

Immobilisation of yeasts for continuous fermentation

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Continuous ethanol fermentation from cheap sugar cane molasses was studied. Two strains of *Saccharomyces cerevisiae* were used, one of them flocculent, in order to evaluate the performance of fluidized tubular reactors, with and without support for yeast immobilisation (polyurethane foam and expanded clay argilate granules were tested). In this heterogeneous continuous system, with three phases, flow conditions are difficult to control, mostly due to CO₂ production. Different long term continuous experiments were performed using three different reactors. Operating parameters such as feedstock medium, temperature and pH were not changed and ethanol productivity was followed up as the dilution rate was increased. Different steady states were reached and the productivity increased with the dilution rate. Using a 1L volume reactor, 25 g L⁻¹h⁻¹ of ethanol were achieved at 0.8 h⁻¹ dilution rate, although in a non stable operation mode. The performance of the two strains is comparable, but the flocculent one raises problems due to fast growth and support particles aggregation.

Keywords bioethanol; fluidized bed; continuous fermentation; cells immobilisation

1. Introduction

The demand for ethanol to be used as fuel additive is increasing due to UE legislation. In fact, the combustion of ethanol produced from renewable sources does not contribute to global warming because the release of carbon dioxide equals its uptake in a cycle of combustion and photosynthesis.

In the European Union the dependence on imported oil is already high (currently 75%) and it is likely to increase even further and exceed 85% by the year 2020. The rising fossil fuel consumption augments the greenhouse gas emissions, in particular those of CO₂. Moreover, in EU about 60% of CO₂ emissions come from transport, accounting for 25% of total energy-related CO₂ (<http://www.nf-2000.org>). This scenario led to the Directive (EU 2003)[1] that promotes the use of renewable transport fuels which include bioethanol. Due to this, the market for fuel ethanol will increase and this is a great opportunity for the European bioethanol industry.

An increase in production, required to fulfil future market demand, can be achieved by replacing the traditional batch fermentation by continuous operation. Nevertheless, it is also important to increase ethanol productivity and this requires a high cell concentration in the fermenter [2]. A productivity increase can indeed be achieved by continuous fermentation. However, this operation mode sets new challenges before it may become a real alternative to commonly used batch fermentation processes. They include the use of immobilised cells to increase the biomass content inside the reactor and preventing contamination during long term operation fermentations.

Nowadays, most ethanol is produced by yeast fermentation of sugar or grains, in a process that is traditionally batch[3]. Although suitable for alcoholic beverage production, the batch process is time and labour consuming for fuel production at an industrial scale. Fermentation from renewable sources, as agro-food-industry residues, e.g. cane and beet molasses, or devoted energetic crops, is an ecological and economic way to fulfil market demands. Alternative feedstocks are being looked for to reduce ethanol production costs[3,4]. To be competitive at a large scale, fuel alcohol production by fermentation requires the introduction of important modifications, namely to be run in continuous mode and the use of immobilised cells[5]. Several operating parameters influence ethanol productivity that also depends on cell concentration. On the other hand, biomass growth is sensitive to ethanol concentration. In a batch fermentation process, the sensitivity of cells to ethanol is not a problem because each batch uses a new

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inoculum, but in continuous operation this limits the maximum concentration of ethanol achievable. Biomass immobilisation on a solid support enables to achieve a high microorganism concentration in a continuous fermenter and improve productivity. Nevertheless, immobilisation cannot risk decreasing the renewal rate of microbial cells, very important in long term operation. In fact, the aim is to achieve a high concentration of viable cells in the fermenter.

Self-aggregating yeast strains[6,7,8], their entrapment in an organic matrix[9,10] or adsorption on organic or inorganic supports[2], are the most common methods for cells immobilisation. Apart a great amount of biomass per mass (or volume) unit of support, they must also have other important features, namely (i) a high mechanical and chemical stability, (ii) they must be non toxic and biocompatible with the cells and (iii) they must promote a high diffusion coefficient for both substrates and fermentation products. The best support does not exist at all; so, a compromise must be established, taking into account the use of the immobilised cells.

Artificial entrapment of cells in organic matrices makes it possible to achieve high cell densities and it allows the attainment of tailor made supports by a somehow control of their properties. This goal can be enough for using the immobilised cells as biocatalysts, as long as substrates and reaction products can diffuse through the support without strong mass transfer resistances. Gel entrapment of cells is probably not the best choice for fermentation processes, particularly for continuous operation. In fact, ethanol is a primary metabolite, that is, a compound associated to cell growth. This means that in a long term continuous fermentation it is essential to preserve cell viability. To favour the ethanol synthesis we are mainly interested in anaerobic conditions, but it is also necessary to guaranty a small growth rate of yeast cells (a facultative anaerobe) and favour its renewal. Therefore it is not only important to trap viable cells but also to allow old ones to be released and dragged by the effluent stream. The best way to achieve this goal is to allow for natural entrapment of cells by colonizing a three dimension net with large pores.

1.1 Natural immobilisation of yeasts

Different solid supports were selected to be tested for natural immobilisation taking into account their ability to immobilise cells and their density. This last property is relevant because our project aims at developing a fluidised bed fermenter. The easy way of immobilising cells is by letting Nature work. Under the right conditions (*e.g.*, reactor dilution rate exceeding the specific growth rate of the cells), most microorganisms will attach to a solid surface (by adherence or adsorption), and form a biofilm, which is a complex structure of cells and cellular products (such as extra cellular polysaccharides, often produced by bacteria) linked to each other and to the support.

The preliminary adsorption tests carried out revealed that Leca[®] (a light expanded clay aggregate in small spherical granules, “www.leca.pt”) and PVC compact foams could be good supports. Coke was mentioned as a good support for *Saccharomyces* adsorption in a synthetic medium but not in a cane molasses medium. Cork is light but not enough resistant to shear in the fluidised flow. PVC foams enable bed fluidisation; however, the quantification of biomass by incineration is not possible because high temperatures reduce PVC foams to ashes.

Leca[®] particles with 1.4-1.7 mm diameter were used in tests (cell growth and fermentation), performed batch wise and continuously, in a bench scale tubular reactor (3 cm diameter and 40 cm height). When this small fermenter is run continuously in down flow, the CO₂ produced enables fluidisation of the top half of the bed height. In the lower half, the CO₂ flows upwards in slugs. Although this does not fully correspond to the desired flow conditions, it will be possible to improve gas/liquid flow hydrodynamics by working on the diameter/length fermenter ratio. Concerning the ability of *Saccharomyces cerevisiae* to adhere, Leca[®] has proved to be an adequate support. During continuous operation the amount of immobilised biomass has reached 90g/kg of Leca[®]. A continuous operation with a dilution rate up to 1.2 h⁻¹ was achieved with yeasts immobilised on Leca[®]. Part of the biomass in the reactor is always in suspension and the equilibrium between biomass produced and biomass washed out is sometimes difficult to be established, particularly in long term operations, very often leading to the blockage of the reactor by cell accumulation.

The natural adsorption of cells on inorganic supports (cells affinity to organic supports can be higher but they are more sensitive to shearing effects), at the form of biofilms, is a good choice for the biomass retention in fluidised bed fermenters. These carriers are usually cheap, with mechanical stability and the internal mass transfer resistance to substrates and products diffusion is very low. Nevertheless, biofilm is not an immobilisation procedure free of limitations. The start-up phase of biofilm reactors is very long, biofilms are not very well established (the kinetics of formation and detachment of biomass is not well understood), reactors tend to show an oscillatory performance and they are very sensitive to sudden changes in the operating conditions, such as inlet concentrations or dilution rate. The use of natural adsorption of yeasts for continuous fermentation has also drawbacks, such as the low capacity of yeasts to attach to solid surfaces, the low density of carriers (to facilitate fluidisation) associated to the CO₂ release turn solid retention inside the reactor difficult (a screen can not be used because it blocks very fast), and the low “buffer” capacity of the reactor to disturbances (the detachment of a large fraction of biomass can collapse the reactor). At bench scale we tried to attenuate these disadvantages by modifying hydrodynamic flow and design of the reactor; however, it seems to be difficult to keep a long term operation of this biological system at a larger scale. Biofilm reactors for continuous ethanol fermentation are not so efficient as they are for effluent treatment, in trickling filters, with fixed solid supports, where organic compounds are just converted into biomass.

At the best of our present knowledge and experience, we believe that the natural allowance for cells to grow in light macro-porous supports, such as polyurethane foams (commercially available), can be a good choice for biomass retention in fluidised bed fermenters. Cells can colonize the three-dimensional structure, being retained and giving very high cell concentrations in the reactor bed, but also allowing cells renewal. The CO₂ release is also easy. Long term operation can be kept by changing a fraction of the biomass loaded foams by fresh foams.

Most of the continuous fermentations for ethanol production described in the literature involve short-term operation[8,10] (indeed a major difficulty is keeping the system operating for a long period without troubles), as well as low dilution rates[2,7,8], to avoid cells wash-out (particularly when they are self-aggregated or adsorbed on supports). An increase in productivity requires a high dilution rate and stable operation for long periods, being an important feature to enable future use at industrial scale.

In this work natural methods of yeasts immobilisation were used, taking as goal the performance of a continuous semi-fluidised bed fermenter. Long term experiments were run using cane molasses and a constant concentration of sucrose in the feed. Two different *Saccharomyces* strains (flocculent and non flocculent) were tested with two different supports (Leca[®] and polyurethane foam), and without support, in three different reactor sizes (0.25, 1 and 5 L). During long term operation, problems of different natures were faced, *e.g.* biological, analytical and hydraulic: solutions have been proposed.

2. Experimental

2.1 Tubular Reactors

The smallest tubular reactor was built with Perspex[®] tubing of 3.2 cm i.d. and 39 cm length, with a working volume of 0.25 L. The medium was pumped by a peristaltic pump (Watson Marlow 101 U/R) and introduced close to the top of the reactor that worked down flow. The effluent was collected close to the bottom of the reactor by a tube with a U. The length of this tube controlled the liquid level in the reactor. A thermostatic bath was used to control the reactor temperature at 30±0.5°C.

The second reactor has a working volume of 1L, it was also built with Perspex[®] tubing (52 cm length and 5.4 cm i.d.) with a jacket, for temperature control, through which circulated water from a water bath, and a conical bottom in stainless steel, to allow for flow distribution in the inlet zone. The reactor usually worked in up-flow operation, but it can also work in down flow. The medium was pumped by a Watson Marlow 505 S peristaltic pump and another peristaltic pump was used for the recirculation stream.

The design of the largest reactor used (5 L working volume) is different as it has an expansion zone in steel at the top. In this reactor temperature control is achieved by means of an external heat exchanger,

through which flows the recirculation stream. It can also work in up or down flow mode. Peristaltic pumps (Watson Marlow 505 U/R) were used to pump both fresh medium and recirculation streams. The gas outlets at the top of the reactors were plugged with cotton. The reactors and tubes were washed with bleach solution (10%) and sterilized water, prior to operation.

2.2 Yeast inoculum

The same procedure was used to cultivate each strain: *Saccharomyces cerevisiae* NCYC 1119, obtained from UKNCC, and *Saccharomyces cerevisiae* used for sparkling wine production (local supplier). Yeasts were cultivated in sterilised Erlenmeyers with a synthetic medium containing malt extract (3 g), yeast extract (3 g), peptone (5 g), glucose (10 g) and water (1L). The inoculum cultures were incubated for about 48 hours at 30°C and 120 rpm. These conditions promote biomass growth and a concentrated yeast culture was obtained for reactor inoculation.

2.3 Supports

Spherical granules of a light expanded clay aggregate, Leca[®] (1-1.4 mm diameter), given by Maxit - Argilas Expandidas, S.A., were used once washed and sterilized. Polyurethane foam was cut into small pieces with *c.a.* 0.1 cm³ volume, washed and sterilized.

2.4 Feedstock medium

The feedstock solution used throughout this study consisted of water (798 g dm⁻³), cane molasses (200 g dm⁻³), and (NH₄)₂SO₄ (2 g dm⁻³). The cane molasses were obtained from R.A.R. (Porto – Portugal) and have the following composition (% w/w): dry matter: 69.2; total sugars: 48.1 (sucrose 45.5; glucose 1.6; fructose 1.0, analysed by Enzymatic Testkit, r-biopharm); crude protein: 1.7; ash content: 7.7; pH: 6.9. The feedstock solution was autoclaved for 80-120 minutes, depending on the volume, and the pH value was approximately 7.6±0.2.

2.5 Analysis

Effluent samples were collected periodically for analysis. Ethanol and sugars concentrations were measured by HPLC. The samples analysed by HPLC were immediately centrifuged in a Hettich Zentrifugen Universal 32, at 3500 rpm for 15 min, to remove biomass, than filtered through 0.2 µm pore membranes and frozen for later analysis. The HPLC equipment consists on a system equipped with a WellChrom K-1001 pump. The oven was set at 60°C and a PL Hi-Plex H Acid 8µm column was used; 100% of 0.01N H₂SO₄ and a flow rate of 1 mL min⁻¹ was used as the mobile phase. The identification of ethanol was carried out using a refractive index detector (Perkin Elmer model LC-25 RI). Ethanol was also measured by distillation according to the norm NP-2143 and a DENIS volumetric alcoholmeter, class II F80 01 851 was used. This technique is widely used in distilleries and could be used in this study as continuous operation allows for large volumes of effluent. pH was measured with a pH meter from WTW-inoLab.

3. Results

The first set of experiments was carried out in the smallest reactor using Leca[®] as support for the immobilisation of a non-flocculent strain of *Saccharomyces cerevisiae*. At the outset of continuous operation Leca[®] occupied 40% of the net reactor volume used, in order to allow for bed expansion due to both CO₂ production and biomass growth. The rate of gas formation was high, therefore down flow was used to avoid support entrainment in this small size reactor due to fluidization by the produced gas. The tubular reactor was placed in a water bath to allow for constant temperature 30±0.5°C. Over a period of 25 days, after steady state had been reached, the flow rate was increased in order to study reactor

performance at different dilution rates, ranging from 0.2 to 1.2 h⁻¹. The results are summarized in Table 1. pH was measured daily and effluent samples were analysed twice a day. Due to the low flow rates used, large volume of samples could not be collected and, consequently, HPLC was used to measure effluent composition. The data in Table 1, runs A to D, are related to the smallest reactor and show that it took eleven days to reach the first steady state (run A). In biological systems, a start up period is required in order to develop a significant concentration of biomass, that explains this long period, also due to the low dilution rate used. The following steady states were reached much faster. Along the 25 days this continuous fermentation lasted, the change in ethanol concentration in the effluent was not significant: 18.1 to 20.9 g L⁻¹. Nevertheless, it was expected a drop in ethanol concentration as the dilution rate increased, due to a shorter residence time, but this is not shown in Table 1. The main reason is the increase of biomass observed during the long term fermentation that, certainly, influenced ethanol production. Biomass built up in this small size reactor and the flow through the bed became unstable, as operation duration increased. CO₂ produced could not escape easily. It formed big slugs and when these burst the support was often swept away blocking the reactor outlet. So, hydrodynamic and flow problems forced to stop the continuous experiment at D=1.2 h⁻¹, before steady state had been reached. Although ethanol concentration remained nearly constant during the continuous fermentation, ethanol productivity reached 20.9 g L⁻¹h⁻¹ at D=1 h⁻¹, that was a good result compared to batch fermentation.

Table 1. Continuous fermentation with a non flocculent *Saccharomyces cerevisiae*, with two different supports and reactor sizes (down-flow operation).

Run	A	B	C	D	E	F	G
Reactor volume, V (L)	0.25	0.25	0.25	0.25	1	1	1
Support	Leca®	Leca®	Leca®	Leca®	Polyurethane foam	Polyurethane foam	Polyurethane foam
V_{support} /V	0.4	0.4	0.4	0.4	1/3	1/3	1/3
Dilution rate, D (h⁻¹)	0.2	0.6	1	1.2	0.2	0.4	0.6
Ethanol concentration (g L⁻¹)	18.1	19.7	20.9	18.8	33.3	26.5	21.2
Ethanol productivity (g L⁻¹h⁻¹)	3.6	11.8	20.9	22.5	6.7	10.6	12.7
pH (average)	5.4	5.4	5.3	5.3	5.1	5.3	5.0
Recirculation stream	no	no	no	no	yes	yes	yes
Operation time (days)	11	8	3	3*	4	6	21
Steady state reached	yes	yes	yes	yes	yes	yes	yes

* Operation troubles

In the 1L reactor, with a jacket, polyurethane foam was used as support, in a continuous fermentation that lasted for a month, runs E to G, and the highest dilution rate tested was D=0.6 h⁻¹. A recirculation stream was introduced to improve fluidization. The larger flow rates used enabled to collect enough effluent to be analysed for ethanol content by distillation. The highest ethanol concentration, 33.3 g L⁻¹, was obtained at D=0.2 h⁻¹. Once steady-state was reached, the dilution rate was increased and during the

experiment it ranged from 0.2 to 0.6 h⁻¹, while the outlet ethanol concentration decreased, as expected, from 33.3 to 21.2 g L⁻¹. Nevertheless, as the flow rate is higher, the maximum productivity 12.7 g L⁻¹h⁻¹ was reached for D=0.6 h⁻¹. Although the desired competitive productivity (25 g L⁻¹h⁻¹), compared to batch fermentation was not yet achieved, experiments in this reactor compare well to equivalent dilution rates in the smallest reactor. For further improvement of the productivity the dilution rate must be increased and, simultaneously, a larger volume of support was used to increase biomass concentration in the reactor. After one month in continuous operation, the pieces of polyurethane foam were completely filled and covered with biomass, and an increased adherence between foam pieces was noticed which rendered the flow difficult. This explains why the experiment was interrupted in run G, before other dilution rates were tested. In spite of the problems faced during a month of continuous operation, both the strain used in this experiment and the polyurethane foam, allowed to achieve promising results.

The flocculent yeast strain *Saccharomyces cerevisiae* NCYC 1119 allows to compare these results to performances of reactors and strains when supports are not used. The 1 L reactor was inoculated and run in batch mode for two days, to increase cell density, before continuous operation was started. In these set of experiments, in up-flow, a recirculation stream was used to improve biomass fluidization. In the first two runs shown in Table 2, operation without support was tested. The reactor worked for six days at a dilution rate of 0.2 h⁻¹ and, at steady state, the ethanol concentration was 36.5 g L⁻¹. In run 2 the flow rate was increased to D=0.4 h⁻¹; however, a few hours later a decrease in activity was registered. The ethanol content in the effluent was low and the biomass had been washed out. The problems faced to attain biomass retention without immobilisation, even with a flocculent strain, confirm this is unpractical and the use of supports is required.

The polyurethane foam was tested afterwards as support for this flocculent strain, runs 3 to 7 in Table 2. The reactor was filled with pieces of foam up to the level of effluent outlet, inoculated and run batch wise for two days. Once again, the first dilution rate tested was 0.2 h⁻¹. After seven days in continuous operation, the effluent analysis for ethanol showed reproducibility confirming steady state had been

Table 2. Continuous fermentation with a flocculent *Saccharomyces* strain (NCYC 1119), with and without polyurethane foam as support for cells immobilisation (up flow operation).

Run	1	2	3	4	5	6	7
Reactor volume, V (L)	1	1	1	1	1	1	1
Support	no	no	yes	yes	yes	yes	yes
V _{support} /V	-	-	1	1	1/3	2/3	2/3
Dilution rate, D (h ⁻¹)	0.2	0.4	0.2	0.4	0.4	0.6	0.8
Ethanol concentration (g L ⁻¹)	36.5	⁽¹⁾	40.7	⁽²⁾	33.7	30.6	31.4
Ethanol productivity (g L ⁻¹ h ⁻¹)	7.3		8.1		13.5	18.4	25.1
Operation time (days)	6		7	4	11	4	3
Steady state reached	yes	no	yes	no	yes	yes	no ⁽³⁾

⁽¹⁾ washing out was observed

⁽²⁾ operation blockage

⁽³⁾ cells blockage, steady state not confirmed

reached at a concentration of 40.7 g L⁻¹. At this stage a big increase in biomass was already noticeable; however, biomass built up as operating conditions were changed to D=0.4 h⁻¹. The reactor had been running at this dilution rate for four days when operation had to be stopped, due to the strong accumulation of biomass, some of it probably non active due to mass transfer resistances. For a long term continuous operation with this strain, it is much better not to fill up the reactor with support. Pursuing a compromise, in the following runs the volume of support used was reduced. Reactor operation was restarted with one third of the volume filled with polyurethane foam at a dilution rate of 0.4 h⁻¹ (run 5). The ethanol concentration in the effluent increased steadily, till a constant productivity of 13.5 g L⁻¹h⁻¹ was achieved. In order to maximize productivity, the dilution rate was increased as well as the volume of foams, which reached two thirds of the reactor volume. In Table 2 it can be confirmed that this strategy led to the expected results. A productivity of 25 g L⁻¹h⁻¹ was reached but this result could not be confirmed due to another operation interruption, once again caused by biomass accumulation.

To avoid biomass accumulation, a long term continuous experiment was carried out in the 1 L reactor, again with the non flocculent strain. The results of run 1 of Table 3 confirm that ethanol productivity increased with dilution rate. During the first period of operation at D=0.6 h⁻¹, 6 days, ethanol productivity did not exceed the maximum value reached before with D=0.4 h⁻¹. Through the transparent acrylic fermenter walls it was observed that biomass was building up and the bed was compacted. To overcome this problem the recirculation flow rate was increased to approximately 10 times the inlet medium flow rate, allowing for bed expansion and the release of inactive biomass. A better contact between active biomass and molasses medium may explain the increase in ethanol concentration and productivity, which reached an average value of 18.6 g L⁻¹h⁻¹. In order to confirm these results a second experiment was carried out in this reactor (run 2 in Table 3), using the same yeast and medium composition and D=0.4 h⁻¹.

Table 3 – Continuous fermentation with non flocculent *Saccharomyces cerevisiae* strain, with two different reactor sizes and two different sugar feed concentrations ($V_{\text{support}}/V=30\%$).

	Run 1			Run 2		Run 3		Run 4	
	A	B	C	D	E	F	G	H	
Reactor volume, V (L)	1	1	1	1	5	5	5	5	
Feed sucrose concentration (g L ⁻¹)	100	100	100	100	100	100	130	130	
Dilution rate, D (h ⁻¹)	0.2	0.4	0.6	0.4	0.2	0.4	0.2	0.4	
Ethanol concentration (g L ⁻¹)	41.6	30.8	31.0	28.4	35.5	27.4	59.9	40.0	
Ethanol Productivity (g L ⁻¹ h ⁻¹)	8.3	12.4	18.6	12.8	7.1	11	12.0	16.1	
pH (average)	5.0	5.3	5.0	5.1	4.3	4.6	4.8	4.7	
Operation time (days)	3	7	20	8	15	11	14	12	
Sugar conversion (%)	-	-	-	-	69.8	55.4	76.0	50.5	

The later scaling-up of the fermentation process was carried out in a larger reactor, 5L, and at different operating conditions: two different sugars concentrations (100 and 130 g L⁻¹), T= 30.5±0.5 °C and pH= 4.3-4.8 (Table 3, experiments E to H). In these experiments ethanol productivity increased both with dilution rate and sugar content in the feed. As the inlet sugar content was increased, the highest ethanol concentration 59.9 g L⁻¹ was obtained, at D= 0.2 h⁻¹ (experiment G). On the other hand the highest ethanol productivity 16.1 g L⁻¹h⁻¹ was obtained in the same run, at D= 0.4 h⁻¹ with a total sugar conversion of 50.5 %. The bioreactor had been operating for 26 days and biomass accumulation on the foams obliged once again to stop the run before dilution rate was further increased to test on the evolution of the productivity pattern.

4. Conclusions

The data gathered in these long term runs confirms that continuous fermentation of cane molasses with yeasts can reach high ethanol productivities, once a high cell density is achieved. Polyurethane foams proved to be able to trap the biomass and allow non-stop continuous mode for 30 days, before becoming clogged. Nevertheless, the density of the foams determined operation mode. Down flow and a recirculation stream were used in order to expand the bed. Different long term experiments were performed using three different reactors and two *Saccharomyces cerevisiae* strains. It was confirmed that, in the range of dilution rates used, ethanol productivity increases with dilution rate. The use of bigger size reactors reduced the hydraulic problems faced when flow rate was increased in this three phase fermentation process. The highest ethanol productivity reached was 25 g L⁻¹h⁻¹ at 0.8 h⁻¹ dilution rate, using polyurethane foam as biomass support. Although this is a good result, it requires further testing to ensure reproducibility. Taking the stability of the biological process as the main parameter to optimise for long term operation, the best results were achieved with a non-flocculent strain of *Saccharomyces cerevisiae* immobilized on polyurethane foam.

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