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Influence of Oxygen Availability on Cell Growth and Xylitol Production by *Candida guilliermondii*

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Abstract

Oxygen availability is the most important environmental parameter in the production of xylitol by yeasts, directly affecting yields and volumetric productivity. This work evaluated the cell behavior in fermentations carried out with different dissolved oxygen concentrations (0.5–30.0% of saturation), as well as a limited oxygen restriction (0% of saturation), at several oxygen volumetric transfer coefficients ($12 \leq k_La \leq 70$ h^{-1}). These experiments allowed us to establish the specific oxygen uptake rate limits to ensure high yields and volumetric productivity. When oxygen availability was limited, the specific oxygen uptake rate values were between 12 and 26 mg of O_2/of g cell·h, resulting in a yield of 0.71 g of xylitol/xylose consumed, and 0.85 g/[L·h] for the volumetric productivity. According to the results, the effective control of the specific oxygen uptake rate makes it possible to establish complete control over this fermentative process, for both cell growth and xylitol production.

Index Entries: Xylitol; xylose; aeration; *Candida guilliermondii*; oxygen availability.

Introduction

Xylitol is a natural carbohydrate with sweetness similar to sucrose. It has anticariogenic properties and is insulin-independent when metabo-

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lized by humans. Currently, xylitol is produced by catalytic hydrogenation, an expensive process mainly because of the number of purification stages required. The bioconversion of xylose by yeasts is an alternative xylitol production route, a technology with great economic potential, since microbial production of xylitol is a simple process, with great specificity and low energy requirements *(1–3)*.

Several variables that affect the bioconversion of xylitol from xylose have been examined with the aim of improving fermentation performance *(4-6)*. However, the oxygen supply is one of the most important environmental factors in this bioconversion, drastically affecting the yield as well as the xylitol production rate. Oxygen availability determines the division of carbon flow from xylose between cell growth and product formation *(7–9)*.

The literature indicates that the fermentation of xylose is strongly affected by a limited oxygen supply owing to the action of oxygen as a terminal electron acceptor. This procedure is required by the cell to alleviate the partial redox imbalance in the initial steps of xylose metabolism. When the specific oxygen uptake rate is reduced owing to the lower oxygen supply, the electron transfer system present in the tricarboxylic acid (TCA) cycle becomes unable to regenerate the complete NAD^+ from the NADH produced. As a consequence, an increase in intracellular NADH levels occurs, reducing the enzymatic reaction rate of the NAD^+-dependent xylitol dehydrogenase, and allowing xylitol to accumulate. This behavior is evidence that oxygen is required for the growth and activation of the mitochondrial functions, consonant to other aerobic and facultative organisms, as well as for the generation of energy involved in xylose transport *(10)*.

The purpose of the present work was to evaluate cell behavior during the bioconversion of xylose by a strain of *Candida guilliermondii* with different oxygen availabilities. These different aeration conditions were obtained by varying the volumetric oxygen transfer coefficient (k_La) between 12 and 70 h^{-1}. By discovering the specific oxygen uptake for this strain, we were able to establish fermentation conditions in which cell growth or xylitol production were maximized. Determining the aeration limit at which yeast begins to excrete xylitol at a high rate in detriment to cell growth without compromising the minimum cell functions is of fundamental importance for the development of an efficient process for producing xylitol on a larger scale by bioconversion.

Materials and Methods

Microorganism

C. guilliermondii IM/UFRJ from the Institute of Microbiology of the Federal University of Rio de Janeiro, UFRJ, R.J., Brazil was used. This strain had been preselected in our laboratory by Sá *(3)* as having great potential for xylitol production. The yeast was maintained at 4°C on semi-synthetic agar slants with the following composition: 3.0 g/L of yeast extract, 5 g/L of peptone, 20 g/L of xylose, 15 g/L of agar.

Preparation of Medium and Inoculum

Three loopfuls of cells were inoculated in a medium containing : 20 g/L of D-xylose, 1.1 g/L of potassium phosphate, 1.25 g/L of urea, 1.5 g/L of yeast extract, 40 mL/L of mineral salts solution and citric acid. The pH of the medium was adjusted to pH 6.0 *(11,12)*. The inoculum was grown in a 500-mL Erlenmeyer flask containing 200-mL of medium, previously sterilized (15 min at 110°C), for 36 h at 30°C on a rotary shaker (300 rpm). The grown cells were then used for inoculating the fermentor.

Fermentations Conditions

The fermentations runs, carried out at dissolved oxygen (DO) levels of 30, 1.0 and 0.5% of saturation, were undertaken in a medium with the same qualitative composition as the inoculum preparation, but the concentrations of nutrients were increased proportionally to the concentration of D-xylose (50 and 80 g/L). The experiments were performed in a 2.6-L fermentator (Bioflo III; New Brunswick), containing baffles and two flat blade turbines. The fermentation system was equipped with temperature, pH, and DO controllers. The air stream was filtered through 0.2-µm membranes (Milllipore). All experiments took place in a 2.2-L volume medium at 30°C and pH 6.0, with mechanical stirring and a variable supply of oxygen according to the k_La required.

Analytical Methods

The fermentation runs were monitored through periodic sampling to determine xylose uptake, xylitol production, and cell growth. Samples of an appropriate dilution were prepared by filtration through a 0.45-µm membrane, followed by another filtration with SEP-PAK C-18 (Millipore) before injection for chromatography analysis.

Xylose and xylitol were analyzed by high-performance liquid chromatography (Waters) using a Shodex Sugar SC1011 column (300 × 8 mm) at 80°C and degasified Milli-Q water as the mobile phase at a flow rate of 0.8 mL/min.

Cell growth was estimated by turbidimetry at 570 nm. The cell concentration was determined using a standard curve, which correlated absorbance and dry cell weight.

The volumetric oxygen transfer coefficient (k_La) was determined by the gassing-out method, as suggested by Moser *(13)*.

Results and Discussion

The use of xylose by *C. guilliermondii* occurs inside the cell through an oxireduction stage and is converted into D-xylulose, which, once phosphorylated, is the precursor for cell metabolism and is mainly used in the Embden-Meyerhof-Parnas and pentose phosphate pathways *(10,14,15)*. However, to obtain D-xylulose, the cell first reduces xylose into xylitol, which is an intermediate substance, and this is subsequently oxidized, form-

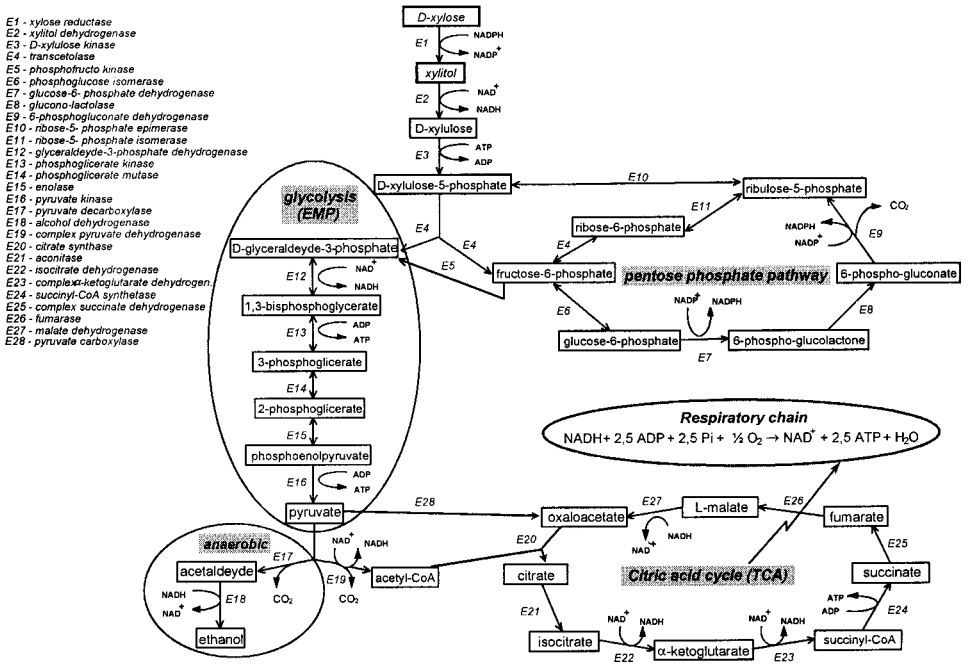

Fig. 1. Metabolism of xylose by *C. guilliermondii*.

ing D-xylulose, Fig. 1. The difference in the rates of these two enzymatic reactions will indicate the accumulation or total consumption of xylitol *(16)*. The first reaction of the oxireduction stage by *C. guilliermondii* shows the preferential action of xylose redutase by NADPH *(17)*, yet the subsequent enzymatic oxidation of xylitol by xylitol dehydrogenase has greater affinity for NAD^+. $NADP^+$ is reduced to NADPH in the pentose phosphate pathway, and NADH oxidation occurs mainly in the respiratory chain *(18)*. The absence or the existence of a small amount of oxygen indicates an intracellular redox imbalance owing to the accumulation of NADH, inhibiting the oxidation of xylitol. This explains the need for oxygen displayed by several xylose-utilizing yeasts that, aiming at cell growth, are unable to regulate this redox imbalance for other metabolic routes.

On the other hand, under limited oxygen supplies, it is possible to guarantee the minimum conditions in which a fraction of the cofactor NADH is regenerated. Thus, the difference in activity between xylose reductase and xylitol dehydrogenase naturally presented by *C. guilliermondii (1)* allows for intracellular accumulation of xylitol up to a limiting concentration, after which the excess xylitol is excreted.

The direct production of xylitol does not bring any energy gain to the cell, and the NADH buildup harms the natural cell metabolism. Thus, it becomes evident that reduced amounts of oxygen are needed to maintain the minimum vital cell functions, and consequently, make xylitol production viable.

The intensity of this "oxygen restriction" will regulate the level of xylitol production. Oxygen restriction should be understood as the aeration condition below which the oxygen supply falls below the minimum required for full growth.

The material balance of the DO concentration (C_{OX}) during a bioreaction can be given by Eq. 1 *(19)*:

$$\frac{dC_{OX}}{dt} = k_L a \left(C_{OX}^{*} - C_{OX}\right) - q_{O_2} \cdot X \quad (1)$$

in which q_{O_2} is the specific oxygen uptake rate (mg of O_2/g of cell·h), C_{OX}^{*} is the saturated DO concentration (mg of O_2) and X is the cell mass (g of cell/L). The term $k_L a(C_{OX}^{*} - C_{OX})$ represents the oxygen transfer rate, while $q_{O_2} \cdot X$ represents the oxygen uptake rate.

Through analysis of Eq. 1, it is possible to see that at the beginning of the bioconversion with fixed $k_L a$, the oxygen transfer rate is dynamically controlled through the variation in DO concentrations in the medium. If the total oxygen demand increases with cell growth, the DO in the medium decreases and the oxygen transfer rate therefore increases. This dynamic operates until the DO concentration in the medium reaches zero. From this moment onward, the oxygen transfer rate becomes constant and no longer assists in the increase of oxygen demand for cell growth. This marks the beginning of the "restriction condition" of oxygen required by the cell to supply its conventional metabolic needs. From this point, there starts to be a redox imbalance, and, thus, the xylitol produced and accumulated inside the cell begins to be excreted.

In abundant oxygen conditions, the term $q_{O_2} \cdot X$ represents the yeast oxygen demand, but under restricted oxygen conditions, it would be more correctly called the "oxygen uptake rate." It should be understood that oxygen uptake rate is the same as the oxygen demand rate until the moment when all the required oxygen is supplied. When this nutrient is restricted, the demand will always be larger than the effective consumption. This conceptual difference can be easily observed by analyzing cell behavior in fermentations with different degrees of oxygenation.

Figures 2–4 depict the kinetic profiles of experiments with DO concentrations of 30, 1 and 0.5% of saturation, respectively. These experiments were carried out with an initial xylose concentration of 50 g/L.

With oxygen abundance (Fig. 2), the cell responds to its natural tendency to grow. Xylose permeates the cell membrane and is converted into xylitol, which, in turn, is transformed into other metabolites to assist other cell functions. All the produced xylitol is consumed, which is characteristic of the balance between the oxidation-reduction reactions involved. This behavior continues throughout the fermentation, resulting in a cell yield ($Y_{X/S}$) of 0.51 g/g.

In the fermentation when the available oxygen was 1% of saturation (Fig. 3), it was observed that the full cell oxygen demand ceased to be met. At this point, less xylitol was excreted. However, this small redox imbal-

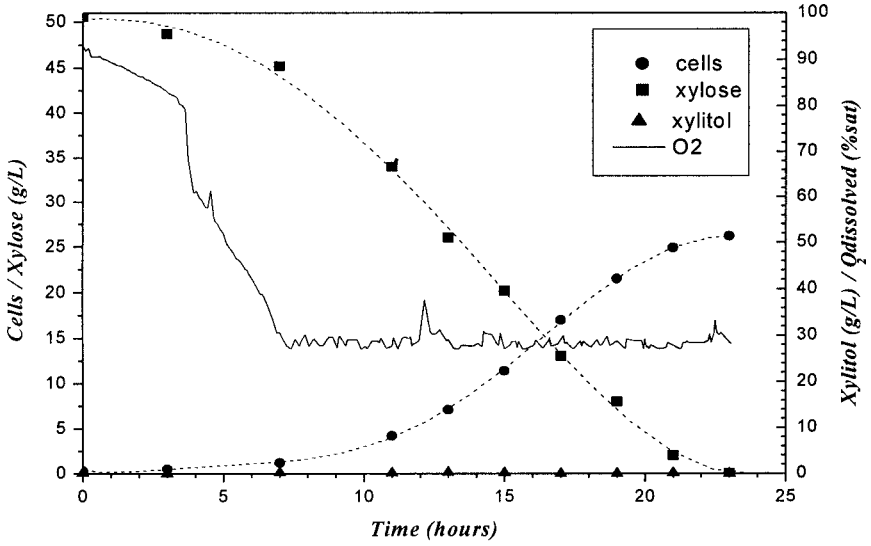

Fig. 2. Xylitol and cell concentrations during fermentation at 30% of DO.

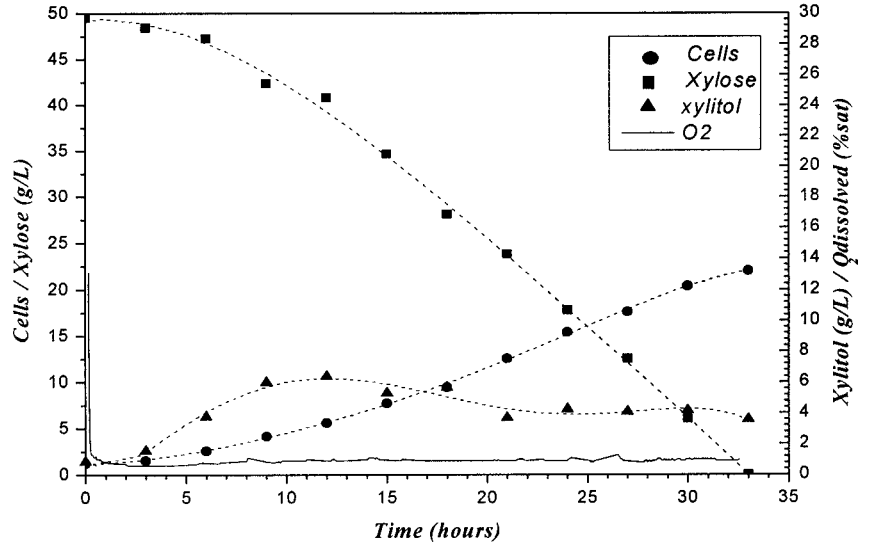

Fig. 3. Xylitol and cell concentrations during fermentation at 1% of DO.

ance tended to be regulated by the cell, which adapted to this aeration condition and reduced its growth pace. Thus, the cell readjusted to a balance between the cofactors' availability since the required rate for regeneration of NAD^+ started to be fulfilled again. This is confirmed through the progressive decrease in xylitol excretion. In these conditions $Y_{X/S}$ was 0.42 g/g.

The reduction in the oxygen supply to 0.5% of the saturation (Fig. 4) considerably increased the oxygen restriction, resulting in a more drastic

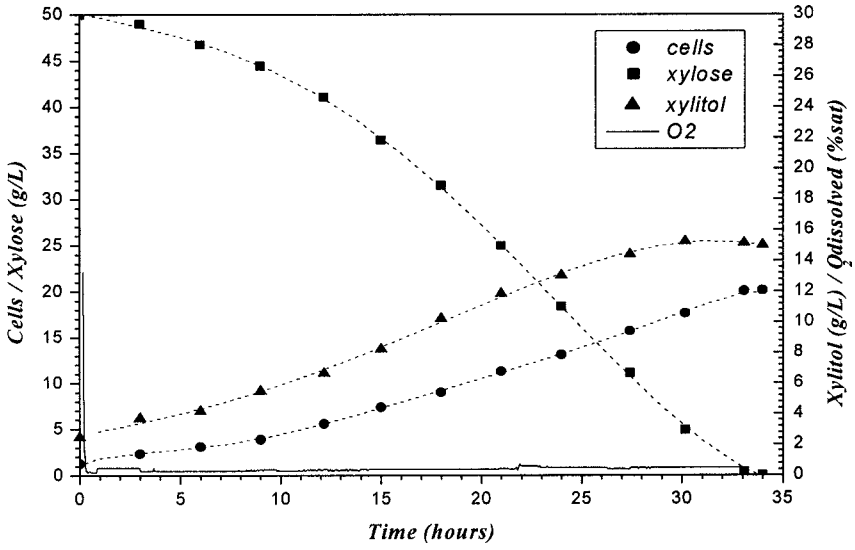

Fig. 4. Xylitol and cell concentrations during fermentation at 0.5% of DO.

reduction in the intracellular consumption of the xylitol produced. Since not all the xylitol produced could be consumed, the excess was excreted. However, unlike in the previous conditions (DO of 1%), the cell was unable to meet its needs in NAD^+ owing to the intense damage in the regeneration of this cofactor, in view of the very low oxygen availability. In this condition, the cell growth rate was significantly reduced ($Y_{X/S}$ = 0.38 g/g), and 15.2 g of xylitol/L was excreted, corresponding to a xylitol yield value ($Y_{P/S}$) of 0.31 g/g.

Figure 5 shows the results of an experiment carried out with different aeration rates and an initial xylose concentration of 109 g/L. Initially, the cells grew in an environment with abundant aeration, in which a DO concentration of 25% of the saturation was maintained. Under these conditions, extracellular xylitol was not detected and there was vigorous cell growth. When the cell concentration reached a value of approx 9 g/L, the automatic DO control system was turned off and k_La reduced to a fixed value of 17.9 h^{-1}, which marked the beginning of restricted oxygen. This shift led to a sharp reduction in the DO concentration, and in <2 h the DO concentration decreased to 0% of the saturation. The presence of xylitol in the medium was detected immediately after this alteration in oxygen availability. Apparently, the intensity of xylitol excretion was greater in the first hours when k_La was 17.9 h^{-1}, after which time a reduction occurred. From this moment on, the excretion rate remained practically constant until there was a new alteration in k_La.

This alteration in the xylitol excretion rate seen in the first hours after a brusque change in oxygen availability can be related to the abrupt alteration in the condition of plenary cell growth, in which all the enzymatic apparatus was working at full activity, for another oxygen condition

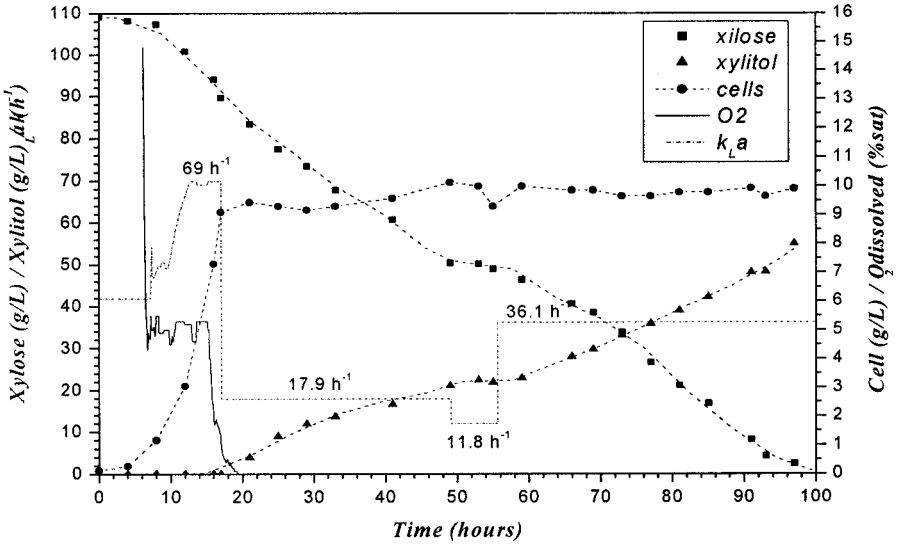

Fig. 5. Xylitol, cell and DO concentrations during fermentation at different aeration rates.

(restricted), below the ideal limit of oxygen supply for the obtainment of an ideal xylitol excretion rate.

After 48 h of bioreaction, the k_La was further reduced to 11.8 h^{-1}. This reduction affected cell activity even further, and growth and production came to a halt. The cells submitted to aeration conditions below the ideal limit of oxygen restriction tended to cease growing, also compromising the pentose phosphate pathway responsible for NADPH regeneration, used in the targeted conversion of xylose into xylitol by xylose reductase.

After this period of high oxygen restriction, the k_La was increased to 36.1 h^{-1}. As the availability of oxygen increased, the enzymatic activities of the pentose phosphate pathway were restored and xylitol was once more excreted. Under this new aeration condition, the xylitol production rate (0.85 g/[L·h]) was higher (0.63 g/[L·h]) than that observed with a k_La value of 17.9 h^{-1}, indicating that it was nearer to the optimum aeration limit for xylitol production. Note that even when aeration was more abundant, the DO concentration remained at zero, characteristic of a restricted condition.

The material oxygen balance in restricted condition when the DO concentration is zero ($dC_{OX}/dt = 0$) yields:

$$k_L a\left(C_{OX}^{*} - 0\right) = q_{O_2} \cdot X \tag{2}$$

Thus, the specific oxygen uptake rate for yeast in restriction conditions can be described by Eq. 3:

$$q_{O_2} = \frac{k_L a \cdot C_{OX}^{*}}{X} \tag{3}$$

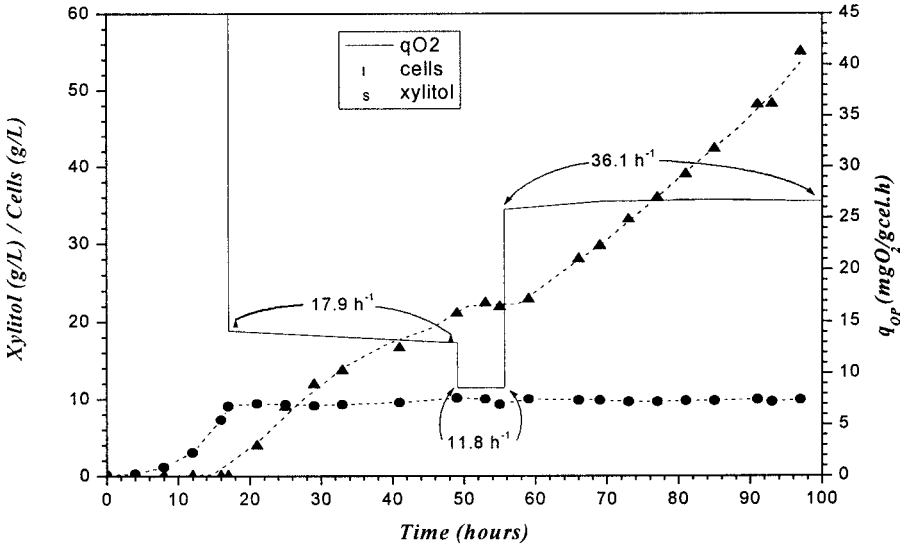

Fig. 6. Specific uptake of oxygen by *C. guilliermondii* at different aeration levels.

Figure 6 describes the variation of specific oxygen uptake rates (q_{O_2}) at different k_La values, in accordance to the experiment displayed in Fig. 5. The reduction in k_La to 17.9 h^{-1} and the consequent reduction in DO to 0% resulted in a mean specific oxygen uptake rate value of 14 mg of O_2/g of cell·h. Diminishing k_La to 11.8 h^{-1} led to a reduction in the specific oxygen uptake rate to 8.5 mg of O_2/g of cell·h, and prevented the cells from maintaining their basic metabolic functions, ceasing their growth, xylitol excretion, and xylose uptake.

With the rise in aeration level (k_La = 36.1 h^{-1}), the yeast's metabolism was gradually restored, and it resumed xylose consumption and associated xylitol excretion, reaching a rate 35% higher than when the k_La was 17.9 h^{-1}, and exhibiting a specific oxygen uptake rate of 26 mg O_2/g of cell·h. Under these conditions, the xylitol yield for consumed xylose ($Y_{P/S}$) was 0.71 g/g, higher than that obtained with a k_La of 17.9 h^{-1} (0.56 g/g).

Conclusion

This work led us to conclude that the specific oxygen uptake rate (q_{O_2}) is the key variable governing xylitol excretion by yeasts, and that it should be controlled within a narrow range if xylitol is to be produced. Values above 30 mg of O_2/g of cell·h favor cell growth, which is detrimental to the amount of excreted xylitol. In these conditions, a low yield of xylitol per consumed xylose (0.3 g/g) was obtained.

The optimum limit of oxygen supply capable of guaranteeing xylitol excretion with satisfactory yields was obtained when the specific oxygen uptake rate ranged between 13 and 30 mg of O_2/g of cell·h. Moreover, the

experiments showed the fast adaptation of *C. guilliermondii* IM/UFRJ 50088, which responded almost immediately to different oxygen availabilities.

Acknowledgments

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Nomenclature

C_{OX}^{*} = saturated DO concentration (mg of O_2/L)
C_{OX} = DO concentration (mg of O_2/L)
k_La = volumetric oxygen transfer coefficient (h^{-1})
P = product: Extracellular xylitol (g/L)
q_{O_2} = specific oxygen uptake rate, mg O2 /(g cell·h)
S = substrate: xylose (g/L)
X = cells (g/L)
$Y_{P/S}$ = xylitol yield coefficient (g of xylitol/g of xylose)
$Y_{X/S}$ = cell mass yield coefficient (g of cells/g of xylose)

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