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Ethanol production by *Pichia stipitis* immobilized on sugarcane bagasse

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Abstract: The search for new ethanol production technologies is due to this biofuel being a renewable and environmentally friendly option. Immobilized cell systems for ethanol production have been studied; however, the phenomenon involved in cell sorption on raw materials has been poorly explored. Therefore, this work evaluates *P. stipitis* immobilization on sugarcane bagasse pre-treated with sulphuric acid, as well as ethanol production in batch culture. The results obtained showed that the Guggenheim-Anderson-de Boer (GAB) model explained the sorption phenomenon. The selected inoculum size for immobilization was the same as the monolayer sorption capability (1.17 gl^{-1}). Using 1:100 g ml^{-1} solid-liquid ratio, at 250 rpm, ethanol yield and productivity of 0.404 gg^{-1} glucose and 0.41 $gl^{-1}h^{-1}$ were obtained, respectively. The immobilized systems were stable for up to twenty-five repeated batches (36 h each). Ethanol production was increased from the first to the twenty-fifth batch (18.1 and 24.7 gl^{-1} ethanol). The use of complex media, such as molasses "B" or sugarcane hydrolyzates, caused an increase in process efficiency 2.4 and 1.8-fold respectively, compared with free cells systems. Biotechnological ethanol production from lignocellulosic hydrolyzates could be improved by the use of the immobilization cell sorption on pre-treated raw materials.

Keywords: yeast sorption, sugarcane bagasse hydrolyzate, molasses "B", ethanol production, sorption immobilization

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1 Introduction

Ethanol has been proposed as a renewable and environmentally friendly biofuel. In recent years, ethanol production has increased dramatically, along with the development of new technologies and processes [1]. The main feedstock employed for its production are sugars and starch [2]. About 95% ethanol production comes from agricultural products, so biotechnological processes are mainly involved. Cell immobilization could be an interesting option for alcoholic fermentation due to ease of handling, good operational stability and low raw material and process costs [3-5]. Additionally, the cells can be easily recovered for their use in other repeated batches [6]. Possible immobilization methods are cross-linking, entrapping, carrier-binding or a combination of these methods [7].

Fermentation processes using immobilized microbial cells have the advantages of increasing volumetric productivity and diminishing production costs. In spite of these advantages, there are several issues that had to be solved, such as the development of new cheaper carrier materials and flocculent strains, in order to mitigate industry resistance to the widespread adoption of such systems [4]. Surface adsorption appears to be more reasonable than membrane retention, microencapsulation or gel entrapment, as when the cells are immobilized by surface sorption, the yeast growth is not significantly affected and some yeasts can also be washed out from the system. The aim of using immobilized systems is related to decreasing bioreactor volume, thus diminishing capital investment [3]. The Guggenheim-Anderson-de Boer (GAB) isotherm model has advantages over others, as improvements by both Langmuir and Brunauer-Emmett-Teller (BET) physical adsorption theories have established it as a tenable basic theory. This model postulates that the state of sorbate molecules in the second layer is identical to the one in superior layers, but different from those of the liquid state [8]. Although the GAB model has been used

in food products (fruits and meats), it has not yet been used for yeast cell sorption.

Lignocellulosic residues such as sugarcane bagasse, rice straw, sorghum bagasse and some others have been employed for cell immobilization [3-4, 9-10]. Therefore, the search for new carrier materials to be used in microbial immobilization for industrial application, is an active topic [11]. Pre-treatments like delignification and diluted acid for raw materials have been proposed as a strategy in order to improve the immobilization process [12]; this causes damage to lignocellulose material integrity, therefore increasing the sorption phenomenon.

On the other hand, yeast strain selection is an important factor in increasing ethanol yield and reducing production costs, due to their higher fermentation performance (osmotolerance, reduced glycerol and foam formation, high viability and respiratory deficient phenotype) [13-15]. The use of respiratory deficient (RD) phenotype in yeasts could be interesting in immobilized cell systems as oxygen availability is less than in free cells systems; the use of this phenotype could therefore be an option in order to increase ethanol production. On the other hand, RD *Pichia stipitis* ACL 2-1 becomes interesting because of its ability to produce ethanol from glucose and xylose, although its acetic acid and 2-furaldehyde tolerance were not the highest compared to other *Pichia stipitis* RD strains [16]. Therefore, the aim of this work was to evaluate ethanol production by *Pichia stipitis* immobilized on sugarcane bagasse pre-treated with diluted sulphuric acid.

2 Methods

2.1 Microorganism

The RD *P. stipitis* ACL 2-1 employed in this study was obtained previously by acrylamide exposure [16].

2.2 Sugarcane bagasse pre-treatment

The sugarcane bagasse was obtained from "El Modelo" factory in January 2012. It was washed in order to eliminate remaining sucrose and subsequently sun-dried. Finally, it was sieved for size particle from 3.36 to 6.36 mm (Retsch Mod. 30) and dried to constant weight at 70°C. The dilute acid pre-treatment was carried out using 2% w/v sulphuric acid (H_2SO_4) at a 1:14 solid: liquid ratio, at 121°C for 40 min. Residual bagasse was washed using water until bagasse neutralization was achieved, and thereafter it was sundried again.

2.3 Culture media

2.3.1 Conservation medium

P. stipitis ACL 2-1 was stored at 4°C in a culture medium with the following composition: glucose, yeast extract and agar (20, 10 and 20 gl^{-1}, respectively).

2.3.2 Preculture and Kinetic medium

Preculture and kinetic medium contained glucose, yeast extract, KH_2PO_4, $(NH_4)_2SO_4$ and $MgSO_4 \bullet 7H_2O$ (60, 2.0, 5.0, 3.0 and 1.0 gl^{-1}, respectively). The initial pH was adjusted to 5.5 using 85% v/v ortho-phosphoric acid. The preculture was made in a 250 ml Erlenmeyer flask with 100 mL liquid medium, stirred at 150 rpm. After inoculation, each Erlenmeyer flask was incubated at 30°C for 24 h. Two precultures were prepared to obtain the inoculum.

2.4 Cell immobilization

Cell immobilization was carried out using 1 g pre-treated sugarcane bagasse (PTSB) sterilized at 121°C for 15 min. Previously, yeast cells were centrifuged at 4500g for 20 min at 15°C, and afterwards suspended in 9 gL^{-1} NaCl solution. After that, under aseptic conditions, an inoculum was added; the inoculum sizes evaluated were 0.27, 0.55, 1.10, 2.75, 6.00, 8.25 and 15.00 g dry cell × g $PTSB^{-1}$ × l^{-1}. In all cases 20 ml inoculum was added to 1 g PTSB. The samples were incubated at 30°C and 80 rpm. Sampling was carried out every 6 h for 24 h. Every sample was carried out using the whole flask, so there were 4 flasks (6, 12, 18 and 24 h) plus a duplicate, for each inoculum size. The adsorbed biomass on PTSB was calculated by dry weight with Whatman paper No. 2 using a vacuum system (Oaklon Mod WP-15) and dried at 70°C. The control sample was 9 gl^{-1} NaCl solution. Free cells were determined by direct cell count using a Thoma Chamber (Brand GmbH + CO KG, Germany) correlated to dry cell weight.

Cell retention on the bagasse (R, g/g) was calculated as the ratio of the mass of cells immobilized on the carrier (g) to carrier mass (g). Immobilized cell concentration (Xi, g/l) was calculated as the ratio of mass of cells immobilized on the carrier (g) to medium volume (l), 20 ml in each case. The mass of cells immobilized on the carrier was evaluated by the difference of cell dry weight between PTSB before and after cell immobilization. Immobilization efficiency (Yi, %) was calculated as the ratio of Xi to total cell concentration (suspended plus immobilized, X_T, g/l) multiplied by 100.

2.5 Guggenheim-Anderson-de Boer (GAB) Model

The experimental data found at equilibrium were modeled using the Guggenheim-Anderson-de Boer (GAB) model, according to the following equation 1:

$$X = \frac{X_m \alpha\, k\, c}{(1 - k\, c)(1 + \alpha\, k\, c - k\, c)} \quad \text{Eq.1}$$

where
X= the amount of cells free at equilibrium (g/L)
X_m= specifies monolayer sorption capability (g/l)
α = the energetic constant, a parameter related with the energy difference between the molecules of the first and other layers (cal/cal) or (energy sorption in first layer/ energy sorption of other layers)
k = measures the chemical potential difference between the molecules of the second layer and the liquid phase (cal/cal) or (energy sorption in the interphase/energy in the liquid phase)
c = the immobilized cells at equilibrium (g/g) or (cells in grams /grams of PTSB)

The parameters involved in this model can be calculated by non-linear regression, the equation obtained being: (Eq.2-5)

$$\frac{X}{c} = C_1 + C_2\, c + C_3\, c \quad \text{Eq.2}$$

where

$$k = -\frac{-C_2 \pm \sqrt{{C_2}^2 - 8C_1C_3}}{4C_1} \quad \text{Eq.3}$$

$$\propto = \frac{C_2 + 2C_1 k}{C_1 k} \quad \text{Eq.4}$$

and

$$X_m = \frac{1}{C_1 \propto k} \quad \text{Eq.5}$$

C_1, C_2 and C_3 are the coefficient values in equation 2 that were solved using the general quadratic equation.

2.6 Solid-liquid ratio and immobilization stability

The ratio of PTSB in grams and aseptic culture medium volume was denominated the solid-liquid ratio. These ratios were prepared using batch cultures in 500 mL flasks using 2 g pre-treated sugarcane bagasse with yeast cells immobilized under the established conditions. The solid-liquid ratios evaluated were 1:50, 1:75 and 1:100. The flasks were incubated at 150 or 250 rpm at 30°C using an incubator (New Brunswick Scientific classic series C24KC Refrigerated Incubator Shaker Edison NJ, USA). Immobilization stability was evaluated by twenty-five sequential cultures using the best solid-liquid ratio found. The culture medium was removed by decantation and fresh culture medium added. All the experiments were carried out in duplicate.

2.7 Free *vs* Immobilized cells

In order to compare free versus immobilized cell systems, batch kinetics were performed using the same inoculum in both cases (free and immobilized cells). Also the culture media was evaluated using glucose, diluted molasses "B" (from "El Modelo" factory, 2012) at 85 gl^{-1} total sugars and acid-hydrolyzed sugarcane bagasse obtained using 2% w/v sulphuric acid, 1:14 solid: liquid ratio at 121°C for 40 min and neutralized using NaOH. The hydrolyzed sugarcane bagasse contained 28, 3.6, 2.4 and 0.8 gl^{-1} of xylose, glucose, acetic acid and 2-furaldehyde, respectively. All these culture media were supplemented with the same salts and yeast extract as the previously mentioned culture medium.

2.8 Analytical techniques

Viability was obtained by the methylene blue staining method [17]. Additionally, the culture medium was centrifuged for 10 min at 10,000 rpm (Eppendorf Centrifuge 5424, Germany) and the supernatant stored at -20°C until analysis. Glucose, glycerol and ethanol were measured by a Waters 600 high performance liquid chromatograph (TSP Spectra System, Waters, Milford, MA, USA) using a Shodex SH1011 column (8 x 300 mm). The temperature was 45°C, 10 mM sulphuric acid mobile phase, 0.6 ml min^{-1} flow rate using an Index Refraction detector (Waters 2414, TSP Refracto Monitor V, Waters, Milford, MA, USA).

3 Results and discussion

3.1 Immobilization conditions

P. stipitis ACL 2-1 was immobilized on sugarcane bagasse previously pretreated with sulphuric acid. The inoculum employed was from 0.27 to 15 g dry cell l^{-1}. The greatest

cell retention was always obtained at 18 h independent of inoculum size (Figure 1). Increasing the inoculum size provokes an increase in cell retention from 0.003 to 0.141 g cells g^{-1} PTSB. However, immobilization efficiency was affected by inoculum size as well as contact time. Using 0.27 and 0.55 g dry cells l^{-1}, the highest efficiency was obtained at 6 and 12 h, respectively. For 1.10, 2.75, 6.00, 8.25 and 15.00 g dry cells l^{-1} inoculum sizes, the highest efficiency was obtained at 18 h. The results shown in Figure 2 demonstrate that increasing inoculum size causes an increase in cell retention. Immobilization efficiency remains unchanged at 64% from 0.27 to 8.25 g dry cell l^{-1}. The least immobilization efficiency (47%) was obtained with the greatest inoculum size (15 g dry cell l^{-1}), possibly due to sugarcane bagasse sorption places already being occupied, so no more yeast cells could be sorpted.

Figure 3 shows the experimental data and modelling with a correlation index (R^2) of 0.94. We found that X_m = 1.17 gl^{-1}, so lesser values cause monolayer sorption. The energetic constants α and k were 5.43 and 6.04, respectively. Although the assumptions of this isotherm included a uniform surface, the absence of interactions between sorpted molecules made it impossible to establish the physical meaning of these values because the system was more complex [18]; the phenomenon involved, however, should be the same. The 6.27 k value is comparable with that previously reported for *S. cerevisiae* ITV-01 RD immobilized on sugarcane bagasse pre-treated with sulphuric acid [18]. Cell immobilization conditions were 18 h contact time with a 1.17 g dry cell l^{-1} inoculum size, the X_m value. These conditions allowed the monolayer sorption to be already formed at the end

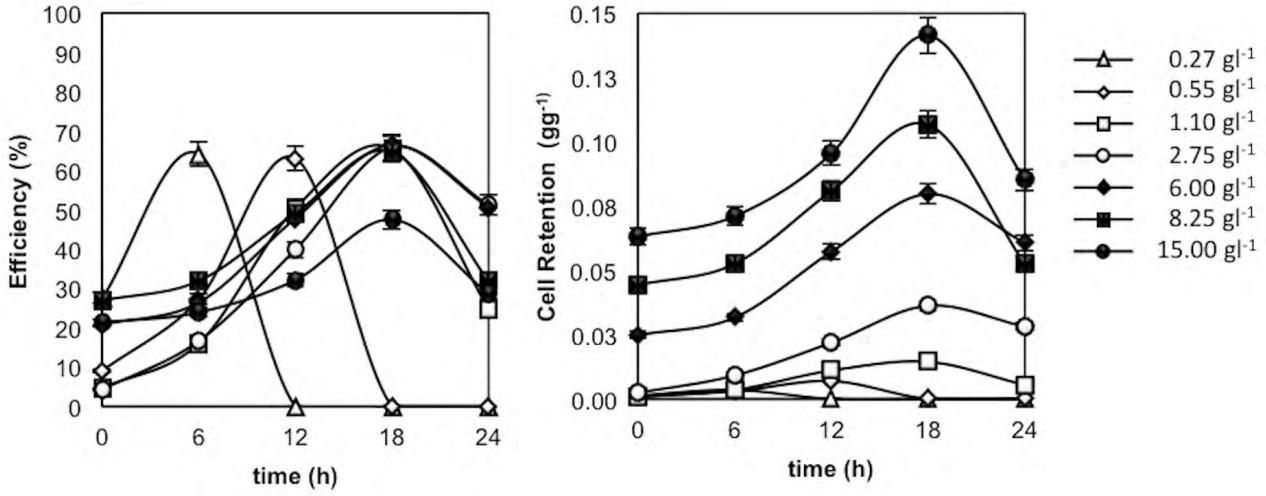

Figure 1. Effect of inoculum size on cell retention and immobilization efficiency of *Pichia stipitis* ACL 2-1 in sugarcane bagasse pre-treat with sulphuric acid.

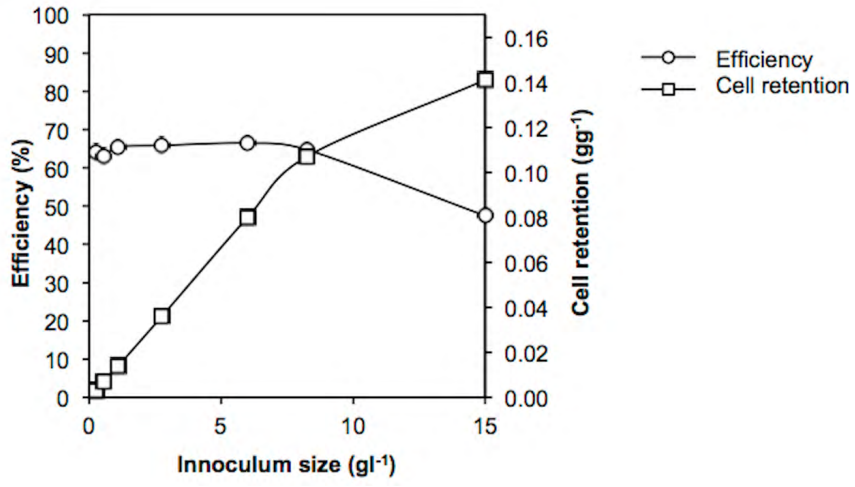

Figure 2. Effect of inoculum size on immobilization efficiency and cell retention of *P. stipitis* ACL 2-1 on sugarcane bagasse pretreated with sulphuric acid.

of immobilization process. The fermentation properties of PTSB could be improved modifying the pre-treatment employed; the damage to sugarcane bagasse structure could be greater in order to facilitate cell sorption, so X_m, α and k values should be modified.

3.2 Solid-liquid ratio

In order to evaluate the metabolic activity of yeast cells immobilized on sugarcane bagasse, the effect of the solid-liquid ratios were evaluated at 1:25, 1:50, 1:75 and 1:100 g bagasse ml^{-1} glucose culture medium, stirred at 150 rpm; the ratios of 1:75 and 1:100 g bagasse mL^{-1} culture medium were also evaluated at 250 rpm. The results showed that at 150 rpm, the highest ethanol yield ($Y_{p/s}$) was obtained using the 1:100 ratio. Fermentation time was 72 h and ethanol productivity (Q_p) was similar using both 1:75 and 1:100 ratios (Table 1). Previously, the solid: liquid ratio had been evaluated in *S. cerevisiae* ITV-01 RD, and the best solid: liquid ratio selected was also 1% [18]. In order to diminish fermentation time, agitation was increased from 150 to 250 rpm, and the results showed a reduction from 72 to 36 h. Furthermore, ethanol yield and productivity was increased from 0.408 gg^{-1} and 0.25 $gl^{-1}h^{-1}$ to 0.430 gg^{-1} and 0.404 $gl^{-1}h^{-1}$, respectively.

3.3 Immobilization stability

In order to evaluate cell immobilization stability, 25 repeated batches were carried out. Each batch was carried out using a 1:100 solid: liquid ratio for 36 h at 250 rpm. After that, the culture medium was removed and fresh medium added. The results are shown in Figure 4. All the parameters monitored remained unchanged after 25 batches although ethanol production increased, possibly due to *P. stipitis* sorption

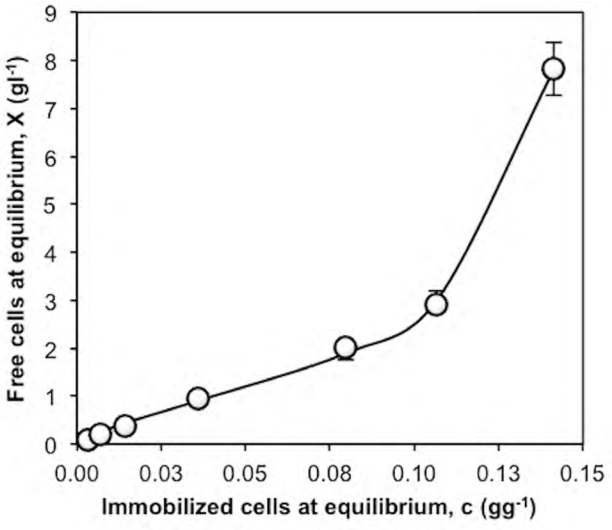

Figure 3. Experimental data adjusted using the GAB model.

continuing in a multilayer process or some ethanol remaining from a previous batch because PTSB was not washed. So *P. stipitis* ACL 2-1 immobilization on acid pre-treated sugarcane bagasse is stable up to 900 hours. The ability of yeasts cells to produce ethanol for many periods could be due to the changes in the plasmatic membrane increasing fatty acid composition, so that, membrane fluidity increases ethanol tolerance [3]. Also, it could be due to the yeasts modifying their cell wall composition, more precisely increasing the ratio of β 1-6: β 1-3 glucans, so that cell wall tortuosity increases as well as ethanol resistance [19].

This immobilization method is more stable than the one previously reported for thermotolerant *Saccharomyces cerevisiae* SV_3 immobilization on sugarcane stalks [20] because that was used for 25 batches, ethanol production increasing for each batch; during batches 9 to 13, however, ethanol production and productivity decreased. The mechanical strength of the immobilizing carrier is a very important phenomenon in repeated batch fermentation

Table 1. Effect of solid-liquid ratio on ethanol production by *P. stipitis* ACL 2-1 immobilized on sugarcane bagasse.

Solid-Liquid Ratio SLR (g ml^{-1})	Agitation (rpm)	Fermentation time (h)	Ethanol production P (gl^{-1})	Ethanol yield $Y_{p/s}$ (gg^{-1})	Ethanol productivity Q_p ($gl^{-1}h^{-1}$)	Residual glucose (gl^{-1})
1:25	150	72	4.0 ± 1.7	0.127 ± 0.04	0.055 ± 0.02	31 ± 0.32
1:50	150	72	11.5 ± 1.2	0.264 ± 0.03	0.160 ± 0.02	21 ± 0.35
1:75	150	72	18.9 ± 1.5	0.353 ± 0.03	0.262 ± 0.02	12 ± 0.07
1:100	150	72	18.0 ± 1.5	0.408 ± 0.02	0.250 ± 0.02	22 ± 0.55
1:75	250	66	14.6 ± 1.1	0.385 ± 0.06	0.222 ± 0.06	31 ± 1.38
1:100	250	36	16.9 ± 0.4	0.404 ± 0.02	0.470 ± 0.01	26 ± 0.74

based operations and is highly dependent on bioreactor volume. The higher the volume, the higher the damage inflicted on immobilization supports [20]. On the other hand, the immobilization method employed in this study showed better results than those previously reported [18] due to the increase in ethanol production for each batch; while using *S. cerevisiae*, however, no effect on ethanol production was obtained. This phenomenon could be related to the inoculum size employed for cell immobilization in *P. stipitis* ACL 2-1 being equal to monolayer sorption capability (X_m = 1.17 gl^{-1}), while in *S. cerevisiae* ITV-01 RD, that value was 1.7 times greater (X_m = 1.99 gl^{-1}).

3.4 Free vs Immobilized Cells

In order to compare the advantages of yeast cell immobilization, kinetics using free and immobilized cells were performed. Inoculum size was adjusted in order to obtain the same size in the free as in the immobilized system. Complete glucose consumption was reached at 36 h using the immobilized system while this was not possible using the free cell system (Table 2). In addition, ethanol production using the immobilized system was higher than in the free cell system (22 and 18.8 g ethanol l^{-1}, respectively) although ethanol yield remained unchanged (0.43 gg^{-1}). Ethanol productivity in the immobilized system was at least twice that of free cell systems. These results agree with those previously established which demonstrate that yeast sorption immobilization on sugarcane bagasse is a strategy for process performance improvement [3-4, 6, 12].

The use of diluted molasses "B" and hydrolyzed sugarcane bagasse was also evaluated. The results showed that sugar consumption and ethanol productivity were always higher in immobilized than in free cell systems. In spite of process efficiency, glucose use remained unchanged in both free and immobilized cell systems (84%); process efficiency using diluted molasses "B" and hydrolyzed sugarcane bagasse increased from 35 to 84% and 27 to 51%, respectively. This could be due to the immobilized system increasing the tolerance to stress conditions, like

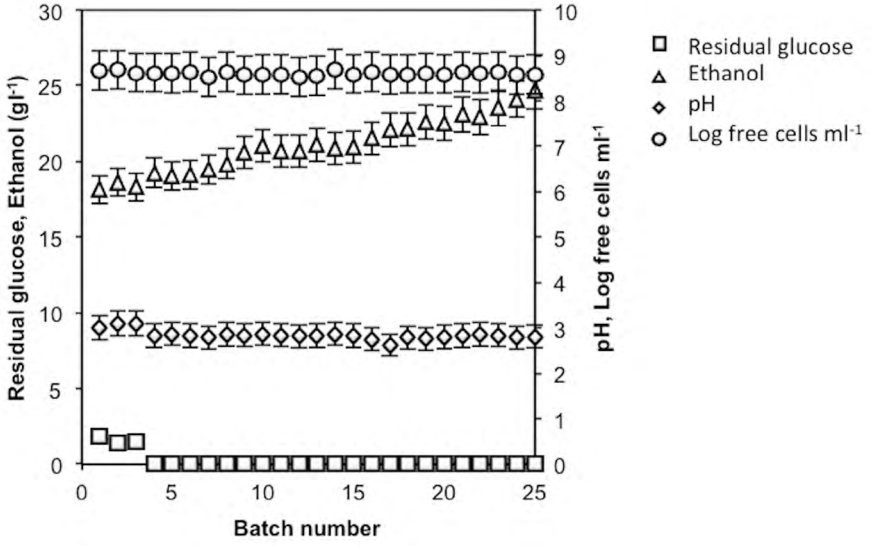

Figure 4. Immobilization stability of *P. stipitis* ACL 2-1 on sugarcane bagasse pre-treated with sulphuric acid.

Table 2. Comparison between free and immobilized cells using different culture media.

Culture media	Free Cells			Immobilized cells		
	Glucose	Molasses B	SBH*	Glucose	Molasses B	SBH*
Fermentation time (h)	72	60	60	36	60	60
Sugar consumption (%)	82 ± 2.3	68 ± 3.4	11 ± 0.5	100 ± 0.0	84 ± 2.8	15 ± 0.8
Ethanol yield, $Y_{p/s}$ (gg^{-1})	0.43 ± 0.01	0.18 ± 0.02	0.14 ± 0.01	0.43 ± 0.01	0.43 ± 0.02	0.26 ± 0.02
Ethanol productivity, Q_p ($gl^{-1}h^{-1}$)	0.26 ± 0.05	0.17 ± 0.03	0.01 ± 0.04	0.61 ± 0.06	0.49 ± 0.05	0.02 ± 0.01
Efficiency process (%)	84 ± 2.1	35 ± 4.2	27 ± 1.1	84 ± 2.1	84 ± 4.2	51 ± 4.2

*SBH: Sugarcane bagasse hydrolyzate

increasing osmotolerance (suspended solids in culture media by the use of diluted molasses "B") and tolerance to toxic compounds from lignocelullosic hydrolysis such as acetic acid and 2-furaldehyde (in the case of hydrolyzed sugarcane bagasse). Furthermore, studies should be carried out in order to establish the phenomenon involved that could be related with yeast sorption.

4 Conclusions

The Guggenheim-Anderson-de Boer (GAB) model explained the yeast sorption phenomenon. The selected inoculum for immobilization was 1.17 gl^{-1} because this is the monolayer sorption capability value. The 1:100 g ml^{-1} solid-liquid ratio, stirred at 250 rpm, produces an ethanol yield and productivity of 0.404 gg^{-1} and 0.41 $gl^{-1}h^{-1}$ respectively. Immobilized systems were stable for up to twenty-five repeated batches (36 h each one); at the end of the 25th repeated batch, ethanol production had increased compared with the first batch (18.1 and 24.7 gl^{-1}, respectively). Culture media with Molasses "B" and sugarcane bagasse hydrolyzates caused a 2.4 and 1.8 fold increase in process efficiency compared with free cell systems (84 and 35% in the case of immobilized systems and 51 and 27% for free cell systems, respectively). The use of immobilized cell systems showed advantages over free cell systems independent of the culture medium employed. Biotechnological ethanol production from lignocellulosic hydrolyzates could be improved by the use of immobilized cell systems on raw materials.

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Conflict of interest: Authors declare nothing to disclose.

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