Food Sci. Biotechnol. 24(4): 1381-1389 (2015)
DOI 10.1007/s10068-015-0177-x

RESEARCH ARTICLE

Effects of Oxygen and Nutrients on Xylitol and Ethanol Production in Sugarcane Bagasse Hydrolyzates

Juan Francisco Castañón-Rodríguez, José Antonio Portilla-Arias, Blanca R. Aguilar-Uscanga, and María Guadalupe Aguilar-Uscanga

Received August 28, 2014; revised November 5, 2014; accepted November 10, 2014; published online August 31, 2015

Abstract The influence of oxygen and nutrient supplementation on xylitol and ethanol production in a synthetic medium and sugarcane bagasse hydrolyzates using *Candida tropicalis* IEC5-ITV and *Saccharomyces cerevisiae* ITV01-RD was investigated for evaluation of bioconversion of pentoses and hexoses present in the lignocellulosic biomass. The best oxygen transfer rate (56.05 mg of O_2/L/h) and xylitol and ethanol yields (0.67 and 0.47 g/g, respectively) were obtained in a synthetic medium. A yeast extract had a positive effect on xylitol and ethanol production (0.64 and 0.44 g/g, respectively) at a concentration of 1 g/L. *C. tropicalis* and *S. cerevisiae* exhibited not previously observed morphological changes depending on the nutrient composition. Use of sugarcane bagasse hydrolyzates requires a good supply of oxygen and addition of a yeast extract to improve xylitol and ethanol production.

Keywords: xylitol, ethanol, nutrient, sugarcane, bagasse

Introduction

In order to enhance the competitiveness of cellulosic ethanol, use of process integration has been proposed to reduce the cost associated with bioethanol production. One promising approach that may be effective is use of xylose-rich hemicellulose hydrolyzates for production of value-added chemicals, like xylitol, that contribute to the overall economy of the process (1). One of the most effective ethanol-producing microorganisms for hexose is *Saccharomyces cerevisiae*, yeast with a high ethanol productivity and tolerance to ethanol and to inhibitory compounds present in hydrolyzates of the lignocellulose biomass (2). A high fermentation performance using this yeast has been achieved, determined based on medium composition and operating parameters, including, substrate and vitamin feeding strategy, oxygen level, and temperature (3). Although *S. cerevisiae* can grow well even at a relatively low pH, which prevents bacterial contamination, strains of *S. cerevisiae* are unable to use xylose for growth or fermentation (4). Due to this metabolic restriction, *S. cerevisiae* can produce neither ethanol nor xylitol using the xylose present in lignocellulose material hydrolyzates. On the other hand, use of respiratory deficient mutants has not been studied for lignocellulosic hydrolyzates.

Biotechnological production of xylitol using crude hemicellulose hydrolyzates instead of pure xylose could be of economic interest due to a reduction in production costs (5). *Candida* sp. is recognized as the best yeast xylitol producer (6). Huang *et al.* (7) listed several strains of *Candida tropicalis* that have been used for fermentation of xylose-rich hydrolyzates from lignocellulose materials for production of xylitol, with maximum yields varying widely from 0.11 to 0.71 g/g depending on the efficacy of the detoxification process, hydrolyzate composition, and oxygen availability. The key enzymes for xylose assimilation in xylitol-producing yeasts are xylose reductase (XR), which catalyses xylose reduction to xylitol and requires NADPH as the main cofactor, and NAD^+-dependent xylitol dehydrogenase (XDH), which catalyses xylitol conversion to xylulose that is then phosphorylated and incorporated

Juan Francisco Castañón-Rodríguez, José Antonio Portilla-Arias, María Guadalupe Aguilar-Uscanga (✉)
Instituto Tecnológico de Veracruz-UNIDA. Av. Miguel A. de Quevedo 2779, Col. Formando Hogar. CP. 91860. Veracruz, Ver. Mexico
Tel: +52 229 9345701; Fax: +52 229 9345701
E-mail: gaguilar@itver.edu.mx

Blanca R. Aguilar-Uscanga
Centro Universitario de Ciencias Exactas e Ingeniería. Universidad de Guadalajara. C.P. 44430, Guadalajara, Jalisco, México

into the pentose phosphate pathway for regeneration of the NADPH that is necessary for XR activity. The NAD^+ cofactor for XDH is mostly supplied by the mitochondrial respiratory chain (7,8). The major metabolic regulator of xylitol production is strongly influenced by several fermentation conditions, among which the most important are initial substrate concentration, inoculum level, and aeration/agitation conditions. Process effectiveness is based on an optimal oxygen flux to balance use of carbon both for growth and xylitol production (9). Many yeast species grow well in a simple aqueous medium containing sugars, ammonium salts, minerals, trace elements, and vitamins. Nevertheless, yeasts have extremely diverse nutritional requirements. Thus, different components present in a culture broth may exert an activating, inhibitory, or neutral effect on microorganismal growth (10).

Hydrolyzates from lignocellulosic materials, such as sugarcane bagasse, the major by-product of the sugarcane industry, an economically viable raw material produced at a rate of approximately 250 kg per ton of sugarcane, is available for xylitol and ethanol production (11). The objective of this study was to evaluate the effects of different oxygen transfer rates and nutritional requirements on xylitol and ethanol production, respectively, by *C. tropicalis* IEC5-ITV and *S. cerevisiae* ITV01-RD (respiratory deficient). The effect of oxygen transfer rates was evaluated based on modification of agitation and aeration levels in a synthetic medium. The influence of addition of a yeast extract (1 g/L), urea (3 g/L), magnesium sulfate (0.4 g/L), and potassium monobasic phosphate (5 g/L) on both growth and metabolism of both *C. tropicalis* IEC5-ITV and *S. cerevisiae* ITV01-RD in sugarcane bagasse hydrolyzates (SCBH) was also evaluated.

Materials and Methods

Strains Wild type yeasts were *C. tropicalis* IEC5-ITV isolated from sugarcane bagasse and *S. cerevisiae* ITV01-RD isolated from sugarcane molasses (12), and a respiratory deficient phenotype (RD) obtained from Ortiz-Muñiz *et al.* (13). Both yeasts are part of the collection strains of the Veracruz Institute of Technology, Veracruz, México.

Culture media Both *C. tropicalis* IEC5-ITV and *S. cerevisiae* ITV01-RD were preserved at 4°C using the following culture medium: yeast extract (10 g/L), agar (25 g/L), and xylose (20 g/L) for *C. tropicalis* IEC5-ITV, and glucose (20 g/L) for *S. cerevisiae* ITV01-RD. These reagents were purchased from Difco (Detroit, MI, USA) and from Sigma-Aldrich (St. Louis, MO, USA), respectively. Microorganisms were subcultured every 3 weeks. The synthetic medium used for evaluation of oxygen effects was prepared using yeast extract (Difco, 1 g/L), urea (Difco, 3 g/L), $MgSO_4 \cdot 7H_20$ (Difco, 0.4 g/L), KH_2PO_4 (Difco, 5 g/L), and either xylose (Sigma-Aldrich, 30 g/L) or glucose (Difco, 50 g/L). For evaluation of the effect of nutrient composition, assays were performed using sugarcane bagasse hydrolyzates containing all nutrients as a control (yeast extract 1 g/L, urea 3 g/L, $MgSO_4 \cdot 7H_2O$ 0.4 g/L, KH_2PO_4 5 g/L, and xylose 30 g/L for *C. tropicalis* IEC5-ITV, and glucose 50 g/L for *S. cerevisiae* ITV01-RD). Several medium preparations were used with the presence or absence of an evaluated nutrient. A xylose-rich sugarcane bagasse acid hydrolyzate composed of 30 g/L of xylose, 4.6 g/L of acetic acid, and 0.5 g/L of furfural was obtained using 2% (v/v) H_2SO_4 at 121°C for 40 min with a liquid:solid ratio (LSR) of 6:1. A glucose-rich enzymatic hydrolyzate neutralized using NaOH and composed of 50 g/L of glucose and 2.8 g/L of acetic acid was obtained using commercial cellulases (0.75 mL of Novozyme 188 and 0.5 mL of Celluclast 1.5, from Sigma-Aldrich) in an acetate buffer of pH 4.8 at 50°C using 200 rpm in an orbital shaker (New Brunswick Scientific Co. Inc., Edison, NJ, USA) for 72 h with a 77:1 LSR ratio. Both hydrolyzates were sterilized in an autoclave (model CV-250; AESA, Monterrey, Mexico) at 121°C and a pressure of 1.055 kg/cm^2 for 15 min.

Culture conditions and preculture Analysis of the effect of oxygen was carried out in a 14 L Bioflo 3000 (New Brunswick Scientific Co., Inc.) bioreactor with a 6 L working volume. The temperature was 30°C at pH 5.5 with agitation levels of 150, 250, and 350 rpm, and aeration rates of 0.05, 0.10, and 0.15 vvm. Qualitative effects of nutrients were evaluated using the pulse technique (14). Fermentations were carried out in a continuous culture using a 3 L Applikon bioreactor equipped with an ADI 1010 Bio Controller and an ADI 1025 Bio Console (Applikon Dependable Instruments, Delft, The Netherlands) bioreactor with a 1.5 L working volume. For nutrient experiments, 250 mL flasks were used with 150 mL of acid hydrolyzate for production of xylitol by *C. tropicalis* IEC5-ITV and 150 mL enzymatic hydrolyzate for ethanol production by *S. cerevisiae* ITV01-RD. In these fermentations, microorganisms were incubated at 30°C and 150 rpm in an orbital shaker (New Brunswick Scientific Classic Series C24KC Refrigerated Incubator Shaker, New Brunswick Scientific Co., Inc.). Yeasts strains were activated in a liquid medium of composition similar to the fermentation medium with only a different sugar concentration (20 g/L). Precultures were carried out in 500 mL Erlenmeyer flasks with 200 mL of synthetic medium and stirred in an orbital shaker (New Brunswick Scientific Classic Series C24KC Refrigerated Incubator Shaker, New Brunswick Scientific

Co., Inc.) at 250 rpm for 24 h for *C. tropicalis* IEC5-ITV and 12 h for *S. cerevisiae* ITV01-RD. Both yeasts were adapted to hydrolyzates following subculturing for 10 adaptation cycles. The inoculum size of each yeast was 6×10^6 cells/mL in all fermentations.

Determination of volumetric oxygen transfer coefficients and OTR Gas-liquid transfer coefficients (k_La) were determined, as described by Aguilar-Uscanga *et al.* (15), at different air flow rates of 18 (0.05 vvm), 36 (0.10 vvm), and 54 L/h (0.15 vvm), and agitation speeds of 150, 250, and 350 rpm following the static gassing out method (16) using a dissolved oxygen probe and measurement in a non-respiring system. The dissolved oxygen transfer rate (OTR) was calculated using the equation $OTR=k_La\ (C^*-C)$. The dissolved oxygen concentration in the liquid phase inside the bioreactor was continuously monitored using an Ingold O_2 dissolved oxygen sensor (Broadley Technologies Ltd., Bedford, England). Analyses were done in duplicate.

Pulse technique The pulse technique was used for identification of growth-limiting nutrients in a chemostat culture with dilution rates of 0.065 L/h for *C. tropicalis* IEC5-ITV and 0.120 L/h for *S. cerevisiae* ITV01-RD. When a steady state was reached, each tested medium component, urea, magnesium sulfate, and potassium monobasic phosphate, was sterilized in an autoclave (model CV-250; AESA) at 121ºC for 15 min and injected using a syringe through a rubber plug into the bioreactor. The feed culture medium was composed of only xylose (30 g/L) or glucose (50 g/L) and yeast extract (1 g/L). Aseptic samples were taken periodically. Analyses were performed in duplicate.

Analytical techniques The amount of biomass was monitored based on measurement of the optical density at 620 nm and direct counting using a Thoma chamber (Blau Brand, Wertheim, Germany) with a correlation against dry weight. Cell viability was assessed following the methylene blue staining method proposed by Lange *et al.* (17). For evaluation of substrates and products, samples were centrifuged for 10 min at 9,391×*g* (Centrifuge 5424; Eppendorf, Hamburg, Germany) and concentrations were determined based on HPLC (600 TSP Spectra System; Waters Corporation, Milford, MA, USA) using a Shodex SH1011 column (8×300 mm) maintained at 55ºC with a mobile phase of 0.05 N sulfuric acid at a flow rate of 0.6 mL/min using an Index Refraction Detector (2414 TSP Refracto Monitor; Waters Corporation). Hydrolyzates were subjected to a detoxification process using 5% (w/v) zinc sulfate and 0.3 M barium oxide before HPLC analysis.

Yield and productivity Xylitol and ethanol yields ($Y_{XylOH/xyl}$, $Y_{EtOH/gluc}$) were defined as the ratio between the final xylitol or ethanol concentration (P_F) and the concentration of xylose or glucose consumed during fermentation (S_0-S_F). The biomass yield ($Y_{Biomass/s}$) was defined as a ratio between the final biomass concentration and the concentration of substrate (xylose or glucose) consumed. Xylitol, ethanol, and biomass productivity values (Q_{XylOH}, Q_{EtOH}, $Q_{Biomass}$) were defined as a ratio between final xylitol, ethanol, and biomass concentrations (P_F) and the end fermentation time (t).

Statistical analysis The means of the data were compared using analysis of variance (ANOVA), to test the significant differences (p=0.05) between the treatments, and Tukey's test for multiple comparisons between all of the possible group mean values. All data analyses were conducted using NCSS (version 7.0; NCSS LLC, Kaysville, UT, USA).

Results and Discussion

Influence of oxygen levels on *C. tropicalis* IEC5-ITV and *S. cerevisiae* ITV01-RD metabolisms The influence of oxygen availability on glucose and xylose metabolisms in wild type yeast was evaluated based on a batch culture at aeration flow rates of 18 (0.05 vvm), 36 (0.10 vvm), and 54 L/h (0.15 vvm), and agitation speeds of 150, 250, and 350 rpm. The volumetric oxygen transfer coefficient (k_La) was first determined and results are shown in Table 1. The lowest values corresponded to the lowest airflow rates from 8.01 to 13.98 L/h, while the highest values were at the highest aeration rate evaluated (0.15 vvm). An increase in k_La values occurred as the agitation speed and aeration rate increased, in agreement with the report of Aguilar-Uscanga *et al.* (15). Thus, aeration and agitation were important variables for production of an effective OTR.

The oxygen uptake rate must be ascertained to control the dissolved oxygen concentration. A proper mechanical bioreactor design is important to provide a controlled system where a homogeneous oxygen concentration in a medium allows optimal growth and/or product formation (18). OTR values at different aeration flow rates and agitation speeds are shown in Table 1 ranging from 56.05 to 672.87 mg of O_2/L/h. Based on all OTR values determined, the values 56.05 mg of O_2/L/h (150 rpm, 0.05 vvm), 111.21 mg of O_2/L/h (150 rpm, 0.15 vvm), and 168.06 mg of O_2/L/h (250 rpm, 0.10 vvm) were evaluated for an effect on xylitol and ethanol production, and xylose and glucose consumption, by *C. tropicalis* IEC5-ITV and *S. cerevisiae* ITV01-RD, respectively. Oxygen rates can be influenced by, in addition to the air flow rate and agitation speed, gas and liquid physical properties, operational conditions, geometrical parameters of the bioreactor, and also by

oxygen consumption by cells (19). Therefore, it is necessary to include these parameters when determining the OTR value.

C. tropicalis IEC5-ITV exhibited different behaviors for xylose consumption and xylitol production based on the OTR value (Table 2). Consumption of xylose was faster at the highest OTR value evaluated (168.06 mg of O_2/L/h). At 35 h, all xylose was consumed and the maximum xylitol concentration (8.39 g/L) was reached. A value of 111.21 mg of O_2/L/h was achieved when 13.04 g/L of xylitol was produced. For *C. parasilopsis,* a range of OTR =65.7-211.7 mg of O_2/L/h and a k_La value=9.0-36.1 L/h were measured in a synthetic medium for xylitol production (20). The highest biomass yield of 0.46 g/g was obtained at an OTR value of 169.06 mg of O_2/L/h, but the lowest yield of xylitol of 0.26 g/g was observed, in agreement with the report of Hahn-Hägerdal *et al.* (21) using xylitol-producing yeasts. Oxygen supply affects the rate and yield of xylitol production, and determines partitioning of the carbon flux from xylose between cell growth and xylitol formation.

Cell viability at all OTR values was above 90%. Therefore, oxygen did not have an effect on cell viability. Kastner *et al.* (22) observed a *C. tropicalis* ATCC 13803 cell viability value of 95% and suggested that to maintain cell viability, periodic glucose-feeding for cofactor regeneration and dissolved oxygen must be carefully controlled because an excess of oxygen enhances biomass formation at the expense of xylitol production. The greatest xylitol productivity in this study of 0.18 g/L/h indicated the best conditions for subsequent studies as an airflow rate of 0.05 vvm, 150 rpm, and an OTR value 56.05 mg of O_2/L/h.

OTR values from 56.05 to 168.06 mg of O_2/L/h were evaluated for ethanol production by *S. cerevisiae* (Table 3). For all OTR values evaluated, all glucose was consumed at 24 h, and the maximum ethanol production and yield were 23.67 g/L and 0.47 g/g, respectively, obtained using an OTR of 56.05 mg of O_2/L/h. The OTR is important because oxygen plays a key role in alcoholic glucose fermentation by *S. cerevisiae,* and the OTR is a key factor in regulation of pyruvate decarboxylase activity, which are present at high levels under aerobic conditions in Crabtree-positive yeasts (23) such as *S. cerevisiae*. In Crabtree-negative yeasts, such as *C. utilis* and *Kluyveromyces lactis*, levels of pyruvate decarboxylase increase only under oxygen-limited conditions. This effect is present during alcoholic fermentation under aerobic conditions (3) and the same condition was probably created at 56.05 mg of O_2/L/h OTR value. *S. cerevisiae* ITV-01 showed the best theoretical ethanol yields of 88-96% from 112 yeast strains isolated from grape juice, sugarcane molasses, and cane juice (12). This same wild type yeast (named above) also demonstrated the best adaptation to both ethanol (5-7% w/v) and glucose (20% w/v), but oxygen requirements have not been evaluated (12). The dissolved oxygen concentration fell to zero as soon as the lag phase ended and remained at zero during the growth and stationary phases. This behavior was observed using *Brettanomyces bruxellensis* with air flows in a range of 30-120 L/h (15)

Table 1. Oxygen volumetric coefficients (k_La)(1/h) and OTR (mg O_2/L/h) at different aeration rates and agitation levels in a synthetic medium

Aeration (vvm)	Agitation (rpm)					
	150		250		350	
	k_La	OTR	k_La	OTR	k_La	OTR
0.05	8.01±0.10	56.05±0.03	11.48±0.80	80.36±0.62	13.98±0.62	97.87±0.40
0.10	12.16±0.14	85.09±0.08	24.01±1.23	168.06±1.64	33.52±0.41	234.61±0.80
0.15	15.89±0.03	111.21±1.04	30.51±1.45	213.55±1.43	49.05±0.30	343.32±1.20

Table 2. Fermentative parameters of different OTR using *C. tropicalis* IEC5-ITV for xylitol production

OTR (mg O_2/L/h)	Agitation (rpm)-Aeration (vvm)	Residual substrate (g/L)	Xylitol (g/L)	$Y_{XylOH/xyl}$ (g/g)	$Y_{Biomass/xyl}$ (g/g)	Q_{XylOH} (g/L/h)	$Q_{Biomass}$ (g/L/h)	Final viability (%)
56.05	150-0.05	6.38±0.85	13.04±0.63	0.67±0.03	0.03±0.014	0.18±0.02	0.029±0.004	93±2.00
111.21	150-0.15	7.73±0.11	10.87±0.41	0.49±0.04	0.09±0.010	0.15±0.01	0.038±0.002	93±1.00
168.06	250-0.10	0±0.00	8.39±0.42	0.26±0.04	0.46±0.03	0.11±0.03	0.17±0.014	92±1.00

Table 3. Fermentative parameters of different OTR using *S. cerevisiae* ITV01-RD for ethanol production

OTR (mg O_2/L/h)	Agitation (rpm)-Aeration (vvm)	Residual substrate (g/L)	Ethanol (g/L)	$Y_{EtOH/gluc}$ (g/g)	$Y_{Biomass/Gluc}$ (g/g)	Q_{EtOH} (g/L/h)	$Q_{Biomass}$ (g/L/h)	Final viability (%)
56.05	150-0.05	0±0.00	23.67±1.48	0.47±0.01	0.09±0.01	0.99±0.01	0.20±0.06	96±1.00
111.21	150-0.15	0±0.80	22.21±1.73	0.45±0.02	0.08±0.01	0.67±0.02	0.13±0.01	95±1.00
168.06	250-0.10	0±0.60	19.00±1.17	0.39±0.01	0.09±0.00	0.63±0.02	0.14±0.01	94±2.00

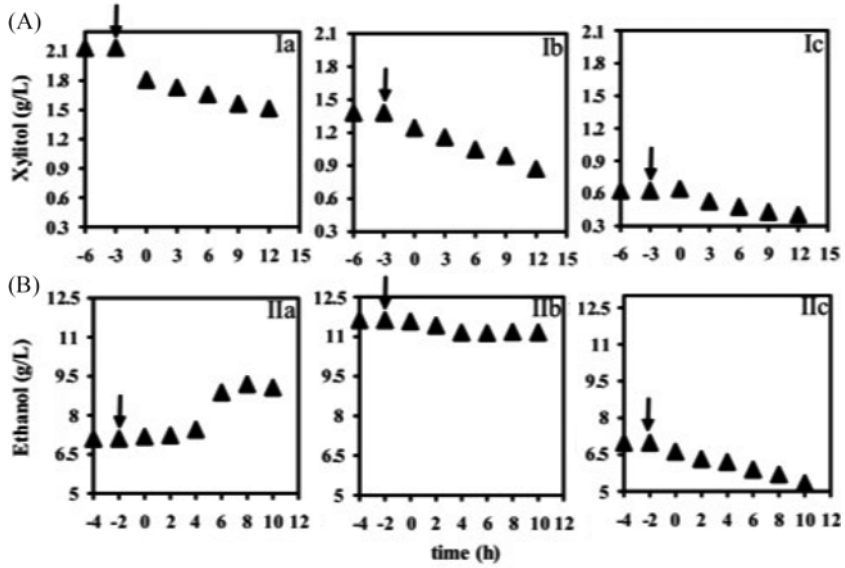

Fig. 1. Response of *C. tropicalis* IEC5-ITV (I) and *S. cerevisiae* ITV01-RD (II) to urea (a), magnesium sulfate (b), and potassium monobasic phosphate (c) pulses for xylitol and ethanol production. Arrows indicate the moment when pulses were applied.

where, at high airflows, k_La values increased from 3.5 to 21.7 L/h at 0.05 to 0.5 vvm, respectively. The biomass yield was similar for the 3 OTR values evaluated (23.5, 90.45, and 145.6 mg O_2/Lh) Biomass production was lower for *B. bruxellensis* under aerobic conditions, compared with the *S. cerevisiae* ITV01 competent strain, perhaps related to damage to the respiration-deficient (RD-petite) oxidative metabolism (24).

A lack of oxygen stimulates alcoholic fermentation. Oxygen stimulates yeast growth and reduces ethanol production; however, the ethanol concentration under aerobic conditions for the RD strain was significantly higher ($p<0.05$) than for the wild type yeast, indicating major repression by the Crabtree effect over the Pasteur effect (25). This phenomenon was previously reported for RD yeasts (21). Ethanol yields were more than 0.39 g/g and there was no residual glucose, with cell viability oscillating between 94-96% at the end of fermentation for all OTR values evaluated. Therefore, RD yeasts showed a predominantly fermentative performance independent of airflow.

For all fermentations, the main byproduct generated was glycerol at concentrations of >2 g/L. Glycerol formation in yeast is well-known as a response to aeration conditions due to a role in redox balance and osmoregulation in yeast cells because yeast cells will increase the rate of glycerol productivity with a decreased extracellular water activity, which causes hyperosmotic stress in the cells. Glycerol is conserved within cells to maintain an osmotic equilibrium with the external environment and the medium osmotic pressure (26).

Effect of nutrients on xylitol and ethanol production using the pulse technique The effect of 3 g/L of urea, 0.4 g/L of magnesium sulfate, and 5 g/L of potassium phosphate were studied on xylitol and ethanol production in a synthetic medium using the pulse technique. Pulse is not frequently used, but it provides information about the metabolic behavior of yeasts when a compound is added to a culture medium. Therefore, in this study pulse was used as an alternative strategy for evaluation of the nutritional requirements of yeasts. Also, pulse is a good way to identify growth-limiting nutrients and allows testing of a large number of nutrients in a short time (27). Urea used as nitrogen source, magnesium sulfate as a magnesium and sulfur source, and potassium phosphate as a potassium and phosphorus source had a great effect on xylitol production. The effect of each component on *C. tropicalis* growth and xylitol production was estimated (Fig. 1A). Addition of each component had a negative effect on xylitol production and a positive effect on biomass production. This behavior was also observed for *B. bruxellensis* using the same technique (27). Thus, potassium phosphate and magnesium sulfate at concentrations of 5 and 0.4 g/L, respectively, did not supply necessary potassium, phosphorus, magnesium, and sulfur to satisfy yeast nutritional requirements due to the presence of these substances in the yeast extract (1 g/L) used in the feeding medium.

When the pulse technique is used at steady state, the amount of biomass is proportional to the concentration of the growth-limiting nutrient. Therefore, if an injected substance is growth limiting, biomass synthesis increases until the pulsed substance is exhausted. On the other hand,

addition of a non-limiting element does not induce a biomass increase. Although phosphate and magnesium sulfate had negative effects in the synthetic medium, the slightly positive response could be related to the presence of other compounds in acid hydrolyzates which have synergistic effects with, or cover the needs of, *S. cerevisiae* RD for phosphate and magnesium sulfate.

When urea (3 g/L) was added to the *S. cerevisiae* growth medium (Fig. 1B), an increase of 3 g/L of ethanol production occurred when urea was used as nitrogen source to increase ethanol production from concentrated sweet sorghum juice. Urea not only promoted a specific growth rate and ethanol tolerance, but also increased the ethanol yield and reduced byproduct formation (28). Addition of magnesium sulfate (0.4 g/L) had no influence on ethanol production. Addition of potassium phosphate showed a negative effect on ethanol production. In fact, magnesium sulfate had a positive effect on biomass formation while cell viability remained at almost 90%. Thus, addition of the evaluated salts (magnesium sulfate and potassium phosphate) influenced the kinetic behavior of *S. cerevisiae* but did not produce an important change in the fermentation performance. The ability of *S. cerevisiae* to survive in a depleted minimum medium is noteworthy.

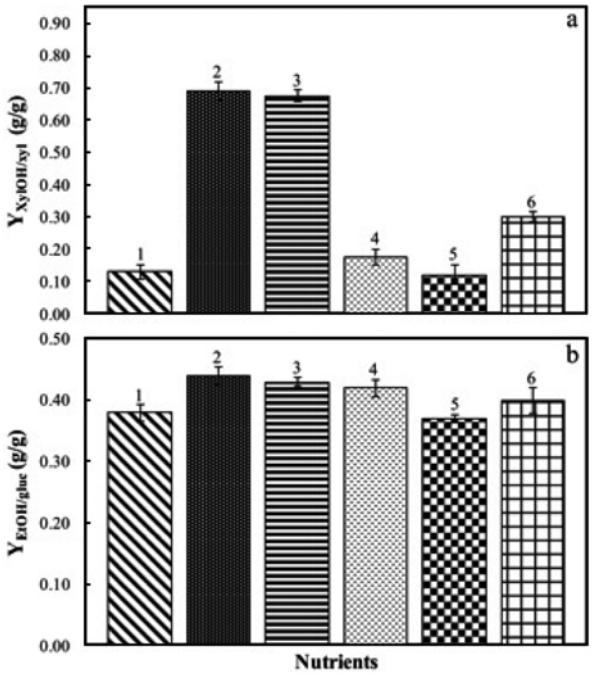

Fig. 2. Effect of nutrient addition in sugarcane bagasse hydrolyzates on xylitol and ethanol yields of *C. tropicalis* IEC5-ITV (a) and *S. cerevisiae* ITV01-RD (b) No nutrients (1), all nutrients (2), yeast extract (3), urea (4), magnesium sulfate (5), and potassium monobasic phosphate (6). Error bars represent the standard deviation (SD) of duplicate measurements.

Effect of nutrients on xylitol and ethanol production using sugarcane bagasse hydrolyzates For evaluation of the influence of additional nutrients on sugarcane hydrolyzate, xylitol and ethanol production, batch culture analyses were carried out in shaken Erlenmeyer flasks using a medium with xylose or glucose without any nutrients, a medium with all nutrients, a medium+yeast extract (1 g/L), a medium+urea (3 g/L), a medium+magnesium sulfate (0.4 g/L), and a medium+potassium phosphate (5 g/L). Xylitol yields obtained with *C. tropicalis* IEC5-ITV shown in Fig. 2a demonstrate that a medium with an exclusive carbon and energy source (xylose) was insufficient for xylitol production. When only urea and magnesium sulfate were added, yields were lower than 0.20 g/g. Gírio *et al.* (29) reported that Ca^{2+}, Mg^{2+}, and Mn^{2+} did not affect the xylitol dehydrogenase enzymatic activity, while Zn^{2+}, Cd^{2+}, and Co^{2+} inhibited the enzyme activity. In contrast, when all nutrients and yeast extract were present, the highest xylitol yield of 0.67 g/g was obtained, suggesting that the yeast extract is an important source of nitrogen, B group vitamins, and carbon compounds sufficient for growth and xylitol production by *C. tropicalis*.

The xylitol yield was similar when a synthetic medium was used, indicating that adaptation to hydrolyzate components enhances the fermentation performance and reduces the possible negative effect of inhibitors present in hydrolyzates. Using adaptation cycles, the xylitol yield was improved from 0.45 to 0.65 g/g in a bagasse hydrolyzate (5), in agreement with this study and with a report of Pessoa *et al.* (30) who did not observe inhibitory effects of furfural and 5-HMF on *C. tropicalis* growth in sugarcane bagasse hydrolyzates. Huang *et al.* (7) using *C. tropicalis* JH030 reported that use of fermenting microorganisms with a high tolerance to inhibitors allows fermentation of xylose-rich hydrolyzates to produce xylitol. Potassium phosphate (5 g/L) yielded a xylitol production value of 0.30 g/g in this study, similar to a stimulatory effect reported previously for *D. hansenii* in which limiting the phosphate amount reduced the activity of xylulose kinase and improved xylitol production (31). For *S. cerevisiae*, different behaviors with regard to ethanol production were observed (Fig. 2b). When the medium was supplemented with all salts simultaneously, a maximum ethanol yield of 0.44 g/g was obtained. The addition of any single nutrient resulted in lower yields: yeast extract, 0.42 g/g, urea, 0.41 g/g, magnesium sulfate, 0.38 g/g and potassium phosphate, 0.38 g/g. Thus, *S. cerevisiae* does not have limited nutritional requirements and the yeast extract was the component with the greatest influence in the nutrient mixture.

Mendes-Ferreira *et al.* (32) suggested that the nitrogen source is a major limiting factor for yeast growth and fermentation performance, and low nitrogen levels are associated with a low cellular activity and a low *S.*

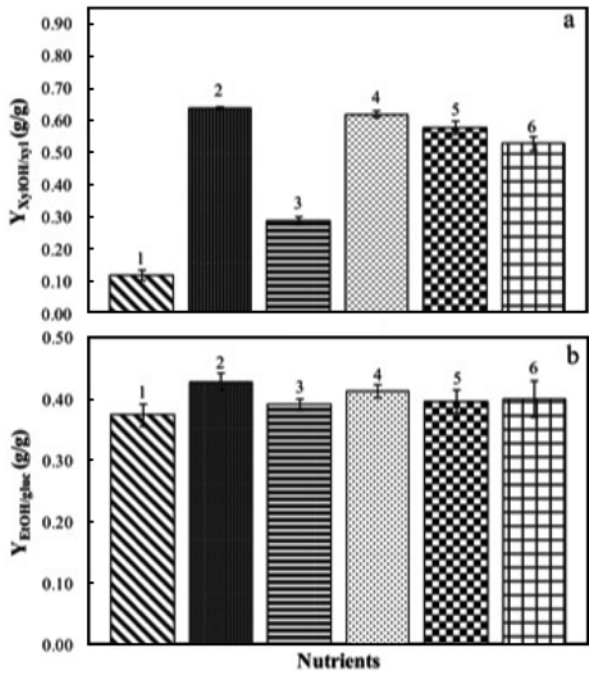

Fig. 3. Effect of a lack of nutrients in sugarcane bagasse hydrolyzates on yields of xylitol and ethanol by *C. tropicalis* IEC5-ITV (a) and *S. cerevisiae* ITV01-RD (b). No nutrients (1), all nutrients (2), without yeast extract (3), without urea (4), without magnesium sulfate (5), and without potassium monobasic phosphate (6). Error bars represent the SD of duplicate measurements.

cerevisiae biomass yield. On the other hand, Ortiz-Muñiz *et al.* (33) reported that a yeast extract stimulates glucose consumption because the important cofactors biotin and riboflavin are present, and high yeast extract concentrations do not affect either *S. cerevisiae* growth or fermentative ability.

The effect of an absence of a medium nutrient on xylitol production was also evaluated (Fig. 3a). To quantify the effect of each nutrient, analyses using a control medium without salts were carried out simultaneously. The control medium xylitol yield was 0.10 g/g. In a medium containing all nutrients, the yield was 0.64 g/g. The yeast extract exhibited the major influence by its absence, followed by potassium phosphate. In a medium where urea and magnesium sulfate were absent, 10 and 15% reductions of xylitol production were observed, respectively. In the absence of all nutrients, a yield of 0.38 g/g was obtained. Winkelhausen and Kuzmanova (34) also reported that some xylitol-producing yeasts of genus *Candida* prefer urea over yeast extract, and xylitol production was higher with urea as a nitrogen source. Therefore, use of urea at a 3 g/L concentration in acid hydrolyzates improved xylitol production in a manner similar to the yeast extract.

S. cerevisiae grown in the absence of a yeast extract, magnesium sulfate, and potassium phosphate, exhibited similar yields (0.40 g/L) (Fig. 3b). On the other hand, *S. cerevisiae* ITV01-RD showed a high degree of adaptation to sugarcane bagasse hydrolyzate, demonstrating that wild yeasts are more tolerant than other strains to inhibitors present in hydrolyzates (13). Strains with respiratory deficient mutation have been little studied involving lignocellulosic hydrolyzates, and this mutation may improve tolerance to inhibitors or toxic compounds present in lignocellulosic substrates. *S. cerevisiae* was the least susceptible when a nutrient was missing. In fact, the enzymatic hydrolyzate alone was sufficient to obtain an ethanol yield of 0.38 g/g and, therefore, addition of any nutrient only increased the yield to 0.40 g/g, attributable to the presence of proteins in hydrolyzates as a nitrogen source necessary for ethanol production. Enzymatic hydrolyzates contained a higher glucose concentration (50 g/L) than substances used in other studies where 6.4 and 17.1 g/L glucose concentrations were achieved using sugarcane bagasse (11,35). Thus, wild type yeasts did not reveal nutritional requirements for improvement of economical ethanol and xylitol production.

Effect of nutrients on yeast morphology Morphological changes were observed in *C. tropicalis* IEC5-ITV and *S. cerevisiae* ITV01-RD based on nutrients present in the culture medium. The classical morphology of *C. tropicalis* (an oval-like form, a multipolar gemation pattern, and a small size) when all nutrients were present is shown in Fig. 4a. In the presence of the yeast extract, elongation of cells such as pseudo-hyphae was observed. This dimorphic transformation of hyphal cells into yeast-like forms and intermediate shapes has been previously reported for *Debaryomyces hansenii* (36). The yeast-to-mycelium transition is associated with unipolar growth, asymmetric divisions, large vacuoles, and repression of cell separation after division. Substrate limitation can also be a key factor for induction of elongation and hyphae development as a method to increase the surface area available for uptake of sugar molecules. Another factor that could influence this morphological trend is a low aeration rate causing transition from oval cells to hyphae.

An increase in the dissolved O_2 concentration resulted in recovery of the classical oval shape. The microscopic morphology of *S. cerevisiae* ITV01-RD is shown in Fig. 5. When grown in the presence of all nutrients (Fig. 5a), the yeast showed a typical elliptical shape with monopolar gemation, similar to the cell morphology when the yeast extract and magnesium sulfate were present. However, urea and potassium monobasic phosphate induced multipolar gemation with formation of small chains. Mycelial growth was not observed, although hyphal growth has been induced in *S. cerevisiae* in a continuous culture ascribed to environmental stimuli perturbation of the culture medium, or modification of the flow rate in a chemostat in the presence of ethanol (37). Potassium monobasic phosphate

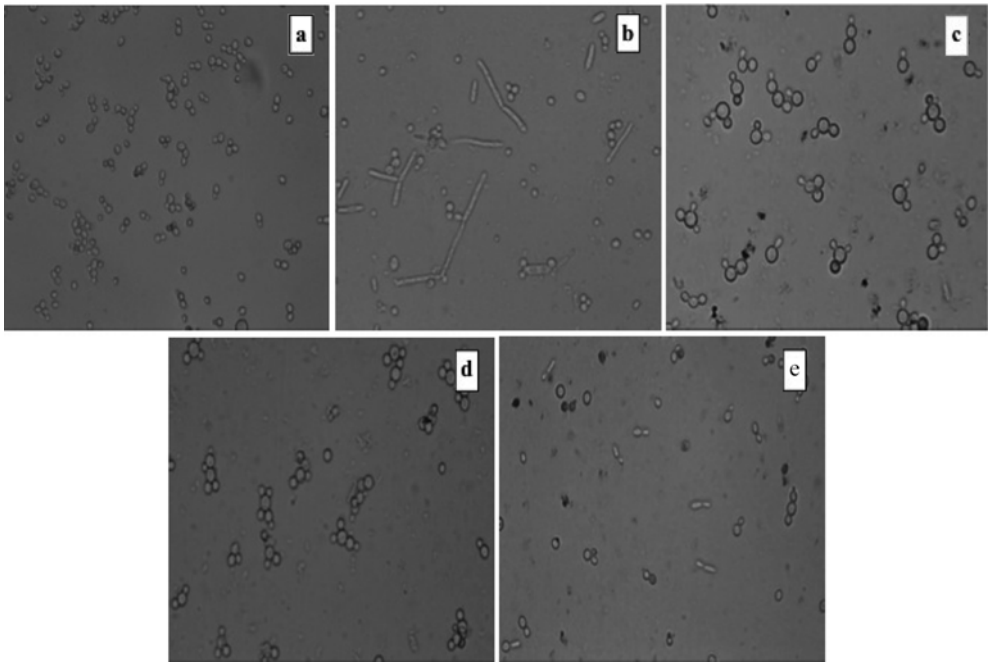

Fig. 4. Morphological changes in *C. tropicalis* IEC5-ITV cells grown in sugarcane bagasse hydrolyzates in the presence of all nutrients (a), yeast extract (b), urea (c), magnesium sulfate (d), and potassium monobasic phosphate (e).

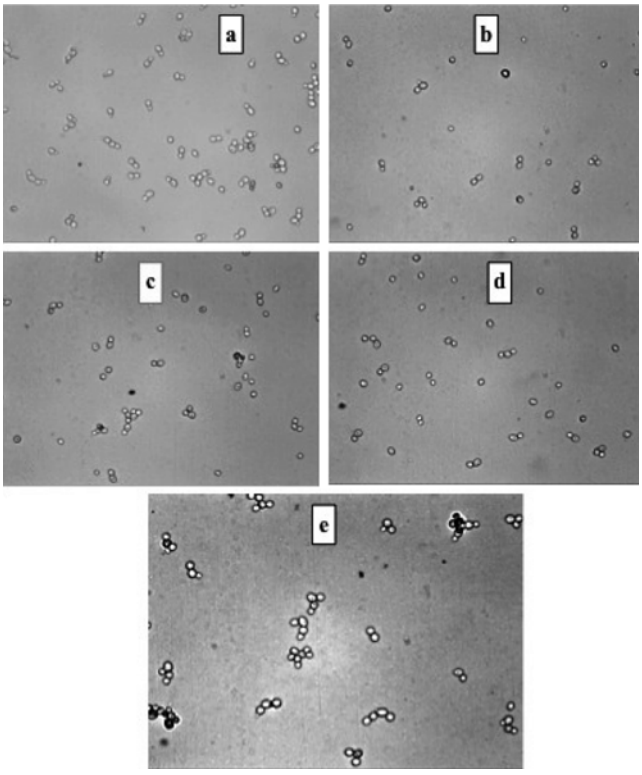

Fig. 5. Morphological changes in *S. cerevisiae* ITV01-RD cells grown in sugarcane bagasse hydrolyzates in the presence of all nutrients (a), yeast extract (b), urea (c), magnesium sulfate (d), and potassium monobasic phosphate (e).

induced monopolar gemation in this study, but mother cells were longer than daughter cells. These alterations in morphology may be related to increased osmolality of the culture due to the presence of salts in the culture medium, or the pH. Although these morphological changes have been observed for other yeasts, they have not been correlated with the presence or absence of nutrients. Behavior of yeasts when exposed to chemicals, nutrients, or inhibitors is important information that can be used to improve fermentation performance or to evaluate strategies for improvements in bioprocesses.

In conclusion, an OTR value=56.05 mg of O_2/L/h was sufficient to satisfy the oxygen demand of *C. tropicalis* IEC5-ITV and *S. cerevisiae* ITV01-RD for production of 0.67 g/g of xylitol and 0.47 g/g of ethanol in a synthetic medium. The presence of urea (3 g/L), magnesium sulfate (0.4 g/L), and potassium monobasic phosphate (5 g/L) exerted a negative effect on xylitol production and induced a stimulatory effect on biomass formation in a synthetic medium. *S. cerevisiae* is not nutritionally demanding and can use either a synthetic medium or an enzymatic hydrolyzate. A yeast extract concentration of 1 g/L was sufficient to satisfy the nutritional needs of *C. tropicalis* IEC5-ITV and *S. cerevisiae* ITV01-RD in sugarcane bagasse hydrolyzates to produce xylitol and ethanol yields of 0.65 and 0.42 g/g, respectively. The presence of salts in hydrolyzates induced morphological changes in *C. tropicalis* IEC5-ITV, but not in *S. cerevisiae* ITV01-RD. However, these alterations did not modify the fermentative performance. Therefore, supplementation with a yeast extract favored xylitol and ethanol production using *C. tropicalis* IEC5-ITV and *S. cerevisiae* ITV01-RD under limited oxygen conditions.

These yeasts can be useful for development of simultaneous or sequential processes for integral biotechnological xylitol and ethanol production using both pentoses and hexoses present in lignocellulosic materials, such as sugarcane bagasse.

Acknowledgments This study was supported by project # 5182.13.14-P of the Dirección General de Educación Superior Tecnológica (DGEST). Critical reading of the manuscript was provided by Patricia Hayward Jones, M.Sc., and Dulce Ma. Barradas Dermitz, M.Sc.

Disclosure The authors declare no conflict of interest.

References

1. Cardona CA, Sánchez OJ. Fuel ethanol production: Process design trends and integration opportunities. Bioresource Technol. 98: 2415-2457 (2007)
2. Olsson L, Nielsen J. The role of metabolic engineering in the improvement of *Saccharomyces cerevisiae*: Utilization of industrial media. Enzyme Microb. Tech. 26: 785-792 (2000)
3. Ishtar Snoek IS, Yde Steensma H. Factors involved in anaerobic growth of *Saccharomyces cerevisiae*. Yeast 24: 1-10 (2007)
4. Matsushika A, Inoue H, Kodaki T, Sawayama S. Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: Current state and perspectives. Appl. Microbiol. Biot. 84: 37-53 (2009)
5. Rao RS, Jyothi CP, Prakasham RS, Sarma PN, Rao LV. Xylitol production from corn fiber and sugarcane bagasse hydrolysates by *Candida tropicalis*. Bioresource Technol. 97: 1974-1978 (2006)
6. Granström TB, Izumori K, Leisola M. A rare sugar xylitol. Part I: The biochemistry and biosynthesis of xylitol. Appl. Microbiol. Biot. 74: 277-281 (2007)
7. Huang CF, Jiang YF, Guo GL, Hwang WS. Development of a yeast strain for xylitol production without hydrolysate detoxification as part of the integration of co-product generation within the lignocellulose ethanol process. Bioresource Technol. 102: 3322-3329 (2011)
8. Aranda-Barradas JS, Garibay-Orijel C, Badillo-Corona JA, Salgado-Manjarrez E. A stoichiometric analysis of biological xylitol production. Biochem. Eng. J. 50: 1-9 (2010)
9. Oh DK, Kim SY, Kim JH. Increase of xylitol production rate by controlling redox potential in *Candida parapsilosis.* Biotechnol. Bioeng. 58: 440-444 (1998)
10. Walker GM. Yeast Physiology and Biotechnology. John Wiley & Sons Ltd., London, UK. pp. 350-355 (1998)
11. Rabelo SC, Amezquita Fonseca NA, Andrade RR, Maciel Filho R, Costa AC. Ethanol production from enzymatic of sugarcane bagasse pretreated with lime and alkaline hydrogen peroxide. Biomass Bioenerg. 35: 2600-2607 (2011)
12. Ortiz-Zamora O, Cortes-García R, Ramírez-Lepe M, Gómez-Rodríguez J, Aguilar-Uscanga MG. Isolation and selection of ethanol-resistant and osmotolerant yeasts from regional agricultural sources in Mexico. J. Food Process Eng. 32: 775-786 (2009)
13. Ortiz-Muñiz B, Carvajal-Zarrabal O, Aguilar B, Aguilar-Uscanga MG. Improvement in ethanol production using respiratory deficient phenotype of a wild type yeast *Saccharomyces cerevisiae* ITV-01. Renew. Energ. 37: 197-201 (2012)
14. Goldberg I, Er-el Z. The chemostat - an efficient technique for medium optimization. Process Biochem. 16: 2-7 (1981)
15. Aguilar-Uscanga MG, Delia ML, Strehaiano P. *Brettanomyces bruxellensis*: Effect of oxygen on growth and acetic production. Appl. Microbiol. Biot. 61: 157-162 (2003)
16. Wang SJ, Zhong JJ. A novel centrifugal impeller bioreactor. II. Oxygen transfer and power consumption. Biotechnol. Bioeng. 51: 520-527 (1996)
17. Lange H, Bavouzet JM, Taillandier P, Delorme C. Systematic error and comparison of four methods for assessing the viability of *Saccharomyces cerevisiae* suspensions. Biotechnol. Tech. 7: 223-228 (1993)
18. Gogate PR, Beenackers AACM, Pandit AB. Multiple-impeller systems with a special emphasis on bioreactors: A critical review. Biochem. Eng. J. 6: 109-144 (2000)
19. García-Ochoa F, Gómez E. Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. Biotechnol. Adv. 27: 153-176 (2009)
20. Aranda-Barradas JS, Delia ML, Riba JP. Kinetic study and modelling of the xylitol production using *Candida parapsilosis* in oxygen-limited culture conditions. Bioprocess Eng. 22: 219-225 (2000)
21. Hahn-Hägerdal B, Jeppsson H, Skoog K, Prior BA. Biochemistry and physiology of xylose fermentation by yeasts. Enzyme Microb. Tech. 16: 933-943 (1994)
22. Kastner JR, Eiteman MA, Lee SA. Effect of redox potential on stationary-phase xylitol fermentations using *Candida tropicalis*. Appl. Microbiol. Biot. 63: 96-100 (2003)
23. De Deken RH. The crabtree effect: A regulatory system in yeast. J. Gen. Microbiol. 44: 149-156 (1966)
24. Guiraud JP, Bourgi J, Stervinou M, Claisse M, Galzy P. Isolation of a respiratory deficient *Kluyveromyces fragilis* mutant for the production of ethanol from Jerusalem artichoke. Biotechnol. Bioeng. 29: 850-858 (1987)
25. Lagunas R, Gancedo C. Role of phosphate in the regulation of the Pasteur effect in *Saccharomyces cerevisiae*. Eur. J. Biochem. 137: 479-483 (1983)
26. Wang ZX, Zhuge J, Fang H, Prior BA. Glycerol production by microbial fermentation: A review. Biotechnol. Adv. 19: 201-223 (2001)
27. Aguilar-Uscanga MG, Delia ML, Strehaiano P. Nutritional requirements of *Brettanomyces bruxellensis*: Growth and physiology in batch and chemostat cultures. Can. J. Microbiol. 46: 1046-1050 (2000)
28. Yue G, Yu J, Zhang X, Tan T. The influence of nitrogen sources on ethanol production by yeast from concentrated sweet sorghum juice. Biomass Bioenerg. 39: 48-52 (2012)
29. Gírio FM, Pelica F, Amaral-Collaço MT. Characterization of xylitol dehydrogenase from *Debaryomyces hansenii*. Appl. Biochem. Biotech. 56: 79-87 (1996)
30. Pessoa Jr. A, Mancilha IM, Sato S. Cultivation of *C. tropicalis* in sugar cane hemicellulose hydrolyzate for microbial protein production. J. Biotechnol. 51: 83-88 (1996)
31. Tavares JM, Duarte LC, Amaral-Colaço MT, Gírio FM. Phosphate limitation stress induces xylitol overproduction by *Debaryomyces hansenii*. FEMS Microbiol. Lett. 171: 115-120 (1999)
32. Mendes-Ferreira A, Mendes-Faia A, Leão C. Growth and fermentation patterns of *Saccharomyces cerevisiae* under different ammonium concentrations and its implications in winemaking industry. J. Appl. Microbiol. 97: 540-545 (2004)
33. Ortiz-Muñiz B, Carvajal-Zarrabal O, Torrestiana-Sanchez B, Aguilar-Uscanga MG. Kinetic study on ethanol production using *Saccharomyces cerevisiae* ITV-01 yeast isolated from sugar cane molasses. J. Chem. Technol. Biot. 85: 1361-1367 (2010)
34. Winkelhausen E, Kuzmanova S. Microbial conversion of D-xylose to xylitol. J. Ferment. Bioeng. 86: 1-14 (1998)
35. Velmurugan R, Muthukumar K. Utilization of sugarcane bagasse for bioethanol production: Sono-assisted acid hydrolysis approach. Bioresource Technol. 102: 7119-7123 (2011)
36. Cruz JM, Domínguez JM, Domínguez H, Parajó JC. Dimorphic behavior of *Debaryomyces hansenii* grown in barley bran acid hydrolyzates. Biotechnol. Lett. 22: 605-610 (2000)
37. Hill GA, Robinson GW. Morphological behavior of *Saccharomyces cerevisiae* during continuous fermentation. Biotechnol. Lett. 10: 815-820 (1998)