Chapter 11

Potential Production of Ethanol by *Saccharomyces cerevisiae* Immobilized and Coimmobilized with *Zymomonas mobilis*: Alternative for the Reuse of a Waste Organic

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Abstract

Fermentation technologies have been developed to improve the production of ethanol and an alternative is the immobilization technology, which offers the possibility of efficiently incorporating symbiotic bacteria in the same matrix. This study analyzes the potential use of immobilized and coinmobilized systems on beads of calcium alginate for ethanol production used mango waste (*Mangifera indica*) by *Zymomonas mobilis* and *Saccharomyces cerevisiae* compared with free cells culture and evaluate the effect of glucose concentration on productivity in coimmobilized system using a Chemostat reactor Ommi Culture Plus. For free cell culture, the productivity was higher for *Z. mobilis* (5.76 g L^{-1} h^{-1}) than for *S. cerevisiae* (5.29 g L^{-1} h^{-1}); while in coimmobilized culture, a higher productivity was obtained (8.80 g L^{-1} h^{-1}) with respect to immobilized cultures (8.45 g L^{-1} h^{-1} - 8.70 g L^{-1} h^{-1}). The conversion of glucose to ethanol for coimmobilized system was higher (6.91 mol ethanol) with 50 g L^{-1} of glucose compared to 200 g L^{-1} of glucose (5.82 mol ethanol); suggesting the immobilized and coimmobilized cultures compared with free cells offer an opportunity for the reuse of organic residues and high alcohol production.

Keywords: *Mangifera indica*, immobilization, coimmobilization, ethanol, *Zymomonas mobilis*, *Saccharomyces cerevisiae*

1. Introduction

1.1. Use of agroindustrial waste in fermentation processes

Alcoholic fermentation is a process by which microorganisms convert hexoses, mainly glucose, fructose, mannose and galactose, in the absence of oxygen and get products as alcohol (ethanol), carbon dioxide and adenosine triphosphate (ATP) molecules. Approximately 70% of the energy is released as heat and the remainder is preserved in two terminal phosphate bonds of ATP, for use in transfer reactions, such as activation of glucose (phosphorylation) and amino acids before of the polymerization. In other words, fermentation is a set of chemical reactions carried out by microorganisms in which an organic compound is oxidized, partially in the absence of oxygen to obtain chemical energy and understood as a partial oxidation when all the carbon atoms of the compound are oxidized to form CO_2. It is a process known since antiquity and is currently the only industrial process for the preparation of ethyl alcohol in all countries. The glucose as raw material is not only used, but other types of raw material much cheaper. However, the process of alcoholic fermentation occurs naturally, originated by the activity of some microorganisms through its anaerobic energy cell metabolism; for a large-scale production process, it is necessary for microorganisms (bacteria, fungi and yeasts) to accelerate the process of alcoholic fermentation and increase the conversion rate [1]. During the twentieth century and until the beginning of the twenty-first century, alcoholic fermentation has focused exclusively on the improvement of fermentation processes and specifically on the optimization of industrial performance through a good selection of yeast strains, which are the most used microorganisms for the production of ethanol by fermentation, due to its high productivity in the conversion of sugars and better separation of the biomass after fermentation. Yeasts are unicellular (usually spherical) microorganisms of size 2–4 μm and are present naturally in some products such as fruits, cereals and vegetables. Different species of fermentative microorganisms have been identified, among which are mainly *Saccharomyces cerevisiae, Kluyveromy cesfragilis, Torulaspora* and *Zymomonas mobilis* [2].

S. cerevisiae is a unicellular organism that is able to follow two metabolic routes to obtain the energy necessary to carry out its vital processes: alcoholic fermentation and aerobic respiration. The first is characterized by the evolution of CO_2 and the production of ethanol out of contact with oxygen, obtaining the energy necessary to carry out its vital processes from metabolizing carbohydrates. The yeast requires glucose to be catalyzed by the glycolysis or Embden-Meyerhof pathway, to obtain pyruvate that is then converted by anaerobically into ethanol and CO_2 by the action of specific enzymes. Its optimal temperature of growth varies between 22 and 29°C and does not survive more than 53°C. It ferments a sugar solution with a concentration of less than 12% and is inactivated when the sugar concentration exceeds 15% due to the osmotic pressure of medium on the cell. On the other hand, *Z. mobilis* is a facultative anaerobic gram-negative bacterium that can ferment certain sugars through a metabolic pathway producing bioethanol, sometimes, more efficiently than yeasts. It has an incomplete Krebs cycle, but it has characteristics to perform the pyruvic synthesis pathways from glucose or glyceraldehyde-3-phosphate. This organism also shows a high rate of sugar uptake and a yield of ethanol as fuel of the 97% [3].

The alcoholic fermentation processes using agroindustrial products present a great challenge given the inconveniences that could arise when using raw material for human consumption or edible vegetable crops for the production of ethanol, and, on the other hand, the change in the use of land destined for the cultivation of vegetables that will be used to produce ethanol and bioethanol, which would sometimes lead to deforestation, food shortages, increase of desert regions and greater inability of soils to retain water, thus disrupting the balance of the hydrological cycle [4].

On a global scale, the use of energy raw materials for energy purposes and in the production of ethanol has led to higher prices for products such as maize or barley, as well as making ethanol production economically unviable. Therefore, it is important to use raw materials that do not compete with food products and that are low cost in the production of biofuels, must also ensure a good profitability and are environmentally sustainable projects. In the energy sector, it has been estimated that the use of all world food surpluses could only produce bioethanol to replace 1% of the oil currently used. Concluding that if food crops were used to produce ethanol, a chain of food imbalances would be generated, which would be unsustainable [5].

An alternative to producing ethanol is through the use of other nontraditional raw materials, which arise as by-products and/or waste from industrial processes. Propose new technologies that allow the production of ethanol from cane residues, solid waste and those materials containing cellulose and hemicellulose, which allows the revalorization of waste from various industries, converting them into raw material for the production of ethanol.

At present, efforts have been made mainly in the search for cheap raw materials, which replace the traditional ones, in order to achieve greater efficiency in the processes of fermentation, recovery and purification of alcohol produced. The importance of the production of bioethanol has as main interest to compete with the use of fossil fuels since ethanol can be used as fuel for motor vehicles increasing the octane number, and therefore the reduction of consumption and contaminants (10–15% less carbon monoxide and hydrocarbons). Ethanol can be mixed with unleaded gasoline from 10 to 25% without difficulty, although some engines have been able to incorporate 100% alcohol as fuel. Thus, ethanol could substitute for methyl tert-butyl ether (MTBE), an oxygenation product with which gasolines have been reformulated in Mexico since 1989, which has reduced CO_2 emissions. This action is very important since MTBE, being a very stable compound, with low degradation and very soluble in water.

The production of bioethanol lost importance at the end of the first half of the XX century, being replaced by the production of synthetic ethanol, from petroleum derivatives, which is cheaper, but cannot be used in food preparation, alcoholic beverages or medications. The rise in oil prices turned our eyes toward the fermentation route of ethanol production, and today, we work mainly in the search for cheap raw materials, replacing the traditional sugary materials. Studies carried out by different researchers suggest that the by-products of mango juice, cane juice and molasses are an efficient alternative for the production of ethanol without affecting the food item, besides increasing the productivity and concentration of ethanol in the fermentation medium, and therefore reduce the costs of ethanol production [6].

Historically, the sugar industry in Mexico is one of the most important, characterized by sugarcane harvests throughout the year, with a production of cane of 46,231,229 tons per year, and the remaining residue derived of sugarcane has been exploited as energy biomass and

for the production of different biotechnological products by fermentation. Other alternatives of raw material are mango juice and its residues, and the fruit is grown in all the countries of Latin America, Mexico being the main exporting country of this fruit, with an annual production of approximately 1 million 452 thousand tons of mango and of which more than 60% of this production is given to the South-Southeast region of our country.

The alternative of using residues or products that can replace the raw materials normally used in ethanol production is now a highly promising possibility, because the cost of production of ethanol is closely related and dependent on the cost of the raw material, the volume and the composition of the same. The existing economy in Mexico related to cane cultivation (experience and sugar tradition) and the export of mango types offers technological alternatives that allow the fermentation of cane juice, molasses and mango juice through *S. cerevisiae* and *Z. Mobilis* as viable sources for the production of ethanol, whether in the manufacture of alcoholic beverages or for the production of biofuels.

1.2. Tecnología de inmovilización

Research has been developed in order to increase the productivity of alcoholic fermentation processes. The productivity, expressed as grams of ethanol produced per hour per unit of fermentation volume, can be increased by optimizing the composition of the culture medium, by the selection of an appropriate microorganism strain or through the adaptation of the design of reactors [7]. One challenge today is to reduce ethanol production costs, and an alternative is to reduce the cost of the culture media, which can represent about 30% of the final production costs of ethanol [8].

Some fermentation technologies have been developed to improve the production of ethanol and its concentration in the culture media [9, 10]. Among these, the immobilization technology offers advantages in contrast to free cell cultures, such as increased retention time in bioreactors, high cellular metabolic activity, high cell load and protection for cells from stress [10, 11]. The immobilization cell technologies have been applied for different purposes as for the production of hydrogen [12] and compounds commercially used in the food industry [13]. Other studies have been developed with immobilized algal cells to remove nutrients (N and P) from wastewater, phenol and hexavalent chromium [14–17]. Similarly, the immobilization of *Zymomonas* and *Saccharomyces* have been used for the bio-ethanol production from waste materials [7–10, 18–20].

On the other hand, the immobilization technology provides the possibility of efficiently incorporating symbiotic bacteria [21, 22]. The interaction between two microorganisms in the same matrix is called coimmobilization, and this association can be positive with higher growth and production. However, there are relatively less applications in the ethanol production involving the immobilization of mixed-culture systems and/or coimmobilized cultures.

In a petroleum deficiency situation, bioethanol from yeast and bacterial fermentation has become a promising alternative source for fuel. Agricultural and industrial waste containing sugar, starch and cellulose, such as cassava peels, fruit bunches, and the effluents from sugar and pineapple

cannery productions have been successfully applied for the bioethanol production [23, 24]. In this context, the municipality of Ciudad del Carmen, Campeche, Mexico, has an annual production of about 2.868 ha mango (*Mangifera indica*), obtained through various forms of cultivation and orchard-based technology, but the lack of local market and the poor product distribution to other locations cause much of the product be wasted, with significant losses in the locality. Hence, the need to seek alternatives to use these wastes and generate added value in the economy of the region.

This study was to determine whether the association between *S. cerevisiae* coimmobilized with *Z. mobilis* improved growth, and ethanol production using a culture medium equivalent to mango juice (*M. indica*) creates an opportunity for a regional fruit for exploitation in the production of ethanol. In this study, both microorganisms were confined in small alginate beads, a practical means of using microorganisms for environmental applications.

2. Materials and methods

2.1. Microorganism and medium

The yeast strain *S. cerevisiae* (ATCC® 2601) and bacteria *Z. mobilis* (ATCC® 8938) were obtained from the laboratory Microbiologis® and used for fermentation in coimmobilized and immobilized systems. Both microorganisms were cultured in a medium containing composition (g L^{-1}), as described by Demirci et al. [25]: 20 g glucose, 6 g yeast extract, 0.23 g $CaCl_2 \bullet 2H_2O$, 4g $(NH_4)_2SO_4$, 1 g $MgSO_4 \bullet 7H_2O$ and 1.5 g of KH_2PO_4, previously sterilized by autoclave. Strains were maintained in 250 mL of culture at 30°C and pH 4.5 with manual shaking three times a day. Transfers of fresh medium were made every 24 h for three consecutive days prior to use in experiments.

2.2. Preparation of immobilized and coimmobilized cells

For the preparation of immobilized cells, we used the technique described by Tam and Wong [26]. Both microorganisms were harvested by centrifugation at 3500 rpm for 10 min. The bacteria and yeast cells were resuspended in 50 mL of distilled water to form a concentrated cell suspension. The suspension was then mixed with a 4% sodium alginate solution in 1:1 volume ratio to obtain a mixture of 2% microorganism–alginate suspension. The mixture was transferred to a 50-mL burette, and drops were formed when "titrated" into a calcium chloride solution (2%). This method produced approximately 6500 uniform algal beads of approximately 2.5 mm in diameter with biomass content for *Z. mobilis*-alginate of 0.0055 g $bead^{-1}$ and for *S. cerevisiae* of 0.00317 g $bead^{-1}$ for every 100 mL of the microorganism–alginate mixture (**Figure 1**).

The beads were kept for hardening in the $CaCl_2$ solution for 4 h at 25 ± 2°C and then rinsed with sterile saline solution (0.85% NaCl) and subsequently with distilled water. A concentration of 2.6 beads mL^{-1} of medium (equivalent to 1:25 bead: medium v/v) was placed in a Chemostat

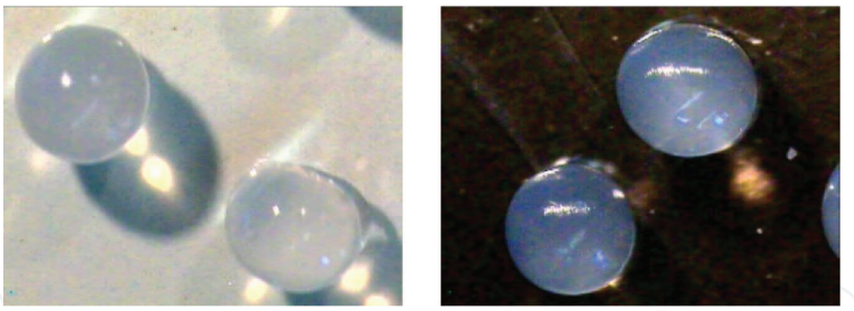

Figure 1. Microorganism–alginate beads suspension.

Ommi Culture Plus (Virtis) containing 2 L of culture medium. The reactor was maintained under stirring at 120 rpm and 30°C (**Figure 2**). A similar procedure was used for coimmobilization, with the difference that the concentrate of bacteria (25 mL) and yeast (25 mL) was mixed and then mixed with 50 mL of alginate; this procedure allowed retaining the same concentration of cells in all experiments.

Figure 2. Chemostat Ommi Culture Plus (Virtis) used for fermentation experimental process.

2.3. Experimental setup and procedure

This study was divided into two parts: (1) the batch experiment consisted of evaluating the growth and ethanol production in a medium equivalent to mango juice in cultures with free cells, immobilized and coimmobilized, and (2) evaluate the effect of glucose concentration in the production of ethanol in the system previously selected in the first experimental part based on the ethanol productivity obtained. Fermentation was performed in a Chemostat Ommi Culture Plus (Virtis) with a volume of 2 L operation, adjusting stirring at 120 rpm and maintaining a temperature of 30°C. The medium equivalent to mango juice was similar to that described by Demirci et al. [25] by adjusting the composition of the medium to a concentration of 200 g L^{-1} glucose, equivalent to that observed in the mango juice (*M. indica*).

The experimental design consisted of triplicate cultures in a Chemostat reactor Ommi Culture Plus for *S. cerevisiae* and *Z. mobilis* in free cell culture, immobilized and coimmobilized. For each experiment, the biomass was collected, as well as samples of the culture medium to the end of the logarithmic phase every 20 h. For the determination of ash-free dry weights, five beads were dissolved and filtered through a GF-C glass fiber filter (2.5 cm diameter), previously rinsed with distilled water, and incinerated at 470°C for 4 h. The samples were dried at 120°C and put to constant weight for 2 h in a conventional oven and then in a muffle furnace at 450°C for 3 h. The soluble solids of each fermenting medium were determined every 20 h by taking 1 mL aliquot from each reactor and testing for the Brix level in an refractometer.

Ethanol content (% v/v) was obtained using the Anton Paar DMA 4100M instrument, which determines the density of the mixture in relation to the standard OIML-STD-90, which can determine the content of distillate ethanol (% v/v); according to the ethanol density recorded, it was possible to obtain an approximate of ethanol content (grams of ethanol per liter of culture) produced for each experiment. Prior to the determination of the ethanol content, a distillation of cultures was conducted with a plate column distiller PS-DA-005/PE of four plates, at small scale. The cooling water flow was 3 L h^{-1} at 15°C. An aliquot of 3 L was distilled for 4 h, maintaining the operating conditions at atmospheric pressure, without reflux and with a temperature ramp in the heating jacket of 30°C up to 80°C.

The STATISTICA 7.0 software for statistical analysis and calculated mean and standard deviation for each treatment were used. The covariance analysis (ANCOVA) with $P \leq 0.05$ was used to evaluate the growth in free cell cultures, immobilized and coimmobilized. The Tukey test ($P \leq 0.05$) was used when significant differences were observed.

3. Results

3.1. Growth

In free cell cultures, the growth was observed immediately after being inoculated in the reactor of 2 L. Growth kinetics shows an exponential phase for *S. cerevisiae* and *Z. mobilis* of 120 h. After this period of cultivation, both species showed a decline in the production of biomass, finalizing treatment after 200 h of culture. The maximum values of biomass concentration

were 14.18 and 11.80 g L^{-1} dry weight for *S. cerevisiae* and *Z. mobilis*, respectively. Both microorganisms grew satisfactorily under the culture conditions used in this study (**Figure 3A**), with a higher growth rate (μ) for *S. cerevisiae* (0.0547 d^{-1}) with respect to *Z. mobilis* (0.0418 d^{-1}). Growth rates in free cell cultures for both microorganisms *S. cerevisiae* and *Z. mobilis* were not significantly different ($P \geq 0.05$).

For immobilized cells, both yeast and bacteria presented immediate growth after adding the beads to the culture medium; in both treatments, the exponential phase of growth reached a maximum of 80 h. It is noteworthy that although both microorganisms were immobilized under the same procedure, the content of biomass per bead at the beginning of treatment was lower for *Z. mobilis* (0.0031 g $bead^{-1}$) compared to *S. cerevisiae* (0.0039 g $bead^{-1}$). Despite these differences, both microorganisms were able to tolerate immobilization (**Figure 3B**), reaching maximum biomass content values of 0.0055 and 0.0047 g $bead^{-1}$ for *S. cerevisiae* and *Z. mobilis*, respectively. In relation to growth, *Z. mobilis* showed a higher growth rate (0.142 d^{-1}) with respect to *S. cerevisiae* (0.106 d^{-1}), but there were no significant differences ($P \leq 0.0001$).

3.2. Glucose-substrate removal

The decrease of substrate showed significant differences ($P \leq 0.0001$) between treatments with free and immobilized cells for both species (**Figure 4**). However, the Tukey test analysis showed that the two species in free culture were not significantly different ($P > 0.05$) in 200 h of treatment. While for the immobilized and coimmobilized cell cultures, only the immobilized *Z. mobilis* bacteria showed no significant differences ($P = 0.245$) during removal of the substrate with the coimmobilized system during 140 h of culture (**Figure 4B**).

It is a fact that consumed substrate was greater in free culture for *S. cerevisiae* and *Z. mobilis* from 200 to 80 g L^{-1} (60% removal) after the 200-h treatment period (**Figure 4A**), compared to the immobilized system with 40% removal for *S. cerevisiae* (from 200 to 120 g L^{-1}) and 30% removal for *Z. mobilis* (from 200 to 140 g L^{-1}), while in those cultures of coimmobilized cells consumption ranged from 200 to 130 g L^{-1} (35% removal) (**Figure 4B**).

The average consumption analysis based on removal rates determined during the exponential growth for both species showed that free culture *S. cerevisiae* and *Z. mobilis* reached removal rates of 2.0 and 2.7 g-substrate per g-biomass d, respectively. This suggested greater productivity for the bacteria (5.76 g h^{-1}) with respect to yeast (5.29 g h^{-1}) (**Table 1**).

In cultures with immobilized cells, the removal rate in the exponential phase (80 h) was greater for *S. cerevisiae* (0.165 g-substrate per g-biomass d) with respect to *Z. mobilis* (0.056 g-substrate per g-biomass d), but in coimmobilized culture it was greater (0.235 g-substrate per g-biomass d) since both species contribute to reducing glucose and increasing the removal rate. Similar results were observed in the productivity, where the coimmobilized cell culture showed higher values (8.80 g L^{-1} h^{-1}) with respect to the immobilized cells of *S. cerevisiae* (8.45 g L^{-1} h^{-1}) and *Z. mobilis* (8.70 g L^{-1} h^{-1} (**Table 1**). In general, the highest productivity levels were recorded in coimmobilized and immobilized cultures with respect to free cell cultures because shorter ethanol production time (80 h) compared to free cultures (120 h).

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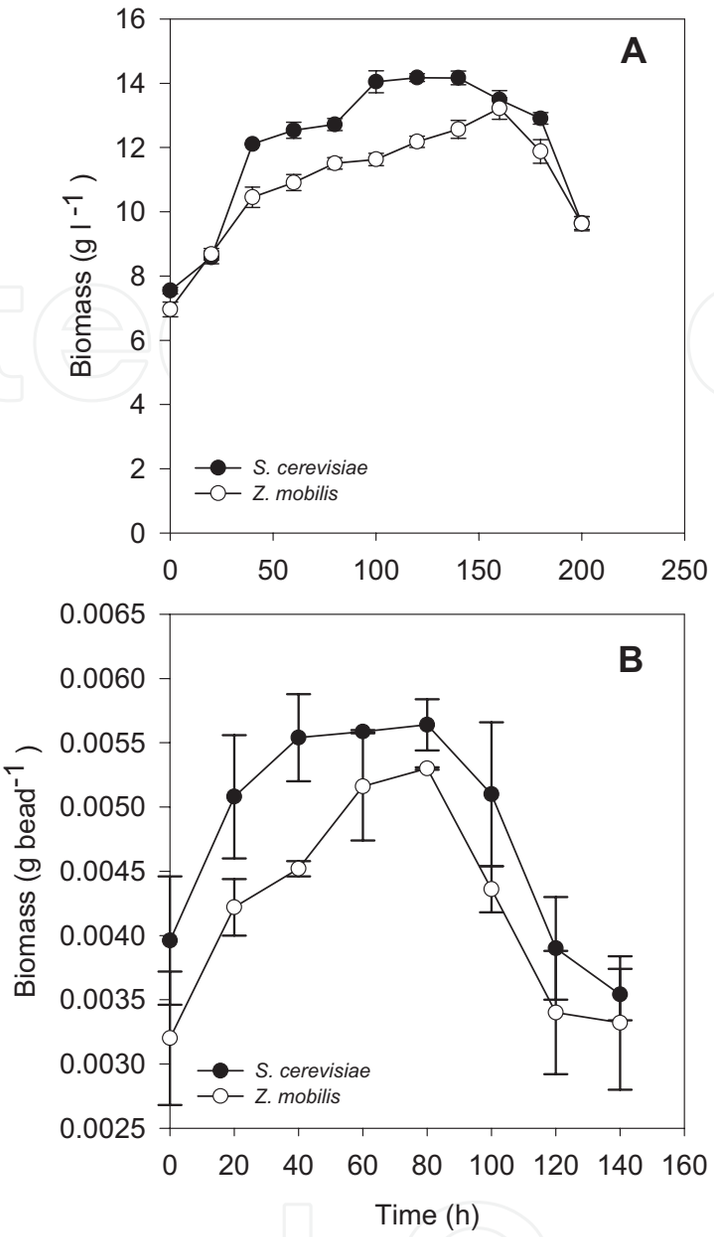

Figure 3. Average increase of biomass for *Saccharomyces cerevisiae* and *Zymomonas mobilis* in free culture (A) and immobilized cells (B).

3.3. Effect of initial concentration of glucose on ethanol production

It is a fact that most cultures from fruits may contain a high concentration of fiber solids that cause problems of mixture in the reactor, and consequently a low contact between cells and substrate. Mango juice is no exception. In this study, we evaluated the growth and alcohol production of *Z. mobilis* coimmobilized with *S. cerevisiae* in cultures with dilutions of 200 and 50 g L^{-1} of substrate in equivalent medium.

For the coimmobilized of *Z. mobilis* and *S. cerevisiae* within alginate beads, an immediate increase in biomass content was observed. Although the biomass content for both treatment

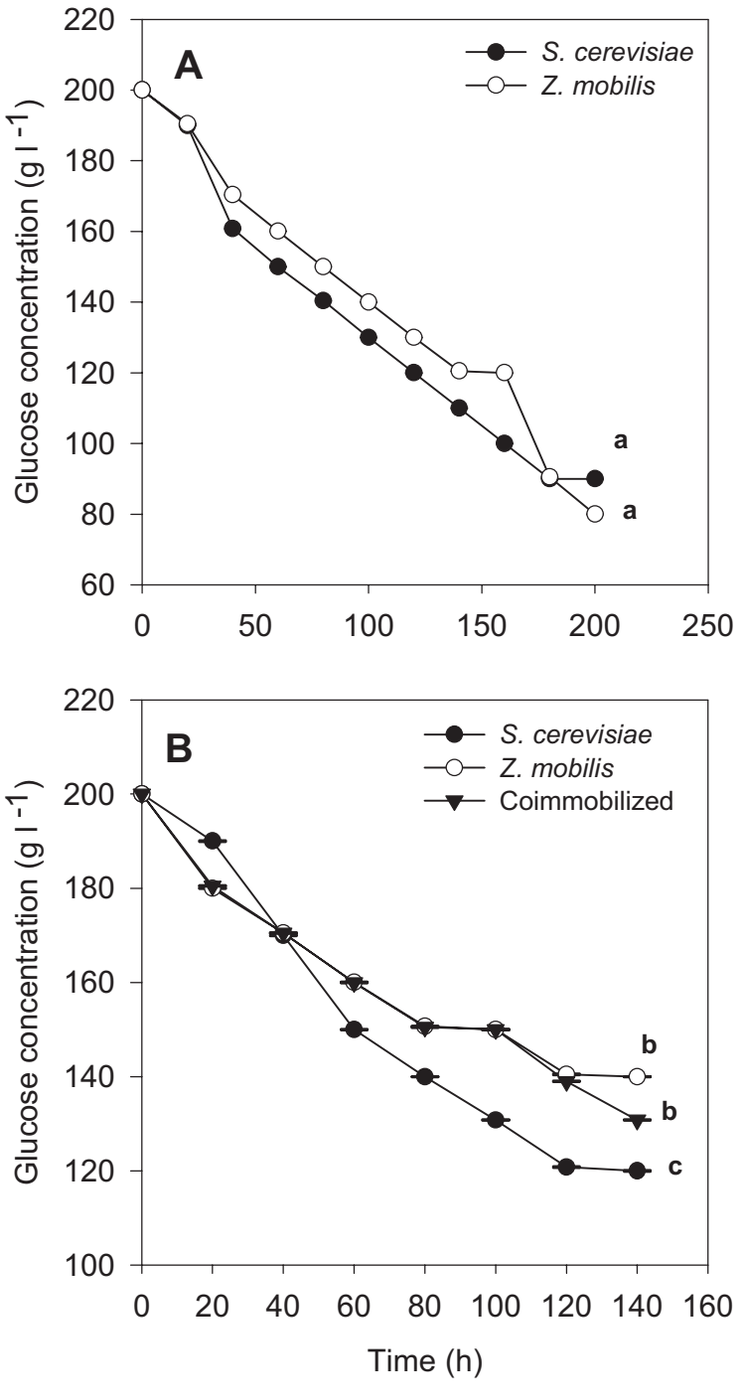

Figure 4. Average reduction of glucose (g L^{-1}) for *Saccharomyces cerevisiae* and *Zymomonas mobilis* in free culture (A) and immobilized culture (B). Different letters indicate significant differences ($P \leq 0.05$).

showed significant differences ($P \leq 0.0024$), the results suggest that the concentration of substrate was not a limiting factor for the growth for bacteria and yeast (**Figure 5**). The maximum biomass content in the treatment of glucose to 50 g L^{-1} (Gl_{50}) was obtained in the first 100 h of culture with about 0.0063 g $beads^{-1}$; while for the treatment of 200 g L^{-1} glucose (Gl_{200}) was of 0.053 g $beads^{-1}$ during a period of 80 h (**Figure 5**).

Culture	Cells	Ethanol formed (% v/v)	Y (g L^{-1} h^{-1})	Glucose consumed (g L^{-1})	Uptake rate (g-substrate removed per g-biomass d)	$\frac{moles\ Eth}{mole\ Glc}$
Free cells	*S. cerevisiae*	80.6	5.29[a]	90	2.71[a]	2.76
	Z. mobilis	87.7	5.76[a]	80	2.0[a]	3.38
Immobilized cells	*S. cerevisiae*	86.0	8.45[b]	60	0.165[b]	4.42
	Z. mobilis	87.7	8.70[b]	43	0.056[b]	6.29
Coimmobilized cells	*Z. mobilis* and *S. cerevisiae*	88.7	8.80[c]	47	0.235[c]	5.82

[a,b,c] Indicate significant differences ($P \leq 0.05$).

Table 1. Uptake rate, productivity (Y) and ethanol mole produced per glucose mole for *Saccharomyces cerevisiae* and *Zymomonas mobilis* in free culture, immobilized and coimmobilized.

The content of alcohol produced had no significant differences ($P \leq 0.05$) with respect to glucose concentration. However, uptake rates exhibit a decline as the glucose content in the reactor decreases (**Table 2**). The highest uptake rate occurred at a concentration of 200 g L^{-1} glucose (0.235 g-substrate per g-biomass d) with a 76.5% removal, compared to 50 g L^{-1} glucose (0.08 g-substrate per g-biomass d). Although the production of alcohol was similar in both treatments, the ratio mol-ethanol produced per consumed mol-glucose was higher in cultures of 50 g L^{-1} glucose with a value of 6.91, with respect to 200 g L^{-1} glucose with a ratio of 5.82 mol-ethanol produced per consumed mol-glucose (**Table 2**). Similarly, higher productivity was obtained (8.85 g L^{-1} h^{-1}) at a lower glucose concentration compared to a medium with high glucose content (8.80 g L^{-1} h^{-1}).

C_0	Ethanol formed (% v/v)	Y (g L^{-1} h^{-1})	Glucose consumed (g L^{-1})	Uptake rate (g-substrate removed per g-biomass d)	$\frac{moles\ Eth}{mole\ Glc}$
50	89.63	8.85	40.0	0.08	6.91
200	88.70	8.80	47.0	0.235	5.82

C_0: Initial concentration of glucose (g L^{-1}).

Table 2. Productivity (Y), uptake rate and ethanol mole produced per glucose with minimum content of glucose for coimmobilized *Z. mobilis* and *S. cerevisiase*.

4. Growth and productivity

To evaluate the capacity of growth in free and immobilized culture, two microorganisms, yeast and bacteria, were subjected to the same culture conditions (**Figure 3A**). In free culture, the yeast *S. cerevisiae* showed a higher cell density and specific growth rate (0.0547 d^{-1}) with respect to

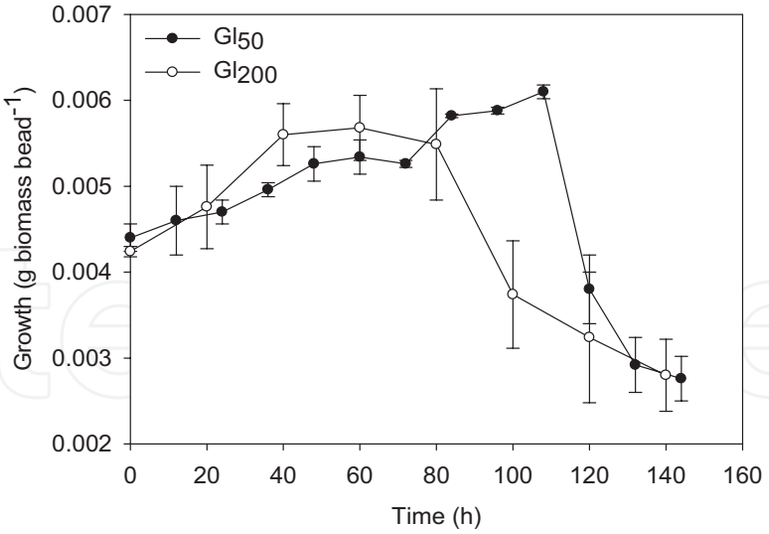

Figure 5. Growth (g biomass bead^{-1}) in coimmobilized system at different substrate concentrations.

bacteria *Z. mobilis* (0.0418 d^{-1}) in a treatment time of 120 h. The immobilized systems are known to have a greater capacity of cell growth and high metabolic activity [27, 28], which is consistent with the results obtained in this study. The result showed a high growth rates for immobilized *Z. mobilis* (0.142 d^{-1}) and *S. cerevisiae* (0.106 d^{-1}) with respect to free cell cultures, suggesting that immobilization did not affect growth in both microorganisms and increased biomass content favorably. Furthermore, the high activity in immobilized cell was observed with in a decrease of substrate in a shorter time of treatment (80 h) compared to free cell cultures (120 h). The short time of treatment for immobilized cell could be attributed to the increase of biomass within the beads and consequently an immediate decay of the substrate; however, this indicates that increasing cell population within the beads can cause a limited effects of nutrients on the cells located at the center of the beads, causing a decrease in cellular activity [28, 29].

Another factor that probably favors the rapid decline in cell density is attributed to the production of CO_2 as result of fermentation activity. Studies suggest the adverse effect of CO_2 gas, because if the diffusion of CO_2 is lower compared to its production, it will accumulate inside of alginate bead [30]. In this study, the CO_2 is observed in the reactor as bubbles attached on the surface of the beads, suggesting that the spread of CO_2 gas in the first 80 h was not a factor that inhibited growth and the production of alcohol; however, after this time, gas saturation in the reactor was probably high, affecting the diffusion of CO_2. This coupled with a limitation in the transport of nutrients and subsequent inhibition of microorganisms and may have caused glucose consumption to be lower compared to free cells (**Table 1**).

In particular, in free cell culture, the lower percentage of alcohol obtained by yeast during the increasing fermentation culture commonly relates to the fact that this is affected by the high concentration of ethanol in the solution, which may inhibit metabolism and decrease efficiency [31], unlike bacteria *Z. mobilis* [30]. In the present study, the lowest biomass produced by the bacteria (0.0047 g L^{-1}) with respect to yeast (0.0055 g L^{-1}) may be practical from the standpoint of waste generation. Similarly, observations were reported by Amin and Verachtert [9] for *Z. mobilis* and *Saccharomyces bayanus* immobilized in carrageenan with 5.6 and 9.9 g L^{-1}, respectively.

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It is evident that ethanol production was not inhibited in immobilized or coimmobilized systems, and even showed higher productivity with respect to free cells (**Table 1**), suggesting that they are more efficient in the conversion of sugar with respect to time. Krishnan et al. [19] reported lower productivity for *Z. mobilis* immobilized in carrageenan (1.6 g L^{-1} h^{-1}) compared to that obtained in this study (8.7 g L^{-1} h^{-1}); this difference may be attributed to the lower amount of glucose content in the culture medium of 32 g L^{-1} with respect to that used in the present study of 200 g L^{-1}.

Interestingly, the immobilized systems showed a higher conversion of substrate of 4.42 and 6.29 mole of ethanol per mole of glucose for yeast and bacteria, respectively, compared to the obtained by free cells, from 2.7 to 3.3 mole of ethanol per mole of glucose. In general, treatments with immobilized cells showed a higher output of ethanol per mole of glucose with respect to that reported by Amin and Verachtert [9] for *Z. mobilis* and *S. bayanus* immobilized in carrageenan with values of 1.8–1.9 mole of ethanol produced per mole of consumed glucose. Gunasekaran et al. [32] and Krishnan et al. [19] suggest that *Z. mobilis* is a good candidate to obtain alcohol with approximately 1.9 mole ethanol per mole of glucose; similarly, Rogers et al. [33] reported that specific productivity of ethanol (g ethanol g^{-1} biomass dry weight) is greater for *Zymomonas* than for *Saccharomyces uvarum*.

According to the results, immobilization and coimmobilization exhibited a lower uptake rate compared to free cells; this shows that there was less consumption of substrate (**Table 1**). Nevertheless, there was greater productivity, which indicates that it is possible to obtain high alcohol content with a lower requirement of substrate, but with the disadvantage of residual glucose in the medium; this problem can be solved with sequenced systems, as suggested by Demirci et al. [25]. Another alternative of solving this problem is to increase the cell number or inoculum size within the reactor. This is reasonable because a high number of cells could create a greater sorption of substrate (glucose) into the cell and eventually consumed substrate. However, Siripattanakul-Ratpukdi [10] suggested that with different cell yeast loads, the same reduction (>90%) of substrate is obtained at the end of a treatment period of 10 h.

The low glucose reduction observed in this study in alginate beads can be attributed to the decline in cell density, but it is likely that the diffusion of substrate could have been prevented. Studies have reported that the adsorption of substrate by the matrix was observed in the first hours of treatment, with a possible decrease of substrate diffusion within the matrix in a continuous process [34].

On the other hand, Robinson et al. [35] suggest that the diffusion rate within the alginate matrix depends on the concentration gradient between the culture medium and matrix; this is, when the nutrient concentration in the culture medium decreases, the diffusion rate occurs within the matrix and therefore the removal rate. In this study, during the first hours of treatment, the matrix is probable a partial saturation with substrate (glucose), because the substrate is decreased during the culture time, and cell growth for both microorganisms was continuous. Clearly, the immobilized cell system successfully decreased glucose by adsorption of the matrix (immobilized glucose) and biodegradation (bioconversion of glucose), being the main process the biodegradation. This suggests that the main factor that could limit glucose removal may have been the high concentration of CO_2 in the reactor.

4.1. Coimmobilization of *Z. mobilis* and *S. cerevisiae* at different glucose concentrations

The biomass content in alginate beads shows that a high concentration of glucose (200 g L^{-1}) leads to a rapid decrease compared to cultures with a low glucose concentration (50 g L^{-1}). This confirms the fact that the high concentration of glucose saturates beads faster, reducing the diffusion between beads and the culture medium; consequently, the diffusion of CO_2 produced can be reduced and remains trapped inside the bead, causing a decrease in growth and substrate consumption.

Conversely, the low concentration of substrate of the culture medium indicates the presence of a soft transport and substrate accumulation within the matrix, allowing a proper consumption and growth of bacteria and yeast. Therefore, a low concentration of substrate may actually increase the production of alcohol with minimal residual glucose, reaching values of 6.91 mol of ethanol per mole of glucose, with respect to a high glucose concentration (**Table 2**).

Previous studies in our laboratories have shown that the fermentation process of mango juice for a coimmobilized system can produce a production ratio of 1.4 L of alcohol (79% v/v ethanol) for every 3 L of mango juice.

5. Conclusions

The present study has shown the existing potential of using coimmobilized systems in the production of ethanol. The association of *Z. mobilis* and *S. cereviase* was positive, obtaining a higher ethanol content and high conversion of substrate compared to free and immobilized cells.

In general, the immobilization technology offers an alternative by increasing productivity and conversion of substrate compared to culture systems with free cells. In the present study, the immobilized systems showed high conversion capacity to obtain high alcohol content with a lower requirement of substrate.

The possible substrate inhibition was not a factor affecting cell growth in both organisms; it is clear that the immobilized cell system successfully reduced glucose by the matrix adsorption (immobilized glucose) and biodegradation (bioconversion of glucose), being biodegradation the main process. This suggests that the main factor that could limit further growth was the high concentration of CO_2 in the reactor. Furthermore, although no significant differences were detected in the alcohol content in immobilized culture in diluted medium, the conversion from glucose to ethanol is greater in those media with a glucose concentration of 50 g L^{-1}. For practical purposes, it is desirable that the fermentation of waste organic be performed through dilutions to increase the homogeneity of alginate beads within the reactor and consequently allow the diffusion of CO_2 and substrate through the beads.

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