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Oxygen Uptake Rate in Production of Xylitol by *Candida guilliermondii* with Different Aeration Rates and Initial Xylose Concentrations

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Abstract

The global oxygen uptake rate (OUR) and specific oxygen uptake rates (SOUR) were determined for different values of the volumetric oxygen mass transfer coefficient (15, 43, and 108 h^{-1}), and for varying initial xylose concentrations (50, 100, 150, and 200 g/L) in shaking flasks. The initial cell concentration was 4.0 g/L, and there was only significant growth in the fermentation with the highest oxygen availability. In this condition, OUR increased proportionally to cell growth, reaching maximum values from 2.1 to 2.5 g of O_2/(L·h) in the stationary phase when the initial substrate concentration was raised from 50 to 200 g/L, respectively. SOUR showed different behavior, growing to a maximum value coinciding with the beginning of the exponential growth phase, after which point it decreased. The maximum SOUR values varied from 265 to 370 mg of O_2/(g of cell·h), indicating the interdependence of this parameter and the substrate concentration. Although the volumetric productivity dropped slightly from 1.55 to 1.18 g of xylitol/(L·h), the strain producing capacity ($Y_{P/X}$) rose from 9 to 20.6 g/g when the initial substrate concentration was increased from 50 to 200 g/L. As for the xylitol yield over xylose consumed ($Y_{P/S}$), there was no significant variation, resulting in a mean value of 0.76 g/g. The results are of interest in establishing a strategy for controlling the dynamic oxygen supply to maximize volumetric productivity.

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Index Entries: Xylitol; *Candida guilliermondii*; xylose-fermenting yeasts; oxygen uptake.

Introduction

Xylitol, a naturally occurring polyol present in some fruits and vegetables, has been found increasingly useful as a nonsucrose sweetener, because of its high sweetening power *(1)*, its anticariogenic properties *(2)*, and its insulin-independent metabolism *(3)*. Xylitol is produced on an industrial scale through the chemical reduction of D-xylose derived from agroindustrial wastes *(1)*. The limiting step in this process is xylitol purification from other polyols and sugars, which causes overall production costs to rise. Biotechnological production of xylitol is attractive because of the specificity of the enzymes involved in D-xylose metabolism, which can lead to high yields and simpler purification steps. In yeasts and filamentous fungi, D-xylose catabolism occurs in two steps: initially, D-xylose is reduced to xylitol by NADPH-dependent xylose reductase, after which xylitol is converted into xylulose, catalyzed by NAD^+-linked xylitol dehydrogenase *(4)*. Xylose-fermenting yeasts show different behavior according to oxygen availability; in aerobiosis, NADH produced in the second reaction is reoxidized in the respiratory chain, with oxygen as the final electron acceptor. However, when oxygen is severely restricted, NADH is accumulated in the cell, causing a redox imbalance, which leads to accumulation of xylitol *(5)*. However, anaerobic conditions are not convenient, because the amount of intracellular coenzymes is limited and the reaction can stop, owing to the unavailability of NAD^+. Additionally, both the aeration rate and the initial D-xylose concentration affect xylose reductase activity *(6)* and fermentation rates *(7)*.

If one is to evaluate the oxygen restriction in a system, especially in shaking flasks, it is necessary to know what the global oxygen uptake rate (OUR) and the specific oxygen uptake rate (SOUR) are and their relationship to the process variables such as aeration, expressed by the oxygen volumetric mass transfer coefficient (K_La), and the initial D-xylose concentration, among others. The OUR is the oxygen required by the cell to have complete aerobic metabolism, when there is a plentiful oxygen supply. When there is limited aeration, this real oxygen requirement is not supplied, resulting in a condition of oxygen restriction. The intensity of this oxygen restriction will command the highest or the lowest xylitol production. Oxygen restriction should be understood as the aeration condition below which the oxygen supply no longer assists the minimum demand requested for full growth. If xylitol production is to be optimized, an optimum aeration rate should be attained to ensure NADH reoxidation at adequate levels. This aeration rate can be calculated through determining the cell OUR and its relationship to the initial D-xylose concentration, which is the aim of the present work.

Materials and Methods

Microorganism and Media

Candida guilliermondii IM/UFRJ 50088 was obtained from the Microbiology Institute of Federal University of Rio de Janeiro (Brazil). The strain was kept on agar slants containing 20 g/L of D-xylose, 5 g/L of peptone, and 3 g/L of yeast extract and stored at 5°C. The growth medium (GM) contained 20 g/L of D-xylose, 1.25 g/L of urea, 1.1 g/L of KH_2PO_4, and 1.5 g/L of yeast extract. The fermentation media contained 50, 100, 150, or 200 g/L of D-xylose, 1.25 g/L of urea, 1.1 g/L of KH_2PO_4, and 0.5 g/L of yeast extract. In all the media, a mineral salt solution, described by du Preez and van der Walt *(8)*, was added in the proportion of 40 mL/L. The pH was adjusted to 6.0 and the media were sterilized at 110°C for 20 min.

Preparation of Inoculum

A loopfull of a 1 to 3 mo stock culture was transferred to a 500-mL Erlenmeyer flask with 200 mL of GM and incubated in a rotary shaker (model G-25 KC; New Brunswick) at 180 rpm with the temperature controlled at 30°C for 24 h. Eighty milliliters of this preinoculum was then transferred to 2-L Erlenmeyer flasks containing 800 mL of GM. The flasks were incubated for 32 h under the same conditions as just described. After cell measurement, the volume to be centrifuged, at 1500*g* for 10 min, was calculated, in order to obtain an inoculum concentration of 4 g/L. The cells were resuspended and inoculated in 500-mL Erlenmeyer flasks containing the fermentation medium.

Fermentation Conditions

All the experiments were carried out in the same rotary shaker used for the inoculum preparation at 250 rpm and temperature controlled at 30°C. All the flasks were closed with polyurethane plugs in an attempt to ensure a uniform aeration rate. In the first set of experiments, the volumes of fermentation media employed were 75, 150, and 300 mL. The volumetric oxygen mass transfer coefficients (K_La), determined by the modified sulfite method *(9)* under the same process conditions, were 15, 43, and 108 h^{-1}, for 75, 150, and 300 mL water, respectively, with a maximum variance of 5%. The experiments to measure the effect of the initial D-xylose concentration on the fermentation parameters were conducted at the highest aeration rate (K_La = 108 h^{-1}).

Analytical Methods

At intervals of 6 or 12 h, two flasks were removed from the shaker for analytical determinations and for *ex situ* oxygen uptake measurements following the Taguchi and Humphrey method modified by Ururahy *(10)*. This method consists of saturating the sample with oxygen, followed by

suppression of aeration and a further monitoring of the decrease in dissolved oxygen concentration after a suitable time interval, with a model DMO-2 polarographic oxygen electrode probe (Digimed, Brazil). For each sample, six oxygen uptake measurements were made. After the statistical treatment of the results, the mean OUR value was calculated as the ratio of the variation in oxygen concentration to the corresponding time interval. The SOUR values were calculated as the ratio of the OUR value to the cell concentration at the corresponding time determined as follows. The samples were centrifuged at 1500*g* for 10 min. The cells were washed and resuspended in distilled water and measured by the linear relationship between the cell dry wt and the optical density, read at 570 nm with 1-cm path cuvets using a Spectronic model 20 MV spectrophotometer (Milton Roy). D-Xylose and xylitol were measured using high-performance liquid chromatography operating with isocratic elution with high-purity water at a flow rate of 0.8 mL/h, using a model 510 pump (Waters), a manual injector (Rheodyne) working with a 20-µL injection volume, a model 410 RI refractive index detector , a model CHM furnace operating at 75°C, a model 746 integrator (Waters), and a model SC 1011 Ca^{+2} cationic-exchange column (Shodex, Japan) operating at 40°C.

Results And Discussion

Effect of Aeration Rate on Oxygen Uptake and Fermentation Parameters

Figures 1–3 show the interdependence of the aeration rate, cell growth, and potential global (OUR) and (SOUR). There was only significant cell growth during the fermentation with the highest oxygen availability (K_La = 108 h^{-1}). Under these conditions, OUR increased proportionally to cell growth, achieving a constant mean value of 2.10 ± 0.05 g of O_2/(L·h). SOUR behaved differently, growing to a maximum value that coincided with the beginning of the exponential growth phase, after which it decreased to a mean value of 175 ± 5 mg of O_2/(g of cell·h). For other K_La values, growth was negligible, and both OUR and SOUR remained constant. The increase in OUR with cell growth was expected, since high cell densities need large quantities of oxygen to develop their metabolic reactions *(11)*. On the other hand, when the oxygen supply was severely restricted, SOUR values were greater than those obtained at the highest aeration rate. This phenomenon could well be related to adenosine triphosphate (ATP) synthesis, as a consequence of the reoxidation of NADH in the respiratory chain *(12)*. According to this hypothesis, when the aeration rate is high, the culture reaches the adequate ATP level required for metabolism, resulting in a reduction in SOUR. Conversely, lower oxygen availability inhibits the reoxidation of NADH, so the ATP synthesis rate decreases. In this case, the increase in SOUR is a cell response, indicating that a suitable ATP level had not yet been achieved. In all aeration rates investigated, the OUR values obtained were always higher than the oxygen

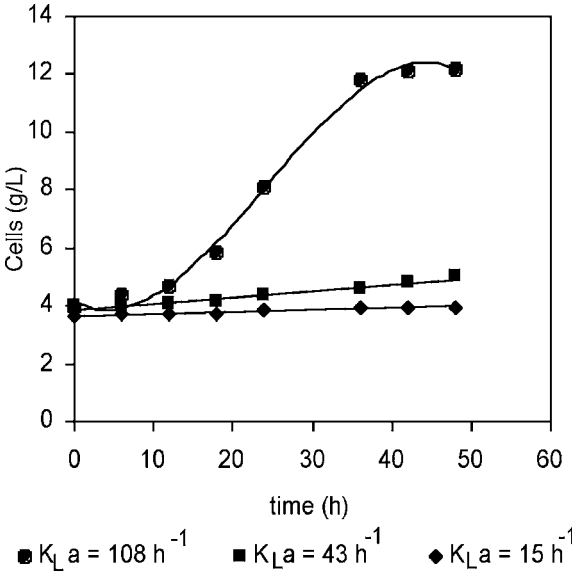

Fig. 1. Effect of K_La on cell growth.

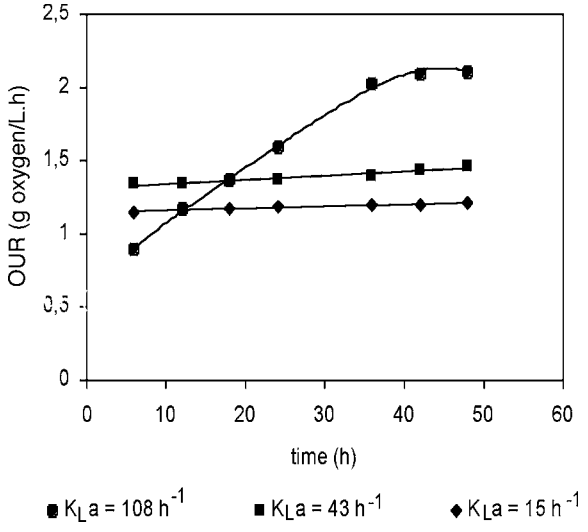

Fig. 2. Effect of K_La on *ex situ* OUR.

supply (Fig. 2 and Table 1), which under ideal gas-liquid mass transfer conditions is calculated as the product $K_La \cdot C_S$, in which C_S is the oxygen saturation in the medium, denoting a condition of "oxygen restriction".

As for the process variables, whose variation did not exceed 2%, D-xylose was depleted at the highest aeration rate after 24 h, resulting in a maximum volumetric productivity of 1.52 g/(L·h) and a xylitol yield ($Y_{P/S}$) of 0.77 g of xylitol/g of xylose consumed. For the intermediate aera-

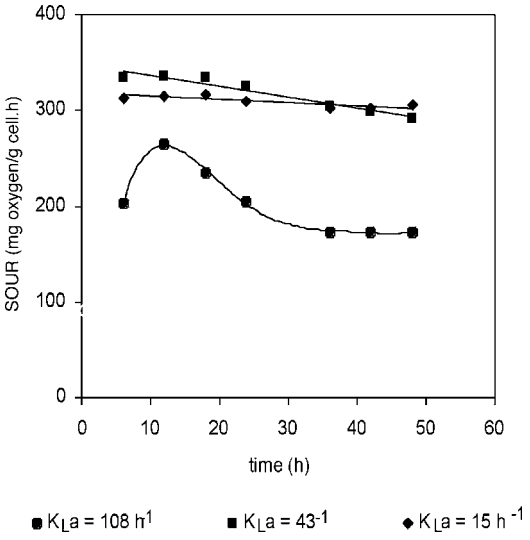

Fig. 3. Effect of K_La on *ex situ* SOUR.

Table 1
Effect of Aeration Rate on Fermentation and Mass Transfer Parameters

V_L (mL)	K_La (h^{-1})	$K_La \cdot C_S$ (mg O_2/[L·h])	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g)	Q_P (g/[L·h])
75	108	814	0.77	8.9	1.52
150	43	367	0.84	40.0	0.84
300	15	113	0.76	77.0	0.51

tion rate (K_La = 43 h^{-1}), when d-xylose depletion occurred after 48 h, the highest xylitol yield was obtained ($Y_{P/S}$ = 0.84 g of xylitol/g of xylose consumed), even though the volumetric productivity was lower (0.84 g/[L·h]). The fermentation rate sharply decreased at the lowest aeration rate, which showed a residual D-xylose concentration of 15.9 g/L after 48 h of fermentation, resulting in volumetric productivity and a xylitol yield of 0.51 g/(L·h) and 0.76 g of xylitol/g of D-xylose consumed, respectively.

In all the experiments, either the volumetric productivity or xylitol yield obtained were compatible with the range of values reported in the literature for xylitol production by *C. guilliermondii* strains, in which volumetric productivity values vary between 0.40 and 2.2 g/(L·h) and xylitol yields between 0.40 and 0.90 g/g *(13)*. The faster consumption of D-xylose when aeration was at its highest rate may have been associated with the occurrence of a relief in the cell redox imbalance, resulting in an increase in the NADH reoxidation rate. In addition, low aeration rates did not lead to a loss of the strain-producing capacity, expressed by the ratio of maxi-

Table 2
Effect of Initial D-Xylose Concentration
on Fermentation and Growth Parameters for K_La = 108 h^{-1}

S_0 (g/L)	t_i (h)	t_f (h)	μ_x (h^{-1})	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g)	Q_P (g/[L·h])
50	0	24	0.039	0.086	0.77	9.0	1.55
100	0	48	0.039	0.086	0.76	8.9	1.51
150	18	90	0.022	0.055	0.77	14.1	1.25
200	24	120	0.020	0.036	0.74	20.6	1.18

mum xylitol concentration to cell growth ($Y_{P/X}$). On the contrary, as can be seen in Table 1, as oxygen was restricted, $Y_{P/X}$ rose from 8.9 to 77.0 g of xylitol produced/g of biomass, a phenomenon that was probably associated with the osmoregulatory mechanism commonly occurring in yeasts, which, in order to preserve their water activity, excludes polyols when submitted to stressful conditions *(14)*. Therefore, since the low aeration rate led to an increase in the contact time between the cells and the fermentation medium, this situation may have also stimulated the cell osmoregulation mechanism.

Effect of Initial Substrate Concentration on Fermentation Parameters and Oxygen Uptake

An evaluation of the effect of the initial D-xylose concentration on the fermentation parameters was attempted (Table 2). There was an inhibitory effect on the initial fermentation stages caused by high substrate concentration, especially for 150 and 200 g/L, when the substrate only started to be consumed after 18 and 24 h, respectively. This delay in D-xylose consumption indicates that the cells experienced a more hostile environment owing to the increase in the osmotic pressure in the fermentation medium. At the lowest initial substrate concentrations (50 and 100 g/L), there was no initial inhibition of D-xylose consumption, which had a linear profile from the beginning of the process. Although the volumetric productivity decreased slightly from 1.55 to 1.18 g of xylitol/(L·h) when the initial D-xylose concentration varied from 50 to 200 g/L, the strain-producing capacity ($Y_{P/X}$) rose from 9 to 20.6 g/g, respectively. This also corroborates the role of xylitol in xylose-fermenting yeasts, which protects cells from adverse medium conditions, whether they are caused by a high substrate concentration or oxygen restriction, much like what took place when the effect of the aeration rate was studied. As for the xylitol yield against xylose consumed ($Y_{P/S}$), it did not vary significantly, resulting in a mean value of 0.76 ± 0.01 g/g. Our results were different from those reported by Nolleau et al. *(7)*, who worked with another strain of *C. guilliermondii* (NRC 5578) cultivated in shaking flasks. They reported

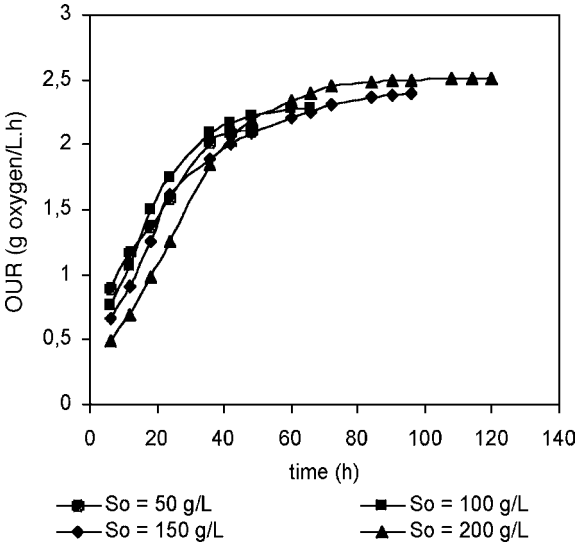

Fig. 4. Effect of initial D-xylose concentration (S_0) on *ex situ* OUR.

an increase in both volumetric productivity and xylitol yield factor for the same range of initial substrate concentration employed in the present work. The values for the specific growth rate, calculated during the exponential phase, and the downward trend in the cell yield factor suggest that the growth was inhibited when there was a high initial D-xylose concentration. However, in all the experiments, growth stopped when the cell concentration reached about 12 g/L. This behavior may be related to the depletion of some nutrient, probably the yeast extract, since only a very small amount was included in the fermentation media.

The previous discussion concerning the influence of aeration rate on *ex situ* oxygen uptake was based on the relationship between ATP synthesis and oxygen uptake, leading to an increase in SOUR. Using the same approach, the relationship between OUR, SOUR, and initial D-xylose concentration may be established. As shown in Fig. 4, during the initial fermentation stages, the OUR values were lower as the initial D-xylose concentration was increased. It could be that this phenomenon was related to substrate inhibitory effects, as mentioned earlier, reducing cell activity and consequently reflecting on OUR values. Furthermore, for all the initial D-xylose concentrations investigated, the OUR increased proportionally to cell growth. When the stationary growth phase was reached, the OUR had been stabilized, reaching mean values of 2.08 ± 0.04, 2.26 ± 0.03, 2.38 ± 0.03, and 2.55 ± 0.03 g of O_2/(L·h), for initial xylose concentrations of 50, 100, 150, and 200 g/L, respectively. This behavior may have been linked to an alleviation of the inhibitory effect owing to the consumption of D-xylose, leading to an improvement in cell metabolic activity.

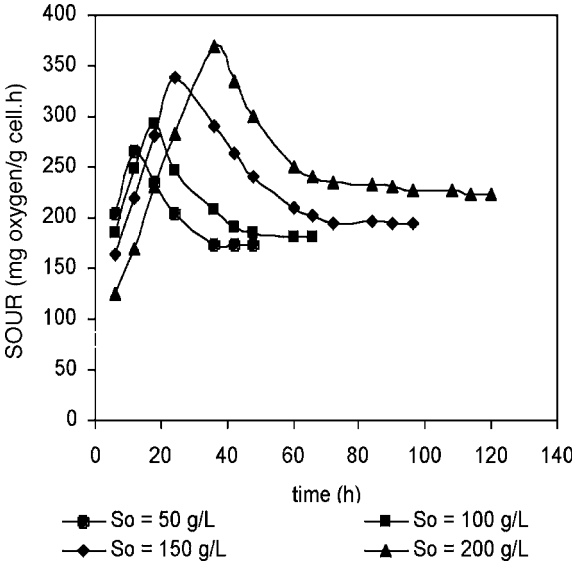

Fig. 5. Effect of initial D-xylose concentration (S_0) on *ex situ* SOUR.

The SOUR reacted differently, increasing to a maximum value that coincided with the beginning of the exponential growth phase, after which point it decreased (Fig. 5). The maximum SOUR values, which varied approx 2%, increased from 265 to 370 mg of O_2/(g of cell·h), when the initial xylose concentration was raised from 50 to 200 g/L, indicating the dependence of SOUR on substrate concentration. This observation can also be related to the ATP synthesis and oxygen requirements. When high ATP production occurred at the beginning of the exponential growth phase, it may have caused an increase in SOUR up to a maximum value. However, because of a probable feedback regulatory mechanism, whereby the intracellular ATP concentration reaches the level necessary for cells to perform their metabolic functions, a decrease in the ATP production rate may also have occurred, consequently decreasing SOUR. Aguiar *(15)* reported similar behavior during the aerobic growth of *C. guilliermondii*, when the existence of a maximum SOUR value was observed, coinciding with the beginning of the exponential phase.

Figure 6 depicts the variations in OUR with cell concentration. During the initial fermentation stages, when cell concentrations were low, the main contribution to the sharp increase in OUR was associated with the preponderance of SOUR. However, in all the experiments, after the beginning of the exponential phase, when the cell concentration exceeded 5 g/L, OUR increased linearly, showing the dominant effect of cell growth on this parameter. It is also important to point out that cells at the same growth stage cultivated under the same oxygen supply conditions had different OURs, corroborating the interdependence among cell growth, D-xylose concentration, and oxygen availability.

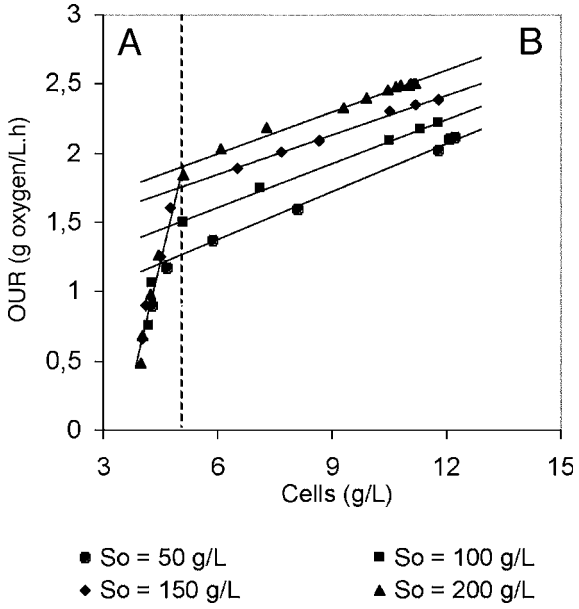

Fig. 6. Influence of cell growth on *ex situ* OUR at different initial D-xylose concentrations (S_0). A; Measurements made before the exponential growth phase; B; measurements made after the beginning of the exponential growth phase.

Conclusions

The interdependence of aeration rate, cell growth, and potential global (OUR) and SOUR was shown. The aeration rate affected both volumetric productivity and xylitol yield, yet for the highest oxygen availability, the gain in Q_P was proportionally higher than the variation in $Y_{P/S}$, which translates into a competitive advantage in terms of industrial production. OUR and SOUR were also influenced by the initial substrate concentration. For the same cell concentration, different values of both parameters were encountered, denoting that as the environment becomes more hostile, the oxygen requirement increases. The increase in the strain-producing capacity corroborates the role of xylitol in xylose-fermenting yeasts, because it is excluded preferentially and protects cells from the adversity of the medium, whether it is caused by a high substrate concentration or oxygen restriction. The results reported in this work are of interest for establishing a strategy for dynamic oxygen supply control, in order to maximize volumetric productivity.

Nomenclature

C_S = oxygen saturation concentration (mg/L).
K_La = volumetric oxygen mass transfer coefficient (h^{-1}).
OUR = oxygen uptake rate (g of oxygen/[L·h])

Q_P = xylitol volumetric productivity (g of xylitol/[L·h])
S_0 = initial D-xylose concentration (g/L).
SOUR = oxygen uptake rate (mg of oxygen/[g of cell·h])
t_i = beginning time of D-xylose consumption (h)
t_f = D-xylose depletion time (h).
V_L = fermentation media volume (L).
X = cell concentration (g/L).
$Y_{P/S}$ = xylitol yield factor on D-xylose consumed (g/g)
$Y_{P/X}$ = xylitol yield factor on cell growth (g/g)
$Y_{X/S}$ = growth yield factor on D-xylose consumed (g/g)
μ_x = specific growth rate at exponential phase (h^{-1})

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