FEASIBILITY OF SUGARS CONSUMPTION FROM HEMICELLULOSE SUGARCANE BAGASSE HYDROLYSATE BY IMMOBILIZED PENTOSE CONVERTING YEASTS IN CALCIUM ALGINATE MATRIX

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ABSTRACT – Hemicellulose, a large available carbohydrate present in sugarcane bagasse, can offer great potential to be used as carbon source on Biotechnology process. However, specific yeasts to convert pentose sugars and operation system are key factors for the hemicellulose hydrolysate converting into bioproducts. This investigation presents the feasibility of sugars consumption from hemicellulosic sugarcane bagasse hydrolysate (HSBH) by two important pentose converting yeasts (*S. stipitis* NRRL Y-7124 and *S. shehatae* UFMG-HM 52.2) immobilized in calcium alginate matrix in different conditions. Results showed 99,64 % and 98,66% of sugars consumption in 48h by *S. stipitis* NRRL Y-7124, when experiments were conducted with 1% and 2% of sodium alginate in the immobilization step. The same experiments using the yeast *S. shehatae* UFMG-HM 52.2 achieved 99,2% and 98,3% in 72h, respectively. Assays showed the potential of these immobilized yeast by this technical for sugars consumption from HSBH.

1. INTRODUCTION

Lignocellulosic biomass, the most abundant raw material on Earth, stand out as an environmental friendly and sustainable carbon source in biotechnological process. These materials are basically composed of cellulose, hemicellulose, lignin, extractives, ash, and other compounds, whereas cellulose and hemicellulose (polymers of sugar monomers) represent around 70% of the total biomass (Balat; Balat, 2009). Thus, the potential of lignocellulosic materials as a sustainable raw material is directly related to its constitution, as can be seen some examples in Table 1.

Table 1 - Composition of different types of lignocellulosic materials

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Sugarcane bagasse	42.8	25.9	22.1	Silva *et al.* (2011)
Sugarcane Straw	40.8	30.8	25.8	Mouta *et al.* (2012)
Rice straw	43.4	22.9	17.2	Roberto *et al.* (2003)
Wheat Straw	33.0	33.0	20.0	Canilha *et al.* (2006)

However, the major obstacle for the use of lignocellulosic biomass is closely linked to its composition. Its three main components (cellulose, hemicellulose and lignin) are arranged in a crystalline structure that is an array of networks of cross-linking polysaccharides and lignin glycosylated proteins, hindering the availability of carbohydrates for fermentation process. (Zhao *et al.*, 2012). Through a pre-treatment step which involves a mechanism to disrupt lignocellulose structure, the fractions can be separated and fermentable sugars such as xylose, glucose and arabinose become accessible to bioconversion (Pandey *et al*. 2000). Pretreatments methods can be divided into physical, chemical, physico-chemical and biological (Sarkar *et al.*, 2012). Among many types of pretreatment, diluted acid hydrolysis is one of the most used, where hemicellulose fraction is released in hemicellulosic hydrolysates, composed primarily by xylose and other sugar monomers sugars. (Girio *et al.*, 2010).

For the use of hemicellulosic fraction, it is necessary microorganisms that convert pentoses sugars into bioproducts (Silva *et al*., 2012). The knowledge about pentose fermenting species is still limited. The search for new pentose-fermenting microorganisms is still a challenge to the viability of lignocellulosic materials use. However, some microorganisms such the yeasts *Scheffersomyces shehatae* (Antunes *et al*.,2013) and *Scheffersomyces stipitis* (Milessi *et al*.2012) have been showed potential for xylose assimilation.

Although seeking the industrial viability of lignocellulosic use, strategies that increase process productivity become interesting. The use of immobilized cells has many advantages such as the ease of separation and recovery of the biocatalyst, use of high cell densities and operation in repeated batches (Silva *et al.*, 2007; Zhao; Xia, 2010). However, the major disadvantage of this technique is related to the possible obstacles in the transport of substrates and products and limitations on the transfer of mass and oxygen.

Taking this into account, this work presents the pentose assimilation from sugarcane bagasse hemicellulosic hydrolysate by two pentose-fermenting yeasts immobilized in calcium alginate gel, *Scheffersomyces stipitis* NRRL Y-7124 (one of the most extensively studied pentose fermenting yeast) and *Scheffersomyces shehatae* UFMG HM-52.2 (a novel pentose fermenting yeast from Brazilian forest).

2. MATERIALS AND METHODS

2.1 Raw material and preparation of hemicellulose hydrolysate

Sugarcane bagasse was kindly provided by Usina São Francisco located in Sertaozinho/SP–Brazil. The hemicelulosic hydrolysate was prepared in hydrolysis reactor (200L) using H_2SO_4 98% as a catalyst in a ratio of 100 mg H_2SO_4/g of dry material for 20 minutes at 121°C. After hydrolysis, the reaction mixture was cooled, recovered and was stored at 4 °C. The hydrolysate was concentrated under reduced pressure by a concentrator with capacity of 32L at 70 °C in order to obtain a xylose concentration of 30 g/L. This concentrated hydrolysate was detoxified (in order to reduce inhibitors compounds generated at acid hydrolysis step) according to the method of Alves et al. (1998) that

involves three stages: raising the pH to 7.0 with calcium oxide; reduction of pH to 5.5 with phosphoric acid and addition of activated charcoal (2.4% w/v) followed by incubation for 1h at 200 rpm and 30 °C. After each stage, the hydrolysate was filtered under vacuum and finally it was autoclaved at 0.5 atm (110 °C) for 15 min and was used in the fermentation reactions.

2.2 Microorganism and inoculum preparation

The yeasts *Scheffersomyces stipitis* NRRL Y-7124 from EEL-USP culture colletion and *Scheffersomyces shehatae* UFMG-HM 52.2, isolated from Atlantic rain forest in Brazil and kindly provided by the Culture Collection of Microorganisms and Cells of the Federal University of Minas Gerais (UFMG), were used in all fermentations assays.

For the inoculum preparation, a loopful of stock culture was transferred to Erlenmeyer flasks of 125 ml containing 50 ml of synthetic medium composed of 30.0 g/L xylose, 10.0 g/L yeast extract and 20.0 g/L of peptone. Cells were grown in an incubator at 200 rpm and 30 ºC for 24 hrs. Following the 24 h growth, the fermented broth was centrifuged at 2000 x g for 20 min. Then, the cells were washed and resuspended in sterile distilled water and used in cell immobilization step.

2.3 Cell immobilization

The cells were immobilized by entrapment in calcium alginate gel. The cell suspension was added to a solution of sodium alginate (sodium salt of alginic acid from brown algae, Fluka analytical, Switzerland). Prior to cells addition, it was autoclaved at 111 °C for 15 min. The cell suspension (3.0 g/L of cells) was added into different concentration of alginate solution in order to obtain final concentration of 1% and 2% . Spheres of gel were produced by dripping this suspension in sterilized calcium chloride solution maintained under gentle stirring with a concentration of 0.10 M, The beads containing immobilized cells were maintained in calcium chloride solution at 4°C for 24 hours of conditioning time. After the exposure periods, the beads were washed by sterile distilled water and used in fermentative processes. Figure 1 shows the immobilization process.

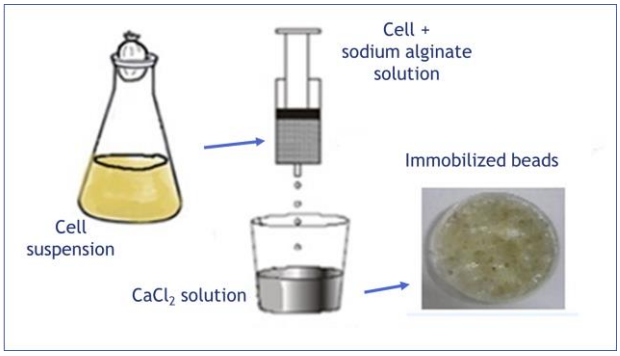

Figure 1 – Process showing the cell immobilization in Ca-alginate gel carrier

2.4 Fermentation medium and conditions

For the experiments with *S. stipitis*, hemicellulosic hydrolysate was supplemented with peptone (5.0 g/L), yeast extract (3.0 g/L), $CaCl_2$ (0.1 g/L) and $(NH_4)_2SO_4$ (2.0 g/L), to increase the nutrient value of medium for the sufficient growth of cells. For the yeast *S. shehatae*, hemicelllosic hydrolysate was supplemented with 5g/L of ammonia sulfate, 3 g/L of yeast extract and 5 g/L of malt extract.

All fermentation assays were carried out in 125 ml Erlenmeyer flasks containing 40 ml of production medium and 10 ml of immobilized biocatalyst. Fermentation assays were performed in an incubator shaker at 200 rpm and 30 °C for 96 h.

2.5 Analytical methods

Sugars (xylose and glucose) concentrations were analyzed by HPLC (Schimadzu LC-10 AD (Kyoto, Japan) with column equipped with BIO-RAD Aminex HPX-87H (300 × 7.8 mm) coupled to a detector of refractive index RID-6A, with eluent 0.01 N sulfuric acid at a flow rate of 0.6 $mL \cdot min^{-1}$, column temperature of 45°C and injected volume of 20 μL. Before HPLC analysis, the samples were filtered through Sep Pak C18 filter.

The photomicrographs were carried out by cutting session pellets of cells immobilized in calcium alginate and stained with methylene blue for light microscopy.

3. RESULTS AND DISCUSSION

Currently, biotechnology process aim the use of renewable sources of raw material and increase of productivity. Taking this into account, this investigation presents in Figure 2 the feasibility of sugars consumption from hemicellulosic sugarcane bagasse hydrolysate (HSBH) by two important native pentose consuming yeasts (*S. stipitis* NRRL Y-7124 and *S. shehatae* UFMG-HM 52.2) immobilized in calcium alginate matrix in different conditions.

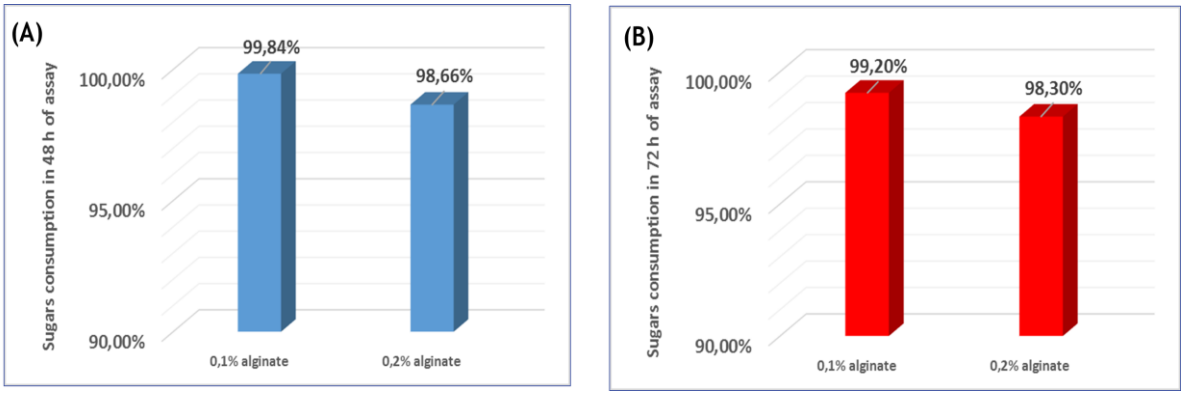

Figure 2 - Sugars consumption of sugarcane bagasse hemicellulosic hydrolysate by immobilized cells of *S. stipitis* NRRL Y-7124 (A) and S. *shehatae* UFMG-HM 52.2 (B).

It was observed for both microorganism a sugar consumption from sugarcane bagasse hemicellulosic hydrolysate close to 100% (*S. stipitis* NRRL Y-7124: 99,84% and 98,66%; *S. shehatae* UFMG-HM 52.2: 99,2% and 98,3%, for use of 1% and 2% of sodium alginate, respectively). These microorganisms are able to convert pentose sugars such as xylose present in HSBH into biomass or bioproducts.

Normally there are two pathways for this conversion. Depending on the microorganism, the metabolism of D-xylose can be initiated by conversion into D-xylulose catalyzed by the enzymes xylose reductase (XR) and xylitol dehydrogenase (XD) or by the action of the enzyme xylose isomerase (XI). For instance, yeasts like these carried out at this work as well *Candida guilliermondii* have XR and XD, while some fungus such *as Piromyces sp* has XI to assimilate xylose (Karhumaa *et al*., 2005). Followed conversion to D-xylulose, metabolism continues with the phosphorylation reaction catalyzed by the enzyme xylulokinase into xylulose 5-P. Thus, xylulose 5-P is metabolized in glycolytic pathway intermediates such as glyceraldehyde 3-P and fructose 6-P. Further, these compounds can be converted into pyruvate by the glycolytic pathway. (Toivari *et al*., 2001; Jeffries, 2006). We enhance that from this pentose sugar assimilation into the key compound pyruvate, biomass and bioproducts can be generated by different pathways.

Moreover, for the sugars consumption, it is necessary the access of nutrients between fermentation medium and the yeast. Systems with immobilized biocatalysts can be subjected to limitations nutrients supply of to the inner cells. The presence of heterogeneous materials can provide no convective flow within the immobilized array and the cells (RILEY et al. 1996). However, the cell immobilization into a solid matrix is an alternative that retains this characteristic with few loss of mass transfer. (Senthuran *et al.*, 1997). Regarding this feature and the simplicity of the method, entrapment in gel is one the most commonly used system of immobilization (Nigam, 2000).

The immobilization procedure is usually performed by gel a suspension of the biocatalyst in a monomer solution and the polymerization is initiated by a change in temperature or by addition of chemical reagent. (Meena and Raja, 2006). In this work, immobilized beads were provided by permutation of sodium ions with calcium ions in solution generating solidification of sodium alginate in solution of calcium chloride. This fact occured due the calcium ions forming cross-linked with the

alginate, increasing its viscosity to gel formation (Santos *et al*, 2005). Figure 3.A shows beads of immobilized cells while Figure 3.B shows microscope picture of S. *stipitis* NRRL Y-7124 into calcium alginate matrix.

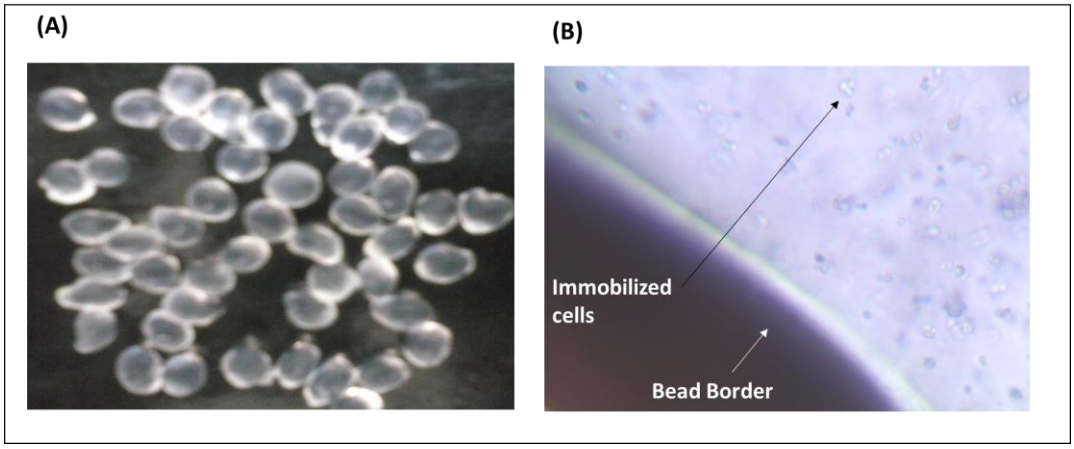

Figure 3 - (A) Immobilized cells of *S. stipitis* NRRL Y-7124 prepared by calcium alginate matrix. (B) Microscope picture of *S. stipitis* NRRL Y-7124 into calcium alginate matrix.

Once it was observed almost totally sugars used in this work for both yeast, we highlight the feasibility of the immobilization method. It was noted that *S. stiptis* consumed sugars faster than *S. shehatae* (48h and 72h, respectively). Once the immobilization technique was the same for both yeasts, this behavior can be attributed from the specific feasibility time of each microorganism to assimilate the carbohydrates when submitted to this method. It was not observed difference between sugars assimilation when used 1% or 2% of sodium alginate in the immobilization preparation regarding twice microorganisms. Regarding this fact, it can be used 1% of sodium alginate, in order of save costs if worked in large scale process.

4. CONCLUSION

This investigations presented the feasibility of sugars consumption from hemicellulosic sugarcane bagasse hydrolysate (HSBH) by two important pentose converting yeasts (*S. stipitis* NRRL Y-7124 and *S. shehatae* UFMG-HM 52.2) immobilized in calcium alginate matrix in different conditions (1% and 2% of sodium alginate). Results showed sugars consumption close to 100% for both yeasts in all establish conditions. Experiments showed the potential of these microorganisms when immobilized by this method for sugars consumption of HSBH, indicating its possible use for large scale process.

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