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COMPARATIVE STUDY BETWEEN YEASTS IMMOBILIZED ON ALUMINA BEADS AND ON MEMBRANES PREPARED BY TWO ROUTES

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ABSTRACT

Alumina channeled beads and rough surface membranes prepared from aqueous sols of fibrillar pseudoboehmite are able to immobilize yeasts for ethanol fermentation of sugar solutions. This paper describes comparative results of assays carried out with yeasts immobilized onto alpha-alumina beads and membranes prepared under two different conditions of processing and firing. The fermentation tests evaluated by the decrease of fermentable sugars, referred as Brix degrees per hour, indicated that the yeasts immobilized on beads had similar performance, probably because their surfaces, even being morphologically different, presented the same value of open porosity. One type of membrane (asymmetrical; precursor: pseudoboehmite; firing temperature 1,150ºC; crystal structure; alpha-alumina) had better performance than the other type (asymmetrical; precursor: fibrillar pseudoboehmite plus aluminum hydroxiacetate mixture; 1,150ºC; alpha-alumina) because the yeast cells entered into their porous interior through the surface slits, were immobilized and their growth was easier than on the external surface.

Key words: Porous alumina; channeled alumina; immobilized yeast; ethanol production

INTRODUCTION

Hamdy *et al.* (5) and Hyndman *et al.* (6) described the use of patented alumina for yeast immobilization. Hyndman *et al.* (6) used the chemical adsorption of organophosphates onto alumina to create linking surfaces for protein immobilization, alumina being elected a carrier due to its physical properties and ability to link with phosphorous organic compounds. For Inloes *et al.* (7) the development of large-scale microbial reactors is limited due to the difficulty to maintain stable cell cultures for long periods. The immobilization and growth over and onto a solid support would improve the availability of the use of yeast and other cells in biochemical reactors. The techniques of immobilization give an increase in the catalyst stability inside the porous texture of the support. The possibility of obtaining higher cell concentrations linked to the carrier in comparison with conventional cell suspension may result in a higher productivity of the biochemical reactor.

Scanning electron microscopy (SEM) is being used to confirm the immobilization of yeast cells on alumina and other solid surfaces (5,8,9,10,19,20).

Souza Santos *et al.* (15,17) reported that alpha alumina channeled beads and membranes specially prepared from aqueous sols of fibrillar pseudoboehmite are able to immobilize yeast cells for ethanol fermentation of sugar solutions. These pseudoboehmite sols were prepared from pure chemicals produced in Brazil. By pseudoboehmite is meant the poorly crystallized Al^{III} compound of composition $Al_2O_3.xH_2O$ ($2.0 > x > 1.0$) with interplanar spacing increased in the [020] direction to 6.7 Å in comparison with 6.12 Å for well-ordered boehmite (12). The adjective "fibrillar" was applied by Bugosh (2,3) to the colloidal pseudoboehmite crystals (with $d_{020} = 6.23$Å) with the shape of long fibrils, first observed by Souza Santos *et al.* (18).

The yeast cells show spontaneous adhesion to the internal and external walls of the surface of the channeled porous

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crystalline texture of the beads followed by a vigorous growth clearly observable by scanning electron microscopy, as reported before by Souza Santos *et al.* (18). Drying at room temperature of this yeast cell-α-alumina bead complex does not destroy the vitality of the immobilized yeast cells. The cell growth was evaluated by the weight increase of the beads.

The purpose of this research is to confirm and to compare the viability of immobilization of pure yeast cells (*Saccharomyces cerevisiae)* on membranes and beads, or beads having channeled surfaces on crystalline surfaces having alpha-alumina crystalline structure prepared from two different precursors. After immobilization and a growth period, nitrogen analysis of the alumina pieces was made in comparison with "blank pieces". Fermentation tests of sugar solutions and scanning electron microscope examination were carried out in parallel to prove the fixation of cells onto membranes and beads.

MATERIALS AND METHODS

Fermentation media

The fermentation tests were conducted in synthetic media prepared with commercial invert sugar syrup containing 76% of total fermentable sugars. The media were prepared by water dilution of the syrup to different concentrations from 17.5º Brix to 6º Brix and addition of 1.0 g/liter of KH_2PO_4; 1.0 g/liter of $MgSO_4.7H_2O$; 1.0 g/liter of $(NH_4)_2SO_4$; the media pH was 4.5-4.6.

Inoculum

A suspension of a pure culture of *Saccharomyces cerevisiae* IZ-1904 from Escola Superior de Agricultura Luiz de Queiroz, University of São Paulo, developed in a 5º Brix fermentation medium as described in literature (13) was used as inoculum, to immobilize yeast cells on the alumina beads and membranes.

Alumina membranes and beads

Two routes were used for their preparation, the batches named 244 and 245, each batch having a dozen membranes or beads.

Route 244

(1 Al: 0.5 HAc: 50 H_2O) The reaction was conducted in a two-liter, three-necks, round bottom stirred flask; the proportion of reagents is: aluminum powder ALCOA 123 – 27.0 g; glacial acetic acid – 30.0 g; water – 900 g; temperature 90ºC ± 1ºC (thermostat). The reaction was conducted under reflux, continuously, for 168 hours. A whitish turbid sol is obtained, containing 70 g/L of AlOOH; left standing at room temperature, it separates spontaneous into two layers; that two-layer separation is fundamental for formation of an asymmetrical membrane. By transmission electron microscopy (TEM) (Fig. 1), it is shown that the sol is constituted only by fibrils. Air-dried at room temperature, the ground powder has the X-ray diffraction (XRD) curve of pseudoboehmite, with a basal reflection of 6.15 Å.

Route 245

(1 Al: 1,0 HAc: 50 H_2O) It is conducted exactly as Route 244, except the proportion of reagents is different: 27.0 g Al 123; 60.0 g of acetic acid and 900 g of water. On standing, after 168 hours, there is spontaneous formation of three layers; the upper two layers are similar to those from route 244 and are composed by pseudoboehmite fibrils; the bottom layer is an opaque white precipitate, which is constituted by so-called faceted microcrystals of aluminum monohydroxide diacetate – $Al(OH)(CH_3.COO)_2$; it was characterized by XRD; its morphology as shown by SEM is similar to a "lettuce" (Fig. 2), as described by Xavier *et al.* (21).

Membrane preparation

For both routes, the same procedure was used. The sols were homogenized by vigorous stirring and the viscous liquid was poured in polystyrene disk moulds, 7>>7-mm diameter and 5 mm high. They were left to dry at room temperature (20ºC-25ºC); that occurred in 5-7 days; asymmetrical membranes were formed due to the layer separation for both routes. After air-drying at 60ºC/70ºC and at 110ºC, 24 hours each, the membranes were ready for firing at 700ºC and 1150ºC for 3 hours in a programmed electric furnace, provided with natural cooling and oxidizing atmosphere. Both membranes, 700ºC and 1,150ºC, have asymmetrical texture, clearly shown in fractured cross sections when observed by SEM.

Bead preparation

For both routes the same procedure was used for channeled bead preparation. The sols were homogenized by stirring and heated at 60ºC ± 5ºC under stirring; by water evaporation, they changed to a plastic body, which can be conformed by extrusion; 12 mm high and 12 mm cylinders are cut from extruded body and rolled into 12 mm diameter beads; the beads were air-dried at 60ºC/70ºC and at 110ºC, being then ready for firing in the same way as membranes.

The fired samples, membranes or beads were kept in polyethylene bags for tests; membranes and beads were named by numbers 244 and 245 according their preparation procedure.

Structural characterization

Samples of the unfired and fired membranes and beads were ground to pass ABNT sieve nº 100 and examined by XRD in Philips X'Pert equipment using operating a 40 kV and 40 mA, using copper K-alpha radiation, between 1º (2 θ) and 90º (2 θ).

The morphological characteristics of the crystals of sols from routes 244 and 245, after adequate dilution, were examined in a Philips model CM 200 transmission electron microscope

(TEM) operating at 200 kV (14). The surface characteristics of the beads and of the membranes, including of their fractures, were examined in a JEOL SM model 840 A SEM.

Immobilization of yeasts

The immobilization test was obtained by the spontaneous fixation of yeast cells using a simple procedure. Membranes and beads, submersed in the fermentation medium, were sterilized at 121ºC for 20 minutes. After cooling, the medium was inoculated with a suspension of the yeast cells and left in an oven at 32ºC until the total fermentation of sugars. Membranes were picked out and rinsed several times with enough sterilized distilled water to remove the free yeast cells from the surface. Same procedure was used for beads. Then, both were considered ready for the next stage; the fermentation tests samples from both were used for total nitrogen measurements, before and after the tests.

Fermentation tests

The tests carried out in two series of experiments had the main objective of proving the immobilization of the yeast cells onto the beads and membranes. Both, membranes or beads, were placed in cylindrical cartridges of perforated stainless steel (65 holes per sq. cm) and hanged 2 cm high from the bottom of fermenting vessel; the space under the cartridges was reserved for settling of the excess of multiplied yeast cells, that is, non-immobilized or free cells.

The evaluation of the fermentations was followed by the decrease of the concentration of the medium, measured by the difference between the initial and final Brix degrees.

Discontinuous fermentations took place in two cylindrical vessels containing sterilized medium where the cartridges were submerged. In the first series of fermentation tests, one cartridge had ten 244 surface channeled beads with 12 ± 2 mm diameter and weighing 0.6 ± 0.3 g each. The other cartridge had ten 245 beads with 12 ± 1mm diameter and weighing 0.5 ± 0.1 g each.

In the second series, one cartridge had ten 244 membranes with 68 mm diameter and 2 mm thick, weighing 2.6 ± 0.6 g each. The other cartridge had ten 245 membranes with 68 mm diameter and 2 mm thick, weighing 1.2 ± 0.2 g each.

Nitrogen determination

It was made by Kjeldahl method according to AOAC (1) in which the nitrogen is transformed in ammonia, recovered by distillation and measured by titrimetry.

Alcohol determination

It was made by distillation of a fermented medium sample diluted with water in same volume of the sample. The distillate was recovered in a same volume as that of the sample. Later the specific weight was determined at 20ºC with picnometer and the amount of alcohol in volume was found in tables.

RESULTS AND DISCUSSION

Characterization of membranes and beads

The 110ºC dried and ground membranes and beads 244 and 245, presented by XRD the same crystalline structures described before for both sols; both, 244 membranes and beads, presented only the 6.15Å basal reflection of fibrillar pseudoboehmite; both, 245 membranes and beads, presented the XRD pattern of a mixture of pseudoboehmite with aluminum hydroxiacetate crystals, as expected from the Al/acetic acid ratio used for the preparation (21). Fig. 1 is a TEM of pseudoboehmite fibrils from 244 sol; Fig. 2 is a SEM micrograph of "lettuce" shaped microcrystals of aluminum hydroxiacetate from 245; these crystals plus the pseudoboehmite fibrils are the precursors of the alumina component of beads and membranes formed by thermal treatment.

After firing at 700ºC, the fibrils from 244 membranes and beads maintained their macro and microscopical morphology; their XRD pattern is of gamma-alumina, as described by Souza Santos and Souza Santos (16) for firing pseudoboehmite at that temperature. After firing at 700ºC, the crystals of 245 membranes and beads also maintained their macro and microscopical morphology; their XRD pattern is of gamma-alumina, because also the aluminum hydroxiacetate crystals, at 700ºC, also produces that transition alumina, as shown by Kiyohara *et al.* (11). Gamma-alumina was used because it is the most important alumina for adsorbents, catalysts and catalyst carriers (14) and was used by Kana *et al.* (9).

After firing at 1,150ºC, the crystals of 244 and 245 membranes and beads kept their original macro and micro morphologies (10,14), but all presented, by XRD, the alpha-alumina crystalline structure. Alpha-alumina was used due to its well-ordered crystallinity, hardness, thermal stability and chemical inertness (4).

Fig. 3 is a micrograph of the surface of a 244 channeled bead showing that the topographic relief is due to sinuous and smooth channels, as described before in beads made from fibrils of pseudoboehmite (15,17). Fig. 4 is a micrograph from the surface of a 245 bead; the mixture of the pseudomorphs, resulting in a roughness in which the channels are thinner and shorter than in 244 bead, forms the surface texture. Fig. 5 is a SEM of the shiny or bright upper surface of a 244 membrane fired at 1,150ºC; Fig. 6 is the dull upper surface of a 245 membrane fired at the same temperature. It seems that the pseudomorphs, even both having alpha-alumina structure, have different effects on the topography of the surfaces, being smooth in 244 (only fibrils) and rough in 245, due to the mixture of fibrils and "lettuce-like" pseudomorphs.

Strength of membranes and beads in the fermentation media

Samples of whole and fragments of membranes 244 and 245 dried at 110ºC, fired at 700ºC and 1,150ºC, were immersed in the fermentation media and left at room temperature (24ºC ± 3Cº) to observe any effect on their mechanical strength. It was observed,

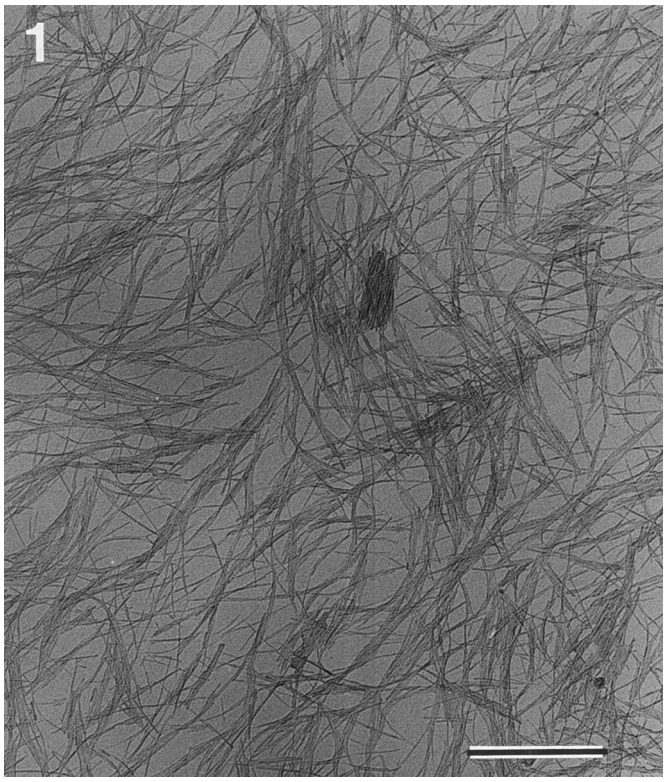

Figure 1. Fibrils of pseudoboehmite (Bar = 250 nm).

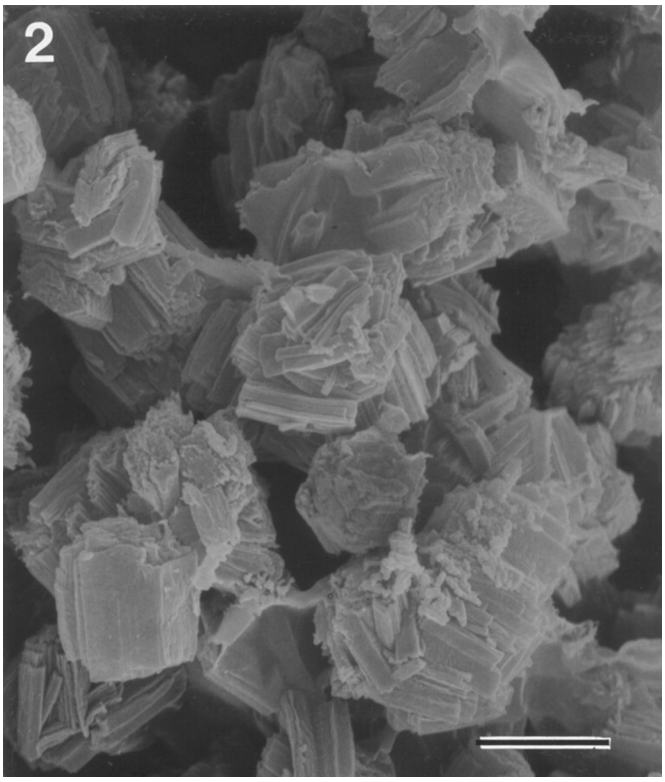

Figure 2. Microcrystals of Al (OH) Ac_2 (Bar = 5 nm).

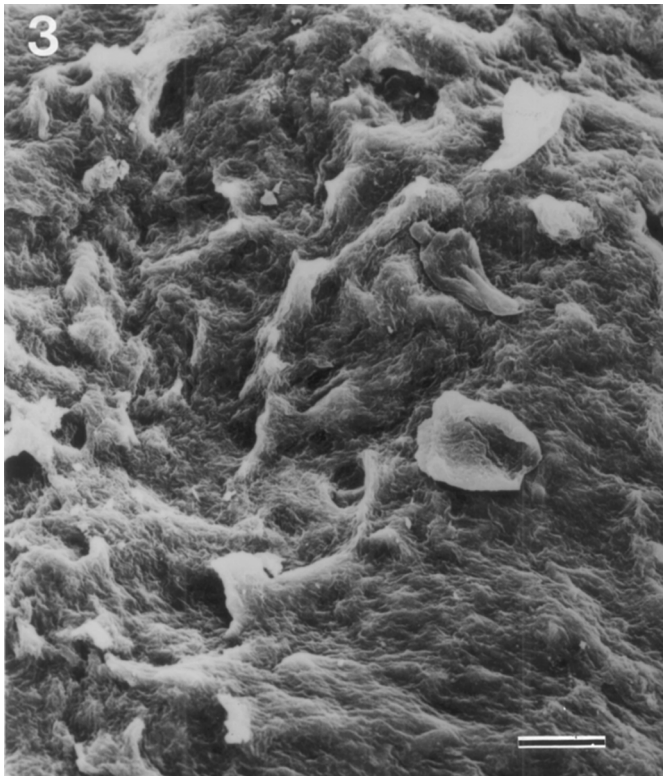

Figure 3. Surface texture of bead (# 244 route) (Bar = 20 nm).

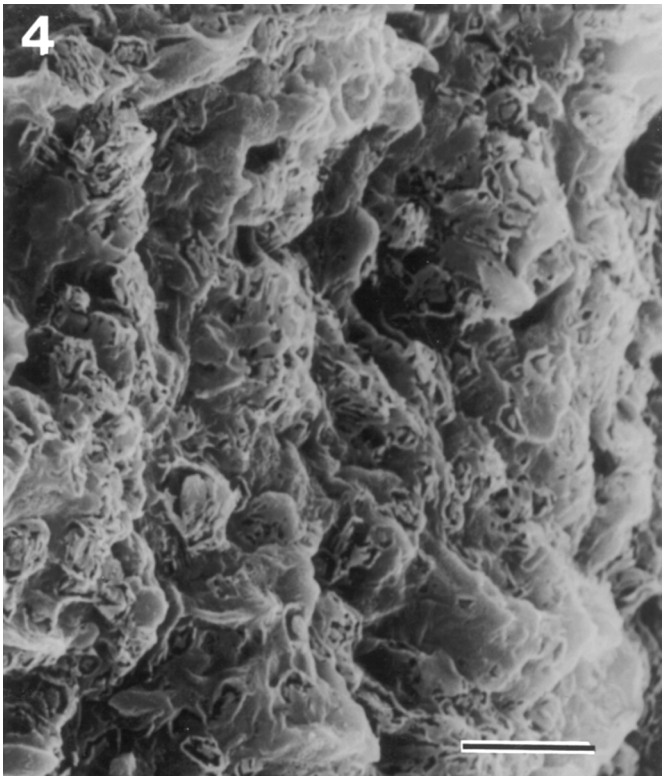

Figure 4. Surface texture of bead (#245 route) (Bar = 5 nm).

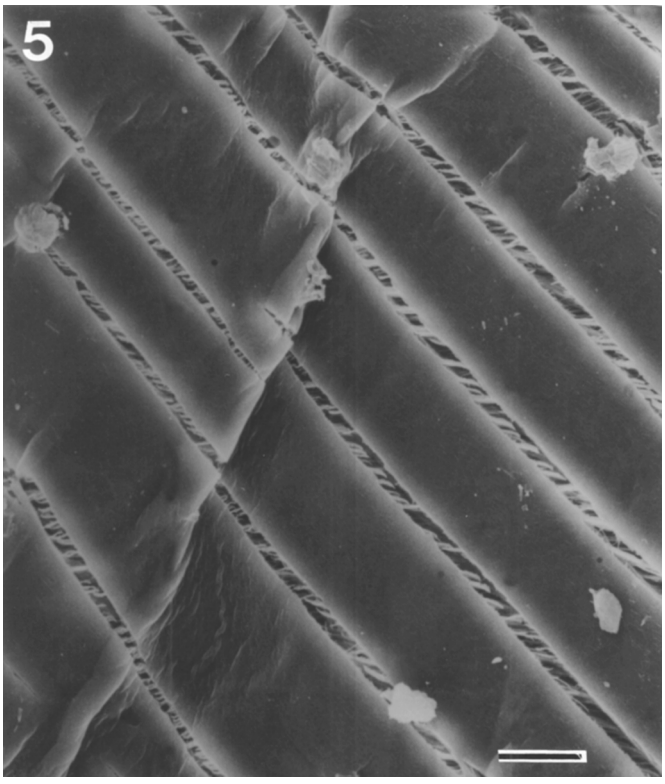

Figure 5. Upper surface of membrane (#244 route) (Bar = 20 nm).

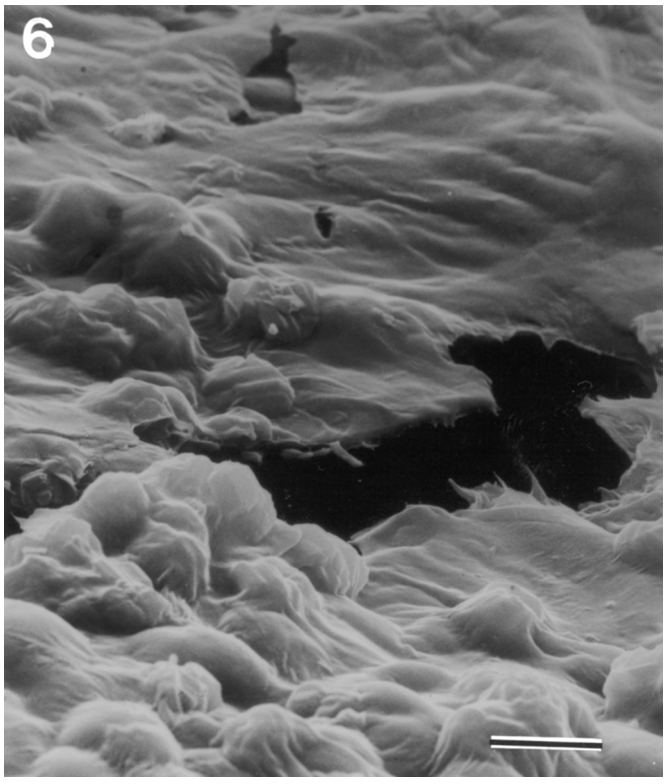

Figure 6. Upper surface of membrane (#245 route) (Bar = 10 nm).

after 8 days, that 244 and 245 membranes either dried at 110ºC (fibrillar pseudoboehmite) or fired at 700ºC (gamma-alumina) became fractured when hand shaken in their flasks. So, only the experiments conducted with 1,150ºC fired membranes and beads (both with alpha-alumina structure) are presented.

Alcoholic fermentation tests with yeasts immobilized onto beads and membranes

The results of several fermentation tests with beads and membranes 244 and 245 all fired at 1,150ºC are summarized in Table 1 to 8, in which are shown the average values of results of fermentation tests carried out at room temperature (24 ± 3ºC) and at controlled temperature (32ºC).

The tests were conducted in media of different sugar concentrations; tests A, B, D, E and G were made with more concentrated fermentation medium to observe the complete fermentative process from the beginning to its end in about 6 days, and tests C, F, G and H were carried out with diluted fermentation medium, searching to complete the fermentation process within one day, that is, in 6 hours. In the test H, it was observed that membranes 244 and 245 were lightly cracked, with a loss of weight of 0.484 g for 244 and 0.280 g for 245.

A comparative evaluation of the arithmetic average values of the decrease of the sugar concentration per hour in tests A to D for beads and E to H for membranes is presented in Table 9.

Table 9 shows that the membranes obtained by 244 route had slightly better performance than 245 route (decrease of 0.13 vs. 0.09 Brix degrees per hour), probably due to their smoother surface, i.e. with less "microrugosities" as it can be seen by comparing Figs. 5 and 6. On the other hand, both routes were equivalent for beads performance (both presented 0.06 Brix degrees per hour), probably because their surfaces, even being morphologically different (Figs. 3 and 4), presented the same degree of open porosity.

To check the latter explanation, the open porosity of spheres was tested using the water absorption test used for ceramic refractory materials, like alumina (ABNT; MB-67); the average values for the spheres were 108,7% for 244 and 108,6% for 245, what strongly supports such explanation

Electron microscopy of yeast growth

Fig. 7 is a SEM of the channeled surface of a 244 bead showing the fixation and growth of yeast cells. Fig. 8 is a transverse fracture of a bead showing the entrapment and growth of the yeast cells, which penetrated from surface slits inside the channeled surface. The same vigorous growth and immobilization is shown in Figs. 9 and 10, which are from the upper surfaces of membranes 244 and 245, respectively. The examination of fractured beads and membranes shows the penetration of yeast cells inside the porous solids and their growth also internally as well externally.

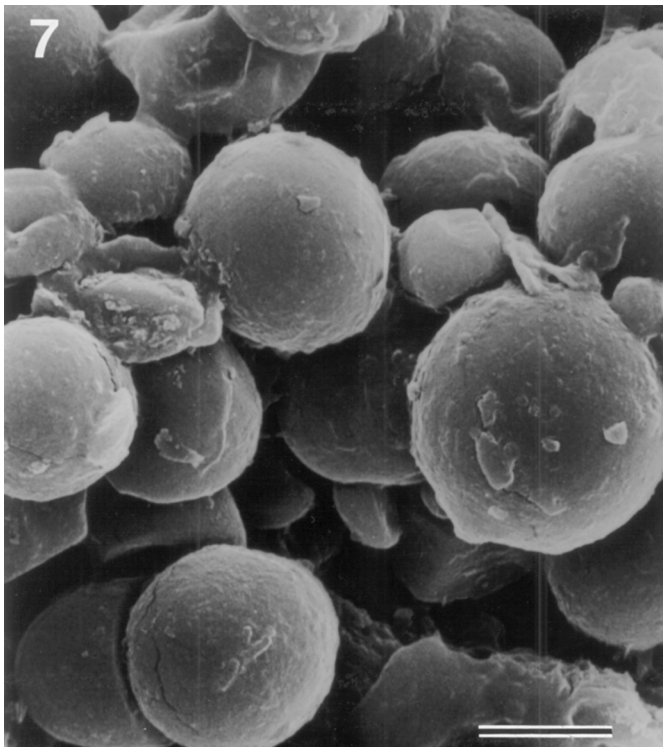

Figure 7. Growth of yeast cells (*Saccharomyces cerevisiae*) on channeled surface of bead (#244 route) (Bar = 8 nm).

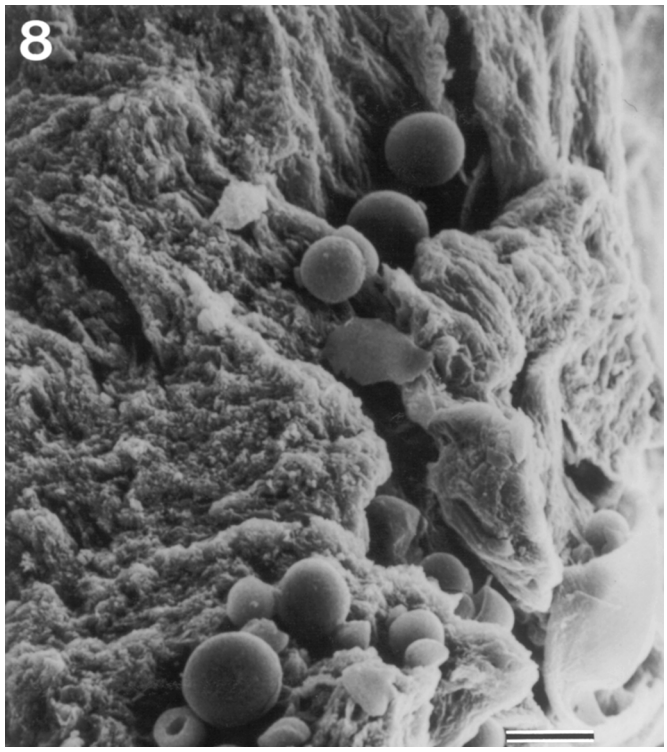

Figure 8. Growth of yeast cells inside a fractured bead (#244 route) (Bar = 2 nm)

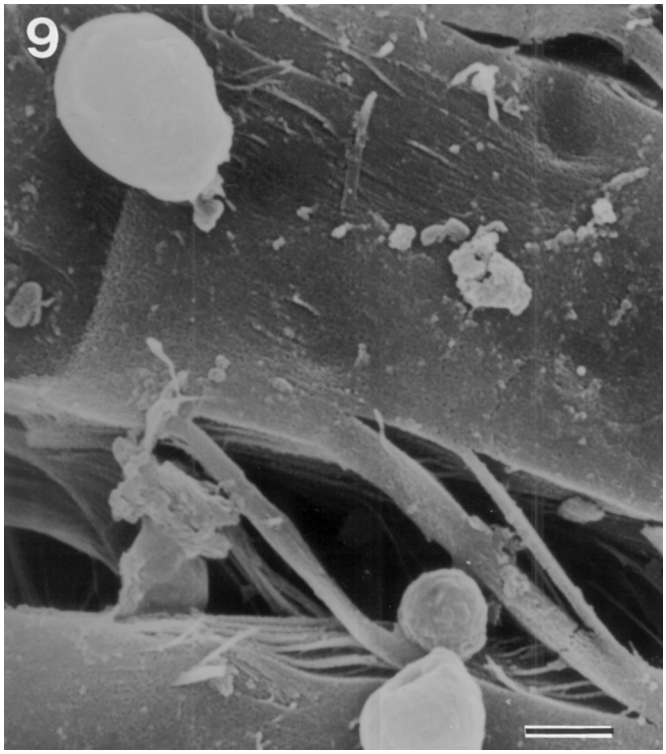

Figure 9. Growth of yeast cells on the upper surface of membrane (#245 route) (Bar = 2 nm).

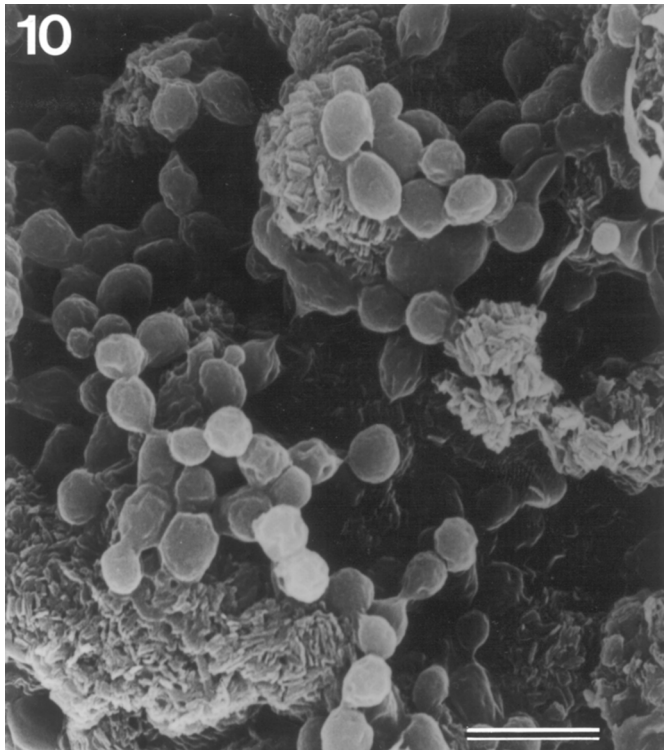

Figure 10. Growth of yeast cells on the upper surface of membrane (#245 route) (Bar = 2 nm).

The better performance of membranes than the beads may be attributed to the protected immobilization and growth inside the porous membranes, probably due to the fact the membranes are only 2 mm thick when the beads have 12 mm diameter.

Membranes 244 showed a better performance than 245; as they have smoother external surfaces (Fig. 3 vs. Fig. 4), the yeast cells probably entered by the surface slits and became immobilized inside the porous asymmetrical texture and their growth took place inside the membranes. So, only few yeast cells grow on the smooth surface (Fig. 4), while the surface growth is larger in 245.

Total nitrogen content

Measurements of total nitrogen content were made in a sample of membrane 244, before and after the end of the fermentation test, to confirm the immobilization and growth of yeast cells. The sample of the membrane before the fermentation had 0.007% of nitrogen and after the fermentation test had 0.157% of nitrogen. As the membrane was rinsed with enough sterilized distilled water to remove all or most of the cells of the external surface, that difference probably is an indication of the preferential yeast cell growth and retention in the internal pore surface, that is, inside the open pores of the asymmetrical membrane.

Considering that, after the fermentation tests, 100 g of dried membranes presented 0.157% of nitrogen, or 0.981 g % of protein (0.157 x 6.25) and assuming that dried yeast cells contain 35% of protein, 100 g of the membranes retained 2.8 g of dried yeast cells or 9.0 g of moist cells internally in the open pores.

Alcoholic content in fermented medium

In the experiment corresponding to Table 1, the fermentation medium with the beads 244, 4.57% of ethanol in weight (5.70% in volume) was measured and with beads 245, was 4.70% in weight (5.86% in volume).

Immobilization vitality

The beads and membranes were picked out of the fermented fermentation medium at the end of each fermentation test and rinsed with water, before being submersed into new fermentation medium for a new assay. After the introduction of the alumina carriers, a decrease of the concentration of fermentable sugars, referred as degree Brix, was observed in each fermentation flask. A satisfactory production of alcohol was also observed, confirming the presence of active yeasts in the carriers.

The water rinsed beads and membranes were used for eight successive tests (Tables 1 to 8) carried out along three months,

Table 1. Fermentation test A at (24 ± 3ºC).

Time of fermentation in hours	Concentration of the medium in Brix degrees	
	Beads 244	Beads 245
Initial	17.50	17.50
24	17.50	16.60
48	17.40	16.50
72	11.25	10.00
168	7.00	7.00
-	-	-
Total decrease of º Brix	10.50	10.50
Decrease per hour of test	0.06	0.06

Table 2. Fermentation test B at (24 ± 3ºC).

Time of fermentation in hours	Concentration of the medium in Brix degrees	
	Beads 244	Beads 245
Initial	15.00	15.00
24	15.00	14.50
48	9.60	8.00
72	5.75	5.75
98	5.75	5.60
120	5.25	5.00
Total decrease of º Brix	9.75	10.00
Decrease per hour of test	0.08	0.08

Table 3. Fermentation test C at (24 ± 3ºC).

Time of fermentation in hours	Concentration of the medium in Brix degrees	
	Beads 244	Beads 245
Initial	6.00	6.00
2	6.00	5.90
4	5.90	5.80
4	5.75	5.75
Total decrease of º Brix	0.25	0.25
Decrease per hour of test	0.06	0.06

Table 4. Fermentation test D at (32ºC).

Time of fermentation in hours	Concentration of the medium in Brix degrees	
	Beads 244	Beads 245
Initial	10.00	10.00
24	9.75	9.50
48	8.25	8.00
144	4.50	3.00
Total decrease of º Brix	5.50	7.00
Decrease per hour of test	0.03	0.04

Table 5. Fermentation test E at (24 ± 3ºC).

Time of fermentation in hours	Concentration of the medium in Brix degrees	
	Membranes 244	Membranes 245
Initial	11.00	11.00
24	10.75	10.75
72	7.50	7.75
144	3.50	4.00
-	-	-
-	-	-
Total decrease of º Brix	7.50	7.00
Decrease per hour of test	0.05	0.05

Table 6. Fermentation test F at (24 ± 3ºC).

Time of fermentation in hours	Concentration of the medium in Brix degrees	
	Membranes 244	Membranes 245
Initial	9.50	9.00
1	9.25	8.90
2	9.00	8.50
4	8.75	8.25
5	8.00	7.80
6	7.75	7.75
Total decrease of º Brix	1.75	1.75
Decrease per hour of test	0.29	0.21

Table 7. Fermentation test G at (24 ± 3ºC).

Time of fermentation in hours	Concentration of the medium in Brix degrees	
	Membranes 244	Membranes 245
Initial	9.00	9.00
24	9.00	8.75
48	7.00	7.50
120	4.00	4.00
144	4.00	4.00
Total decrease of º Brix	5.00	5.00
Decrease per hour of test	0.04	0.04

Table 8. Fermentation test H at (24 ± 3ºC).

Time of fermentation in hours	Concentration of the medium in Brix degrees	
	Membranes 244	Membranes 245
Initial	8.00	8.00
1	7.50	8.00
4	7.00	8.00
6	7.00	7.50
-	-	-
Total decrease of º Brix	1.00	0.50
Decrease per hour of test	0.16	0.08

having uniform performance of fermentation and in cell immobilization. The sequence of fermentations, washings, air drying at room temperature and the repetition of the fermentation results show the vitality of the entrapped yeast cells inside the membranes and beads.

CONCLUSIONS

a) It is possible to produce alpha-alumina membranes and beads with special texture surfaces and crystalline alpha-alumina structures from pseudoboehmite fibrils and aluminum monohydroxide diacetate as precursors, fired at 1,150ºC, from aqueous sols prepared by two different routes using pure chemicals produced in Brazil and to immobilize and grow yeast cells of *Saccharomyces cerevisiae* for ethanol fermentation of sugary solutions;

b) The best immobilization supports are asymmetrical alpha-alumina membranes obtained from fibrillar pseudoboehmite aqueous sols as the only precursor, prepared by reaction of

Table 9. Average decrease of the sugar concentration in degrees Brix per hour.

Decrease of the sugar concentration in Brix degrees per hour			
Beads fired at 1150ºC			
Test	Number 244	Number 245	Temperature in ºC
A	0.06	0.06	24 ± 3ºC
B	0.08	0.08	24 ± 3ºC
C	0.06	0.06	24 ± 3ºC
D	0.03	0.04	32ºC
Average	**0.06**	**0.06**	
Membranes fired at 1150ºC			
Test	Number 244	Number 245	Temperature in ºC
E	0.05	0.05	24 ± 3ºC
F	0.29	0.21	24 ± 3ºC
G	0.03	0.03	24 ± 3ºC
H	0.16	0.08	24 ± 3ºC
Average	**0.14**	**0.10**	

pure Al powder with aqueous acetic acid solution at 90ºC and fired at 1,150ºC.

c) The yeast cells immobilized inside and on the surface of alpha-alumina membranes and beads keep their fermentation capacity after eight cycles of air-drying at room temperature.

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RESUMO

Esferas de superfície canelada ou estriada e membranas de textura porosa e superfície rugosa preparadas com sóis aquosos de pseudoboemita fibrillar são capazes de imobilizar leveduras para fermentação alcoólica de soluções aquosas açucaradas. Este artigo descreve os resultados de ensaios levados a efeito com leveduras imobilizadas em esferas e membranas de alumina-alfa, preparadas sob duas diferentes condições de processamento e queima. A fermentação alcoólica dos meios foi acompanhada pelo decréscimo do teor de sólidos solúveis em graus Brix por hora e indicou que as leveduras imobilizadas nas esferas obtidas pelas duas rotas apresentaram desempenho similar, provavelmente por causa de suas superfícies que, mesmo morfologicamente diferentes, apresentaram o mesmo valor de porosidade aberta. Um tipo de membrana (assimétrica; pseudoboemita fibrilar como precursor; temperatura de queima de 1.150ºC; estrutura cristalina de alumina-alfa), levou a melhor desempenho do que outro (assimétrico; mistura de pseudoboemita fibrilar e hidroxiacetato de alumínio como precursor; 1.150ºC; alumina-alfa), provavelmente porque as células de leveduras penetraram no seu interior poroso pelas fendas superficiais, imobilizaram-se e cresceram mais facilmente do que na superfície externa.

Palavras-chave: alumina porosa, alumina estriada, levedura imobilizada, produção de etanol

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