0273-2289/02/101/0015/$13.75

Effect of Starting Xylose Concentration on the Microaerobic Metabolism of *Debaryomyces hansenii*

The Use of Carbon Material Balances

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Received April 4, 2001; Revised September 12, 2001; Accepted September 13, 2001

Abstract

Xylitol production by *Debaryomyces hansenii* NRRL Y-7426 was performed on synthetic medium varying the initial xylose concentration between 50 and 300 g/L. The experimental results of these tests were used to investigate the effect of substrate level on xylose consumption by this yeast. Satisfactory values of product yield on substrate (0.74–0.83 g/g) as well as volumetric productivity (0.481–0.694 g/L·h) were obtained over a wide range of xylose levels (90–200 g/L), while a worsening of kinetic parameters took place at higher concentration, likely due to a substrate inhibition phenomenon. The metabolic behavior of *D. hansenii* was studied, under these conditions, through a carbon material balance to estimate the fractions of xylose consumed by the cell for different activities (xylitol production, biomass growth, and respiration) during the lag, exponential, and stationary phases.

Index Entries: Xylitol; *Debaryomyces hansenii*; starting substrate concentration; synthetic medium; carbon material balance.

Introduction

Owing to its dietetic and clinical properties and sweetness comparable with that of sucrose *(1–3)*, xylitol is arousing great interest in the food and pharmaceutical industries. Although currently manufactured by hydrogenation of xylose from hemicellulose hydrozylates, the organoleptic

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characteristics of xylitol are superior when produced by natural fermentation *(4)*. In addition, its biotechnological production would meet the international guidelines concerning food additives.

The best xylitol producers are yeasts, mainly belonging to the species *Candida guilliermondii*, *Pachysolen tannophilus*, and *Debaryomyces hansenii (5)*, which metabolize xylose through a two-step oxido-reductive route. First, xylose reductase (XR), in the presence of NADH and/or NADPH as cofactor, reduces D-xylose to xylitol *(6)*. Through a subsequent reaction, this pentitol is oxidized to D-xylulose by either NAD^+-linked or $NADP^+$-linked xylitol dehydrogenase (XDH), depending on the microorganism *(7,8)*. Among these microorganisms, *D. hansenii* is of particular interest because of the negligible formation of ethanol under microaerobic conditions, which ensures particularly high xylitol yields *(9)*.

Close relationships between the oxygen availability, the cofactor linked to XR, and the products of xylose metabolism were reported *(4)*. Under both anaerobic and oxygen-limited conditions, the yeasts with XR activity linked to both NADH and NADPH can regenerate the NAD^+ consumed in the second step of xylose metabolism (i.e., *Pichia stipitis* and various species of *Candida* genus) *(8,10–12)*. In this case, the major reaction product is ethanol owing to the redox balance between the cofactors of XR and XDH. On the other hand, yeasts that metabolize xylose by XR activity dependent only on NADPH (i.e., *D. hansenii*) cannot accumulate xylitol under anaerobic conditions, because of their inability to use $NADP^+$ in the second step *(9,13–15)*. Under aerobic conditions, the NADH formed in the oxidation of xylitol by XDH can be re-oxidized in the respiratory chain and the carbon source is utilized for growth, but, under semiaerobic conditions, a redox imbalance limits the rate of the oxidative step, leading to xylitol accumulation *(9,14–17)*.

Because the effect of initial substrate concentration, S_0, on fermentation parameters is important in the design of batch and fed-batch processes, much experimental work has been done to clarify its influence on xylose metabolism in xylitol accumulating yeasts. As a general rule, very low starting xylose levels favored yeast growth *(18,19)*, while an evident inhibition took place at very high S_0 values *(20,21)*. As a consequence, maximum xylitol productivities were obtained, under semiaerobic conditions, at intermediate starting xylose levels ranging from 60 to 200 g/L, depending on the microorganism *(19–23)*. However, the effect of this condition on xylitol fermentation by *D. hansenii* is practically unknown, therefore a set of batch runs was performed at $50 < S_0 < 300$ g/L to estimate the related kinetic parameters. Batch conditions were preferred because it is generally possible to obtain higher final product concentrations with respect to continuous culture, which is the major advantage in processes characterized by high product recovery costs *(24)*.

A certain number of studies have recently been performed on the metabolism of different yeasts using simple carbon material balances *(25–27)*, by which the xylose fractions consumed for biomass growth, for-

mation of different metabolic products, and respiration have been evaluated on different synthetic and complex media. This approach proved a powerful tool for the study of microbial metabolism under batch conditions and is used in this work to evaluate the effect of initial xylose concentration on the microaerobic behavior of *D. hansenii*.

Materials and Methods

Microorganism and Growth Media

The experiments were carried out in triplicate with the yeast *D. hansenii* NRRL Y-7426. The cells were maintained on slants of potato dextrose agar (PDA) with 20 g/L xylose, 5 g/L peptone, 3 g/L yeast extract, and 3 g/L malt extract added and then incubated at 30°C for 48 h. A loopful of cells was transferred into 500 mL Erlenmeyer flasks containing 250 mL of growth medium, formulated with the same components and concentrations as the previous one without agar. The cells were cultivated in a rotatory shaker at 30°C and 250 rpm for 72 h, then collected by sterile centrifugation (3800*g* for 15 min) and finally used for inoculum.

Fermentation Conditions

The fermentations were carried out in a 3-L Applikon Z61103CT04 fermentor containing 1 L of medium at 30 ± 0.2°C, agitation of 250 rpm, average oxygen flow rate of 4.98 mg/s (corresponding to 0.2–0.5% of the saturation value in water). The corresponding oxygenation conditions were reported as the optimum for xylitol production by yeasts, in terms of both volumetric productivity and xylitol yield *(27,28)*. The operative conditions were automatically controlled through the electronic device Applikon ADI 1030. The pH was kept at the selected value (5.5 ± 0.1) by addition of a stream of 1.0 *M* NaOH solution. The oxygen level throughout the fermentations was maintained within 0.2–0.5% of the saturation value in water by means of a polarographic sensor, connected with the above electronic control device and with an electrovalve to regulate the air supply.

Batch tests were performed in a synthetic medium containing 5 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \cdot 7\ H_2O$, 1 g/L KH_2PO_4, whereas the initial xylose concentration was varied from 50 to 300 g/L.

Analytical Methods

Xylose and xylitol concentrations were determined at 35°C by HPLC (Hewlett Packard 1100) equipped with a SUPELCOGEL H 300 × 7.8 column; a 0.1% H_3PO_4 water solution was used as eluent at a flow rate of 0.5 mL/min. Cell mass concentration was determined by dry weight after filtration of known-volume aliquots of the fermentation broth through 0.45 µm membrane filters. As previously described *(27)*, carbon dioxide development during fermentation was followed monitoring the exhaust gas with a CO_2 gas analyzer and the total amount of CO_2 produced at each

fermentation time was calculated by integration of the time course varying in CO_2 concentration.

Estimation of Kinetic Parameters

The average volumetric productivity, Q_P, was calculated as the ratio of the final xylitol concentration to the time necessary to complete each fermentation, t_f. The average specific productivity, q_P, was calculated as the ratio of Q_P to the average biomass concentration during each run. The yield of xylitol on starting xylose, $Y_{P/S0}$, was calculated as the ratio of the final xylitol concentration to the starting xylose concentration. The average specific growth rate, μ, was calculated as $\mu = (1/t_f)\ln(X_f/X_0)$, where X_f and X_0 are the final and starting biomass concentrations. The average specific rates of xylose consumption, q_S, and CO_2 formation, q_{CO2}, were calculated as the ratios of the difference between the starting and final substrate concentrations or the CO_2 amount produced per unit reactor volume, respectively, to the average biomass level and t_f.

Carbon Material Balance

The microaerobic behavior of *D. hansenii* was studied at different starting substrate levels through simple carbon material balances, using the batch experimental data of xylitol, biomass, and CO_2 production as well as those of xylose consumption. It is well known that metabolic flux analysis through biomass synthesis and product formation would be a more useful tool for this purpose, but is better applied to continuous culture (*29,30*). The contribution of alcoholic fermentation to total xylose consumption was not considered in the carbon balance because *D. hansenii* did not produce appreciable amounts of ethanol under the selected oxygen-limited conditions (*9*).

The proposed balance is based on the models reported for the fermentation of xylose by yeasts only with NADPH-dependent XR activity *(16)* and for yeast composition *(31)*. It starts with the following: a) the reduction of D-xylose to xylitol by XR is linked only to the cofactor NADPH in *D. hansenii (9)*, whereas the subsequent slow oxidation of xylitol to D-xylulose by XDH is linked only to the cofactor NAD^+ *(32)*; b) NADPH is regenerated by the pentose-phosphate shunt, whereas NADH is regenerated by the respiratory chain *(16)*; c) *D. hansenii* is unable to make the extra-mitochondrial interconversion of NADH and NADPH because of the absence of transhydrogenase activity; nevertheless, this is possible during the respiration, which is an intra-mitochondrial phenomenon *(16)*; d) the amount of xylitol oxidized to D-xylulose is only that needed to regenerate NADPH, the remaining part being accumulated as main product; e) as suggested by Roels *(31)*, the actual ATP yield by the regeneration of reducing cofactors could be lower (about 1.5) than the theoretical values (2 for $FADH_2$ and 3 for NADPH).

Biomass Growth

Under oxygen-limited conditions in a rich medium, the carbon, nitrogen, and energetic sources are partly utilized for the construction of biomass precursors. One can assume for these precursors the average composition $CH_{1.61}O_{0.52}N_{0.15}$ and for their formation the general relationship *(31)*:

$$1.095\ CH_2O + 0.15\ NH_3 + 0.051\ ATP \longrightarrow CH_{1.61}O_{0.52}N_{0.15} + 0.13\ \text{“}H_2\text{”} + 0.095\ CO_2 + 0.334\ H_2O + 0.051\ ADP + 0.051\ P_i \quad (1)$$

where “H_2” stands for reducing equivalents in the form of NADH or any other equivalent form of metabolic reductant, which are consumed for ATP generation by the oxidative phosphorylation. Assuming an ATP yield of 1.5, we obtain:

$$0.13\ \text{“}H_2\text{”} + 0.065\ O_2 + 0.195\ ADP + 0.195\ P_i \longrightarrow 0.325\ H_2O + 0.195\ ATP \quad (2)$$

Neglecting the little difference in composition between biomass and precursors, that is, not considering in this balance the excess elements (H, N, and O), biomass formation from polymerization of precursors can be described by the simple reaction *(31)*:

$$CH_{1.61}O_{0.52}N_{0.15} + 1.5\ ATP + 1.5\ H_2O \longrightarrow CH_{1.79}O_{0.50}N_{0.20} + 1.5\ ADP + 1.5\ P_i \quad (3)$$

Summing from Eq. (1) to Eq. (3), the global reaction for biomass production from xylose according to this model can be obtained:

$$1.095\ C_5H_{10}O_5 + 0.75\ NH_3 + 6.78\ ATP + 0.325\ O_2 + 4.205\ H_2O \longrightarrow 5\ CH_{1.79}O_{0.50}N_{0.20} + 0.475\ CO_2 + 6.78\ ADP + 6.78\ P_i \quad (4)$$

Xylitol Formation

Consistently with the lack of a cytoplasmic NADH-linked XR activity in *D. hansenii (9)*, xylose reduction to xylitol by this yeast has been theorized to take place according to the model proposed by Barbosa et al. for yeasts utilizing only NADPH as XR reducing cofactor *(16)*:

$$6\ xylose + 6\ NADPH_2^+ \longrightarrow 6\ xylitol + 6\ NADP^+ \quad (5)$$

$$6\ xylitol + 6\ NAD^+ \longrightarrow 6\ xylulose + 6\ NADH_2^+ \quad (6)$$

$$6\ NADH_2^+ + 3\ O_2 + 9\ ADP + 9\ P_i \longrightarrow 6\ NAD^+ + 9\ ATP + 15\ H_2O \quad (7)$$

$$6\ xylulose + 6\ ATP \longrightarrow 6\ xylulose\text{-}5\text{-}P + 6\ ADP \quad (8)$$

$$6\ xylulose\text{-}5\text{-}P + H_2O \longrightarrow 5\ glucose\text{-}6\text{-}P + P_i \quad (9)$$

$$5\ glucose\text{-}6\text{-}P + 60\ NADP^+ + 35\ H_2O \longrightarrow 60\ NADPH_2^+ + 30\ CO_2 + 5\ P_i \quad (10)$$

Net (Eqs. 5–10):

$$6\ xylose + 54\ NADP^+ + 3\ ADP + 3\ P_i + 21\ H_2O + 3\ O_2 \longrightarrow 54\ NADPH_2^+ + 3\ ATP + 30\ CO_2 \quad (11)$$

$$54\ NADPH_2^+ + 54\ xylose \longrightarrow 54\ xylitol + 54\ NADP^+ \quad (12)$$

Net (Eqs. 11–12):

$$60 \text{ xylose} + 3 \text{ ADP} + 3 P_i + 21 H_2O + 3 O_2 \longrightarrow 54 \text{ xylitol} + 3 \text{ ATP} + 30 CO_2 \quad (13)$$

Carbon dioxide produced according to Eq. (11) comes from the complete oxidation of xylose through the glucose-6-phosphate pathway, whereas Eq. (12) shows xylose reduction to xylitol by the excess NADPH produced by the same route, and Eq. (13) the complete process in which the energy balance implies an ATP yield of 1.5. According to this balance the theoretical xylitol yield from xylose is 0.91 g/g.

Respiratory Chain

Under microaerobic conditions, a fraction of xylose is completely oxidized to carbon dioxide by the TCA cycle coupled to the respiratory chain, thus reducing the actual xylitol yield. In the mitochondrion, xylitol from xylose reduction can partially be oxidized to xylulose to regenerate NADH. Then xylulose is transformed into xylulose-5-phosphate according to the following scheme:

$$6 \text{ xylose} + 6 NADH_2^+ \longrightarrow 6 \text{ xylitol} + 6 NAD^+ \quad (14)$$

$$6 \text{ xylitol} + 6 NAD^+ \longrightarrow 6 \text{ xylulose} + 6 NADH_2^+ \quad (15)$$

$$6 \text{ xylulose} + 6 \text{ ATP} \longrightarrow 6 \text{ xylulose-5-P} + 6 \text{ ADP} \quad (16)$$

xylulose-5-phosphate is then transformed into glucose-6-phosphate by the pentose–phosphate shunt and this product is dephosphorylated to glucose, releasing ATP:

$$6 \text{ xylulose-5-P} + H_2O \longrightarrow 5 \text{ glucose-6-P} + P_i \quad (17)$$

$$5 \text{ glucose-6-P} + 5 \text{ ADP} \longrightarrow 5 \text{ glucose} + 5 \text{ ATP} \quad (18)$$

Considering an actual ATP yield only of 1.5 ATP mole per mole of "H_2" (in the form either of NADH or $FADH_2$) regenerated by oxygen in the respiratory chain *(31)*, we obtain from 5 glucose moles:

$$5 \text{ glucose} + 30 O_2 + 110 \text{ ADP} + 110 P_i \longrightarrow 30 CO_2 + 140 H_2O + 110 \text{ ATP} \quad (19)$$

Combining Eqs. (14–18) with Eq. (19):

$$6 \text{ xylose} + 30 O_2 + 109 P_i + 109 \text{ ADP} \longrightarrow 30 CO_2 + 109 \text{ ATP} + 139 H_2O \quad (20)$$

Carbon Balance Calculations

All carbon material balances accounted for the consumption of xylose between two selected fermentation times, therefore they could be referred either to a whole batch run or only to a part of it (log, exponential, or stationary phases). For these balances, we used the experimental values of xylose, xylitol, CO_2, and biomass concentrations measured at the end and the beginning either of a given selected phase or of a whole fermentation, according to circumstances. First, the amount of xylose consumed for biomass growth was estimated by Eq. (4) using the experimental final and

Table 1
Effect of the Initial Xylose Concentration (S_0)
on the Main Average Kinetic Parameters
of Batch Xylitol Production by *Debaryomyces hansenii*

S_0 (g/L)	Q_P [a] (g/L·h)	q_P [b] (g/g·h)	Y_{P/S_0} [c] (g/g)	q_S [d] (g/g·h)	μ [e] (h^{-1})	q_{CO_2} [f] (g/g·h)
50	0.058	0.029	0.24	0.086	0.0057	0.0640
90	0.694	0.069	0.83	0.081	0.0043	0.0120
125	0.503	0.040	0.82	0.048	0.0032	0.0071
200	0.481	0.022	0.74	0.032	0.0024	0.0063
300	0.393	0.018	0.69	0.022	0.0012	0.0047

[a]Q_P, volumetric productivity; [b]q_P specific productivity; [c]Y_{P/S_0}, xylitol yield on starting xylose; [d]q_S, specific rate of xylose consumption; [e]μ, specific growth rate; [f]q_{CO_2}, specific rate of carbon dioxide formation.

starting values of biomass concentration and assuming the dry biomass composition proposed by Roels (*31*) for yeasts: $CH_{1.61}O_{0.52}N_{0.15}$. The amount of xylose consumed for xylitol production was subsequently estimated by Eq. (13) using the experimental final and starting values of xylitol concentration. The amount of carbon dioxide produced was estimated by Eq. (20) assuming that the remaining amount of xylose not consumed either for cell growth or for xylitol production was used by the yeast, under microaerobic conditions, for respiration. These theoretical values of CO_2 production were finally compared with those experimentally measured as described in the *Materials and Methods* section.

Results and Discussion

To study the effect of starting substrate concentration on xylitol production by *D. hansenii*, a set of batch fermentations was performed at $50 < S_0 < 300$ g/L. Previous work demonstrated that S_0 values higher than 300 g/L were not feasible *(33)* because of a strong inhibition effect, therefore they were not considered in this study. An evaluation of the main average kinetic parameters of these fermentations, listed in Table 1, allows us to make some important observations. The average volumetric, Q_P, and specific, q_P, productivities as well as the product yield on starting substrate, Y_{P/S_0}, were very low under conditions of substrate shortage (S_0 = 50 g/L), reached maximum values at an optimum (S_0 = 90 g/L) and strongly decreased at $S_0 > 200$ g/L, as previously observed also by Silva et al. *(33)*. A different behavior can be observed, on the other hand, for both specific rates of substrate consumption, q_S, and CO_2 formation, q_{CO_2}, that progressively decreased from 0.086 to 0.022 g/g·h and from 0.0640 to 0.0047 g/g·h, respectively, with increasing S_0 from 50 to 300 g/L. The average specific growth rate, μ, was always very low because of the very high starting biomass levels used in this study to favor xylitol production at the expense of the other metabolic activities. In addition, μ progressively decreased

with S_0 (Fig. 1), following, with excellent correlation ($r^2 = 0.989$), the exponential decay:

$$\mu = \mu_o \exp(-aS_0) \quad (21)$$

where $\mu_o = 0.0074\ h^{-1}$ is the theoretical specific growth rate at $S_0 = 0$ and $a = 0.0060$ L/g a correlation coefficient. These dependences of the kinetic parameters can be better evidenced within three different ranges of S_0.

At relatively low starting substrate level ($S_0 = 50$ g/L), xylitol formation was very slow ($Q_P = 0.058$ g/L·h; $q_P = 0.029$ g/g·h), therefore the yeast could have not taken enough energy to grow and preferred to consume the carbon source for respiration and ATP production. This situation seems to be confirmed by the behavior of the average specific rate of CO_2 formation, that reached a maximum value ($q_{CO_2} = 0.0640$ g/g·h) under these conditions. It is possible to identify another initial xylose concentration range (90–125 g/L) where Y_{P/S_0} was rather high (0.82–0.83 g/g), which constitutes a very interesting result for a potential industrial development of this fermentation process. Within the same range, xylitol production was particularly quick ($0.503 < Q_P < 0.694$ g_P/L·h) and, as a consequence, biomass and CO_2 productions decelerated ($0.0032 < \mu < 0.0043\ h^{-1}$; $0.0071 < q_{CO_2} < 0.0120$ g/g·h). These results compare reasonably with those reported in the literature for this microbial system *(28)*. In particular, the mean product yield obtained in this work (0.80 g/g over $90 < S_0 < 200$ g/L) was similar to that reported by Domínguez et al. *(17)* for the same strain (0.79 g/g at $S_0 = 279$ g/L) and by Gírio et al. *(34)* for *D. hansenii* DTIA77 (0.70 g/g at $S_0 = 90$ g/L). The volumetric productivity achieved a maximum value (0.694 g/L·h; at $S_0 = 90$ g/L) that was practically the same as that reported by Parajó et al. *(35)* but lower than that (0.84 g/L·h; at $S_0 = 90$ g/L) obtained by Roseiro et al. *(14)*. Lower product yields and productivities were often obtained with other yeasts *(28)*. As for other fermentation processes *(36)*, xylose bioreduction to xylitol showed a progressive decrease in volumetric and specific productivities for $S_0 \geq 90$ g/L. At very high starting substrate levels ($S_0 > 200$ g/L), the fermentation was strongly inhibited, so the yeast consumed a higher xylose fraction for respiration to get enough energy. Since in batch processes cell density continuously grows with time, it is nearly impossible to control the oxygen mass transfer rate at a constant level without varying some other operative condition; therefore, the negative effect of S_0 on xylitol production observed in this study could be due either to substrate saturation inhibition or to oxygen mass transfer limitations.

On the basis of the above theoretical considerations, carbon material balances were performed for each batch fermentation using the final and starting experimental values of xylitol, ethanol, CO_2, biomass, and xylose concentrations. As previously explained, the fraction of xylose consumed for growth was calculated by Eq. (4), then the amount of xylose consumed for xylitol production was estimated by Eq. (13). The experimental values of total amount of CO_2 produced were finally compared with those esti-

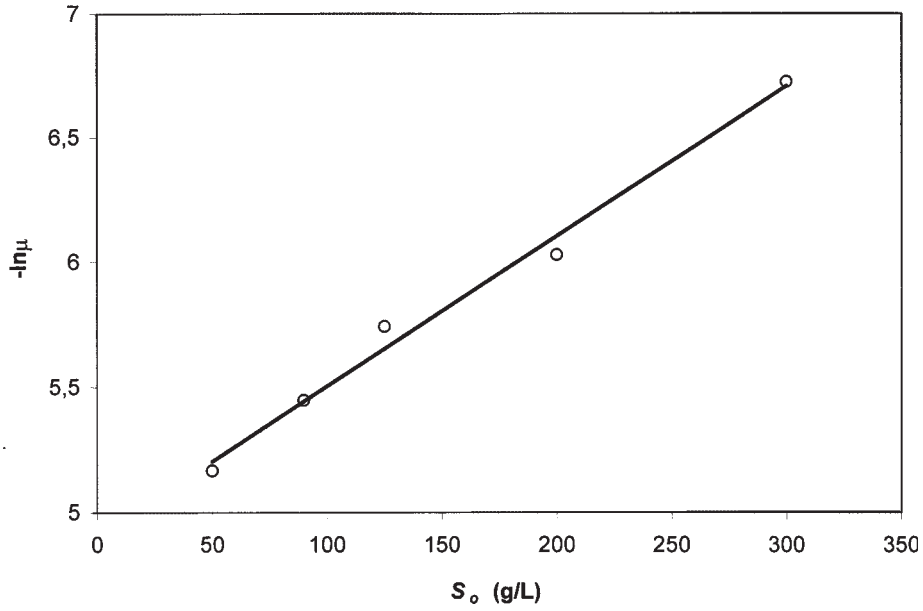

Fig. 1. Influence of the starting substrate concentration on the average specific growth rate on synthetic medium.

mated assuming that the amount of xylose not consumed for biomass and xylitol productions was utilized by the cell for respiration. The time-averaged errors between theoretical and experimental values gradually decreased from about 10% to 5% with increasing S_0 from 50 to 300 g/L because of the growing accuracy of the CO_2 determinations. Anyhow, these errors can be considered satisfactory for the purposes for the present study.

Using the experimental data versus time, we also estimated by the same equations the fractions of the carbon source consumed by *D. hansenii* for xylitol production, cell growth and respiration during the typical phases of a batch run (lag, exponential, and stationary phases). In addition, the variations of these relative consumptions consequent to a progressive increase in the initial xylose concentration from 50 to 300 g/L provided interesting information about the influence of the carbon source level on the metabolic behavior of this yeast.

As shown in Fig. 2, at very low substrate concentration (S_0 = 50 g/L), xylose was consumed during the lag phase in a larger proportion (35%) than at higher S_0 values (about 10%), probably because of the above mentioned necessity to take energy from the respiratory activity under these conditions. Nevertheless, xylose consumption during the exponential phase was always much higher than in the other phases and achieved a minimum (55%) at the lowest S_0 value. On the other hand, the carbon source fraction consumed during the stationary phase did not show any significant dependency on S_0 and it was always less than 10% of total amount of xylose consumed.

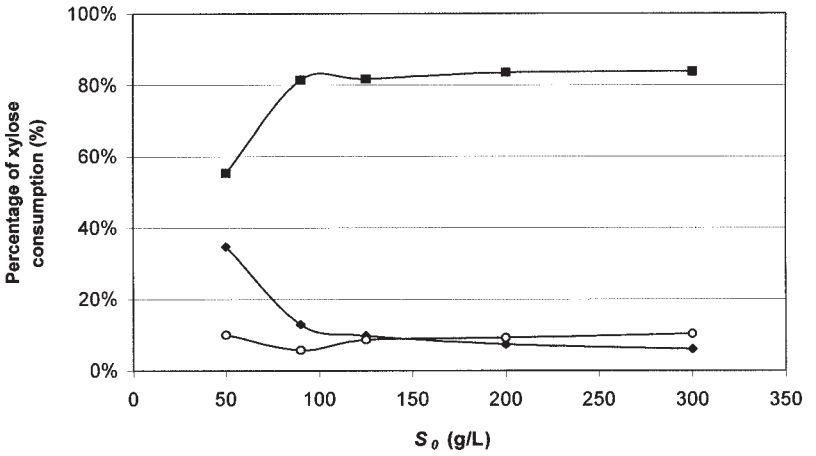

Fig. 2. Influence of the starting substrate concentration on the percentages of xylose consumed during each fermentation phase. (◆) Lag phase; (■) exponential phase; (○) stationary phase.

This effect can be seen more clearly in Fig. 3, that shows the carbon source splitting for biomass growth, xylitol formation and respiration. During the lag phase (Fig. 3A), it is evident at S_0 = 50 g/L the importance of respiration for the energetic requirements of the microorganism. In fact, under conditions of substrate shortage and high starting biomass levels, this is the only activity able to ensure enough energy in the form of ATP for maintenance requirements. At starting xylose levels higher than the optimum (S_0 > 90 g/L), the activity of XR could be inhibited and the fractions of xylose consumed for activities more strongly dependent on oxygen availability (cell growth and respiration) increased. This evidence seems to demonstrate that the observed worsening of xylitol production with increasing S_0 could likely be due to a substrate inhibition phenomenon rather than to limitations of oxygen mass transfer. The percentage of xylose consumed during the exponential phase (Fig. 3B) for respiration was still important at S_0 = 50 g/L, confirming its significance under substrate shortage. On the other hand, a nearly negligible fraction was used for respiration at higher substrate concentrations, with no dependence on S_0. Xylitol formation consumed more than 90% xylose at $S_0 \geq 90$ g/L in this phase, which is a significantly higher proportion with respect to the other phases. Although less important, because referring to a nearly negligible residual xylose amount, the stationary phase (Fig. 3C) showed similar trends to those of the exponential phase, except for the respiratory activity which was again predominant at S_0 = 50 g/L.

Figure 3 also shows the carbon source consumption variations in the different fermentation phases at a given S_0 value. At low substrate availability (S_0 = 50 g/L), xylose consumption for xylitol production was higher than for respiration only during the exponential phase, being the respiratory activity predominant in the other two phases. At higher S_0 values,

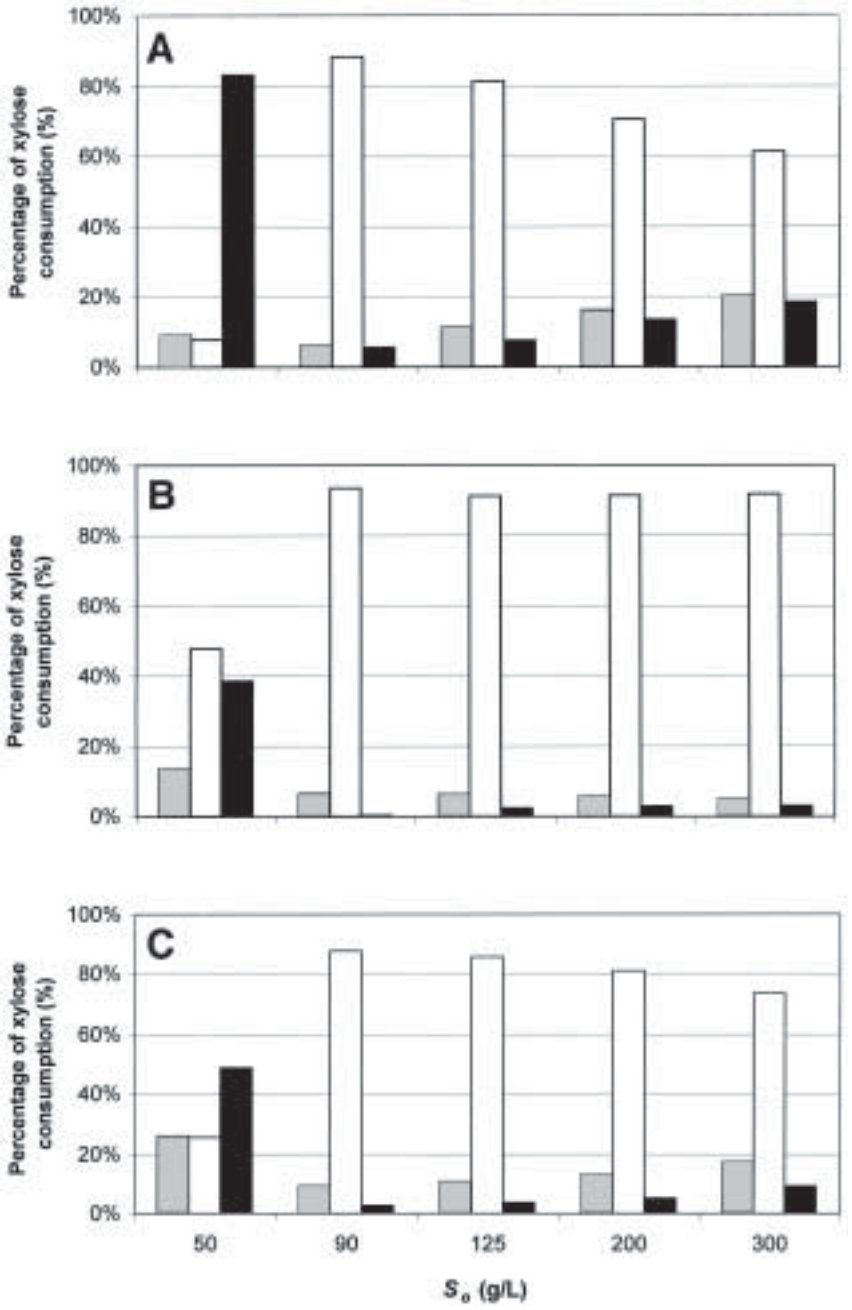

Fig. 3. Effect of starting substrate concentration on the percentages of xylose consumed by each metabolic activity during different fermentation phases. **(A)** lag phase; **(B)** exponential phase; **(C)** stationary phase. (▒) Biomass growth; (□) xylitol production; (■) respiration.

xylitol production was the main metabolic pathway (consuming about 90% xylose in the central period of the runs), while a slow decrease of this consumption occurred in the other phases, which was obviously counter-

balanced by increases in biomass growth and respiration. This effect could be considered as an answer of the system to an energy inquiry at low S_0 values and to an inhibiting effect at high S_0 values. This latter phenomenon, likely due to excess substrate inhibition, became significant in particular during the lag phase, because the microorganism needed to adapt to new fermentative conditions (high osmotic pressure and high substrate concentration) which were very different from those of the preculture. As a consequence, an increased importance was acquired by both respiration and biomass growth.

The relative significance of these metabolic activities could be better explained analyzing the influence of starting carbon level on the percentages of each product formation on its total production during the different phases (Fig. 4). None of the three phases implied relevant variations in xylitol production with varying S_0, the highest percentage of xylitol production (82–87%) having been recorded during the exponential phase, while less than 10% was produced during the other two phases (Fig. 4A). On the contrary, biomass production was strongly influenced by starting xylose concentration (Fig. 4B). The fraction of biomass produced during the exponential phase was always lower than that observed for both produced xylitol and consumed xylose. Moreover, biomass fractions produced in the other fermentation periods were, at extreme S_0 values, higher than 20%, while most of biomass (80%) was produced at S_0 = 90 g/L in the central phase of the fermentation. Also this effect could probably be the consequence of the above phenomena of inhibition and insufficient energy supply at high and low S_0 values, respectively. Since most of CO_2 was produced by respiration, an analysis of the percentage of carbon dioxide production on its total amount (Fig. 4C) can provide a rough indication of the respiratory activity behavior; no significant difference between lag and exponential phases is evident at low xylose concentration, while nearly 80% of total CO_2 production took place in the exponential phase at higher S_0 values. This trend can be explained with the significant contribution of xylitol formation on CO_2 production in this phase. No relevant changes occurred, on the other hand, during the stationary phase, where carbon dioxide production was about 10% of the total amount.

Since the experimental values of CO_2 production were always less than the ones estimated by this carbon balance, although the relative errors never exceeded 10%, it is possible that a) the proposed model is not able to perfectly describe the actual metabolic behavior of the system, even if it provides undoubtedly a good approximation; b) a small but systematic loss of total CO_2 produced volume could have taken place using the selected analytical procedure.

Finally, an overall analysis of the carbon source sharing among the different metabolic activities at the end of fermentation (Table 2) shows that, at S_0 = 50 g/L, respiration was predominant to the detriment of biomass growth and, partially, of xylitol formation. This trend was very similar to the one shown for the exponential phase referred to the specific

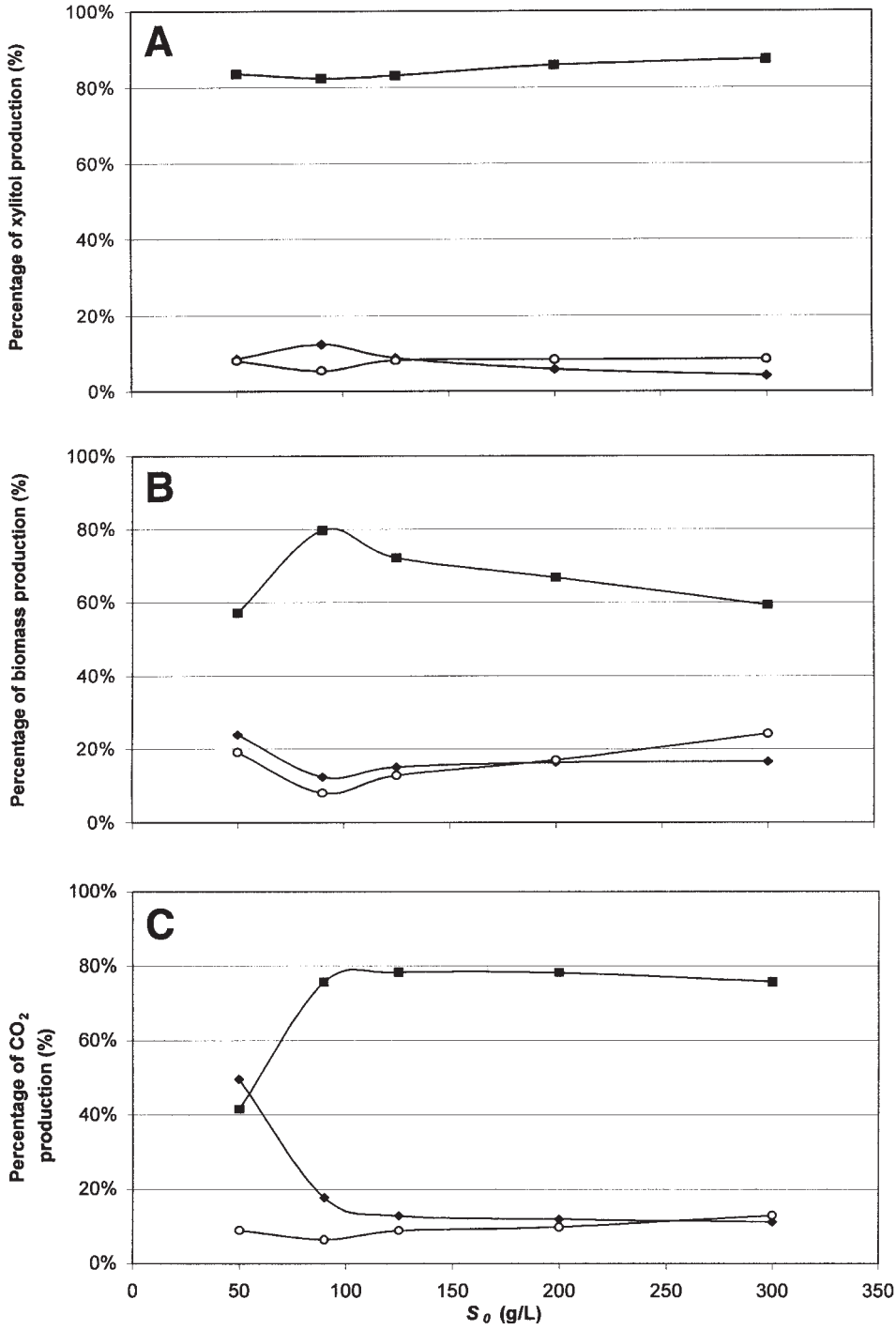

Fig. 4. Influence of the starting substrate concentration on the percentages of **(A)** xylitol, **(B)** biomass, and **(C)** CO_2 produced during each fermentation phase with respect to total final productions. (◆) Lag phase; (■) exponential phase; (○) stationary phase.

Table 2
Influence of Starting Xylose Concentration
on the Estimated Percentages of Xylose Consumed
for Different Metabolic Activities in *Debaryomyces hansenii*

S_0 (g/L)	50	90	125	200	300
Biomass growth	13.31%	6.57%	7.19%	7.23%	7.28%
Xylitol production	31.64%	92.40%	89.85%	88.89%	88.15%
Respiratory chain	55.05%	1.03%	2.96%	3.87%	4.57%
Xylose consumed (g/L)	25.3	89	123.5	191.5	255

xylose consumption, because the widest portion of the carbon source was metabolized just during this period (except at S_0 = 50 g/L). Besides, a relevant xylose residual concentration was detected only at extreme values of starting substrate concentration. At S_0 = 50 g/L it was likely due to the intrinsic slowness of the respiratory activity (which is predominant under these conditions) combined with a very low xylitol productivity (Table 1), while at S_0 = 300 g/L possible inhibition due to excess substrate could be added to these effects.

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