitis* CBS6054 (Table 2). The maximum ethanol production was observed after 192 h of fermentation for *P. kudriavzevii* CBA-519, indicating that the mechanism for ethanol conversion took more time to adapt. A major problem with the hydrolysis of lignocellulosic materials is the release of fermentative inhibitors that negatively influence the bioconversion of sugars into the desired products.[12] The usual strategy to minimize the inhibitory effect caused by acid during hydrolysate fermentation is to adjust the, decrease instantaneous sugar concentrations and improve fermentability of hemicellulosic hydrolysates.[24]



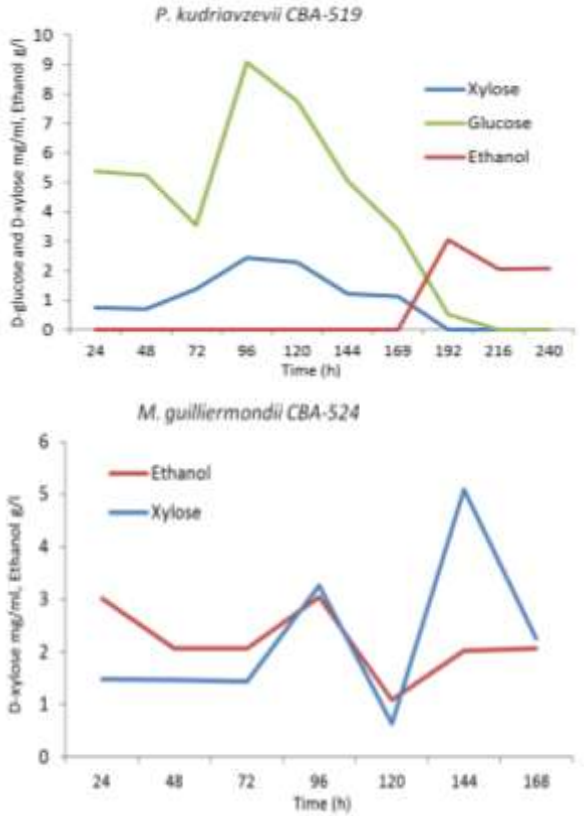


Fig. 1: Time course for the hydrolysis of sugarcane bagasse and ethanol production of *Pichia stipitis, P. kudriavzevii* and *Meyerozyma guilliermondii*

Table 2: Comparison of ethanol production between newly isolated strains

Strain	Time (h)	Ethanol (g/l)	$Y_{P/S}$ (g/g)	Q_P (g/l/h)
P stipitis CBS6054	48	4.00	0.34	0.083
P kudriavzevii CBA-519	192	3.05	0.26	0.015
M guilliermondii CBA-524	24	3.01	0.26	0.125

The sugars-to-ethanol conversion yields ($Y_{P/S}$) and the volumetric productivities (Q_P) presented in Table 2, were 0.34 g/g and 0.083 g/l/h for *P. stipitis CBS6054*, 0.26 g/g and 0.015 g/l/h for *P. kudriavzevii CBA-519*, and 0.26 g/g and 0.125 g/l/h for *M. guilliermondii* CBA-524. These results are similar to the ones reported for *P. stipitis* DSM 3651, with a yield of 0.30 g/g and a productivity of 0.16 g/l/h from sugarcane bagasse hydrolysate,[14] for *P. stipitis* BY2, with a yield of 0.45 g/g and productivity of 0.33 g/l/h from sugarcane bagasse hemicellulosic hydrolysate,[3] and for *P. stipitis* NRRL Y-7124 with a yield of 0.32 g/g and productivity of 0.065 g/l/h from sunflower seed hull hydrolysate.[10] *Pichia stipitis* NCIM-3497, with an ethanol yield of 0.425 g/g and productivity of 0.176 g/l/h from an acid hydrolysate of water-hyacinth (*Eichhornia crassipes*),[22] to *Scheffersomyces stipitis* UFMG-IMH 43.2, the best results to ethanol yield and productivity was 0.19 g/g and 0.13 g/l/h, respectively from sugarcane bagasse hemicellulosic hydrolysate,[19] and ethanol yield of 0.33 g/g and a productivity of 0.17 g/l/h to *Scheffersomyces (Pichia) stipitis* CBS6054 from giant reed (*Arundo donax* L.).[24] Thus, the isolated CBA-524 seems to be a promising yeast when compared to published data.

After reaching a maximum, the ethanol concentration was notably reduced, probably due to its consumption by the yeast as a consequence of the fast exhaustion of sugars from the growth medium. Similar behavior has already been observed for other yeast strains,[21] and this might result from the use of ethanol for maintaining cell viability after glucose depletion,[8] and might indicate that this strain also utilizes ethanol as carbon source.[19]

The results from the current study are comparable to those obtained with adapted *S. stipitis* CBS strains, suggesting that, under these conditions, bagasse hydrolysate processing does not inhibit growth of *M. guilliermondii CBA-524*. Although the ethanol yield and productivity that we obtained (Table 2) were not very high, they are still relevant, since this is the first study on ethanol production by this yeast strain, and the fermentation conditions were those standard for *P. stipitis CBS6054*. Fermentation parameters can be altered in order to improve the performance comparison among microorganisms. This procedure can allow the identification of variations, the prediction of optimal conditions for strain cultivation aimed at maximum yields, and the prediction of the most adequate time to stop the process, to avoid unnecessary waste of time and energy at the final fermentation phase.[22] This demonstrates the importance of establishing the best fermentation medium and conditions to achieve high ethanol yield and productivity by *Meyerozyma guilliermondi* CBA-524 and *Pichia kudriavzevii* CBA-519.

Conclusion

Meyerozyma guilliermondi CBA-524 and *Pichia kudriavzevii* CBA-519 native microorganisms showed potential for ethanol production from sugarcane bagasse hemicellulosic hydrolysate, a promising feedstock for second generation bioethanol production, especially *M. guilliermondi,* which was able to adapt quickly to the inhibitors that are commonly formed during acid catalyzed pretreatment.

Improvements in culture media formulation and modified fermentation methods could lead to higher ethanol yields from these microorganisms growing on lignocellulose hydrolysates, and also the development of robust strains able to produce ethanol at an industrial scale.

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