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Yeast immobilization in LentiKats®: a new strategy for xylitol bioproduction from sugarcane bagasse

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Summary

A new PVA-hydrogel matrix for yeast cell immobilization for xylitol bioproduction from sugarcane bagasse was studied. Five repeated-batch fermentation runs were carried out in medium based on sugarcane bagasse hemicellulosic hydrolysate with reuse of the entrapped biocatalyst. The system performance as well as the metabolic behaviour of cells entrapped into the matrix were evaluated. The biocatalyst remained stable and exhibited a similar fermentative profile in all the successive batches, demonstrating the viability of the system. At the end of the run, an average xylitol production was observed of 35.1 g l^{-1} and average xylitol yield and productivity of 0.58 g g^{-1} and 0.49 g l^{-1} h^{-1}, respectively.

Introduction

Xylitol is a five-carbon sugar alcohol, which has become very attractive as a sugar substitute because of its high sweetening power (similar to sucrose) and anticariogenic properties (Ikeuchi *et al.* 1999). It has many advantages as a food ingredient and does not undergo the Maillard reaction, which is responsible for both darkening and reduction of the nutritional value of proteins. Xylitol incorporation in food formulations improves the colour and taste of its preparations, without implying undesirable changes in its properties during storage (Parajó *et al.* 1998a). Xylitol is suitable for diabetics, because of its insulin-independent metabolic utilization and is recommended for obese people (Emodi 1978; Pepper & Olinger 1988). Moreover, xylitol appears to be an attractive alternative to prevent acute otitis media (Uhari *et al.* 2000), improves the biomechanical properties of bones in cases of osteoporosis (Mattila *et al.* 2002) and is very well received in post-surgery infusions by patients with difficulty in metabolizing sugar (Mäkinen 2000). Therefore, this polyol has great potential for use in both the food and pharmaceutical industries, possessing high aggregate value.

Although xylitol is naturally found in many fruits (yellow plums, raspberry, strawberry, blueberry, grape, banana) and vegetables (lettuce, cauliflower, carrot, aubergine, pumpkin, onion), it cannot be extracted from these sources due to their low content. On a large scale, xylitol is currently produced by chemical hydrogenation of xylose derived from hemicellulosic hydrolysates, in the presence of a nickel catalyst. However, this process requires high temperature and pressure (Winkelhausen & Kuzmanova 1998) as well as xylose purification prior to chemical reaction (Hyvönen *et al.* 1982), since the presence of impurities reduces xylitol yield. As a consequence, it is characterized by high production costs and high product price (Silva *et al.* 1996).

The use of lignocellulosic materials for high value chemical production by biotechnological processes is very promising, since they represent an abundant and renewable source of carbohydrate. Natural polysaccharides present in lignocellulosics such as cellulose and hemicellulose can be converted into simple sugars such as glucose and xylose and then used as substrates in fermentation processes. Sugarcane bagasse deriving from the sugar-alcohol industry is an abundant lignocellulosic material in Brazil and is composed approximately of 50% cellulose, 25% hemicellulose, and 25% lignin (Zanderson *et al.* 1999). Although used in sugar factories as fuel for boilers, large quantities of this material are accumulated in the mills, leading to environmental problems. Its use as a solid support for bioprocesses (Pandey *et al.* 2000) and biofiltration of polluted airstreams (Sene *et al.* 2002; Zilli *et al.* 2004) or its chemical hydrolysis to provide hemicellulosic hydrolysate for xylitol bioproduction (Parajó *et al.* 1998b) are additional ways of utilizing this material.

It is widely known that xylitol is produced from D-xylose as a metabolic intermediate in some xylose-metabolizing yeasts (Yahashi *et al.* 1996), and various research groups have carried out studies in order to

produce xylitol by biotechnological processes. In xylose-fermenting yeasts (Figure 1), xylose is reduced by NADPH- and/or NADH-dependent xylose reductase (XR) to xylitol, which is then oxidized to xylulose by mainly NAD^+-dependent xylitol dehydrogenase (XDH). Afterwards, xylulose is phosphorylated by xylulose kinase to xylulose 5-phosphate, which can then enter the pentose phosphate and consequently the glycolytic pathways (Sene *et al.* and 2001).

The main advantage of the microbial process of xylitol production over chemical synthesis, is that there is no necessity for previous xylose purification steps. In addition, agricultural byproducts or residues such as rice straw, sugarcane bagasse and other low cost raw materials can be used as substrates.

Immobilized biocatalysts offer some advantages over free-cell systems, such as protection of the entrapped biocatalysts against adverse environmental conditions, possibility of cell recycling, utilization of high cell densities that usually make possible higher processing rate and high dilution rate in continuous operation. Moreover, immobilization of cells or enzymes can improve the bioprocess efficiency. Due to poor information on the use of immobilized-cell systems for xylose-to-xylitol bioconversion, the authors have dedicated efforts to allow better evaluation of these systems. Some studies have recently been reported on the use of Ca-alginate entrapped-cells (Carvalho *et al.* 2004) and porous glass adsorbed-cells systems (Santos *et al.* 2005) for xylitol bioproduction.

A new support based on polyvinyl alcohol was investigated in this study. Cells of *Candida guilliermondii* were immobilized into LentiKats® hydrogel for xylitol production from sugarcane hemicellulosic hydrolysate and the system performance was investigated.

Material and methods

Preparation of the sugarcane bagasse hydrolysate

The sugarcane bagasse hemicellulosic hydrolysate was obtained by acid hydrolysis of sugarcane bagasse in a 250 l steel reactor at 121 °C for 10 min, using 100 mg of sulphuric acid per g of bagasse (dry wt) at a solid:liquid ratio of 1:10. The liquid fraction was separated by centrifugation and concentrated five-fold at 70 °C under vacuum. To minimize the amounts of the main fermentation inhibitors, the concentrated hydrolysate was treated according to the method proposed by Alves *et al.* (1998): the pH was raised to 7.0 with calcium oxide and acidified to pH 5.5 with phosphoric acid. Subsequently, 2.4% (w/v) activated charcoal was added to the hydrolysate, which was then left under agitation

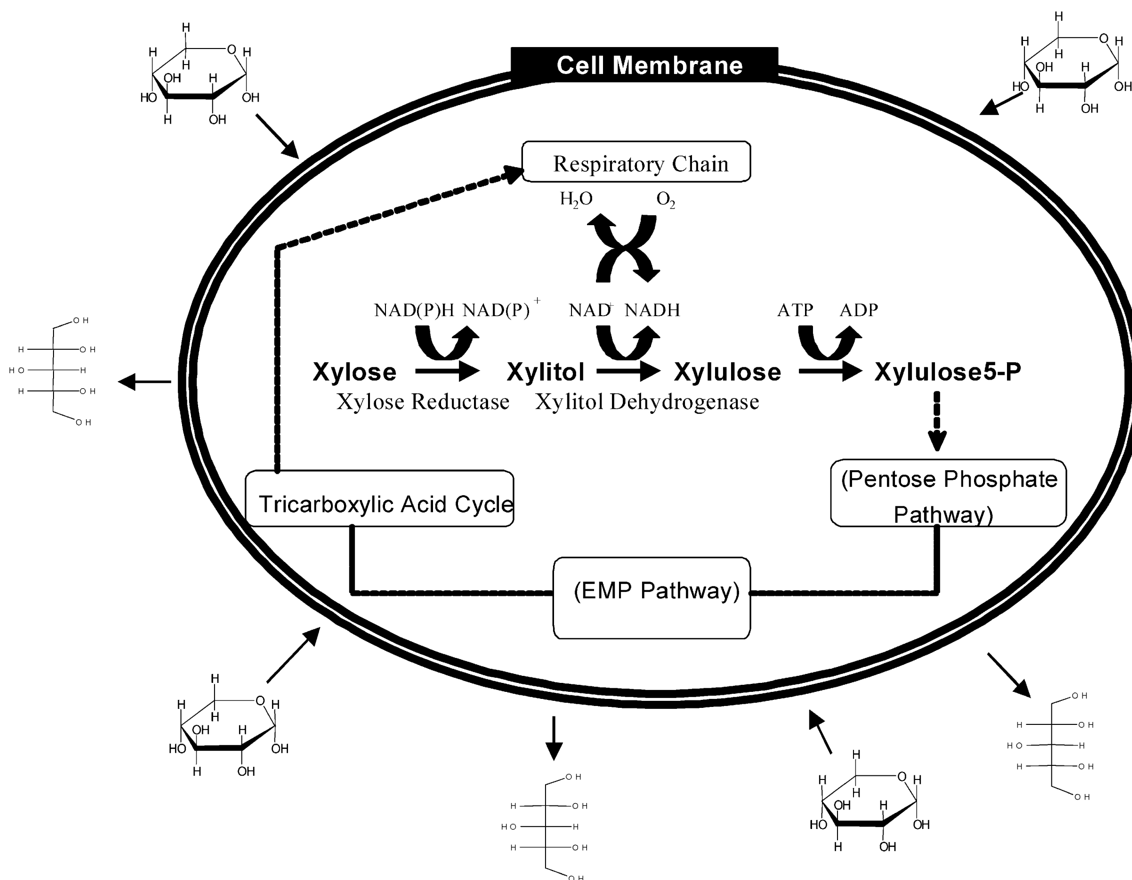


Figure 1. Simplified scheme of xylose metabolism by xylose-fermenting yeasts.

(200 rev/min) at 30 °C for 1 h. The precipitates resulting from all the stages of the treatment were removed by vacuum filtration.

Microorganism and inoculum cultivation

Cells of the yeast *Candida guilliermondii* FTI 20037, described by Barbosa *et al.* (1988), were maintained on malt-extract agar slants at 4 °C.

A loopful of cells was transferred to 125-ml Erlenmeyer flasks containing 50 ml of medium consisted of xylose (30 g l^{-1}), ammonium sulphate (3 g l^{-1}), calcium chloride (0.1 g l^{-1}) and rice bran extract (20% v/v). The inoculum was cultivated on a rotary shaker at 200 rev/min and 30 °C for 24 h. Afterwards, the cells were collected by centrifugation (2000 × *g*, 15 min), rinsed with sterile distilled water, centrifuged, and re-suspended in sterile distilled water to give a suspension with high cell concentration.

Cell immobilization

The yeast cells were immobilized by entrapment in a hydrogel based on polyvinyl alcohol (PVA). About 80 ml of "LentiKats Liquid" (LentiKats®, GeniaLab, Braunschweig, Germany) available as gel were previously melted in a water bath at 90 °C, cooled to 35 °C, and then mixed with 20 ml of cell suspension by a magnetic stirrer, in order to reach a cell concentration of 6 g l^{-1}. The PVA-Hydrogel/yeast cell suspension was allowed to drip through a syringe over Petri plates. The Petri plates containing the droplets were put in a laminar airflow hood (Pachame, Piracicaba, Brazil) under a downwards-vertical airstream at room temperature. Under these conditions, the droplets formed gels and there was a decrease in the initial mass because of water evaporation. The gel particles, obtained in the form of lenses (diameter between 3 and 4 mm, thickness between 300 and 400 μm) were stabilized and re-swelled in stabilizing solution (GeniaLab) for 2 h and then used in the fermentation experiments.

Medium and fermentations conditions

The concentrated and detoxified hydrolysate was heated at 111 °C for 15 min and supplemented with ammonium sulphate (3.0 g l^{-1}), calcium chloride (0.1 g l^{-1}) and rice bran extract (10% v/v), in order to be used as fermentation medium.

The repeated batch fermentations were carried out in duplicate in 125-ml Erlenmeyer flasks containing 45 ml of fermentation medium and 5 g (corresponding to 5 ml) of LentiKats® lenses. The flasks were maintained in a rotary shaker at 200 rev/min and 30 °C. Unless specified otherwise, all the repeated-batch runs were performed for 72 h, where approximately 90% of xylose was consumed. At the end of each run, the fermented medium was unloaded and the system was re-fed with fresh medium. Five successive repeated batch fermentations were performed utilizing the biocatalyst from the preceding run.

Analytical methods

The concentrations of sugars, xylitol, acetic acid, ethanol and glycerol were determined by HPLC with a refractive index (IR) detector and Bio-Rad HPX-87-H (300 × 7.8 mm) column at 45 °C, using 0.005 M sulphuric acid as the eluent, flow rate of 0.6 ml min^{-1} and sample volume of 20 μl. The concentrations of furfural, hydroxymethylfurfural and phenolic compounds were also analysed by HPLC with a UV-VIS detector at 276 nm and a waters 11740 RP18 column using acetonitrile/water (1:8) with 1% acetic acid and phosphoric acid up to pH 2.5 as the eluent, flow rate of 0.8 ml min^{-1}, column temperature of 25 °C and sample volume of 20 μl.

Free cell concentration was determined by a UV-VIS spectrophotometer (Beckman DU 640B, California, USA) at 600 nm using a calibration curve obtained through correlation between cell dry weight and optical density. The immobilized-cell concentration was estimated by the same method after dissolution of lenses by heating at 70 °C.

Kinetic parameters

The experimental data of the initial and final concentrations of products and substrates in a given period of time were used to calculate their respective average volumetric variation rates. These were then divided by the average total cell concentration in the same period to obtain the corresponding specific rates, which were finally expressed as g g_{DM}^{-1} h^{-1} or C-mol C-mol_{DM}^{-1} h^{-1}, according to circumstances. The xylitol yield ($Y_{P/S}$) was defined as the ratio between final xylitol concentration (P_F) and xylose consumption during the run (S_o-S_F). The biomass yield ($Y_{X/S}$) was considered as the ratio of cell concentration variation ($X-X_0$, considering both free and immobilized biomass) to S_o-S_F. The xylitol productivity (Q_P) was considered as the ratio between final xylitol concentration (P_F) and fermentation time. The bioconversion efficiency (η) was considered as the ratio of the xylitol yield on consumed xylose ($Y_{P/S}$) to the theoretical yield of xylitol (0.917 g g^{-1}) defined by Barbosa *et al.* (1988).

Results and discussion

Sugarcane bagasse hydrolysis

Aiming to obtain xylose from sugarcane bagasse, the first step of this study consisted in the preparation of the sugarcane bagasse hemicellulosic hydrolysate, as well as its concentration and treatment for partial removal of toxic compounds. The compositions of sugarcane hemicellulosic hydrolysates, in terms of sugars and

compounds acting as inhibitors of the yeast metabolism, either before or after vacuum concentration, are listed in Table 1.

As it can be seen in this table, the hydrolysis conditions provided a hydrolysate rich in xylose (17.4 g l^{-1}) and having low concentration of glucose (1.30 g l^{-1}), thereby demonstrating a selective extraction of the hemicellulosic fraction. Similar values of xylose and glucose concentrations in sugarcane bagasse hydrolysate were found by Carvalho *et al.* (2002b) and Morita & Silva (2000). Low glucose concentrations in the hydrolysate are desirable for xylose-to-xylitol bioconversion, since glucose is consumed in preference to xylose and even enhances the uptake of this pentose.

In addition to sugars (xylose, glucose and arabinose), the hydrolysate contained hydrolysis by-products like acetic acid, furfural, hydroxymethylfurfural and phenolic compounds (*p*-hydroxybenzoic acid, syringic acid, vanillin, syringaldehyde, ferulic acid) that are known to inhibit yeast metabolism (Parajó *et al.* 1998b). When the hydrolysate was concentrated five-fold, a proportional increase in the levels of sugars (glucose, xylose and arabinose) took place after concentration. Acetic acid and hydroxymethylfurfural levels also increased, but not proportionally to the concentration factor, whereas furfural level even decreased, possibly due to volatilization of this compound during vacuum evaporation at 70 °C. Similar behaviour was reported by Rodrigues *et al.* (2001) for sugarcane bagasse hemicellulosic hydrolysate concentrated under comparable conditions. Although vanillin is a non-volatile compound, a proportional increase was not observed in its level consequent on hydrolysate concentration, probably due to precipitation during the cooled storage of the hydrolysate.

Significant reduction was also observed of the concentrations of furfural and hydroxymethylfurfural after treatment with calcium oxide, phosphoric acid and activated charcoal. Because of the inhibiting effects of these substances, this reduction is very interesting for better microbial development in the hemicellulosic hydrolysate medium and, consequently, for better xylitol production.

Repeated-batch xylose-to-xylitol bioconversion

The operational stability of the proposed system for xylitol production by *C. guilliermondii* cells entrapped in LentiKats lenses has then been evaluated by a set of five repeated-batch runs performed in duplicate with sugarcane bagasse hemicellulosic hydrolysate, using the biomass recovered from the preceding run as a biocatalyst for the subsequent one.

From the results of these bioconversions listed in Table 2, it can be observed that the biocatalyst exhibited a similar fermentative profile in all the five successive batches, without significant losses in xylitol yields, productivity and bioconversion efficiency. In particular, the percentage of xylose consumed after 72 h achieved a maximum value (92.5%) after batch 2 and a minimum one after batch 5 (86.4%), while xylitol production progressively improved from the first batch (33.4 g l^{-1}) to the fourth (36.7 g l^{-1}) and then decreased. Although the standard deviations of these data in relation to the mean values were too low to consider the observed effects significant from the statistical point of view, the general trends suggest a very slight performance decrease. Such a stability of the system indicates that the repeated-batch operation could be profitably exploited for the long-term production of xylitol with only a little loss of the biocatalyst activity. According to Jekel *et al.* (1998), the process with immobilized biocatalyst could offer some advantages over the free cell system, such as protection of the entrapped biocatalyst against environmental effects, higher yields and high process and storage stabilities.

The xylitol yield and productivity also varied very little and showed values comparable to those reported by Carvalho *et al.* (2003) (0.54 g g^{-1} and 0.44 g l^{-1} h^{-1}, respectively) in Erlenmeyer flasks fermentation with *Candida guilliermondii* cells entrapped in Ca-alginate gel in the same medium. The bioconversion efficiency in relation to the theoretical value (0.917 g g^{-1}) estimated by Barbosa *et al.* (1988) increased from 61 to 65% passing from batch 3 to batch 5, thus confirming the results of Carvalho *et al.* (2002c) obtained with the same microorganism in Ca-alginate; however, these authors ascribed the lowest values observed at the end of the first

Table 1. Chemical composition of sugarcane hemicellulosic hydrolysate before and after vacuum concentration.

Component (g l^{-1})	Raw hydrolysate	Concentrated hydrolysate	Concentrated and detoxified hydrolysate
Xylose	17.4	85.0	79.2
Glucose	1.30	7.20	5.55
Arabinose	1.81	9.72	8.74
Acetic acid	2.34	4.94	3.58
Furfural	0.113	0.025	0.003
Hydroxymethylfurfural	0.005	0.028	0.001
p-Hydroxybenzoic acid	0.442	0.588	0.007
Syringic acid	0.020	0.087	0.010
Vanillin	0.041	0.066	0.004
Syringaldehyde	0.464	0.737	0.060
Ferulic acid	0.050	0.202	0.020

Table 2. Results of repeated-batch bioconversions of xylose-to-xylitol by *C. guilliermondii* immobilized in LentiKats lenses on sugarcane bagasse hemicellulose hydrolysate.

Batch N.	1	2	3	4	5	Average	σ range (%)[a]
Y_C (%)[b]	88.5	92.5	89.3	88.0	86.4	88.9	−2.9/ + 4.0
P_F (g l^{-1})[c]	33.4	35.6	35.6	36.7	34.2	35.1	−4.8/ + 4.6
$Y_{P/S}$ (g g^{-1})[d]	0.56	0.58	0.58	0.60	0.57	0.58	−3.4/ + 3.4
$Y_{X/S}$ (g g^{-1})[e]	0.13	0.13	0.13	0.15	0.15	0.14	−7.7/ + 6.7
Q_P (g l^{-1} h^{-1})[f]	0.46	0.49	0.50	0.51	0.48	0.49	−6.1–4.0
η (%)[g]	0.61	0.63	0.63	0.65	0.62	0.63	−3.2/ + 3.2
X_{ri} (g l^{-1})[h]	1.84	1.82	1.84	1.80	1.90	1.84	−2.2/ + 3.3
X_i (g l^{-1})[i]	18.4	18.2	18.4	18.0	19.0	18.4	−2.2/ + 3.3
X_{rm} (g l^{-1})[j]	6.83	7.20	7.18	8.31	8.14	7.53	−9.3/ + 10.4
X_{rt} (g l^{-1})[k]	8.67	9.02	9.02	10.1	10.0	9.36	−7.4/ + 7.9

[a]σ = Error percentage in relation to the mean value; [b]Y_C = Percentage of xylose consumption; [c]P_F = Final xylitol concentration; [d]$Y_{P/S}$ = Yield of xylitol on consumed xylose; [e]$Y_{X/S}$ = Yield of biomass on consumed xylose; [f]Q_P = Xylitol productivity; [g]η = Yield of xylitol in relation to the theoretical value (0.917 g g^{-1}) (Barbosa *et al.* 1988); [h]X_{ri} = Maximum immobilized-cell concentration in relation to the total volume of reactor; [i]X_i = Maximum immobilized-cell concentration in relation to the lenses volume; [j]X_{rm} = Maximum suspended-cell concentration in relation to the total volume of reactor; [k]X_{rt} = Maximum total cell concentration in relation to the total volume of reactor.

and the second batches (53.2 and 59.5%, respectively) to cell adaptation to the hydrolysate.

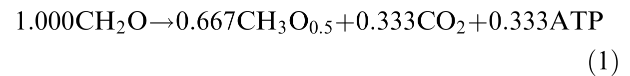
A different situation seemed to take place in the presence of LentiKats lenses, i.e. the low variations of these parameters observed in this study at the end of all the five repeated batches suggest no cell adaptation during the fermentations and that the PVA matrix could have contributed to simple protection of the cells against the adverse conditions present in the hydrolysate.

Cell growth was considered as the increase in the concentration of free cells in the fermentation medium (X_{rm}) and of cells immobilized in the gel lens (X_i). It was observed a maximum cell concentration in the lenses at the end of each batch close to 18 g l^{-1} and average xylose consumption around 90%, whereas there was no further increase in cell concentration inside the gel when the fermentations were prolonged for 84 h (data not shown). This result suggests that the pores of the gel matrix were saturated by immobilized cells after 72 h and that any additional growth could only occur in the fermentation broth. The cells released from the lenses also proliferated in medium, reaching a final concentration that varied from 6.83 g l^{-1} at the end of the first batch until 8.31 g l^{-1} at the end of the fourth.

Metabolic study

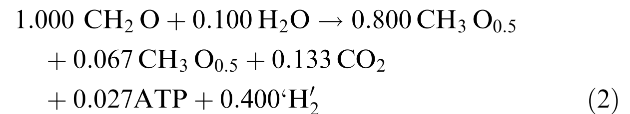
The average results obtained from these bioconversions have then been used in a metabolic study to obtain information on the physiological state and the metabolic behaviour of *C. guilliermondii* cells entrapped in LentiKats lenses. The same approach previously described (Carvalho *et al.* 2005) has been adopted to make carbon balances and estimate the related bioenergetic parameters. For this purpose, the following metabolic model has been proposed, where ‘H_2’ stands for reducing equivalents in the form of NADH or any other form of metabolic reducer:

Glucose-to-ethanol fermentation



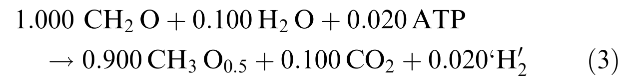
$$1.000CH_2O \rightarrow 0.667CH_3O_{0.5} + 0.333CO_2 + 0.333ATP \quad (1)$$

Anaerobic pentose-to-pentitol conversion



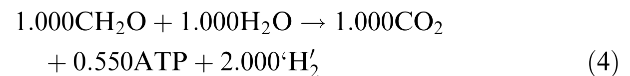
$$1.000\,CH_2O + 0.100\,H_2O \rightarrow 0.800\,CH_3O_{0.5} + 0.067\,CH_3O_{0.5} + 0.133\,CO_2 + 0.027ATP + 0.400`H_2' \quad (2)$$

Semi-aerobic pentose-to-pentitol conversion



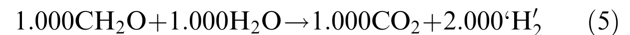
$$1.000\,CH_2O + 0.100\,H_2O + 0.020\,ATP \rightarrow 0.900\,CH_3O_{0.5} + 0.100\,CO_2 + 0.020`H_2' \quad (3)$$

Aerobic pentose oxidation by the TCA cycle



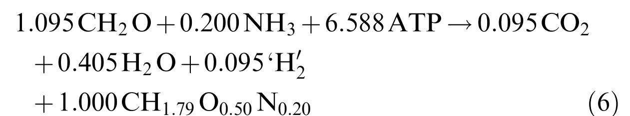
$$1.000CH_2O + 1.000H_2O \rightarrow 1.000CO_2 + 0.550ATP + 2.000`H_2' \quad (4)$$

Aerobic acetate oxidation by the TCA cycle



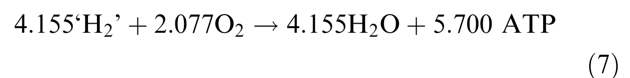
$$1.000CH_2O + 1.000H_2O \rightarrow 1.000CO_2 + 2.000`H_2' \quad (5)$$

Biomass growth on pentoses



$$1.095\,CH_2O + 0.200\,NH_3 + 6.588\,ATP \rightarrow 0.095\,CO_2 + 0.405\,H_2O + 0.095`H_2' + 1.000\,CH_{1.79}O_{0.50}N_{0.20} \quad (6)$$

Reducing power regeneration by the electron transport system



$$4.155`H_2' + 2.077O_2 \rightarrow 4.155H_2O + 5.700\ ATP \quad (7)$$

Applying this model to the experimental data of the concentrations of products (xylitol, arabitol, ethanol, biomass and carbon dioxide) and substrates (xylose, glucose, arabinose, acetic acid) vs. time, the specific rates of variations of metabolite concentrations in the different activities assumed for the metabolism of *C. guilliermondii* have been estimated as earlier described (Table 3).

The comparison of these results to those obtained with the same yeast entrapped in Ca-alginate (Carvalho *et al.,* 2005) suggests a crucial impact of the immobilizing support structure on the physiological state of the cell. In particular, while in Ca-alginate the semiaerobic xylose-to-xylitol bioconversion (Equation 3) was the main activity responsible for either pentose uptake (66.6 $C\text{-mmol}_S\ C\text{-mol}_{DM}^{-1}\ h^{-1}$) or pentitol formation (59.9 $C\text{-mmol}_P\ C\text{-mol}_{DM}^{-1}\ h^{-1}$), in LentiKats lenses it decreased in favour of the corresponding anaerobic activities (Equation 2) (pentose uptake of 68.9 $C\text{-mmol}_S\ C\text{-mol}_{DM}^{-1}\ h^{-1}$ and pentitol formation of 55.1 $C\text{-mmol}_P\ C\text{-mol}_{DM}^{-1}\ h^{-1}$) and the simultaneous formation of ethanol. The rate of anaerobic formation of ethanol from pentoses (Equation 2) (4.59 $C\text{-mmol}_{EtOH}\ C\text{-mol}_{DM}^{-1}\ h^{-1}$) nearly achieved that from glucose fermentation (4.89 $C\text{-mmol}_{EtOH}\ C\text{-mol}_{DM}^{-1}\ h^{-1}$) (Equation 1), as consequence of more anaerobic conditions inside the support with respect to Ca-alginate gels.

According to previous results (Santos *et al.* 2003) and supposing a negligible influence of the immobilization technique on the oxidative phosphorylation efficiency, bioenergetic balances were also performed. Substituting into Equations (1)–(7) the experimental data of the specific rates of product formation and substrate consumption and assuming a P/O ratio of 1.37 $\text{mol}_{ATP}\ C\text{-mol}_O^{-1}$, we calculated the specific rates of ATP variations (q_{ATP}) associated with all the activities listed in Table 3. The specific rates of ATP and reducing power ('H_2') variations linked to the electron transport system, biomass synthesis and catabolic reaction of pentoses increased by at least 50% in relation to the Ca-alginate system. These results demonstrate the better bioenergetics of this system.

Substituting into Equations (1)–(6) the experimental data of the carbon source (glucose, xylose, arabinose and acetate) consumption in five repeated-batch runs, we calculated the average fractions of xylose progressively consumed by each of the assumed metabolic activities, namely anaerobic and semi-aerobic xylose-to-xylitol bioconversions, biomass synthesis from xylose, and xylose consumption by the TCA cycle (Figure 2). These results show that, using the PVA matrix, the fraction of xylose consumed by anaerobic xylitol production increased during the fermentations, exceeding one half the overall uptake. In contrast, such a metabolic pathway was responsible for only 15% of xylose uptake at the beginning of the bioconversion performed with Ca-alginate, the semiaerobic bioconversion to pentitols being the main activity (Carvalho *et al.* 2005). This different behaviour can be explained by the quick saturation of lenses with immobilized cells, whose intense overall activity could have led to the progressive oxygen depletion and the occurrence of oxygen-limited conditions inside the matrix.

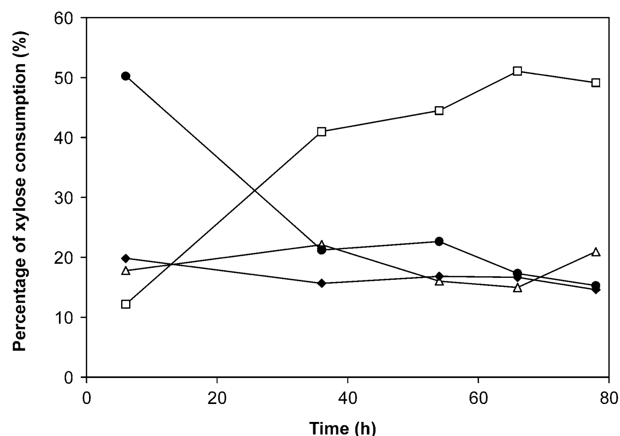


Figure 2. Time variations of the average fractions of xylose progressively consumed by *Candida guilliermondii* cells in five repeated-batch bioconversions. Anaerobic xylose-to-xylitol conversion (□); semi-aerobic xylose-to-xylitol conversion (Δ); biomass synthesis from xylose (♦); catabolic reaction of xylose by the TCA cycle (●).

Such a situation is described even better by the results of q_{ATP} associated with each one of the assumed pathways (Figure 3). These kinetic parameters exhibited high mean values at the start of the run mainly because of typical aerobic activities (reducing power regeneration by the electron transport system and catabolic reaction of pentoses by the TCA cycle) and, in smaller extent, because of the glucose anaerobic fermentation. The subsequent decrease of these parameters took place mainly due to both xylose starvation and the occurrence of more anaerobic conditions. This trend is completely

Table 3. Specific variation rates, q_i ($C\text{-mmol}_i\ C\text{-mol}_{DM}^{-1}\ h^{-1}$ or $\text{mmol}_i\ \text{mol}_{DM}^{-1}\ h^{-1}$)[a], of metabolites' (*i*) concentrations according to the different activities involved in the metabolism of *Candida guilliermondii* cells immobilized in PVA matrix.

Activity (Eq.)[b]	1	2	3	4	5	6	7
Pentoses	0	68.9	29.2	21.6	0	20.5	0
Pentitols	0	55.1	26.3	0	0	0	0
EtOH	4.89	4.59	0	0	0	0	0
ATP	2.45	1.84	−0.58	11.90	0	−94.6	79.0
'H_2'	0	2.76	0.58	43.1	9.3	1.78	−57.6

[a]The units $C\text{-mmol}\ C\text{-mol}_{DM}^{-1}\ h^{-1}$ and $\text{mmol}\ \text{mol}_{DM}^{-1}\ h^{-1}$ are used for the carbon and the non-carbon compounds respectively; [b]The activity numbering is the same as for Equations (1)–(7).

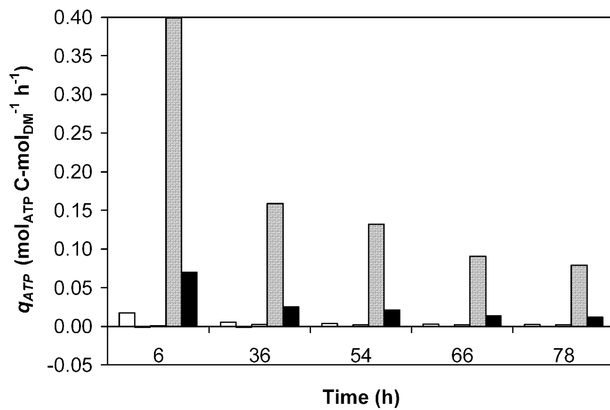


Figure 3. Influence of the fermentation time on the specific rates of ATP generation by glucose fermentation (□), semi-aerobic (■) and anaerobic (■) pentitols productions, electron transport system (■), and catabolic reaction by the TCA cycle (■).

different from that observed in Ca-alginate gel, where the quick substitution of the initial anaerobiosis with semiaerobic conditions within the support was probably responsible for the worse kinetics (Carvalho *et al.* 2005).

We can assume that the cells, during a batch bioconversion, experience a progressive series of pseudo-steady-state conditions, therefore any excess production of energy in the form of ATP has to be consumed for both growth and maintenance (Roels 1983). So the abundant cell growth observed during the first fermentation phases would be the logical consequence of the very high q_{ATP} values. Contrary to previous observations in Ca-alginate (Carvalho *et al.* 2002a, 2005), the carbon sources other than xylose present in the hydrolysate had no crucial role in this bioprocess, thus confirming the earlier supposed effect of cell protection by the support.

The different microenvironments occurring in PVA and Ca-alginate are also evidenced by the time behaviours of the yield of ATP consumption for biomass growth and maintenance ($Y_{ATP/X}$), which was calculated as the ratio of the overall specific rate of ATP generation

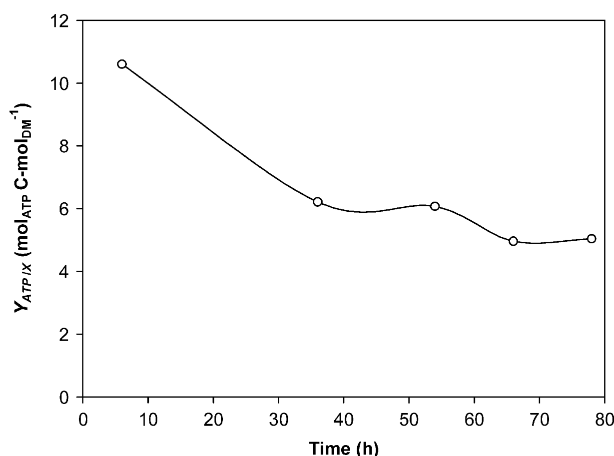


Figure 4. Time behaviour of the yield of ATP consumption for biomass growth and maintenance.

(q_{ATP}) to the experimental specific growth rate (μ). This parameter progressively decreased in the former support (Figure 4) and increased in the latter (Carvalho *et al.* 2005), as the result of increasingly anaerobic and aerobic conditions, respectively. In particular, at the start of the run, this parameter reached a very high value (10.6 mol$_{ATP}$ C-mol$_{DM}^{-1}$) and then decreased to values (5.0 mol$_{ATP}$ C-mol$_{DM}^{-1}$) comparable to those of typical anaerobic fermentations (Zeng *et al.* 1990) and little higher than those of other immobilized-cell systems (Santos *et al.* 2003). These results seem to confirm that xylitol accumulation in this yeast is a process favoured by stress conditions and that oxygen optimization is the most crucial regulatory factor. Finally, it should stand out that, although the final batch xylitol production was lower than using Ca-alginate, the PVA matrix allowed better kinetics, thus suggesting its advantageous use in continuous operation systems.

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