Modelling xylitol and ethanol fermentation using D-xylose and D-glucose mixtures on different aeration conditions by *Debaryomyces hansenii*

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Abstract

We have performed a comparative analysis of the fermentation for solutions of mixtures containing D-glucose and D-xylose with the yeast *Debaryomyces hansenii*, with the aim of producing xylitol and bioethanol. All the experiments were performed in batch bioreactors, with different aeration levels, temperature of 30 ºC, and a culture medium with an initial pH of 5.5. For both aeration levels, the comparison was established on the basis of the following parameters: maximum specific growth rate, biomass productivity, specific rate of substrate consumption (q_s), specific rate of xylitol production, q_{Xi}, and overall xylitol and ethanol yields. For the calculation of the specific rates of substrate consumption and xylitol production, differential method was applied to the kinetic data. From the experimental results, it is deduced that *D. hansenii* sequentially consume the two substrates, first D-glucose and then D-xylose. Highest xylitol yields have been obtained with an extra supply of oxygen (0.5 v/v/min) and just the opposite that happens with the ethanol yield.

Keywords: xylitol, bioethanol, fermentation, aeration, *Debaryomyces hansenii*.

1. Introduction

The characterization of yeasts capable of directly producing substantial concentrations of ethanol from D-xylose has attracted growing interest in the lignocellulose wastes that could be used for industrial production. These wastes, after acidic or enzymatic hydrolysis, can be transformed by fermentation into interesting bioproducts. In the fermentation of hydrolyzed lignocellulose, two primary problems emerge: the fermentation of pentose and the presence of inhibitory compounds from the yeasts. The yeast traditionally used in the fermentation processes, principally of the genera *Saccharomyces* and *Schizosaccharomyces*, can ferment a wide range of sugars, although not D-xylose. Therefore, the integral use of lignocellulose material, among which D-xylose is the major pentose of the hemicellulose fraction, must involve the transformation of this sugar. Recently, some studies have been identified *Debaryomyces hansenii* as a potential good yeast. In the present study, a comparative analysis is made of the fermentation, with this microorganism, for different mixtures of the two main monosaccharides (D-glucose and D-xylose) in any lignocellulose hydrolysate, under two different aeration conditions.

2. Material and methods

2.1 Microorganism

Debaryomyces hansenii NRRL Y-7426 used in this research was obtained from the Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill. USA.

2.2 Fermentation equipment and operation

All the experiments were carried out at laboratory scale in a batch-culture reactor described elsewhere [1] comprising temperature-controlled and magnetically-stirred fermenters with a usable volume of 2 dm^3. The volume of culture medium used was 0.5 dm^3; the stirring speed was 500 rpm, and the stirring rod was 4 cm long and 0.8 cm in diameter. Under these conditions, the overall volumetric mass transfer coefficient (K_{La}), at the beginning of each experiment, was 2.9 h^{-1}. This value of K_{La} was calculated using a dynamic gassing out method.

2.3 Maintenance medium and inoculum preparation

The yeast was stored at 10 ºC in 100 cm^3 test tubes on a sterilised solid culture medium composed of (kg m^{-3}): yeast extract (3); malt extract (3); peptone (5); D-xylose (10); agar-agar (20). Before the start of each experiment the microorganism was inoculated aseptically into glass test tubes containing the solid culture medium described above. These tubes were then kept in an incubator at 30 ºC for 60 h in order to obtain cells at the same growth stage for every experiment. To prepare inocula, cells were resuspended with fresh medium and an appropriate

volume was transferred to the bioreactor. The biomass concentration at the beginning of each experiment was, on a dry weight basis, approximately 0.1 kg m^{-3}.

2.4. Culture medium and procedure

The composition of the culture medium was (kg m^{-3}): $MgSO_4 \cdot 7H_2O$ (1); KH_2PO_4 (2); $(NH_4)_2SO_4$ (3); peptone (3.6) and yeast extract (4). The sum of initial concentrations of D-xylose and D-glucose in the mixture was always 25 kg m^{-3}. Culture conditions were a temperature of 30 ºC and an initial pH of 5.5. The complete culture medium was sterilised using cellulose nitrate filters (Sartorius, type 11307-47-N) with a 0.2 µm pore size.

2.5 Analytical Methods

The quantification of carbohydrates (D-glucose and D-xylose) as well as acetic acid concentrations (in order to estimate the acetyl groups content) were determined by high-performance liquid chromatography (HPLC) using a WATERS instrument, in the conditions: a BIO-RAD Aminex HPX-87H (300×7.8 mm) column at 45 ºC, 0.005 M sulfuric acid as eluant, flow rate of 0.6 cm^3 min^{-1}, refraction index (RI) detector and 0.02 cm^3 sample volume.
Furfural and HMF were analyzed by HPLC using a WATERS instrument with a UV detector (at 276 nm), in the following conditions: a Waters Resolve C18 5 µm (300×3.9 mm) column at ambient temperature, acetonitrile/water (1/8 with 1% of acetic acid) degassed with addition of the phosphoric acid for pH correction to 2.5 as eluant, flow rate of 0.8 cm^3 min^{-1} and 0.02 cm^3 sample volume. The samples were previously diluted with ultrapure water and filtered through membranes HAWP 04700 with 0.45 µm pores. The concentrations of these compounds were calculated from calibration curves obtained from standard solutions.

3. Results and discussion

3.1 Biomass production

The representation of the logarithm values of the dimensionless biomass concentration, $\ln(x/x_0)$, versus time let us to have the growth curves for all cultures; Fig. 1 shows, as an example, the representations obtained for experiments in which D-glucose was the only substrate.

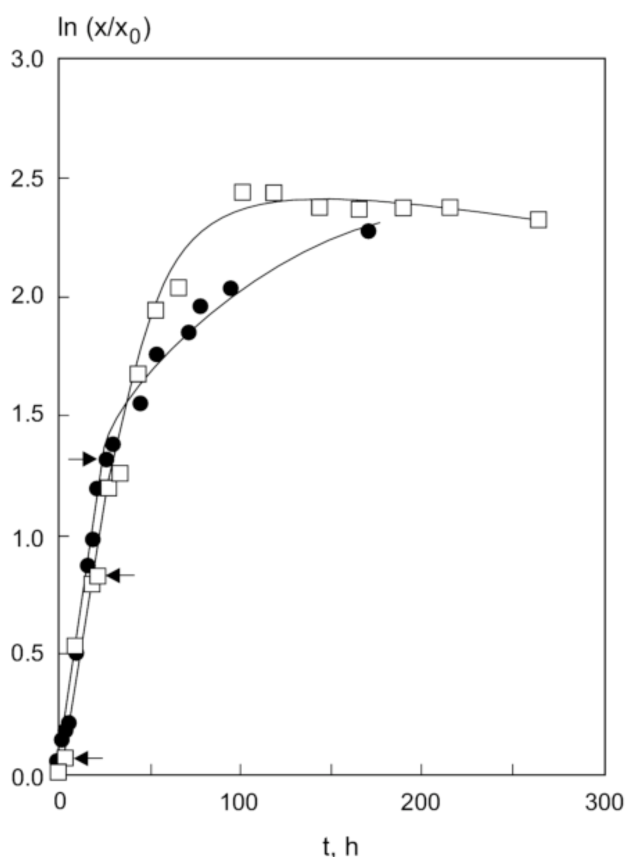

Fig. 1 Growth curves for the experiments Dh-S6 (●) and Dh-S6a (□)

The values of the maximum specific growth rates, μ_m, determined in the exponential phase for each of the experiments, are listed in Table 1 together with the corresponding linear regression coefficients, appreciating that adjustments are acceptable.

Table 1 Specific growth rates

Culture	D-xylose	D-glucose	Q	μ_m	r^2	b	r^2
	kg m^{-3}		v/v/min	h^{-1}		kg m^{-3} h^{-1}	
Dh-S1	25.0	0.0	0.0	0.0147	0.970	0.00512	0.966
Dh-S2	24.0	1.0	0.0	0.0219	0.980	0.00871	0.902
Dh-S3	22.5	2.5	0.0	0.0283	0.980	0.00373	0.989
Dh-S4	20.0	5.0	0.0	0.0325	0.924	0.0140	0.941
Dh-S5	15.0	10.0	0.0	0.0352	0.990	0.0143	0.990
Dh-S6	0.0	25.0	0.0	0.0498	0.988	0.00900	0.989
Dh-S1a	25.0	0.0	0.5	0.0487	0.998	0.0018	0.989
Dh-S2a	24.0	1.0	0.5	0.0545	0.981	0.0114	0.983
Dh-S3a	22.5	2.5	0.5	0.0573	0.981	0.0132	0.967
Dh-S4a	20.0	5.0	0.5	0.0687	0.980	0.0137	0.980
Dh-S5a	15.0	10.0	0.5	0.0693	0.964	0.0153	0.992
Dh-S6a	0.0	25.0	0.5	0.0810	0.987	0.0180	0.983

Experiments conducted with an additional air flow caused by the agitation vortex show μ_m values markedly greater than those obtained when no extra supply of oxygen. The contribution of a larger amount of air may benefit the appearance of aerobic conditions in the system favoring respiratory processes in which the biomass generation is promoted thereby increasing its specific growth rates.

Fig. 2 shows the variation of μ_m versus the initial concentration of D-xylose, appreciating the value of μ_m is greater for cultures where *D. hansenii* used D-glucose (0.050 and 0.081 h^{-1}) as unique carbon source compared to the use of D-xylose (0.015 and 0.049 h^{-1}) as a substrate, both in simple processes as when an additional supply of air was used, respectively. The largest growth of the yeast using the hexose more than the pentose has also been exposed [2,3].

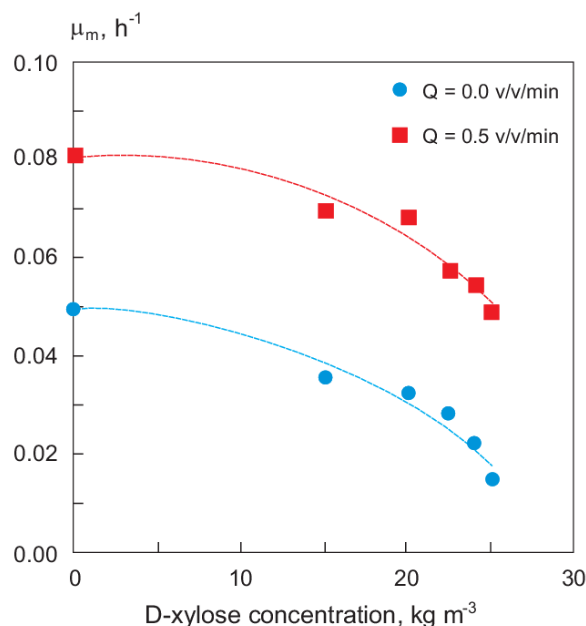

Fig. 2 Variation of μ_m with the initial concentration of D-xylose

Converti et al. [4] set values of μ_m =0.0057 h^{-1} for this yeast growing on concentrations of D-xylose 50 g dm^{-3} and working in reactors 1 dm^3 of medium and oxygen flow of 4.98 mg s^{-1}. For *Candida boidinii* NRRL Y-17213 growing on D-xylose, Vandeska et al. [5] obtained a value of μ_m=0.023 h^{-1} while increasing the ratio D-glucose/D-xylose, growth accelerated reaching 0.067 h^{-1}.

On the other hand, the period of growth after the exponential phase has quantified by the parameter b, determined by Eq. (1), since a behavior linear biomass over time can be admitted. The values calculated for each experiment are also shown in Table 1 along with the corresponding linear regression coefficients.

$$x = c + b\ t \qquad (1)$$

According to these data, biomass productivity is slightly higher when D-glucose is used as carbon source against the use of D-xylose, and furthermore with mixtures of both sugars; the highest values of b were found in the cultures with higher proportions of D-glucose in the fermentation medium, both in the experiments with additional air and when there was no contribution of extra oxygen. The increase of productivities when D-glucose/D-xylose ratio was higher has been also reported [3] and it could be justified by minors requirements for cell growth during the assimilation of hexose than growing on D-xylose.

In general, the values obtained for this parameter are relatively small compared to those obtained with other yeasts as *C. tropicalis* [6] or *P. tannophilus* [1] growing on D-xylose.

3.2 Substrate consumption

As far as substrate consumption is concerned, *D. hansenii* did it sequentially, initially using up the D-glucose quite rapidly. After this, there was a period during which biomass production, substrate consumption and the formation of ethanol ceased or progressed only very slowly; the duration of this second period varied according to the initial sugar concentrations in the substrate but was especially significant in those cultures using similar quantities of both. Subsequently, the consumption of D-xylose begins when practically all the D-glucose present in the medium has disappeared and further production of biomass, ethanol and xylitol occurred. The present observations concur with those of Panchal et al. [7] who reported similar findings of a diauxic (sequential) consumption of D-glucose and D-xylose in the same order by *C. shehatae* and *P. stipitis* when using mixtures of these sugars in the culture medium. Nobre et al. [2] indicate that D-xylose only begins to assimilate when the amount of D-glucose present in the medium is 20%. However, for *D. hansenii* CCMI 941, other authors point to a simultaneous consumption of D-glucose and D-xylose, although hexose is depleted earlier [8].

To calculate the specific rate of total substrate consumption the differential method for the treatment of kinetic data was applied. Among the equations tried, that giving an acceptable reproducibility of the substrate concentration data over the greatest intervals of time was Eq. (2) that linearizes according to the Eq. (3) allows to determine the parameters α and β. As an example of this linearization, Fig. 3 a) shows the representation of $\ln[\ln(s_0/s)]$ vs ln t in the experiment Dh-S4, observing a good adjustment of the experimental data. Once the parameters α and β have been determined for each experiment, the values of residual substrate concentration have been recalculated using the Eq. (2) in the time interval in which this expression can be applied. Fig. 3 b) shows the values reproduced by a solid line while the points correspond to the experimental data.

$$s = s_0\, \alpha^{-t^{\beta}} \qquad (2)$$

$$\ln \ln \frac{s_0}{s} = \ln(\ln \alpha) + \beta \ln t \qquad (3)$$

Once the parameters α and β has been determined and with the Eq. (4), the substrate consumption specific rate has been determined for each experiment in the range of the validity of this equation. In all cases in which D-glucose and D-xylose are mixed in the medium, a unique value of q_s has been determined in the course of the experiment without finding differences between the period of consumption of the two sugars.

$$q_s = \frac{s_0\, \beta\, (\ln \alpha)\, (t^{\beta-1})\, (\alpha^{-t^{\beta}})}{x} \qquad (4)$$

On the other hand, it has been observed that for both simple and additional aeration, D-glucose is entirety consumed but between 68 and 80% of D-xylose remains in the medium. In the case of mixtures, there is a greater assimilation of the substrate especially when the D-glucose/D-xylose ratio is approximately 10%, the

pentose totally depleting. For the other ratios tested the percentage of remaining D-xylose is between 52-72% for simple aeration and less than 32% with 0.5 v/v/min of supplemental air supply.

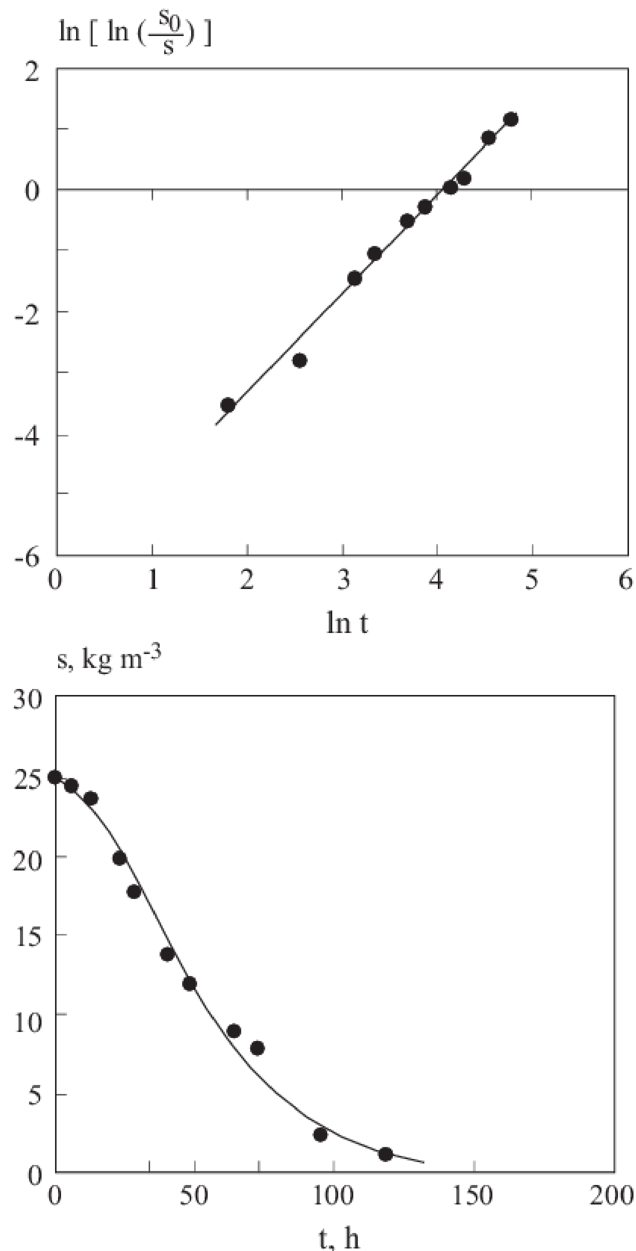

Fig. 3 a) Application of Eq. (3) for the experiment Dh-S4; b) Application of Eq. (2) for the same experiment

Table 2 show the values q_s calculated at various times. In general, higher values of this parameter are obtained with simple substrates than with mixtures and with D-glucose than with D-xylose, both in the experiments that were carried out without additional air supply and in those to which an extra oxygen supply is applied. The fact that *D. hansenii* grows more slowly in pentoses than in hexoses has also been revealed by Nobre et al. [2].

The highest values of specific substrate consumption rate corresponded to the tests in which D-glucose was used as the sole carbon source, 0.29 and 0.27 kg kg^{-1} h^{-1} at 50 hours for Dh-6 and Dh-6a cultures, respectively.

In the experiments performed without external air supply, the highest specific rate of substrate consumption was obtained in the culture with 20% of D-glucose, whereas in which an external flow rate of 0.5 v/v/min was introduced, the maximum value of this parameter corresponds to the experiment with 10% hexose. However, the q_s values are lower for mixtures of both sugars than when yeast grows on D-xylose as the sole carbon source;

this is possible due to a repression of the enzymes involved in the catabolism of this pentose by the presence of D-glucose [3].

Table 2 Specific substrate consumption rates

Culture	D-xylose	D-glucose	Q	t	q_s
	kg m^{-3}		v/v/min	h	kg kg^{-1} h^{-1}
Dh-S1	25.0	0.0	0.0	50	-
				100	0.0289
Dh-S2	24.0	1.0	0.0	50	0.0207
				100	0.00823
Dh-S3	22.5	2.5	0.0	50	0.0397
				100	0.0226
Dh-S4	20.0	5.0	0.0	50	0.209
				100	-
Dh-S5	15.0	10.0	0.0	50	0.0620
				100	0.0226
Dh-S6	0.0	25.0	0.0	50	0.292
				100	0.148
Dh-S1a	25.0	0.0	0.5	50	0.0527
				100	0.0374
Dh-S2a	24.0	1.0	0.5	50	0.0628
				100	0.0297
Dh-S3a	22.5	2.5	0.5	50	0.108
				100	0.0459
Dh-S4a	20.0	5.0	0.5	50	0.0270
				100	0.0136
Dh-S5a	15.0	10.0	0.5	50	0.0688
				100	0.0228
Dh-S6a	0.0	25.0	0.5	50	0.274
				100	0.0875

In addition, the values of q_s decreased with the time of the experiment in all cultures fermented; this decrease has also been reported in other studies over synthetic medium with D-xylose as a single source of substrate, such as *Pachysolen tannophilus* ATCC 32691 [1] or *Hansenula polymorpha* ATCC 34438 [9]. Finally, for all D-glucose/D-xylose mixtures under similar conditions to the embodiments in this work and fermenting with *Candida tropicalis*, values of the substrate consumption rate in the same range have been obtained [6].

To calculate the overall yields in biomass, $Y_{x/s}$, representations of the net biomass formed versus the net substrate consumed concentrations have been made. It has been possible to assume that this parameter is constant, so that the values can be determined by least squares adjustment, Table 3.

Table 3 Overall biomass yields

Culture	D-xylose	D-glucose	Q	$Y_{x/s}$
	kg m^{-3}		v/v/min	kg kg^{-1}
Dh-S1	25.0	0.0	0.0	0.131
Dh-S2	24.0	1.0	0.0	0.141
Dh-S3	22.5	2.5	0.0	0.150
Dh-S4	20.0	5.0	0.0	0.179
Dh-S5	15.0	10.0	0.0	0.185
Dh-S6	0.0	25.0	0.0	0.0642
Dh-S1a	25.0	0.0	0.5	0.0881
Dh-S2a	24.0	1.0	0.5	0.111
Dh-S3a	22.5	2.5	0.5	0.133
Dh-S4a	20.0	5.0	0.5	0.144
Dh-S5a	15.0	10.0	0.5	0.160

Dh-S6a	0.0	25.0	0.5	0.0673

As it can be seen in Fig. 4, when *D. hansenii* grows on D-xylose, there is a major part of the substrate that is invested in the generation of cell mass versus the use of D-glucose as a carbon source. In addition, in mixtures of both sugars, the overall yield in biomass increases with the amount of D-glucose present, so it could be said that the addition of this hexose to the fermentation medium favors the formation of biomass, both in cultures without external air addition as in those in which the supplied air flow rate, in addition to that generated by the stirring vortex, was 0.5 v/v/min.

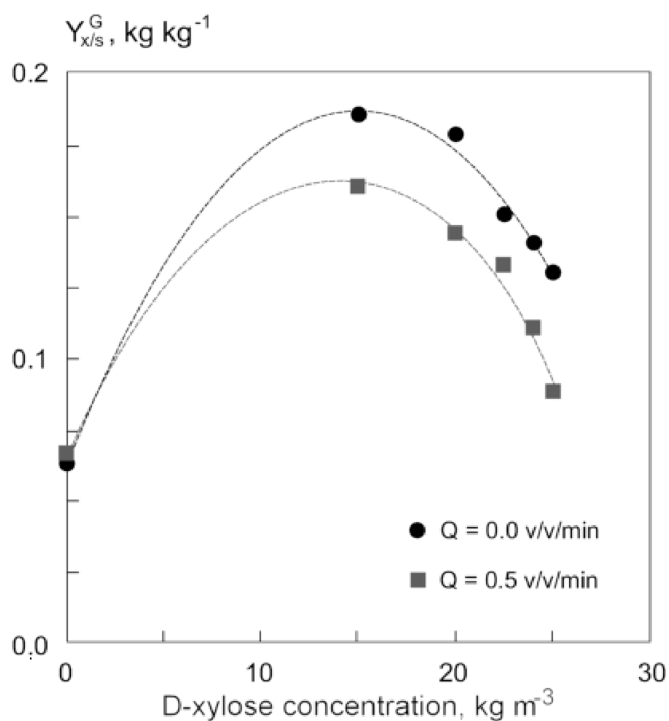

Fig. 4 Variation of overall biomass yields with D-xylose initial concentration

3.3 Ethanol and xylitol production

The formation of the major bioproduct, xylitol, has been studied by the following parameters: specific rate of production, q_{Xi}, and overall xylitol yield, $Y_{Xi/s}$. On the other hand, in cultures where the D-glucose ratio exceeded 10%, ethanol was also detected as a bioproduct, which has allowed to calculate its overall yield, $Y_{E/s}$.

To calculate the specific production rate of xylitol, q_{Xi}, the differential treatment method of kinetic data has been applied, whereby the xylitol concentration data, Xy, against time, t, have been adjusted to the Eq. (5). Once the parameters G and H have been determined using least squares adjustments, the corresponding values of q_{Xi} at different times can be determined by the Eq. (6). Table 4 collects the results obtained for the specific production rate of xylitol at two different times, close to 50 and 100 h. As it can be seen, the specific production rates of xylitol are reduced by the addition of D-glucose in the medium when this hexose is in amounts of 1% to 10% relative to the total sugars content.

$$Xy = H + G\,t \tag{5}$$

$$q_{Xy} = \frac{G}{x} \tag{6}$$

Table 4 Specific xylitol production rates

Culture	D-xylose	D-glucose	Q	t	$Y_{x/s}$
	kg m^{-3}		v/v/min	h	kg kg^{-1}
Dh-S1	25.0	0.0	0.0	50	0.349
				100	0.277
Dh-S2	24.0	1.0	0.0	50	0.0677
				100	0.0450
Dh-S3	22.5	2.5	0.0	50	0.0712
				100	0.0582
Dh-S4	20.0	5.0	0.0	50	0.717
				95	0.491
Dh-S5	15.0	10.0	0.0	50	0.295
				100	0.160
Dh-S6	0.0	25.0	0.0	50	0.182
				100	0.111
Dh-S1a	25.0	0.0	0.5	50	1.25
				100	1.08
Dh-S2a	24.0	1.0	0.5	50	1.68
				90	1.27
Dh-S3a	22.5	2.5	0.5	50	1.29
					-
Dh-S4a	20.0	5.0	0.5	50	0.484
				100	0.380
Dh-S5a	15.0	10.0	0.5	50	0.552
				100	0.302
Dh-S6a	0.0	25.0	0.5	50	0.226
				100	0.112

When D-glucose concentrations are raised (between 20% and 40%), the value of q_{Xi} becomes very close to that obtained when the fermentation medium was composed of D-xylose as the only substrate. On the other hand, from the slopes of the lines that have adjusted the concentrations of xylitol and ethanol produced against the unconsumed substrate, the overall yields in xylitol, $Y_{Xi/s}$, and ethanol, $Y_{E/s}$, respectively have been calculated.

When D-xylose was used as carbon source, cultures Dh-S1 and Dh-S1a, xylitol was obtained as the only bioproduct, with a higher yield ($Y_{Xi/s}$ = 0.23 kg kg^{-1}) working at a higher oxygen flow rate compared to 0.086 kg kg^{-1} when no extra air was supplied beyond that provided by the stirring vortex. In these experiments, no ethanol production was detected and only acetic acid (0.49 kg m^{-3}) was detected when the oxygen input was the lowest of those used.

Small quantities of acetic acid (0.51 kg m^{-3}) were produced in culture Dh-S6 and it was observed that, when D-glucose was almost completely consumed, the ethanol produced was used as a secondary source of substrate. However, when additional oxygen is supplied, Dh-S6a cullture, considerable amounts of acetic acid (3.5 kg m^{-3}) are detected and it is evaporated or oxidized when the primary substrate is almost entirety depleted.

On the other hand, it is observed that the addition of D-glucose to synthetic D-xylose media does not improve xylitol yields under any of the conditions tested since $Y_{Xi/s}$ increase progressively with the relationship pentose/hexose, Fig. 5. This fact demonstrates that the presence of D-glucose in the fermentation medium could act as a limiting factor in the xylitol production processes, causing an inhibitory effect on the production of this bioalcohol, the more important as the smaller the relationship between both sugars, possibly due to a decrease in the activity of xylose reductase which could affect the amount of xylitol produced [10].

In addition, acetic acid concentrations up to 1.5 kg m^{-3} have been obtained when D-glucose added to the fermentation medium was greater than 5 kg m^{-3} in medium without no additional air supply; however this acid is not detected if aeration used was 0.5 v/v/min.

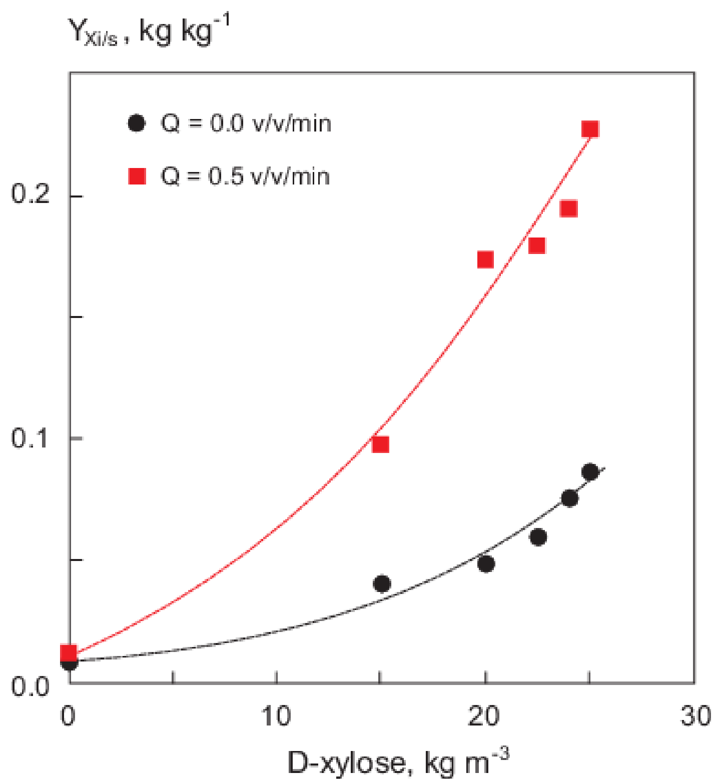

Fig. 5 Overall xylitol yield vs initial D-xylose concentration

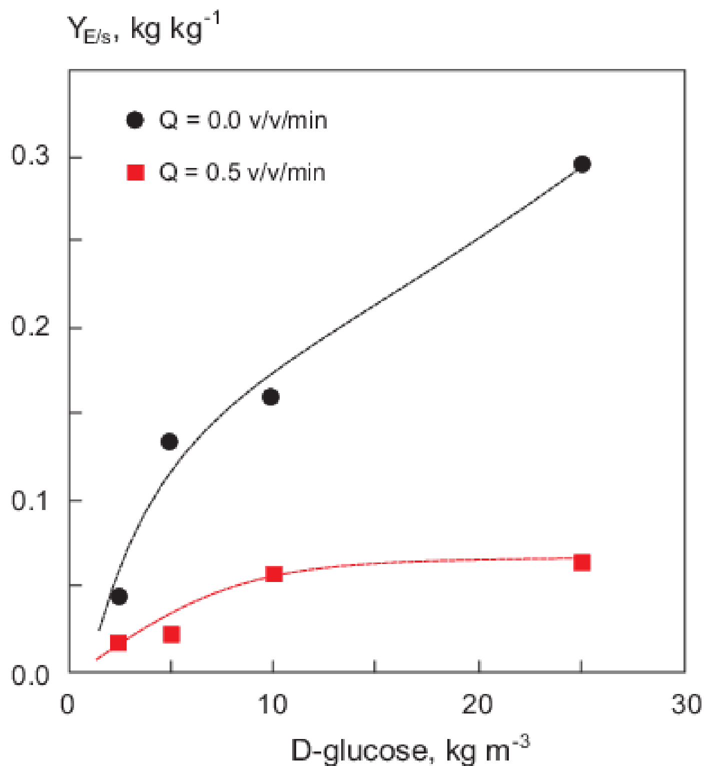

Fig. 6 Overall ethanol yield vs initial D-glucose concentration

Finally, indicate that with D-glucose/D-xylose mixtures at similar concentrations to those used in the present investigation, the maximum yields in xylitol (0.194 kg kg^{-1}) are slightly lower than those obtained with *C. tropicalis* NBRC 0618 (0.28 kg kg^{-1}) for mixtures with 4% of D-glucose [6]. $Y_{Xi/s}$ values obtained are also smaller than those produced with *D. hansenii* CCMI 941 (close to 0.50 kg kg^{-1}) in cultures with a hexose/pentose ratio of about 10%, for total substrate concentrations in the medium of 22 kg m^{-3}, an aeration of 2 dm^3 min^{-1} and an average volume of 1.4 dm^3 [3].

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