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Batch and Continuous Culture of Hemicellulosic Hydrolysate from Sugarcane Bagasse for Lipids Production

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Lignocellulosics, including the sugarcane bagasse, have considerable potential to the future production of biofuels and mitigation of carbon dioxides emissions. Microbial lipid produced by yeast fermentation using inexpensive and renewable carbon sources such as hemicellulosic hydrolysate from sugarcane bagasse is an alternative feedstock for biodiesel production and a potential solution for a bio-based economy. Hemicellulosic hydrolysate (H-H) from *in natura* sugarcane bagasse produced by acid hydrolysis was the carbon source (H-H medium) for batch and continuous fermentations of a previously selected strain of *Lipomyces starkeyi*. This yeast is able to use H-H as substrate to grow and to accumulate lipids; and cell growth is not inhibited by furfural, hydroxymethylfurfural (HMF) and acetic acid. Contrary, these compounds were consumed during the fermentation. During continuous culture at low dilution rate, 0.03 h^{-1}, higher lipid production, 3.7 g/L, is achieved when compared to batch culture, 2.3 g/L. In both cultures, arabinose was not consumed even after depletion of xylose and glucose. These results indicate the large potential of using the hemicellulose sugars of sugarcane bagasse for microbial oils production for additional biofuels manufacturing. Furthermore, *L. starkeyi* becomes a promising strain for lipid production from H-H and holds a great promise for biodiesel & biokerosene in a sugarcane biorefinery.

1. Introduction

Microbial oils, named single cell oils (SCO), are considered promising candidates for the production of biodiesel, because its fatty acid composition is similar to vegetable oils and furthermore, do not show competition with food and limitation of supply . However, the high cost of culture medium makes microbial oils less economically competitive (Huang C. et al., 2012a). Low-cost raw materials are essential to reduce the production of microbial lipids and the lignocellulosic biomass is regarded as inexpensive sources of carbon which can be used as substrates for SCO production (Chen X.F. et al., 2012). Sugarcane bagasse, mainly composed of cellulose, hemicellulose and lignin, can be hydrolysed into fermentable sugars to be used as carbon source by yeasts producing lipids (Zhao X. et al., 2012). The pretreatment of lignocellulosic materials is carried out to remove the hemicellulose and/or lignin in order to reduce the polymerization and crystallinity of cellulose to facilitate the enzymatic hydrolysis (Tsigie Y. A. et al., 2011). However, the hemicellulose fraction, rich in pentoses, can be an excellent carbon source for the production of microbial oil. Although, the difficulty of several microorganisms to ferment pentoses and tolerate the inhibitors originated in the pretreatment of biomass has limited the use of hemicellulosic fraction which would improve the production of biofuels and other chemicals (Mussatto S. I. et al., 2010, Chandel et al., 2011). Since the toxic compounds can inhibit the growth and sugar utilization of microorganisms during fermentation, several detoxification methods such as physical, chemical and biological, have been employed in order to improve the fermentability of lignocellulose materials and thus, extend its biotechnological applications (Chandel et al., 2011). Finding a promising strain that assimilates efficiently pentoses and shows high tolerance to inhibitor is a challenge and could increase the feasibility of hemicellulosic hydrolysates as carbon source for the production of biofuels and high-added-value chemicals. *Lipomyces starkeyi*, lipid-producing yeast, has shown to possess an ability to utilize a variety of carbon sources for growth and lipid production (Zhao X. et al., 2008).

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In this work aimed to employ cheaper raw material for microbial oil by using *L. starkeyi* DSM 70296 cells and investigated the cell growth, lipid accumulation and fatty acid profiles when H-H was used as substrate in batch and continuous cultures under nitrogen-limited. Non-detoxified H-H from sugarcane bagasse was used as carbon and energy source in order to reduce the cost of lipid production.

2. Material and methods

2.1. Raw material

Sugarcane bagasse *in natura* was kindly provided by CTBE (National Laboratory of Bioethanol Science and Technology, Campinas-SP, Brazil). Chemical composition (dry basis) of *in natura* sugarcane bagasse used in this study is cellulose 49.5%, hemicellulose 24.3%, lignin 22.7% and ash 0.3%. It was dried under sunlight and stored in plastic bags until use. The humidity was around 10%.

2.2. Preparation of hemicellulose hydrolysate from sugarcane bagasse

Sugarcane bagasse was hydrolyzed with H_2SO_4 1.5% (w/v) at solid-to-liquid ratio of 1:10 at 120°C for 20 min in autoclave (Aguilar R. et al., 2002). After hydrolysis, the mixture was separated by centrifugation at 2980 G, 7 min and stored at -20°C. Hemicellulosic hydrolysate (H-H), liquid fraction, was used as substrate for fermentation.

2.3. Strain and medium

A previously isolated strain of oleaginous yeast *L. starkeyi* DSM 70296 was used. Agar slants (YPX medium: xylose 20 g/L; peptone 10 g/L; yeast extract 10 g/L; agar 20 g/L) were stored in refrigeration and propagated monthly or stored by cryopreservation at −80ºC with 10% (v/v) of glycerol. The composition of the inoculum and fermentation mediums were (per liter): yeast extract, 0.51 g; $(NH_4)_2SO_4$, 0.36 g; Na_2HPO_4, 1 g; KH_2PO_4, 3.5 g; $Mg_2SO_4{\cdot}7H_2O$, 0.4 g; $CaCl_2{\cdot}2H_2O$, 0.04 g; $ZnSO_4.7H_2O$, 0.08 g; $CuSO_4.5H_2O$, 0.001 g; $CoCL_2.6H_2O$, 0.001 g; $(NH_4)_2Mo_2O_7$, 0.005 g. H-H was used as carbon source and the pH was adjusted to 5.5 with CaO at room temperature. The precipitate was separated by centrifugation at 6000 G for 10 minutes. The medium was named H-H medium. The inoculum was prepared in the H-H medium in order to previously adapt the yeast to the hydrolysate. All chemicals and reagents used were of analytical grade.

2.4. Cultivation conditions

Inoculum was prepared in H-H medium at 28 ºC, 200 rpm and pH 5.5 in orbital shaker incubator model SI6 Shel Lab (USA). 3 g/L of inoculum, C/N ratio of 50 (Garzón, 2009), 1 vvm of aeration and 400 rpm of agitation at 28ºC were used in the batch and continuous culture of H-H. The working volume for batch and chemostat culture was of 1.0 L and 0.5L, respectively, in a 3.0 L and 1.3L bioreactor (New Brunswick, USA), respectively. The pH was controlled at 5.5 by the automatic addition of 2M NaOH or 1M HCl. Dissolved oxygen (DO) was monitored online by a DO electrode and to prevent foam formation, an antifoam agent (Dow Corning FG-10) 20% (v/v) was automatically added to the vessel when needed. For continuous culture the dilution rate (D, volume of incoming medium per unit time/volume of medium in the culture vessel) was $0.03h^{-1}$. Continuous culture started after 15h of batch cultivation. Aliquots were taken at various intervals and stored at –20°C to estimate substrate concentrations, cell dry weight and lipid content. Steady−state conditions were obtained after a continuous flow of at least two working volumes of the culture medium.

3. Analytical methods

3.1. Biomass assessment

Cell optical density was measured at 600 nm with a Nanophotometer (Implen GmbH, Munich, Germany), using H-H medium as calibration reference fermentation. The cell density was also expressed as cell dry weight (CDW). Biomass was harvested by centrifugation and its weight was determined in its lyophilized form. The freeze-dried cells were quantified using samples in triplicates for each time.

3.1.2. Carbohydrate determination

Glucose, xylose and arabinose concentrations were analyzed by ion chromatograph at a Metrohm system (polystyrene/divinylbenzene copolymer column, particle size 5mm, dimensions of 150 x 4.0mm, 871 Advanced Bioscan detector). Eluent: NaOH (0,1mM/L) at 1.0 mL/min, temperature of column and detector: 30°C. During fermentation, the sugar concentration was also monitored by a colorimetric method (Somogy-Nelson) in order to known the sugars depletion.

3.1.3. Acetic acid determination

Acetic acid was also measured by ion chromatograph using a Metrosep Organic Acid column (250 x 7.8 mm Metrohm AG CH 9101), mobile phase 0,5mM H2SO4 and injection volume of 196 µL.

3.1.4. Lipids determination

To provide better extraction efficiency, a previous acid treatment was performed by adding hydrochloric acid (4 mL of HCl 2M for each 300 mg of freeze-dried yeast biomass) and incubating at 80ºC for 1 hour, after that the solution was centrifuged (6000 G, 4ºC, 15 min). The lipids were gravimetrically quantified, from this wet digested pellet, by Bligh-Dyer's method.

3.1.5. Nitrogen determination

Ammoniacal nitrogen was determined by Berthelot reaction. 100 µL of sample (diluted with water when necessary), 2 mL of solution A (10 g/L phenol, 10 mg/L sodium nitroprussiate) and 2 mL of solution B (35.7 g/L Na_2HPO_4, 6 g/L NaOH and 10 mg/L NaOCl) were added to a test tube which was then sealed with a cap. This solution was incubated for 30 minutes at 37ºC and absorbance was determined at 630 nm.

3.1.6. Fatty acids determination

To determine its composition, the oil from lyophilized biomass was derivatized to methyl esters by direct transesterification (Lewis T. et al., 2000). The light phase (hexane with methyl esters) was separated, dried over anhydrous sodium sulfate and filtered through a membrane with a 0.45 µm pore size. Samples were kept at −20°C until injection into chromatograph. Fatty acid composition was assayed by gas chromatography using a GC-2010 Plus, mass detector MS (Shimatzu), capillary column Stabilwax, 30m, ID 0.25 millimeters, 0.25 µm. It was used Helium gas as mobile phase (15 psi, split 1/100), injector and detector temperatures at 250°C and 300°C, respectively. Temperature ramp to the column: initial temperature at 50°C for 2 minutes, gradient of 10°C/min at 180°C with standby time of 5 minutes, gradient of 5°C/min until 240°C. Mass detector operating at scan mode at 0.30 sec of m/z=35 to m/z=400, ionization temperature of 200°C and interface of 240°C.

4. Results and discussion

4.1. H-H from *in natura* sugarcane bagasse

The composition of hemicellulosic hydrolysate from sugarcane bagasse was 13.5 g/L xylose, 2.86 g/L glucose, 2.62 g/L arabinose, 2.64 g/L acetic acid, 0.02 g/L HMF and 0.04 g/L furfural. The xylose is the main hemicellulosic sugar followed by arabinose and glucose released into the liquor during acid hydrolysis. The low concentration of glucose indicates that acid hydrolysed the hemicelluloses and almost did not damage the cellulose fraction, since the glycoside bonds of hemicelluloses are weaker than the ones found on cellulose (Aguilar R. et al., 2002).

Toxic compounds for microorganism growth were found such as acetic acid, furfural and 5-hydroxymethylfurfural (HMF). These inhibitors were formed or released, besides to sugars monomers, by thermochemical degradation of hemicelluloses. Furfural is originated by pentoses degradation in higher temperature and pressure values, while HMF is formed by hexoses degradation. In general, furfural concentration is higher than HMF concentration in acid hydrolysates because hexose concentration is low in hemicellulose acidic saccharification. Acetic acid is formed by hydrolysis of acetyl groups bound to the hemicellulosic monomers and its generation mainly depends on the temperature and residence time of dilute-acid pretreatment until the acetyl groups are fully hydrolysed. These inhibitors affect the microbial metabolism hindering the fermentative process and its toxicity is the major limiting factor to bioconversion of lignocelluloses materials (Chandel et al., 2011).

Detoxification procedures are used to reduce or eliminate inhibitors in order to obtain hydrolysates less toxic to be used as fermentation medium in bioconversion processes. Several methods have been employed to detoxify H-H, as physical, chemical and biological methods (Chandel, 2010). However, the carbohydrates and inhibitors concentrations of H-H do not significantly vary before and after treatment with CaO. Thus, in this work, the neutralization of H-H with CaO at room temperature was not considered as a detoxification step

4.2. Fermentations

It was observed that glucose and xylose were simultaneously consumed with depletion of sugars at 20 hours, although Zhao X. et al. (2008) reported that glucose and xylose consumptions were apparently sequential by *L. starkeyi*. Arabinose wasn't consumed even when the other sugars were already depleted indicating that yeast cannot easily assimilating this carbohydrate (Figure 1A) as it was founded by Zhao X. et al. (2012) in the bagasse hydrolysate cultivation using *R. toruloides*. In the batch culture, *L. starkeyi* produced 13.3 g/L of biomass and 2.3 g/L of lipids representing yields of 0.52 g/g and 0.13 g/g,

respectively. The maximum specific growth ($\mu_{máx}$) achieved was 0.09 h^{-1}. It is worth noticing that the acetic acid, the most abundant aliphatic acid on the H-H, was consumed at 12 hours followed by furfural and HMF indicating high tolerance to inhibitors by yeast. *L. starkeyi* was not apparently inhibited indicating the importance of prior adaptation to the H-H (Figure 1B).

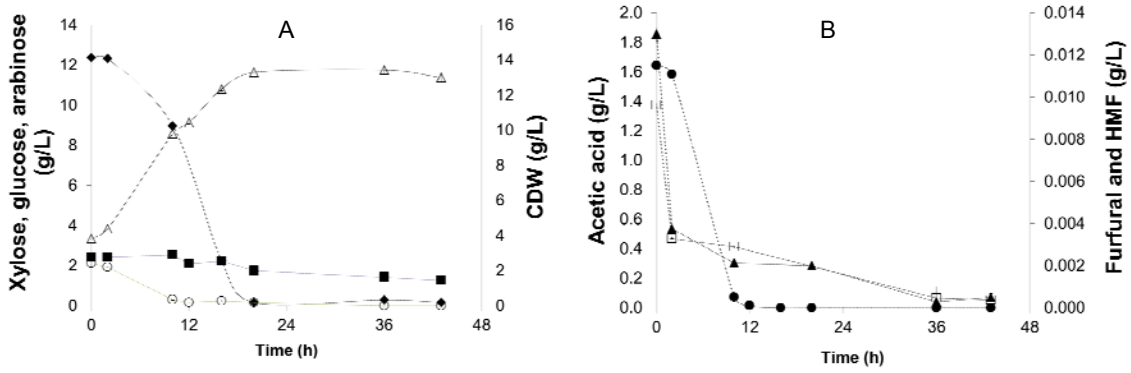

Figure 1: Kinect profile of cell growth and inhibitors consumption during batch cultivation of L. starkeyi on H-H medium. (♦) Xylose; (○) Glucose; (■) Arabinose; (∆) CDW; (●) Acetic acid, (▲) Furfural, (□) HMF.

Continuous cultivation started after a batch stage lasting 15 hours when xylose concentration was lower than 5 g/L. During the continuous feed of H-H was observed that glucose and xylose was assimilated constantly remaining about 2 g/L residual arabinose (Figure 2). The most of inhibitors were consumed during the batch stage and the residual acetic acid was exhausted at 5 hours of continuous feed. The cell dry weight reached 13.9 g/L with 3.7 g/L of intracellular lipids achieving yield of lipids of 0.21 g/g. Normally, low rate dilution (D) is required for optimum conversions, since the microbial cells need to remain within the chemostat for at least 12–24 h in order to consume the available nitrogen and then convert the remaining sugar to oil. To the batch and continuous cultivations the total depletion of nitrogen was achieved at 10 and 15 hours, respectively (no shown). At low dilution rate the lipid production was favored beyond the inhibitors consumption that contributes as carbon source.

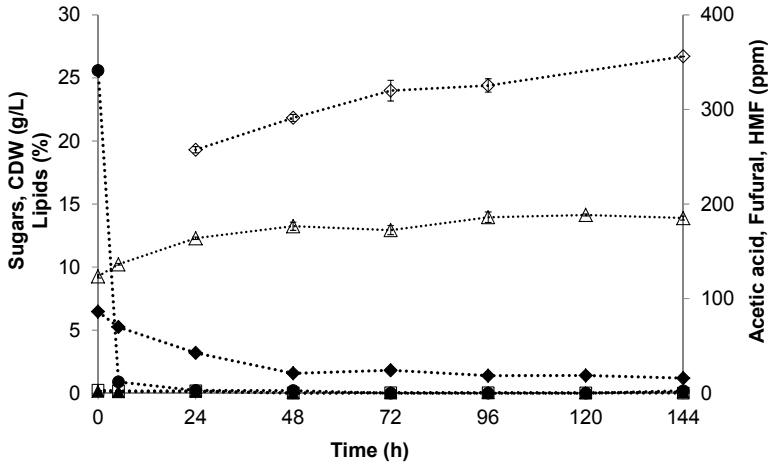

Figure 2: Continuous cultivation of L. starkeyi at 0.03 h^{-1} using H-H medium. (♦) Total sugars, (∆) CDW, (◊) Lipid content, (●) Acetic acid, (▲) Furfural, (□) HMF.

The presence of aldehydes, as such furfural and HMF, on the hemicellulosic hydrolysate can affect the cell growth and lipid accumulation. Interestingly, acetic acid, furfural and HMF, known as potential inhibitors for fermentation, apparently no suppressed the sugar consumption and cell growth on the H-H cultivation. These compounds were consumed or bioreduced by *L. starkeyi* during the fermentation of hydrolysate (Figures 1B and 2). The tolerance to aldehyde compounds is most likely due to the ability of microorganisms to convert these compounds to the corresponding less toxic alcohols (Nilsson A. et al., 2005) and, in general, this reduction happens in a prolonged lag phase (Li et al., 2011). Bioreduction of

furfural and HMF may shorten the lag phase highlighting the importance of prior adaptation of yeast to the hydrolysate. In general, the effects of furans can be explained by a re-direction of yeast energy to fixing the damage caused by furans and by reduced intracellular ATP and NAD(P)H levels, either by enzymatic inhibition or consumption/regeneration of cofactors (Almeida J. R. M. et al., 2007). This fact could explain the reduction of lipids accumulation when H-H was used as substrate in the batch cultivation.

However, the continuous feed of hydrolysate under nitrogen-limited condition could allow higher reductive activity of inhibitors favoring the lipids synthesis from the available sugars (Nilsson A. et al., 2005). Papanikolaou S. et al. (2002) reported that lipid production by *Y. lipolytica* was favored at low dilution rates during growth on raw glycerol under nitrogen-limited continuous cultures. The highest lipid productivity achieved was 0.12 g/L.h at dilution rate of 0.03 h^{-1}. In continuous cultures, the extent of substrates utilization and also biomass and lipid concentrations are the highest at low D values and decrease with the increase of D. Thus, C/N ratio and low D values must be thoroughly tuned to maximize lipid productivity of continuous processes (Rossi M. et al., 2011).

The fatty acid composition of lipids from *L. starkeyi* showed no remarkable changes for the batch and continuous cultivations of hydrolysate (Table 1). Oleic acid (C18:1) was the most abundant fatty acid of lipid, representing about 47% of total fatty acids, followed by palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (C18:2), palmitioleic acid (C16:1) and myristic acid (C14:0). However, the use of H-H, increased the content of palmitic acid and decreased the content of oleic acid, compared with the lipid composition obtained by studies of (Garzón, 2009), in our laboratory, when the same yeast was fed with xylose culture medium. The presence of organic acid, as acetic acid, on the medium is able to induce the bio-synthesis of saturated acids fatty acids. Huang C. et al. (2012b) reported that the contents of palmitic and stearic acids were increased when the *T. fermentans* was cultivated on presence of organics acid.

Microbial lipids from *L. starkeyi* have long-chained fatty acid containing between 14 and 18 carbons and low degree of unsaturation (Table 1). Differences in the relative proportions of palmitic and oleic acids are perhaps due to the differences in carbon sources and medium composition. The nature of substrate affects the composition of fatty acids in yeast lipids (Wild R. et al., 2010). The relative fatty acids composition of the lipids from HH obtained on this study is similar to vegetable oils, such as palm oil which mainly contains oleic acid (46.1%), palmitic acid (36.7%), linoleic acid (8.2%) (Ramos M.J. et al., 2009). Thus, lipids from *L. starkeyi* fermentation on H-H could be used for biodiesel.

Table 1. Comparison of fatty acids profiles of L. starkeyi on xylose and hydrolysate cultivations.

	Lipids of *L. starkeyi* (%, weight)						
Substrate	**C14:0**	**C16:0**	**C18:0**	**C16:1**	**C18:1**	**C18:2**	**Reference**
H-H (Batch)	-	27.8	6.0	3.7	48.2	10.1	This work
H-H (Continuous)	-	30.3	12.6	1.3	46.7	7.0	This work
Xylose	0.8	20.4	10.8	-	56.1	11.9	(Garzón, 2009)

5. Conclusions

Lipomyces starkeyi DSM 70296, previously adapted in our laboratory to H-H culture media, showed ability to consume sugars from non-detoxified hemicellulosic hydrolysate without appreciable inhibition. The continuous cultivation of H-H enhanced the accumulation of lipids at low dilution rate and under nitrogen-limited condition. The tolerance to HMF, furfural and acetic acid (normally considered cell growth inhibitors) here observed by our selected yeast indicates that it is not necessary to detoxify this H-H feedstock to be used as carbon source, increasing the feasibility of microbial oil production from lignocellulosic materials. The similarity of fatty acid composition of lipids produced by *L. starkeyi* with the vegetable oils shows the potential of this lipid to biodiesel production. In this way, sugarcane bagasse is a promising alternative feedstock for microbial oils production and provides the possibility of converting lignocellulosic materials into biodiesel or other chemicals

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