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Rice husk (*Oryza sativa*) as support in the immobilization of yeast cells

Cascarilla de arroz (*Oryza sativa*) como soporte en la inmovilización de células de levadura

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Abstract

The present study aims to evaluate the rice husk (*Oryza sativa*; RH), using H_2SO_4 and NaOH (concentration of 2% v/v, solid to liquid ratio 1:6 and temperature and reaction time of 121 °C and 40 min, respectively, for both treatments) as pretreatment to determine if this material can be used as support in the immobilization of *Saccharomyces cerevisiae* cells. Fermentation kinetics were evaluated in cell buffer solution solid to liquid ratio 1:20 and sampling was performed every 8 h for 32 h. The highest cellular retention of RH pretreated with H_2SO_4 (PretAcid) was at 24 h (98.35 mg g^{-1}), while RH pretreated with NaOH (PretAlka), was at 16 h (63.21 mg g^{-1}), having a cell count of 4.5×10^7, and 1.2×10^7, cell per mg of rice husk respectively, meanwhile, the treatment that was not subjected to chemical treatment (Control), was at 24 h (8.56 mg g^{-1}) and 0.6×10^6 cell per mg of RH. On the other hand, the maximum support efficiency was reached at 36 h in the order of 44.99, 23.25 and 23.43 % for PretAcid, PretAlka and Control, respectively. These results indicate that due to the structural modification that RH underwent during chemical pretreatments, may be an ideal substrate for the adsorption of yeast cells.

Keywords: rice husk, cell immobilization, yeast cells, acid and alkaline pretreatment.

Resumen

El presente estudio tiene como objetivo evaluar la cascarilla de arroz (*Oryza sativa*; RH), utilizando H_2SO_4 y NaOH (concentración de 2% v/v, relación sólido:líquido 1:6 y temperatura y tiempo de reacción de 121 °C y 40 min, respectivamente, para ambos tratamientos) como pretratamiento para determinar si este material puede ser utilizado como soporte en la inmovilización de células de *Saccharomyces cerevisiae*. Se evaluaron cinéticas de fermentación en una solución tampón de células en relación sólido:líquido de 1:20 y se realizó un muestreo cada 8 h durante 32 h. La mayor retención celular de la RH pretratada con H_2SO_4 (PretAcid) fue a las 24 h (98.35 mg g^{-1}), mientras que la RH pretratada con NaOH (PretAlka), fue a las 16 h (63.21 mg g^{-1}), con un recuento celular de 4.5×10^7 y 1.2×10^7, células por mg de cascarilla de arroz, respectivamente, mientras tanto, el tratamiento que no fue sometido a tratamiento químico (Control), se alcanzó a las 24 h (8.56 mg g^{-1}) y 0.6×10^6 células por mg de RH. Por otra parte, la máxima eficiencia de soporte se alcanzó a las 36 h en el orden de 44.99, 23.25 y 23.43% para PretAcid, PretAlka y Control, respectivamente. Estos resultados indican que debido a la modificación estructural que sufrió la RH durante los pretratamientos químicos, esta puede ser un sustrato ideal para la adsorción de células de levadura.

Palabras clave: cascarilla de arroz, inmovilización de células, células de levadura, pretratamiento ácido y alcalino.

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1 Introduction

The rice husk (*Oryza sativa*) is a lignocellulosic waste used as a raw material in different biotechnological processes which through chemical or enzymatic treatments can be transformed into sugars capable of being assimilable by different microorganisms. The rice husk (RH) is a protective covering of rice grains that is separated during milling process, its size oscillates between 5 and 11 mm, in addition, it represents around 20% of rice weight and it is usually considered as waste where grain is processed and grown (Pode, 2016; Goodman, 2020). Traditionally, RH maintains its importance as a supply of chicken litter (Encalada-Paredes, 2011). Liberalesso *et al*. (2021) demonstrated the effects of using RH as an aggregate material in engineered substrates for extensive green roofs and findings that RH used like substrates on monocultivo of *Sedum rupestre* has the potential to improve some physicochemical properties, such as water holding capacity, bulk density, and porosity. Valverde (2007) states that rice husk has energy potential for generating clean energy, although other researchers affirm that this residue is sometimes handled as fuel, but as it has high resistance to fire, it is an inappropriate material for this purpose. Kamari and Ghorbani, (2020) conclude that the use of RH is a promising alternative for use as renewable energy or for the extraction of silica, in addition Lopéz-Ayala *et al*. (2021) mention that from the incineration of the RH, high purity SiO2 can be obtained with the aim of providing added value to this by-product and minimizing the damage to the environment caused by uncontrolled incineration of these wastes. In recent years, this residue has been used the construction industry as a geopolymer for partial replacement of cement and as an additive in manufacture of bricks due to its pozzolanic and thermal properties (Marques *et al*., 2021), RH stands out for its content of silicon dioxide nanoparticles in the ashes, unlike other organic fibers (Orrabalis *et al*., 2019), it is also used in decontamination processes and removal of dyes and heavy metals (Villada-Villada *et al*., 2014; Doria Herrera *et al*., 2016; Llanos-Páez *et al*., 2016; Rodríguez *et al*., 2019). In tests carried out by Piñeros Guerrero *et al*., (2020) found that RH has high activity as an antioxidant extract to obtain biodegradable films despite having a low content of total polyphenols that are highly reactive. On the other hand, Mirmohamadsadeghi and Karimi (2020) report that RH has a high content of cellulose (28.6-43.3%), hemicellulose (22.0-29.7%), lignin (19.2?24.4%) and ash (17?20%) which makes it in a material of low nutritional value, which is rarely used as animal food, due to its low digestibility (Goodman, 2020). Prada and Cortez, (2010) mention that it is difficult to disintegrate RH by biological means due to its high silicon content, however, in order to improve the accessibility and release fermentable sugars from intracellular wall of lignocellulosic residues, pretreatment with sulfuric, hydrochloric and nitric acid has been mainly used (Saucedo-Luna *et al*., 2010; Chen *et al*., 2015), causing the rupture of the recalcitrant structure so that microorganisms can access them (Shahabazuddin *et al*., 2018).

Pretreatment with dilute acid is considered one of the promising methods, because it improves anaerobic digestibility of lignocellulosic material, allows solubilization of hemicelluloses and makes the cellulose more accessible to enzymes, thus avoiding ethanol production (Hendriks and Zeeman, 2009), dilute sulfuric acid is the acid commonly used commercially to pretreat various lignocellulosic materials, such as those derived from corn (*Zea mays*; Li *et al*., 2016), rice straw (*O. sativa* ; Kim *et al*., 2013), among others.

Meanwhile, with the alkaline pretreatment a solvation and saponification reaction is carried out, which causes breakdown of the intermolecular ester bonds between hemicelluloses and lignin, causing a specific area of biomass increases and accessibility of carbohydrates to hydrolysis and fermentation processes heightens (Camesasca *et al*., 2015), however, this procedure is more favorable for biomass with low lignin content (herbaceous crops and agricultural residues) and less efficient for hardwoods (Baruah *et al*., 2018).

Cell immobilization is a biotechnology process where cells are located in a defined region of space to give rise to insoluble forms that retain their catalytic activity and that can be reused, in addition to improving their stability, which makes it possible to handle them in the chemical, pharmaceutical or food industries, in the treatment of waste or in the diagnosis and treatment of diseases (Kunthiphun *et al*., 2017; Maliki *et al*., 2021). In this sense, Martínez-Trujillo and García-Rivero (2012), focused their study on different supports for the immobilization of cells of different microorganisms for the degradation of pollutants, finding polyurethane as more efficient due to its resistance and high porosity. The commonly used yeast immobilization system is calcium alginate

beads (López-Menchero *et al*., 2021). The popularity of this system in alcoholic fermentation is usually due to its ease of preparation (Clementz *et al*., 2015), where the yeasts are mixed in a Na+ alginate solution and dripped into a solution with Ca^{2+} that makes droplets form spheres that contain cells (López-Menchero *et al*., 2021). Another method of cell immobilization is that by adsorption on solid supports, particularly those porous materials, such as those obtained from agricultural residues such as sugarcane bagasse (*Saccharum officinarum*; Sanchez-Herrera *et al*., 2018), corn cob (Manzoor *et al*. al., 2019), sorghum bagasse (*Sorghum bicolor*; Nuanpeng *et al*., 2018) which provide a large surface area and microtubes for cell entrapment; these lignocellulosic materials can be used as support material due to their low cost and simplicity of the microorganism immobilization procedure (Djordjevi? *et al*., 2017). Therefore, the objective of this study was to evaluate the retention capacity of the rice husk used as support in the immobilization of yeast cells to be used in fermentation kinetics.

2 Materials and methods

2.1 Support medium for immobilization

The rice husk (*O. sativa*) was donated by Arrocera del Mante S.A. de C.V., located on Carretera Nacional México-Laredo km 553 in El Mante city, Tamaulipas (Production autumn-winter 2019). The rice husk (RH) was stored directly in hermetically sealed containers to avoid fluctuations in humidity and until subsequent analysis.

2.2 Microorganisms

The yeast strain *Saccharomyces cerevisiae* encoded as "UAT-JC12" and used in this study was selected from the culture collection of the Unidad Académica Multidisciplinaria Mante of the Universidad Autónoma de Tamaulipas, isolated from clear cane juice, and preserved in glycerol and stored to -18 ° C.

2.3 Conservation and reactivation medium

A synthetic nutrient medium of yeast extract peptone dextrose (YPD-agar; Yeast extract 10 g L^{-1}, casein peptone 20 g L^{-1} and dextrose 20 g L^{-1}) was prepared for the reactivation of the UAT-JC12 strain. The microorganisms were activated by streak on slant tube technique and incubated in an orbital shaker (Brand IKA-KS 4000, USA) at 29 ± 1 ° C for 48 h. Subsequently, strains that showed normal and characteristic yeast growth were labeled and kept refrigerated at a temperature of 4 ± 1 ° C for later evaluation.

2.4 Characterization of rice husk

The moisture content of RH was determined by the gravimetric method, dehydrating the sample in an oven (FELISA Mod. FE-291, Jalisco, Mexico) at 105 ° C. Ash content was determined at 550 °C, using a muffle (Thermo Lyne 600, Model F6018, USA) (AOAC, 2010). While structural components (cellulose, hemicellulose and lignin) were calculated after quantitative acid hydrolysis according to the procedure described by Bustos *et al*. (2004), which consists on two stages of treatment with H_2SO_4. The first stage at 72% at 30 °C for 1 h., while the second stage was developed by diluting the medium until reaching a sulfuric acid concentration of 3% at 121 °C for 1 h. The solid residue after hydrolysis was considered as Klason's lignin. Liquors obtained were analyzed by high performance liquid chromatography (HPLC) (Waters Mod. 2695, Milford, USA), using a flow of 0.5 mL min^{-1}, ion-exclusion column 50 at 7 μm 7.8 x 300 mm, 37 ° C, IR detector (refractive index) at 40 ° C, mobile phase H_2SO_4 at 0.01 N, to know the concentration of sugars (glucose, xylose and arabinose) and acetic acid.

2.5 Acid and alkaline pretreatment

To obtain a greater number of cells adhered to the support, two types of pretreatment were tested, following the methodology described by Rezende *et al*. (2011) with slight changes consisting of a acid treatment, where RH ground was treated using a dilute solution of sulfuric acid (H_2SO_4) at 2% (v/v); solid to liquid ratio (S/L) 1:6 (g mL^{-1}) at 121 °C, for 40 min, since sulfuric acid has greater efficiency in dissolving hemicellulose (Geddes *et al*., 2010), subsequently, the pretreated solids (residual scale), were separated by filtration, rinsed with distilled water until obtaining a neutral pH and they were dried in an oven at 60 ° C for 12 hours until reaching a constant weight. While for the alkaline pretreatment a dilute solution of sodium hydroxide (NaOH) at 2% (v/v) was used, using the same S/L ratio, time, temperature and the subsequent conditioning of the residual scale as described for

acid pretreatment. Acid and alkaline pretreatment were coded "PretAcid" and "PretAlka", respectively. In order to compare the effect of the pretreatments, RH was used without a pretreatment using this as a control and it was coded as "Control".

2.6 *Immobilization kinetics*

2 g/100 mL of RH (PretAcid, PretAlka, Control) were weighed into a 125 mL Erlenmeyer flask and sterilized in an autoclave (Felisa, model FE-398, Mexico) at 121 ºC, 15 psi for 15 min. Subsequently, under aseptic conditions, 0.5×10^6 cell/support in buffer solution was added at a solid to liquid ratio of 1:20 and a sampling was carried out every 8 h for 32 h, the system was kept stirring at 100 rpm at a temperature of 30 °C.

2.7 *Contact time on cell retention and efficiency*

In order to evaluate the effect of the immobilization conditions (type of pretreatment used) on the number of cells that adhere to the tested supports (PretAcid, PretAlka, Control), an initial yeast cell concentration of 1 g L^{-1} was used taking as reference the retention values [mg of immobilized cells (g of support) $^{-1}$] and the % efficiency [immobilized cells (total cells) $^{-1}$], with established conditions of 30 °C and a stirring constant of 100 rpm.

2.8 *Biomass analysis*

During the immobilization process, solids from RH are suspended in the cell solution, which makes quantification of free cells by means of density measurement unreliable, therefore, a culture was carried out in synthetic medium and the growth was followed by counting cells under the microscope in a hemocytometer (Neubauer chamber), taking aliquots of known volume (10 mL) at different fermentation times (0, 6, 12, 18, 24 h). Subsequently, a linear regression analysis was performed relating the yeast dry weight data (g L^{-1}) as a function of the cell count (cells mL^{-1}) (Fig. 1).

The culture samples were diluted in order to count a maximum number of 300 cells and a minimum of 30 each time. Each count was made from *n* large squares of the Neubauer chamber. The volume of each square was 4×10^{-6} cells mL^{-1}. The cell concentration (X) per milliliter is given by equation 1.

$$X = \frac{N_d}{4_n} 1\times10^{-6} \tag{1}$$

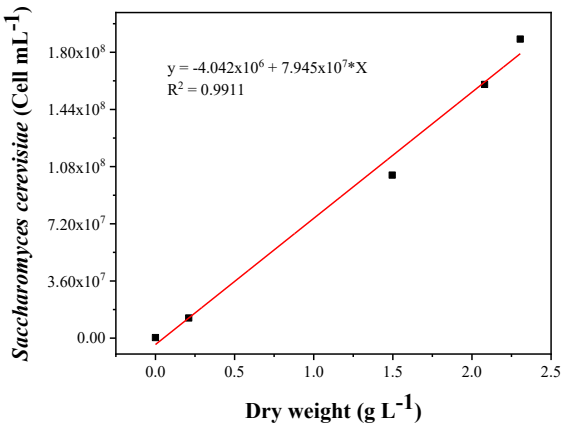

Figure 1. *Saccharomyces cerevisiae* cell count as in function of biomass dry weight. The solid line shows the linear relationship between these two variables.

where N is number of cells counted, d is dilution and n are number of frames counted in the Neubauer chamber.

2.9 *Water absorption index (WAI)*

The water absorption index (WAI) was determined using the method of Anderson *et al.*, (1970). The mixture (1.25 g of support) was suspended with a volume of 15 mL of distilled water in 50 mL Eppendorf^{TM} tubes, the suspension was stirred for 1 min. In a vortex at room temperature (25 °C) and centrifuged (Centrifuge 5810 R, Eppendorf, AG, Hamburg, Germany) at 5000 rpm for 10 min. The WAI was determined according to Equation 2:

$$WAI = \frac{F_l}{F_S} \tag{2}$$

where F_l is the liquid phase (g) and F_s is the solid phase (g).

2.10 *Analysis of data*

All experiments were carried out in triplicate of three independent experiments using a randomized experimental design and the values were expressed as mean values ± standard deviation and the data were subjected to simple classification analysis of variance and in relevant cases to comparison analysis of means by means of the Tukey test. Significance was established as $p \leq 0.05$. The data analysis was carried out in the statistical package Statgraphics Centurion XVI (StatPoint Technologies, Inc., Warrenton, Virginia, USA).

Table 1. Chemical composition of rice husk.

Components	Dry matter (%)*
Cellulose	32.37 ± 0.64
Hemicelluloses	9.30 ± 0.32
Xylan	6.72 ± 0.42
Araban	2.58 ± 0.03
Acetyl groups	1.00 ± 0.02
Lignin	32.94 ± 3.13
Other components	27.95 ± 3.63

*Average values ± standard deviation of tests performed in triplicate.

3 Results and discussion

3.1 Characterization of raw material

Moisture and ash content of the RH in this study was 7.25 ± 0.03 and 18.46 ± 0.02 respectively; these results are like those reported by Prada and Cortés (2010) for rice husks from the Colombian region, who report values of 7.94 ± 0.63% humidity and 17.80 ± 0.86% ash. High percentage of ash in RH can be an indication of high content of silicon oxide (SiO_2), which leads it to being a material with low biodegradability (Mirmohamadsadeghi and Karimi 2020).

Structural components of this agro industrial waste by quantitative acid hydrolysis are shown below. Cellulose and lignin were the main polymeric sugars found in RH, representing 65%, while non-cellulosic structural polysaccharides such as hemicellulose, represented around 10% (Table 1).

Results obtained in the quantitative acid hydrolysis show that the percentage content is close to those expressed by Kumar *et al.* (2010) who reported values of 31.12% cellulose, 22.34% lignin and 22.48% hemicelluloses. Ranges obtained for chemical analysis worldwide for RH are typically 25.89 to 43.3% for cellulose, 18.10 to 29.37% for hemicellulose and 18.20 to 24.6% for lignin (Valverde *et al.*, 2007; Mirmohamadsadeghi and Karimi, 2020). This variability in the results may be due to the color, durability, adherence, drying, discoloration and possible environmental problems of the region where the lignocellulosic material was obtained (Bahera *et al.*, 2014).

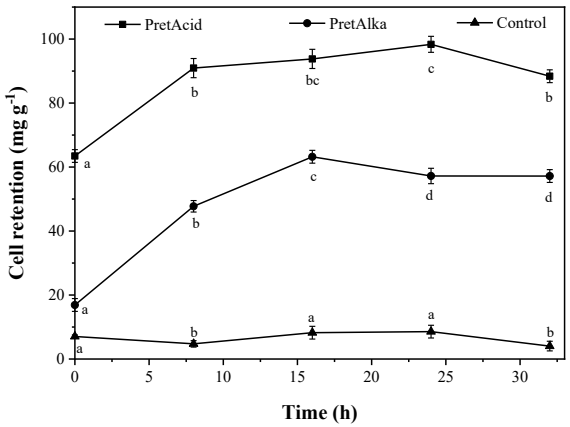

Figure 2. Effect of acid and alkaline pretreatment and control on cell retention in rice husk as function of time. Different lowercase letters indicate significant differences between means ($p \leq 0.05$).

3.2 Cell retention

The highest cell retention with acidic pretreatment (PretAcid) occurred at 24 h with a value of 98.35 mg of cells for each gram of rice husk (mg g^{-1}), while with the alkaline pretreatment (PretAlka) the highest retention of cells was reached at 16 h with a value of 63.21 mg g^{-1}, these values diminishing as time passed (24 and 32 h) until reaching 57 mg g^{-1}. In Fig. 2 it can be seen that the rice husk that was not subjected to a chemical pretreatment (Control) had a very low cell retention (approximately 8.0% at 16 or 24 h), thus showing that the first step for utilization of RH as raw material for fermentation processes is decomposition of structural mass, showing with this, that the pretreatment (acid or alkaline) alters the lignocellulosic material, changes the structure of the cell wall, increases the surface area, potentiates enzymatic hydrolysis and increases porosity and improves digestibility (Dagnino *et al.*, 2013; Sánchez-Herrera *et al.*, 2018). Hemicelluloses, due to their composition, as they present five-carbon sugars, are not easy to ferment by common microorganisms, therefore, a pretreatment stage is required to ?release? carbohydrates from the structural impediments they present (Hendriks and Zeeman 2009; Goodman, 2020).

3.3 Cell efficiency

Regarding the effect of pretreatment on cell efficiency, it was observed that the acid pretreatment obtained up to 45% at 32 h, while the alkaline treatment and

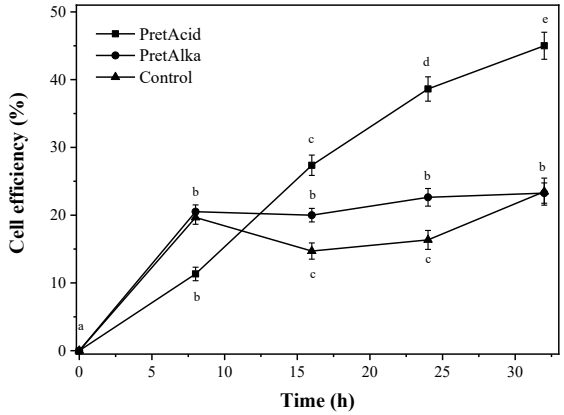

Figure 3. Effect of acid and alkaline pretreatment and control on cell efficiency in rice husk as function of time. Different lowercase letters indicate significant differences between means ($p \leq 0.05$).

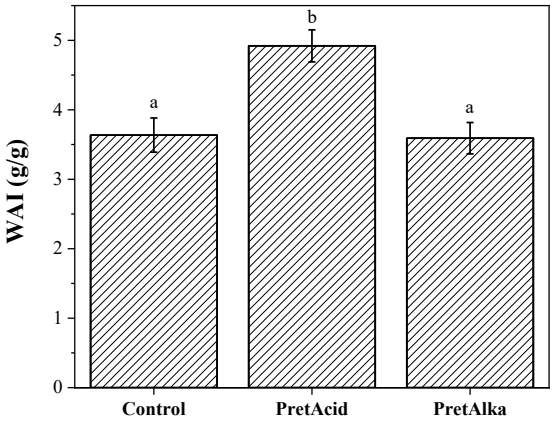

Figure 4. Water absorption index (WAI) g/g. Different lowercase letters indicate significant differences between means ($p \leq 0.05$).

the control had a similar behavior, presenting the highest cell efficiency at 8 and 32 h ($p \leq 0.05$), with values close to 23%, however, the Control exhibited a decrease in cell efficiency at 16 and 24 h, recovering its highest value at 32 h (Figure 3). Differences in cell efficiency between Control compared to PretAcid and PretAlka are since both, the acid pretreatment and the alkaline pretreatment increase the concentration of monosaccharides, the digestibility of the substrate, damage the lignin structure, improve the dissolution of hemicellulose and they help with decomposition of cellulose into simple sugars (Hendriks and Zeeman 2009; Kucharska *et al*., 2018).

3.4 Water absorption index (WAI)

According to the results achieved, PretAcid treatment generated the highest WAI (4.92 ± 0.23 g of water g^{-1} of support) followed by the Control (3.63 ± 0.25 g of water g^{-1} of support) and finally the one that exhibited the lowest WAI was PretAlka (3.59 ± 0.23 g of water g^{-1} of support), however, in these last two no significant differences were observed ($p \leq 0.05$) (Figure 4).

Kunthiphun *et al*., (2017) using cassava (Manihot esculenta) residues as a support in the immobilization of *Saccharomyces cerevisiae* by natural adsorption, reported values of 5.93 ± 0.22 g of water g^{-1} of residue in dry weight, these WAI values in cassava are similar to those found in this study for RH (PretAcid) and according to these authors, said IAA values indicate that this lignocellulosic material is suitable for using as a support in yeast cell immobilization.

Ekielski *et al*., (2020) used the water adsorption index to understand the impact on the sensory qualities of the composition and processing parameters of corn grain extrudates and propylene glycol on their physicochemical characteristics. For their part, Mussatto *et al*. (2009b) and Orzua *et al*. (2009) mention that the water absorption index is a highly relevant parameter that must be considered when evaluating the potential of different materials used as support in a solid-state fermentation. The monolayer sorption capacity is directly correlated to the water absorption index (g water/g dry matter). Mussatto *et al*. (2009a) and Brousse *et al*. (2012) reported that the water absorption index indicates the amount of water that can be absorbed by the support and correlates with the adsorption capacity of a support for cell immobilization and Cardoso *et al*., (2014) mention that the water adsorption properties relate the humidity and the energy of the product under conditions of relative humidity and temperature, they also infer that the amount of energy used to preserve the product in a suitable environment for long periods of time.

Some agro-industrial products present serious disposal problems and are the object of study to turn them into useful products as supports, such is the case of sawdust, which is used as support for the removal of pollutants or sugarcane bagasse also used as support together with a microbial source and nutrients that accelerate the degradation of hydrocarbons (Rodríguez *et al*., 2007). The use of organic waste is a priority to reduce the environmental impact caused by this waste, Mejías-Brizuela *et al*., 2016.

Conclusions

Rice husk is a lignocellulosic material that can be used as a support material in the immobilization of yeast cells, when their structure is modified by chemical processes. The pretreatment with sulfuric acid at 2% (v/v), allows the rice husk to have a cellular efficiency and water absorption index of more than 50% and 25%, respectively, compared to the control treatment. This makes it possible to potentiate the value of rice husk as an agro industrial waste to be used successfully in fermentation kinetics, when it is treated with dilute sulfuric acid solutions.

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