J Ind Microbiol Biotechnol (2011) 38:1099–1107
DOI 10.1007/s10295-010-0889-0

ORIGINAL PAPER

Cashew apple bagasse as a source of sugars for ethanol production by *Kluyveromyces marxianus* CE025

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Received: 1 June 2010 / Accepted: 3 October 2010 / Published online: 30 November 2010

Abstract The potential of cashew apple bagasse as a source of sugars for ethanol production by *Kluyveromyces marxianus* CE025 was evaluated in this work. This strain was preliminarily cultivated in a synthetic medium containing glucose and xylose and was able to produce ethanol and xylitol at pH 4.5. Next, cashew apple bagasse hydrolysate (CABH) was prepared by a diluted sulfuric acid pretreatment and used as fermentation media. This hydrolysate is rich in glucose, xylose, and arabinose and contains traces of formic acid and acetic acid. In batch fermentations of CABH at pH 4.5, the strain produced only ethanol. The effects of temperature on the kinetic parameters of ethanol fermentation by *K. marxianus* CE025 using CABH were also evaluated. Maximum specific growth rate (μ_{max}), overall yields of ethanol based on glucose consumption Y^{G}_{P/S_1} and based on glucose + xylose consumption ($Y_{P/S}$), overall yield of ethanol based on biomass ($Y_{P/X}$), and ethanol productivity (P^{E}) were determined as a function of temperature. Best results of ethanol production were achieved at 30°C, which is also quite close to the optimum temperature for the formation of biomass. The process yielded 12.36 ± 0.06 g l^{-1} of ethanol with a volumetric production rate of 0.257 ± 0.002 g l^{-1} h^{-1} and an ethanol yield of 0.417 ± 0.003 g g^{-1} glucose.

Keywords Cashew apple bagasse · Cashew apple bagasse hydrolysate · Ethanol · Temperature · *Kluyveromyces marxianus* CE025

List of symbols

CAB	Cashew apple bagasse
CABH	Cashew apple bagasse hydrolysate
MXG	Synthetic medium containing D-glucose and D-xylose as carbon source
X	Biomass concentration (g l^{-1})
X_i	Initial biomass concentration (g l^{-1})
X_{max}	Maximum biomass concentration (g l^{-1})
S	Xylose + glucose concentration (g l^{-1})
S_1	Glucose concentration (g l^{-1})
S_2	Xylose concentration (g l^{-1})
P	Ethanol concentration (g l^{-1})
t	Time of fermentation (h)
Y^{G}_{P/S_1}	Yield of ethanol based on glucose consumption (g g^{-1})
$Y_{P/S}$	Yield of ethanol based on glucose and xylose consumption (g g^{-1})
$Y_{P/X}$	Yield of ethanol based on biomass production (g g^{-1})
P^{E}	Volumetric ethanol production rate (g l^{-1} h^{-1})

This article is based on a presentation at the 32nd Symposium on Biotechnology for Fuels and Chemicals.

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P_{max} Maximum ethanol concentration (g l^{-1})
P^X Biomass productivity (g l^{-1} h^{-1})

Greek letters

μ_{max} Maximum specific growth rate (h^{-1})
μ_X Specific rates of biomass production (h^{-1})
μ_{S_1} Specific rates of glucose uptake (g g^{-1} h^{-1})
μ_{S_2} Specific rates of xylose uptake (g g^{-1} h^{-1})
μ_P Specific rates of ethanol production (g g^{-1} h^{-1})

Introduction

Biofuels such as ethanol are gaining worldwide acceptance essentially to overcome problems associated with exploitation and depletion of fossil fuels and environmental pollution. Therefore, the development of bioprocesses based on easily available substrates, such as lignocelluloses and/or hemicelluloses, and suitable microorganisms, which could convert those substrates to ethanol, could be very useful. In the state of Ceará and Rio Grande do Norte (northeast Brazil), the cashew agroindustry has an outstanding role in the local economy and the cashew apple bagasse (CAB), a lignocellulosic raw material, appears as an alternative for ethanol production [22, 23]. CAB, a by-product of the cashew apple juice industry, represents approximately 20% of the total peduncle weight [22, 23, 29]. The official estimate for the Brazilian cashew nut crop for 2008/2009 was around 300,000 tons which accounts for 11% of the world production and corresponds to more than 6 million tons of cashew apple. The industrial peduncle processes for juice production result in 40% (w/w) of bagasse, which essentially has no commercial value and is usually discarded by the local industry. In a previous work [23], CAB contents in terms of cellulose, hemicellulose, and lignin were determined to be 19.21 ± 0.35%, 12.05 ± 0.37%, and 38.11 ± 0.08%, respectively.

The primary sugars found in cellulosic biomass are D-glucose and D-xylose, although other sugars such as L-arabinose, mannose, galactose, and rhamnose are also present [31, 32]. Those sugars represent potential sources of carbon and energy for several microorganisms which could convert them into biofuels.

In cellulosic ethanol processes, pretreatment of lignocelluloses to disrupt their recalcitrant structures is needed in order to increase the digestibility of materials. Although many pretreatment methods (uncatalyzed steam explosion, liquid hot water, diluted acid, and ammonium fiber/freeze explosion—AFEX) have been investigated, few can be used on an industrial scale based on economical and environmental considerations. In addition, most of these methods require high temperatures, which are usually achieved through convection or conduction based on heating [22, 23].

There are many paper [5, 9, 21, 24, 25] and patents [16] discussing the optimization of the fermentation aimed at the industrial scale for ethanol production from lignocellulosic biomass. These efforts comprise searching for new native or genetically engineered microorganisms and new or improved processes. Wild-type strains of *Saccharomyces cerevisiae*, the main microorganism used for commercial ethanol production, are unable to utilize xylose, an abundant sugar in nature, limiting its use in biofuel production [25]. To overcome this problem several researchers have genetically modified *S. cerevisiae* strains to produce ethanol from xylose [5, 9]. However, there are native species of yeasts that ferment xylose to ethanol, including several *Pichia* and *Candida* species as well as some strains of *Kluyveromyces marxianus* [33, 34].

Strains belonging to the yeast species *Kluyveromyces marxianus* have been isolated from a great variety of habitats and this suggests a high metabolic diversity and a substantial degree of intraspecific polymorphism. As a consequence, several different biotechnological applications have been investigated by using this yeast including production of enzymes (β-galactosidase, β-glucosidase, inulinase, and polygalacturonases, among others), single-cell protein, aroma compounds, and ethanol [3, 32]; reduction of lactose content in food products [4, 15]; besides applications in bioremediation and medicine [7]; and as a host for heterologous protein production [7]. *K. marxianus* is one of the most promising yeasts for biotechnological applications, since it supports high temperature and shows moderate tolerance to ethanol, thus being suitable for simultaneous saccharification and fermentation (SSF) of lignocellulosic materials [2, 17].

Operation of the alcoholic fermentation in a continuous mode is desirable since higher productivity, improved yields, and better control are attained [1]. However, industrial implementation of a continuous process requires a previous study of the process behavior to develop an efficient control strategy. The influence of temperature on the kinetic parameters must be considered since it is very difficult to support a constant temperature during large-scale alcoholic fermentation. The process is exothermic and small deviations in the temperature (2–4°C) can dislocate it from optimal operational conditions. Also, the effect of temperature on ethanol fermentation kinetics is useful information when process optimization is desired [1].

Therefore, the aim of this work was to evaluate the potential of *Kluyveromyces marxianus* CE025 to produce ethanol from cashew apple bagasse hydrolysate (CABH). Furthermore, the influence of temperature (30, 34, 37, and 40°C) was investigated and the kinetic parameters (specific rates and yields) were determined.

Materials and methods

Microorganism and inoculum preparation

Kluyveromyces marxianus CE025 was previously isolated from the effluent of a LubNor—Petrobrás petroleum refinery, Ceará, Brazil, and deposited in the culture collection of the Microbial Ecology and Biotechnology Laboratory (LEMBiotech), Biology Department, Federal University of Ceará, Brazil. For the experiments, three colonies were transferred from the stock culture, which was grown in Sabouraud agar (D-glucose 20 g l^{-1}, peptone 10 g l^{-1}, and agar 17 g l^{-1}), to a 250-ml Erlenmeyer flask containing 50 ml of inoculum medium prepared according the following composition (in g l^{-1}): glucose, 20.0; urea 0.4; KH_2PO_4, 1.2; Na_2HPO_4, 0.18; yeast extract, 10; and pH 5.0. The flasks were incubated at 37°C and 150 rpm for 24 h. Afterwards, the optical density (600 nm) of the culture was adjusted to 1.0 and an aliquot of 1 ml of inoculum (2%) was transferred to a 250-ml Erlenmeyer flask, containing 49 ml of the desired culture medium.

Raw material

Cashew apple (*Anacardium occidentale* L.) bagasse (CAB), without any pretreatment, was kindly provided by Jandaia Industry of Juice (Ceará, Brazil). The CAB was washed five times with water, and then it was dried at 60°C for 24 h and milled to pass through 20–80 meshes. The milled CAB was stored at room temperature.

Preparation of cashew apple bagasse hydrolysate

Cashew apple bagasse hydrolysate (CABH) was obtained from the treatment of dried cashew apple bagasse (CAB), 7.40 ± 0.19% of humidity, with diluted acid sulfuric. The treatment was conducted in autoclave at 121°C for 15 min, using 0.2% m H_2SO_4/m CAB, in 250-ml Erlenmeyer flasks with 100 ml of reaction volume and a solid percentage of 30% w/v [22]. Afterwards, the liquid fraction was collected by vacuum filtration (GAST Manufacturing, Inc., Model DOA-P704, Michigan, USA), the pH was adjusted to 4.5 ± 0.2 with $Ca(OH)_2$, and it was filtrated to separate the precipitate. The filtrate, herein named CABH, was used as culture media for ethanol production.

Culture media

In this work, two culture media were used for *K. marxianus* CE025 growth and ethanol production. Since glucose and xylose are the main products of diluted acid hydrolysis of CAB, a synthetic medium named MXG, which contained D-glucose (28 g l^{-1}) and D-xylose (30 g l^{-1}), as carbon source, yeast extract (20 g l^{-1}), and $(NH_4)_2SO_4$ (5 g l^{-1}) at pH 4.5, was used to investigate the capability of *K. marxianus* CE025 in fermenting those sugars. After, the fermentation of the cashew apple bagasse hydrolysate (CABH), without any nutritional supplement, was studied.

Batch fermentation

All assays were conducted in 250-ml Erlenmeyer flasks with 50 ml of culture medium on a rotary shaker TE240 (Tecnal, São Paulo, Brazil) at 200 rpm. Experiments were initiated by transferring 2% (v/v) of inoculum to the prepared medium and they were carried out for 3 days in isothermal conditions. Samples (1 ml) were collected at predefined intervals of time.

First, an exploratory experiment, using MXG, was conducted at 40°C. Afterwards, batch experimental observations at four temperatures (30, 34, 37, and 40°C) were used to evaluate the influence of the temperature on the parameter kinetics of the alcoholic fermentation of the CABH by *K. marxianus* CE025.

Analytical methods

Cell growth (biomass) was determined by measuring the optical density of samples, using a UV–visible spectrophotometer (20 Genesis, BR) at 600 nm, and biomass concentration (in g l^{-1}) was determined by a calibration curve of dry weight (g l^{-1}) versus optical density (600 nm). Glucose, xylose, arabinose, ethanol, xylitol, and inhibitors [organic acids, furfural, and hydroxymethylfurfural (HMF)] were analyzed by high-performance liquid chromatography (HPLC) using a Waters HPLC system (Waters, Milford, MA, USA) equipped with a refractive index Waters 2414 detector using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). The eluent was 5 mmol l^{-1} H_2SO_4 in Water MiliQ (Simplicity 185, Millipore, Billerica, MA) at a flow rate of 0.5 ml min^{-1} at 65°C. Samples were identified by comparing the retention times with those of carbohydrates, xylitol, inhibitors, and ethanol standards.

Kinetics of substrate utilization, biomass production, and product formation: alcoholic fermentation of CABH

In this work, the kinetics of substrate utilization, biomass production, and product formation was investigated only during the alcoholic fermentation of cashew apple bagasse hydrolysate. The estimated kinetic parameters were: specific rates of cell growth (μ_x); glucose uptake $\left(\mu_{S_1}\right)$; xylose uptake $\left(\mu_{S_2}\right)$; and ethanol production (μ_P), defined on Eqs. 1–5, respectively:

$$\mu_X = \frac{1}{X}\frac{dX}{dt} \quad (1)$$

$$\mu_{S_1} = -\frac{1}{X}\frac{dS_1}{dt} \quad (2)$$

$$\mu_{S_2} = -\frac{1}{X}\frac{dS_2}{dt} \quad (3)$$

$$\mu_P = \frac{1}{X}\frac{dP}{dt} \quad (4)$$

$$\ln\frac{X}{X_i} = a + \mu_{max}t \quad (5)$$

where X is cell concentration (g l^{-1}), S_1 is glucose concentration (g l^{-1}), S_2 is xylose concentration (g l^{-1}), P is ethanol concentration (g l^{-1}), and μ_{max} is maximum specific growth rate.

The overall yields of ethanol based on glucose consumption (Y^G_{P/S_1}), based on glucose and xylose consumption ($Y_{P/S}$), and based on biomass production ($Y_{P/X}$) were estimated according to Eqs. 6, 7, and 8.

$$Y^G_{P/S_1} = \frac{dP}{-dS_1} \quad (6)$$

$$Y_{P/S} = \frac{dP}{-dS} \quad (7)$$

$$Y_{P/X} = \frac{dP}{dX} \quad (8)$$

Volumetric ethanol production rate (P^E) and biomass productivity (P^X) were determinate by using Eqs. 9 and 10:

$$P^E = \frac{P}{t} \quad (9)$$

$$P^X = \frac{X - X_i}{t - t_i}. \quad (10)$$

Maximum concentration of biomass (X_{max}) and ethanol (P_{max}) was defined as the highest concentration achieved during the course of fermentation.

Results and discussion

Cashew apple bagasse and cashew apple bagasse hydrolysate composition

Cashew apple bagasse used in this investigation contained 20.54 ± 0.70% cellulose, 16.33 ± 3.0% hemicellulose, 33.62 ± 5.28% lignin, 5.64 ± 0.07% extractives, and 0.20 ± 0.07% ashes. Ferreira et al. [6] quantified cellulose, hemicellulose, and lignin of cashew apple bagasse and obtained 24.3, 18.5, and 22.5%, respectively.

Cashew apple bagasse hydrolysate (CABH) was prepared, by a diluted sulfuric acid pretreatment, and characterized. CABH contained 5.24 ± 0.31 g l^{-1} of cellobiose, 29.08 ± 0.47 g l^{-1} of glucose, 24.48 ± 1.30 g l^{-1} of xylose, 11.33 ± 1.78 g l^{-1} of arabinose, 2.90 ± 0.63 g l^{-1} of formic acid, and 2.73 ± 0.26 g l^{-1} of acetic acid.

It is also worth mentioning the production of toxic compounds such as furfural, HMF, levulinic acid, and formic acid, together with phenolic compounds derived from degraded soluble lignin during pretreatment. These are examples of compounds that inhibit the yeast growth [24, 26, 30]. In this study the contents of HMF and furfural were taken as examples of toxic compounds in CABH. The analysis showed contents of 0.12 ± 0.06 g l^{-1} of furfural and 0.10 ± 0.05 g l^{-1} of HMF. However, when adjusting the pH to 4.5 with $Ca(OH)_2$, the formation of a precipitate was observed, probably due to the low solubility of calcium salts. According to the literature [18, 19, 24], these salts are able to form precipitating complex with some of the toxic compounds, such as furfural, acetic acid, and HMF, present in the biomass hydrolysates. Therefore, before CABH sterilization, the precipitate was removed by filtration and the resulting liquid fraction (the filtrate) contained no detectable HMF and furfural (concentration less than 0.001 g l^{-1}).

Other authors [12, 19, 30] observed that the fermentation of a softwood hydrolysate at low concentrations of acetic, formic, and levulinic acids favored ethanol production, whereas high concentrations of these compounds inhibited its production.

Nevertheless, not only inhibitors were removed by adjusting the pH to 4.5 with $Ca(OH)_2$; sugar and organic acid concentration was also affected. The filtrate contained 25.13 ± 1.87 g l^{-1} of glucose, 21.61 ± 2.00 g l^{-1} of xylose, 11.33 ± 1.78 g l^{-1} of arabinose, 0.4 ± 0.04 g l^{-1} of formic acid, and 1.94 ± 0.34 g l^{-1} of acid acetic.

Ethanol production by *Kluyveromyces marxianus* CE025

First, the fermentative performance of *K. marxianus* CE025 on MXG to produce ethanol was evaluated at pH 4.5, 40°C, and 200 rpm. Figure 1 shows the experimental results of glucose and xylose consumption, product formation, and cell growth. Biomass went through a 2-h lag phase, then increased exponentially and entered a stationary phase between 8 and 24 h. No death phase was observed until 72 h, when biomass concentration was 5.51 ± 0.3 g l^{-1}. The yeast used glucose and xylose present in MXG at pH 4.5. Glucose was completely consumed before 24 h of fermentation. Xylose, on the other hand, presented a slow uptake rate while glucose was available. After glucose in the medium was exhausted, xylose was consumed and reached 10.16 ± 0.5 g l^{-1} at 72 h. *K. marxianus* CE025 produced 8.00 ± 0.2 g l^{-1} of ethanol at 24 h of fermentation, giving an ethanol yield of 0.288 ± 0.04 g ethanol/g glucose and 0.231 ± 0.04 g ethanol/g of total sugar

(glucose + xylose) at 24 h (Fig. 1). Other authors [11] achieved smaller concentrations of ethanol (4.9 ± 0.3 g l^{-1}), compared to this work, at the optimal temperature of growth (50°C) of *Kluyveromyces* sp. IIPE453 using around 20 g l^{-1} of initial glucose concentration.

Xylitol was also produced, see Fig. 1, under the same conditions yielding 1.69 ± 0.3 g l^{-1} at 24 h of fermentation. The highest xylitol production occurred at 72 h, reaching a concentration of 4.77 ± 0.2 g l^{-1}. It is well known that the first step in the metabolism of D-xylose is the transport of the sugar across the cell membrane which is mediated by glucose transporters in the absence of a specific transporter for xylose [10]. Subsequently, the internalized xylose is converted to ethanol by a series of three enzymes: D-xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK). XR reduces xylose to xylitol, XDH oxidizes xylitol to xylulose which is then phosphorylated to xylulose-5-phosphate by XK. Xylulose-5-phosphate is then metabolized through the pentose phosphate pathway into ethanol [8, 21].

Figure 2 shows the CABH fermentation by *K. marxianus* CE025 at 40°C, 200 rpm, and initial pH 4.5. The growth curve (data not shown) revealed that the strain went through an appreciable lag phase (more than 8 h). After this period, an increase in biomass was observed, indicating that the yeast was capable of growth in this medium. Biomass concentration reached 10.13 ± 0.5 g l^{-1} at 72 h. As observed in the fermentation of MXG, glucose uptake rate was faster than xylose uptake rate. Xylitol production, however, was not observed in the fermentation of CABH at pH 4.5, probably due to the inhibition of specific enzymes. However, this fact is not a drawback, since the desired product is ethanol.

The highest ethanol concentration (6.37 ± 0.5 g l^{-1}) was achieved at 48 h. The volumetric ethanol production rate was 0.13 g l^{-1} h^{-1} and the ethanol yield based on glucose was 0.273 ± 0.017 g/g glucose. When compared to synthetic sugars (MXG), the ethanol yield based on glucose and xylose was lower (21 and 48%, respectively). The same behavior was observed by other authors [35], when studying the fermentation capability of *K. marxianus* 6556 in utilizing different sugars to produce ethanol. Similar ethanol concentrations were obtained by other authors [14, 25, 32]. Margaritis and Bajpai [14] evaluated the direct fermentation of xylose (20 g l^{-1}) to ethanol by *Kluyveromyces marxianus* 80-SM-16-10 and obtained 5.2 g l^{-1} of ethanol. Rouhollah et al. [25] obtained an ethanol concentration of 5.02 and 8.24 g l^{-1} when *K. marxianus* used xylose (20 g l^{-1}) or glucose (20 g l^{-1}), respectively, as carbon source. Wilkins et al. [32] studied the fermentation of xylose by the thermotolerant *Kluyveromyces marxianus* IBM2 and obtained an ethanol concentration of 0.48 g l^{-1}. Kumar et al. [11] studied the use a wide range of substrates, such as glucose, xylose, mannose, galactose, arabinose, sucrose, and cellobiose, either for growth or fermentation to ethanol by *Kluyveromyces* sp. IIPE453 (MTCC 5314). They obtained an ethanol concentration of 1.75 ± 0.05 g l^{-1} from an initial xylose concentration of 20 g l^{-1}.

A gradual reduction in ethanol concentration was observed after 48 h (Fig. 2), when glucose was exhausted. If the experimental error is considered, it can be said that ethanol concentration remained almost constant. The decrease in ethanol concentration from this point on can be attributed to its volatilization [14, 25], while the increase of biomass might be guaranteed by the xylose present in the medium. A similar behavior was obtained by other authors [11]

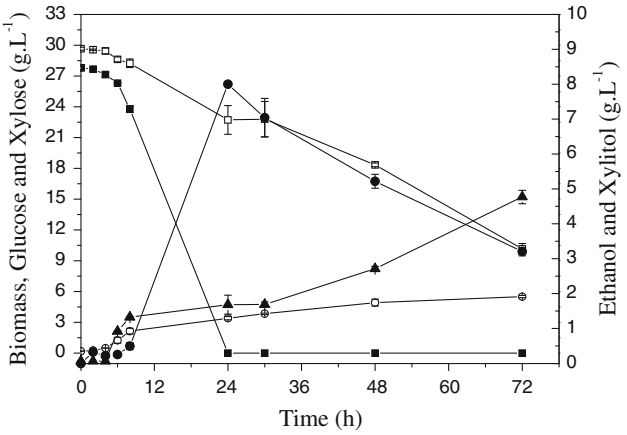

Fig. 1 MXG (glucose and xylose mixture) fermentation by *K. marxianus* CE025 at 40°C, 200 rpm, and initial pH 4.5: *open circles* biomass, *filled squares* glucose, *open squares* xylose, *filled circles* ethanol, and *filled triangles* xylitol

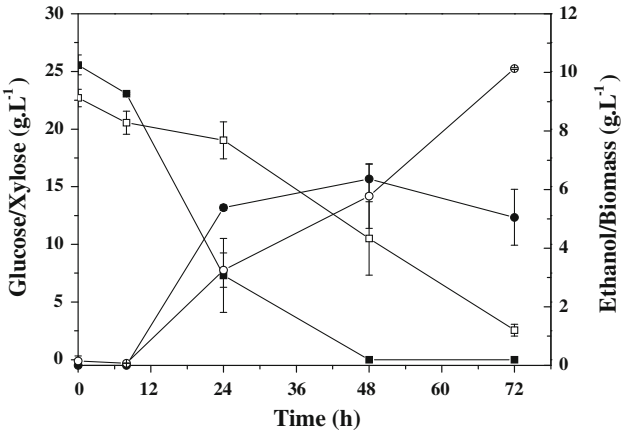

Fig. 2 Profiles of substrate consumption, biomass and ethanol production during the fermentation of CABH by *K. marxianus* CE025 at 40°C, 200 rpm, and initial pH 4.5: *open circles* biomass, *filled squares* glucose, *open squares* xylose, and *filled circles* ethanol

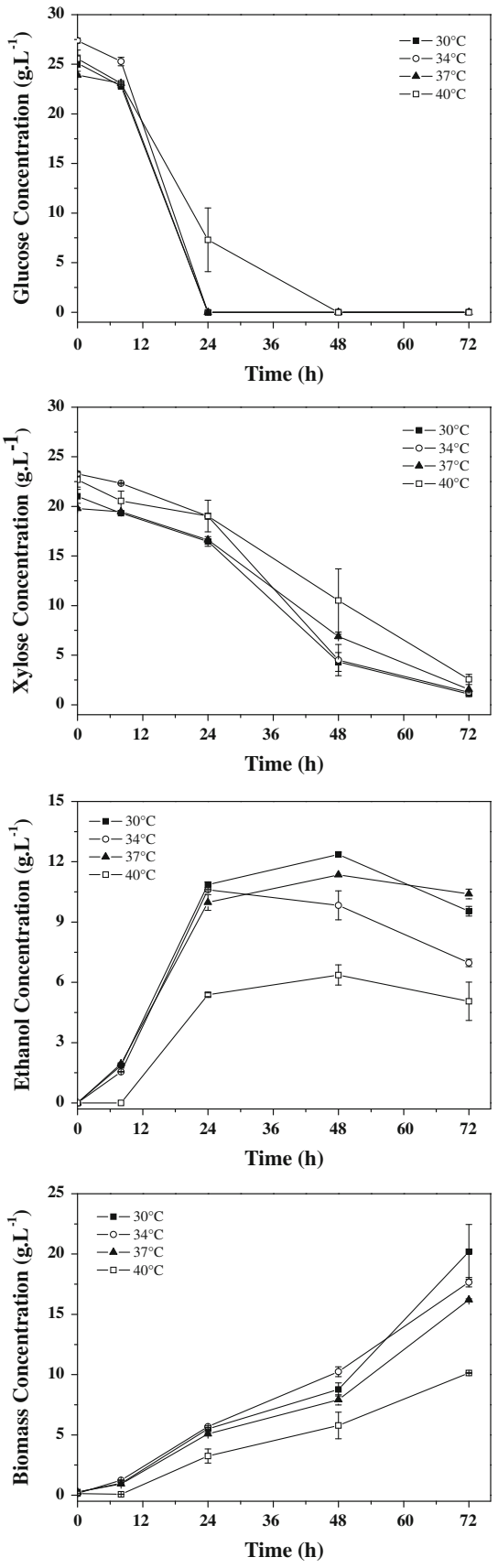

Fig. 3 Profiles of substrate consumption, biomass and ethanol production during the fermentation of CABH by *K. marxianus* CE025 at 200 rpm, initial pH 4.5 at different temperatures: *filled squares* 30°C, *open circles* 34°C, *filled triangles* 37°C, and *open squares* 40°C. Data points represent the mean and standard deviation from at least three separate experiments

when *Kluyveromyces* sp. IIPE453 was used in fermentation of a glucose and xylose mixture (synthetic medium). The authors observed that an ethanol concentration of 38 ± 0.5 g l^{-1} was achieved before 30 h of fermentation, remaining almost constant until the end of the assay (within the experimental error). This time (30 h) coincides with the exhaustion of glucose in the fermentation media, while xylose continued to be consumed without apparent ethanol production.

These results indicated that the bioconversion of cashew apple bagasse hydrolysate into ethanol accomplished by *K. marxianus* CE025 represents a promising alternative for fuel production and also contributes to recycle the cashew apple waste from the juice industry. Further optimization, however, is needed to develop an efficient route for ethanol production from CAB for commercial applications. Therefore, the influence of temperature, one of the operating variables capable of exerting considerable influence on the formation of useful bioproducts [7, 27], on the fermentation of CABH by *K. marxianus* CE025 was investigated.

Influence of temperature on ethanol production by *K. marxianus* CE025 in CABH

The influence of temperature (30, 34, 37, and 40°C) on the fermentation of CABH by *K. marxianus* CE025 was studied, regarding the kinetic parameters related to biomass and ethanol production and substrates consumption (Fig. 3). Biomass and ethanol production was not affected by increasing the isothermal control from 30 to 37°C. However, cell growth and ethanol concentrations declined considerably when the fermentation was conducted at 40°C. All the glucose was consumed after 24 h of fermentation at 30, 34, and 37°C, but 7.30 ± 3.21 g l^{-1} of glucose remained at 40°C at this time. At the optimal temperature for cell growth and ethanol production, 30°C, glucose was exhausted before 24 h of fermentation and the residual xylose was less than 2.5 g l^{-1}, at 72 h.

The maximum concentration of ethanol ($P_{max} = 12.44 \pm 0.1$ g l^{-1}) was obtained at 30°C after 48 h. The highest volumetric production rate ($P^E = 0.5$ g l^{-1} h^{-1}) was achieved at the same temperature but at 24 h. The production of ethanol started to decline after 48 h, in some experiments. As discussed before, the decrease of ethanol production may be attributed to volatilization [14, 32]. The highest ethanol

Table 1 Alcoholic fermentation of CABH by *K. marxianus* CE025: effect of temperature on kinetic parameters

Parameters	30°C	34°C	37°C	40°C
Y^{G}_{P/S_1} (g ethanol/g glucose)	0.417 ± 0.003	0.375 ± 0.004	0.385 ± 0.017	0.273 ± 0.017
$Y_{P/S}$ (g ethanol/g sugar)	0.341 ± 0.017	0.275 ± 0.067	0.302 ± 0.007	0.190 ± 0.020
$Y_{P/X}$ (g g^{-1})	1.515 ± 0.107	1.064 ± 0.120	1.535 ± 0.119	1.234 ± 0.330
P^{E} (g l^{-1} h^{-1})	0.257 ± 0.002	0.205 ± 0.015	0.236 ± 0.001	0.132 ± 0.010

Overall yields of ethanol based on glucose consumption Y^{G}_{P/S_1} and based on glucose + xylose consumption ($Y_{P/S}$), overall yield of ethanol based on biomass ($Y_{P/X}$), and ethanol productivity (P^{E})

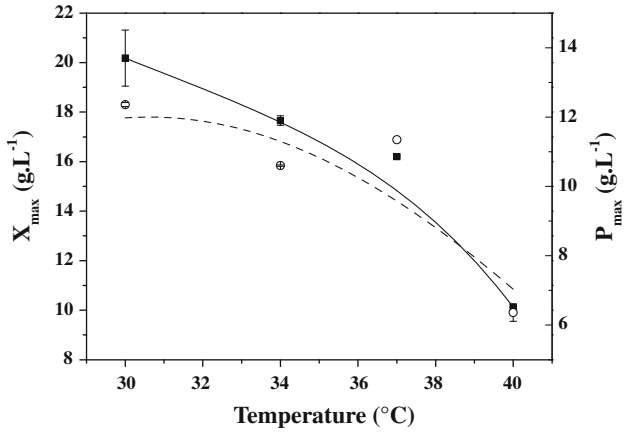

Fig. 4 Alcoholic fermentation of CABH by *K. marxianus* CE025: effect of temperature on X_{max} *filled squares* and P_{max} *open circles*. *Solid line* tendency line for X_{max} and *dotted line* tendency line for P_{max}

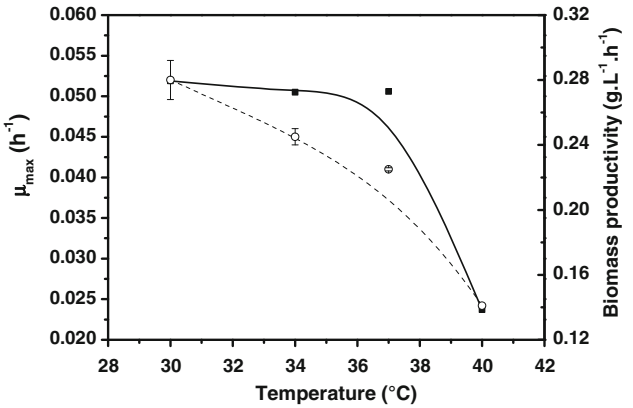

Fig. 5 Alcoholic fermentation of CABH by *K. marxianus* CE025: effect of temperature on μ_{max} (*filled squares*) and biomass productivity (*open squares*) at 72 h. *Solid line* tendency line for μ_{max} and *dotted line* tendency line for biomass productivity

production by *K. marxianus* IMB4 using xylose as carbon source was achieved at 40°C by other authors [14]; however, very few studies have been carried out on ethanol production from lignocellulosic biomass using this yeast. Most of the studies have focused on biochemical and metabolic aspects of different strains and their potential [7, 15, 17, 35], as well as evaluating the yeasts' capability of utilizing different sugars present in synthetic media [4, 14, 25, 32].

Similar results were obtained by some authors [13, 28] using different substrates. Sansonetti et al. [28] studied ethanol production by cheese whey fermentation at 34–45°C and observed that the experiments performed at 34 and 37°C resulted in high ethanol yields, 79 and 84%, respectively, with complete lactose depletion achieved in 18 h only. Limtong et al. [13], who cultivated *Kluyveromyces marxianus* in sugar cane juice media at 30, 37, 40, and 45°C, observed no significant difference between ethanol concentrations achieved at 30 and 37°C, but it was reduced when the fermentation was conducted at 40 and 45°C.

Volumetric ethanol production rates were high at all temperatures tested in this study (more than 0.20 g l^{-1} h^{-1}), except at 40°C (0.13 g l^{-1} h^{-1}) after 48 h of fermentation, i.e., the time at which maximum ethanol production occurred (Fig. 3). Wilkins et al. [32] also obtained low volumetric ethanol production rates (less than 0.02 g l^{-1} h^{-1}) at 40°C (48 h) during the fermentation of xylose by *K. marxianus* IMB4, with initial cell concentrations between 4.1 and 4.5 g l^{-1} and initial pH of 5.5.

Table 1 shows the kinetic parameters of the alcoholic fermentation accomplished by *K. marxianus* CE025 using CABH, and Fig. 4 shows the values of X_{max} and P_{max} as a function temperature. The values for the specific glucose-uptake rate were similar for all evaluated temperatures. The increase in temperature was not significant (95% of significance determined by Microcal origin, 8.1) based on $Y_{P/X}$ but exerted a negative effect on yield of ethanol based on consumed sugar Y^{G}_{P/S_1} (Table 1). The lowest values for X_{max} and P_{max} were obtained at 40°C, whereas these values doubled at 30°C (Fig. 4). Furthermore, there seems to be a relationship between biomass and ethanol production, since the pattern of the two curves is similar. Other authors observed that ethanol production by *K. marxianus* can be further improved by optimizing its growth conditions [35].

Once the exponential growth phase for each culture was established, μ_{max} could be calculated by using Eq. 1, and results are shown in Fig. 5. Temperature slightly affected μ_{max}, except for the temperature of 40°C. The maximum

specific growth rate was achieved at 30°C ($\mu_{max} = 0.0519\ h^{-1}$), under the same conditions that the highest values for X_{max} and P_{max} were observed, while the lowest value ($\mu_{max} = 0.0237\ h^{-1}$) was obtained at 40°C. Biomass productivities after 72 h of fermentation are also pictured in Fig. 5. It can be seen that these decrease as temperature is enhanced, following the same pattern as X_{max} and P_{max}.

Other authors [27] tried to explain the decrease of the fermentation capability of *P. tannophilus* to produce ethanol with increasing temperatures by the fact that at higher temperatures the solubility of the dissolved oxygen in the medium is reduced and also that there tends to be more evaporation of ethanol. As for the cause of the inhibitory effect of temperature on cell growth, Phisalaphong et al. [20] stated that a high temperature could result in changing the transport activity or saturation level of soluble compounds and solvents in the cells, which might increase the accumulation of toxic compounds inside cells.

Specific rates of substrate consumption, biomass and ethanol production by *Kluyveromyces marxianus* CE025 in CABH

Figure 6 pictures the specific rates of growth (μ_X), glucose consumption $\left(\mu_{S_1}\right)$, xylose consumption $\left(\mu_{S_2}\right)$, and ethanol production (μ_P) for the fermentation of CABH by *K. marxianus* CE025 at 30°C. Specific rates followed a typical pattern for ethanol fermentation [7]. Similar profiles were obtained for the other temperatures studied in this work (data not shown). The specific rate of glucose consumption $\left(\mu_{S_1}\right)$ was higher than the specific rate of xylose consumption $\left(\mu_{S_2}\right)$. Specific rates of ethanol production (μ_P) and glucose consumption $\left(\mu_{S_1}\right)$ present similar profiles, thus correlating them very well. The specific growth rate (μ_X) is, approximately, constant for the first 10 h of fermentation, within the experimental error. According to the results pictured in Fig. 6, ethanol formation is associated with growth, and consumption of substrate (glucose), which is a typical behavior of a primary metabolite. Specific rates of xylose consumption $\left(\mu_{S_2}\right)$ also remained, approximately, constant for the first 10 h of fermentation, within the experimental error. This is an indicator that glucose was the main carbon source used by *Kluyveromyces marxianus* CE025 for growth and to produce ethanol in CABH. However, further experiments must be conducted to investigate this behavior.

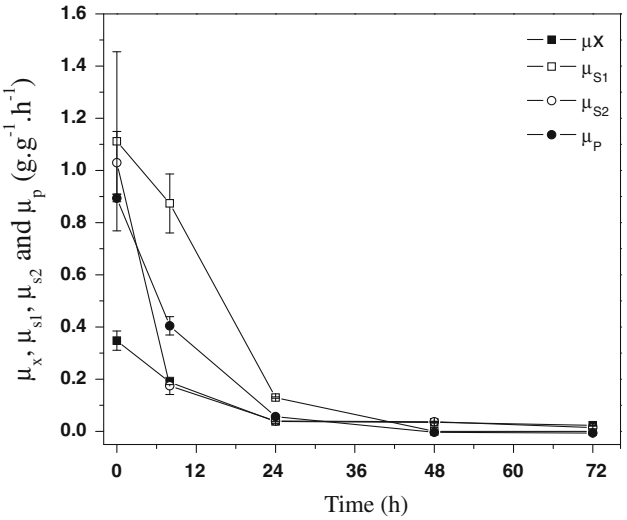

Fig. 6 Specific rates of growth (μ_X, *filled squares*), glucose consumption (μ_{S_1}, *open squares*), xylose consumption (μ_{S_2}, *open circles*), and ethanol production (μ_P, *filled circles*) during the fermentation of CABH by *K. marxianus* CE025 at 200 rpm and 30°C

Conclusion

The results described in this work, compared to results in the literature, show the potential of using cashew apple bagasse hydrolysate for the production of ethanol by *Kluyveromyces marxianus* CE025. The yeast strain *K. marxianus* CE025 was able to consume both glucose and xylose, which are the major constituents of lignocellulosic biomass, either for growth or ethanol fermentation. The maximum specific growth rate on CABH was slightly influenced by temperature between 30 and 37°C, but decreased considerably at 40°C. P_{max} and X_{max} were negatively influenced by increasing temperature of fermentation. Maximum ethanol and biomass concentrations produced by this strain were achieved at 30°C. But, further optimization of other environmental and operational conditions, such as initial pH, agitation, and aeration, must be conducted in order to develop an alternative route for industrial production of ethanol from CABH.

Acknowledgments The authors acknowledge Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Agência Nacional do Petróleo (ANP from Brazil) for the financial support that made this work possible.

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