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Influence of temperature on the fermentation of D-xylose by *Pachysolen tannophilus* to produce ethanol and xylitol

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Abstract

The influence of temperature between 283 and 313 K on the fermentation of D-xylose with *Pachysolen tannophilus* ATTC 32691 to produce ethanol and xylitol was studied. All experiments were made in a batch-culture reactor keeping the aeration level constant and the pH of the culture medium at 4.5. In each experiments the maximum specific net growth rate (μ_m), biomass productivity (b), the specific rates of xylose uptake (q_s) and ethanol and xylitol production (q_E and q_{Xy}) and overall yields in biomass ($Y^G_{x/s}$), ethanol ($Y^G_{E/s}$) and xylitol ($Y^G_{Xy/s}$) were determined. A fitting of the experimental values of $\mu_m - T$, within the wide temperature range studied lead to the equation

$$\mu_m = 2.2 \times 10^9 e^{-6839/T} - 9.1 \times 10^{22} e^{-16\,702/T}$$

which allows a determination of the apparent activation energies for cell growth (E_a=56.9 kJ/mol) and death (E_d=138.9 kJ/mol) and an optimum growth temperature of 306 K. The value of q_E remained virtually constant during the linear growth phase whilst q_s fell throughout the whole culture. The highest values for overall ethanol yield ($Y^G_{E/s}$=0.39 kg/kg) and the specific ethanol-production rate (q_E=0.06 kg/kg h) were obtained for fermentations carried out at 303 K, which is also quite close to the optimum temperature for the formation of biomass. Maximum overall xylitol yield ($Y^G_{Xy/s}$=0.14 kg/kg) occurred at 288 K.

Keywords: D-Xylose; Fermentation; *Pachysolen tannophilus*; Temperature; Ethanol; Xylitol

1. Introduction

The yeasts traditionally used in fermentation processes are able to consume hexoses as substrate but not pentoses. Nevertheless, to obtain the fullest return from lignocellulose waste, within which there is a considerable hemicellulose fraction (18–34%), composed mainly of D-xylose, it is essential to resort to yeasts which also have the capacity to consume this pentose [1]. To this end, *Pachysolen tannophilus* is capable of transforming D-xylose into useful bioproducts such as ethanol and xylitol [2].

Fermentation with *P. tannophilus* has already been studied with regard to such parameters as the pH of the culture medium [3], specific aeration rate [4] and the initial concentrations of the inoculum and the substrate, and the composition of the substrate [5]. Nevertheless, one of the more important operating variables capable of exerting considerable influence upon the generation of useful bioproducts is that of temperature, due to its direct effect on the solubility of oxygen and carbon dioxide dissolved in the medium and the rate of oxygen transfer within the culture.

As far as the growth rate of *P. tannophilus* is concerned, the literature reports that, this yeast shows net growth over temperatures ranging from 288 to 313 K [6–8]. Other authors claim that variations in temperature may affect the concentrations of ethanol and xylitol produced [9]. Temperature is an important parameter not only in terms of growth and yield but also because, from the industrial point of view, lower temperatures imply lower costs and more manageable conditions, particularly with regard to contamination of the culture medium. Thus, we have made experiments within a wide range of temperatures in order to study its influence upon the fermentation of solutions of D-xylose with *P. tannophilus* and to determine the optimum temperature to

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Nomenclature

a	parameter in Eq. (1)
A	parameter in Eq. (10)
B	biomass productivity (kg biomass/m^3 h)
B	parameter in Eq. (10)
c	parameter in Eq. (4)
E	ethanol concentration (kg/m^3)
E_a	apparent activation energy of cell growth (kJ/mol)
E_d	apparent activation energy of cell death (kJ/mol)
E_T	maximum theoretical ethanol production (kg/m^3)
m	parameter in Eq. (13)
n	parameter in Eq. (13)
q_E	specific ethanol-production rate (kg ethanol/kg biomass h)
$\bar{q}_E^D$	mean specific ethanol-production rate, differential method (kg ethanol/kg biomass h)
q_s	specific xylose-uptake rate (kg xylose/kg biomass h)
q_s^D	specific xylose-uptake rate, differential method (kg xylose/kg biomass h)
q_{Xy}	specific xylitol-production rate (kg xylitol/kg biomass h)
$s_0 - s$	substrate consumed (kg/m^3)
t	time (h)
T	temperature (K)
T_{opt}	optimum temperature (K)
x	biomass concentration (kg/m^3)
x_0	initial biomass concentration (kg/m^3)
Xy	xylitol concentration (kg/m^3)
$Y_{E/s}$	instantaneous ethanol yield, defined in Eq. (14)
$Y_{E/s}^G$	overall ethanol yield (kg ethanol/kg xylose)
$Y_{E/x}$	instantaneous ethanol production
$Y_{x/s}$	instantaneous biomass yield
$Y_{x/s}^G$	overall biomass yield (kg biomass/kg xylose)
$Y_{Xy/s}$	instantaneous xylitol yield, defined in Eq. (15)
$Y_{Xy/s}^G$	overall xylitol yield(kg xylitol/kg xylose)

Greek letters

μ_d	kinetic parameter in Eq. (2), (1/h)
μ_m	maximum specific growth rate (1/h)
μ_0	kinetic parameter in Eq. (2), (1/h)

achieve maximum specific rates for the production of ethanol and the highest yields in ethanol and xylitol.

2. Materials and methods

2.1. Microorganism

The yeast *P. tannophilus* ATCC 32691 was supplied by the American Type Culture Collection.

2.2. Experimental device

All experiments were carried out on a laboratory scale in a batch-culture reactor consisting basically of two temperature-controlled, magnetically stirred fermenters with an usable volume of 2 dm^3 [10]. The volume of culture medium was 0.5 dm^3; the stirring speed 500 rpm, and the stirring rod 4 cm long and 0.8 cm in diameter.

2.3. Culture medium

The composition of the culture medium in kg/m^3 was: $MgSO_4$, 1; KH_2PO_4, 2; $(NH_4)_2SO_4$, 3; peptone, 3.6; and yeast extract 4. The initial concentration of D-xylose was 25 kg/m^3.

2.4. Maintenance medium and inoculum preparation

The yeasts were stored at 288 K in 100 cm^3 test tubes on a sterilised solid culture medium composed of (in kg/m^3): yeast extract 3; malt extract 3; peptone 5; D-xylose 10;

agar–agar 20. Before the start of each experiment the microorganisms were inoculated under sterile conditions into glass test tubes containing the solid culture medium described above. These tubes were then kept in an incubator at 303 K for 60 h to obtain cells at the same growth stage for every experiment. The concentration of the inoculum at the beginning of each experiment was approximately 0.01 kg/m^3.

2.5. Procedure

The most favourable operating conditions had been determined in previous studies [3,10]: pH 4.5, initial D-xylose concentration 25 kg/m^3 and aeration supplied by the stirring vortex alone at a speed of 500 rpm. In these experiments the temperature was altered throughout the range from 283 to 313 K. The complete culture medium was sterilised using 0.2 μm-pore cellulose–nitrate filters.

2.6. Analytical technique

Dry-weight (x, kg/m^3) was calculated by the absorbance of the suspension at a wavelength of 620 nm measured against a previously obtained absorbance versus dry-weight calibration line. The residual concentration of D-xylose (s, kg/m^3) was calculated using Miller's reducing sugar method [11]. Ethanol and xylitol concentrations (E, kg/m^3 and Xy, kg/m^3) were quantified according to the methods described by Beutler [12] and Beutler and Becker [13], based on the enzymes alcohol dehydrogenase and polyol dehydrogenase.

3. Results and discussion

The influence of temperature on the fermentation of D-xylose solutions by *P. tannophilus* was studied with regard to the kinetic parameters related to biomass, ethanol and xylitol production and substrate consumption. As an example the primary results for two of the temperatures assayed are set out in Fig. 1.

3.1. Specific growth rate

The growth curves [ln(x/x_0) versus t] reveal that only those cultures grown between 283 and 288 K went through any appreciable lag phase. At the lower temperature the lag phase was around 7 h whilst at temperatures above 293 K it decreased to less than 2 h. Following this there was a relatively short exponential growth phase of between 3 and 48 h for temperatures of between 313 and 283 K, respectively. Subsequently there was a considerably longer period of between 75 and 314 h during which growth remained fairly linear. Growth finally entered a stationary phase coinciding with the total consumption of the substrate. At 313 K there was only a short exponential phase followed immediately by the stationary phase, during which time just 1 g of D-xylose was consumed.

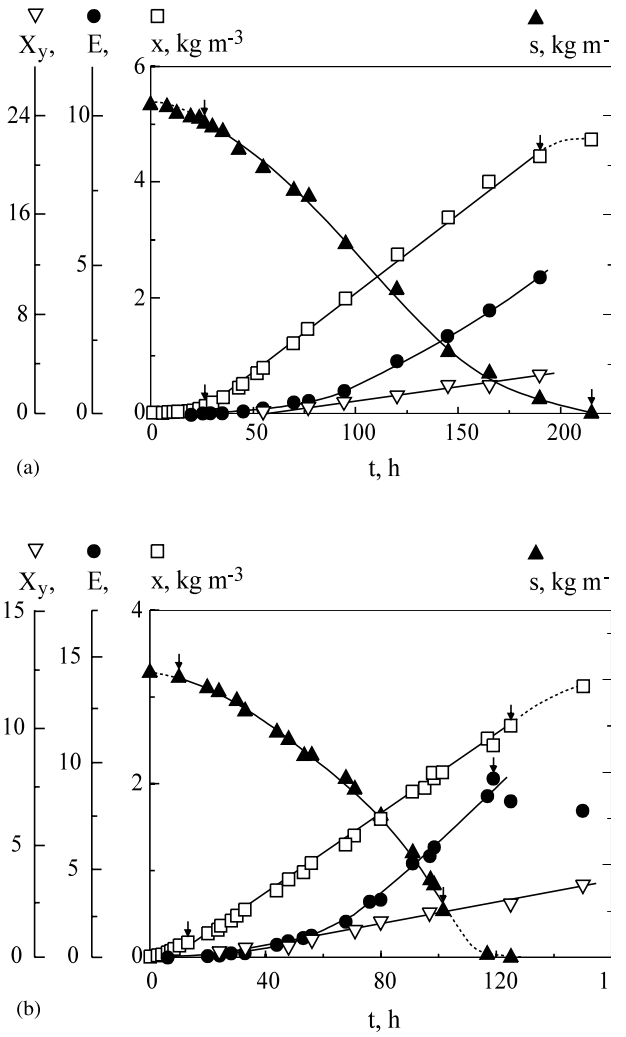

Fig. 1. Variation in the concentrations of biomass (□), ethanol (●) and xylitol (▽) produced, and D-xylose consumed (▲) throughout the experiments made at 288 K (a) and 313 K (b).

Once the exponential growth phase for each culture was established, μ_m could be calculated using the equation

$$\ln \frac{x}{x_0} = a + \mu_m t \quad (1)$$

The μ_m values versus temperature are shown in Fig. 2, where it can be seen that maximum specific net growth rates were achieved at between 303 and 308 K.

Experimental values for the maximum specific net growth rates obtained within this temperature range were fitted to the equation

$$\mu_m = \mu_0 e^{-E_a/RT} - \mu_d e^{-E_d/RT} \quad (2)$$

where μ_m represents the difference between the specific-growth and cell-death rates, E_a and E_d represent the apparent energies of cell growth and death, respectively, and μ_0 and μ_d the corresponding pre-exponential factors. The re-

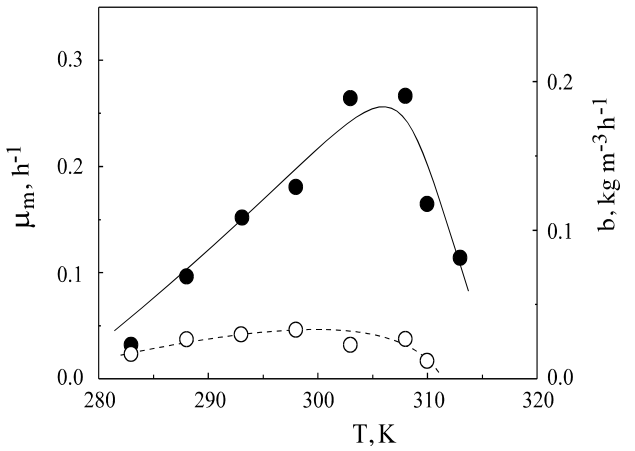

Fig. 2. Maximum specific growth rates (●) and biomass productivities (○) versus temperature.

sults obtained via non-linear regression are set out in Table 1, where it can be seen that the values for E_d are considerably higher than those for E_a, as is normal with most microorganisms. This fact explains the asymmetry of the curve in Fig. 2, where for comparison's sake the experimental values and those obtained from Eq. (2) are shown. The two sets of results fit each other acceptably.

The derivative of μ_m according to T in Eq. (2) allows the determination of the optimum temperature

$$T_{opt} = \frac{E_d - E_a}{R \ln \frac{\mu_d E_d}{\mu_0 E_a}} = 305.8 \text{ K} \quad (3)$$

which leads us to a maximum value for μ_m of 0.25/h.

During the long period after the exponential growth phase, biomass concentration increased linearly. Representations of the experimental results of $x-t$ allow a determination of the duration of this growth period and also, via least-square adjustments to Eq. (4)

$$x = c + bx \quad (4)$$

to obtain the values for biomass productivity, b, which are set out together with those for μ_m in Fig. 2, where it can be seen that temperature only affects this parameter very slightly, with productivity decreasing at both ends of the range. It is worth noting, however, that the duration of the linear growth phase decreased concomitantly with a rise in temperature.

This linear growth period after the exponential phase has also been reported by other authors [1,6,14] and is characteristic of fermentation processes controlled by physical stages, because kinetic control of the bioprocess resides in the transfer of oxygen within the cell suspension. This observation coincides with similar results obtained by Slininger et al. [6] with the same yeast, who report that at the end of the exponential phase there was scarcely any oxygen dissolved in the medium, even in those experiments where the culture was well aerated. In the same way it was found that at the end of our experiments the concentration of oxygen dissolved in the medium was practically zero, in the range of 0–0.3 mg O_2/dm^3.

The linear growth period begins earlier as the temperature rises; thus, for example, at 283 K it begins after 50 h whilst at 310 K it begins after half that time, at 25 h. This behaviour may be explained by the fact that the solubility of oxygen in the culture diminishes concomitantly with a rise in temperature. The duration of the linear growth phase also decreases versus temperature: at 283 K it lasts for 314 h whilst at 310 K it goes on for no more than 75 h, which might be expected if we bear in mind that the coefficient of oxygen diffusion within the medium, and thus the coefficient of oxygen transport, increases exponentially with temperature.

Table 1
Apparent activation energies and pre-exponential factors

E_a (kJ/mol)	E_d (kJ/mol)	μ_0 (1/h)	μ_d (1/h)
56.9	138.9	2.2×10^9	9.1×10^{22}

3.2. Specific xylose-uptake rate and biomass yields

To determine the specific xylose-uptake rate (q_s) both differential and integral methods were applied to the kinetic data.

The calculation of q_s via the differential method is based on the equation

$$q_s = \frac{1}{x}\frac{d(s_0 - s)}{dt} \quad (5)$$

which requires the previous calculation of the volumetric rate of D-xylose consumption, $d(s_0-s)/dt$.

In all experiments the substrate concentration was filled to the equation

$$s = s_0 \alpha^{-t^\beta} \quad (6)$$

which fulfils the initial condition that $s=s_0$ when $t=0$.

To determine the values of parameters α and β in Eq. (6) least-square fittings were made to be able to calculate analytically the derivative $d(s_0-s)/dt$ and thus to obtain the specific consumption rate of D-xylose versus time. These values are set out in Table 2 for three points of time during each experiment, except for that conducted at the lowest temperature in the range, for which we decided that it was only worth working out two values. q_s^D diminishes throughout the experiment and it is noteworthy that the highest values for the specific consumption rate of D-xylose were reached in the cultures made at the lowest temperatures.

Apart from this, q_s can be determined via the integral method using the values for instantaneous biomass yield, $Y_{x/s}$, and the definitions of the specific growth and xylose-uptake rates

$$Y_{x/s} = \frac{dx}{d(s_0 - s)} = \frac{\mu}{q_s} \quad (7)$$

Table 2
Overall biomass yields and specific xylose-uptake rates

T (K)	$Y^G_{x/s}$ (kg/kg)	t (h)	q_s^D (kg/kg h)	q_s (kg/kg h)
283	0.30	50	0.42	0.64
		100	0.11	0.14
288	0.19	30	0.53	0.74
		50	0.20	0.19
		100	0.086	0.066
293	0.17	30	0.32	0.44
		50	0.19	0.17
		100	0.079	0.068
298	0.18	30	0.24	0.39
		50	0.16	0.17
		100	0.069	0.063
303	0.098	30	0.34	0.49
		50	0.24	0.25
		100	0.086	0.11
308	0.13	30	0.24	0.34
		50	0.22	0.17
		100	0.069	0.068
310	0.18	30	0.17	0.15
		50	0.13	0.10
		100	0.085	0.052

Thus, if the hypothesis of constant biomass yield is accepted then q_s can be calculated during the linear growth period by

$$q_s = \frac{1}{x}\frac{b}{Y_{x/s}} \tag{8}$$

the result of which confirms that q_s diminishes concomitantly with an increase in biomass concentration, that is to say versus time.

To verify this hypothesis net biomass production ($x-x_0$) with substrate consumption (s_0-s) was compared in each culture and a linear relationship between both was found, as we had predicted. Thus the overall biomass yields was calculated as set out in Table 2.

The lowest value for $Y^G_{x/s}$ was obtained at 303 K and rose somewhat on either side of this temperature, to the extent that at the lowest temperature assayed (283 K) this value had increased to three times that at 303 K.

Finally it can be seen in Table 2 that the values for the specific xylose-uptake rate calculated by the differential method (q_s^D) agree satisfactorily with those determined by the integral method (q_s). These values are very close to those obtained by Slininger et al. [6], working with *P. tannophilus* NRRL Y-2460. These authors used a different culture medium, an initial D-xylose concentration of 50 kg/m^3, pH 4.5 and a temperature range of 298–305 K and arrived at specific xylose-uptake rate values within the range of 0.3–0.5 kg/kg h.

3.3. Specific rates of ethanol and xylitol production

To determine the specific ethanol-production rate, q_E, both differential and integral methods were applied to the kinetic data. Given that the aim was to determine the specific ethanol-production rate

$$q_E = \frac{1}{x}\frac{dE}{dt} \tag{9}$$

various empirical equations that fit only the phase during which ethanol concentration increases with time were applied. Of these equations that which best fitted the experimental variations was

$$\frac{E_T}{E_T - E} = A^{t^B} \tag{10}$$

where E_T represents the maximum concentration attainable if the yield for the experimental conversion of D-xylose into ethanol matches the theoretical one.

Eq. (10) fulfils the initial condition that when t=0, E=0 and it can be linearized so that the values of parameters A and B can be determined via least-squares adjustments.

Once A and B have been calculated the derivative dE/dt can be determined analytically from Eq. (10) and thus, by substituting into Eq. (9), it was possible to calculate the values for q_E versus time for each of our experiments. It can be deduced that a mean value for q_E is acceptable in the phase during which the ethanol concentration increases and these values, $\bar{q}_E^D$ are shown for each experiment in Table 3.

To apply the integral method, the concept of instantaneous ethanol production was used:

$$Y_{E/x} = \frac{dE}{dx} = \frac{q_E}{\mu} \tag{11}$$

If the specific ethanol-production rate was assumed to be constant, then, according to the definition of b and Eq. (11), it can be deduced that, during the linear growth phase

$$\frac{dE}{x dx} = \frac{dE}{d\left(x^2/2\right)} = \frac{q_E}{b} \tag{12}$$

Table 3
Specific production rates of ethanol and xylitol production

T (K)	$\bar{q}_E^D$ (kg/kg h)	q_E (kg/kg h)	t (h)	q_{Xy} (kg/kg h)
283	0.0028	0.0032	150	0.0011
288	0.012	0.011	150	0.0057
293	0.024	0.021	150	0.0054
298	0.024	0.025	50	0.0061
			150	0.0015
303	0.053	0.062	50	0.025
308	0.037	0.038	50	0.0095
310	0.032	0.029	50	0.012

so that a graph of E versus $x^2/2$ confined to the linear growth phase should give a straight line, from the slope of which we can determine the value of q_E.

According to Eq. (12), the slopes of the corresponding least-square adjustments give the values of q_E/b, and using the values of b in Table 2 those for the specific ethanol-production rate corresponding to the integral method, q_E, were determined. These are given in Table 3 together with the values obtained by the differential method. In general the values of q_E obtained by either method coincide to an acceptable degree.

It is noteworthy that the highest value for the specific ethanol-production rate was obtained at 303 K and that this value diminished considerably at lower temperatures. The values for this parameter are very similar to those obtained by Slininger et al. [6] and Ligthelm et al. [15], although these authors were working with another yeast strain, *P. tannophilus* NRRL Y-2460, and different culture media and operating conditions. Ligthelm et al. [15], for example, using an initial concentration of 40 kg/m^3 under aerobic, microaerobic and anaerobic conditions, arrived at values for q_E of 0.04, 0.1 and 0.07 kg/kg h, which are very similar results to our own. On the other hand, Slininger et al. [6] obtained higher values for q_E (0.1 and 0.2 kg/kg h at temperatures of 301 and 305 K) but, did not take into account that q_E remains virtually constant during the linear growth phase and thus did not determine mean values for the specific ethanol-production rate.

The data concerning xylitol concentrations and culture time fit quite satisfactorily to the linear equation

$$Xy = n + mt \tag{13}$$

This fitting also allows the calculation of the specific xylitol-production rate (q_{Xy}) for any moment of time. It can be seen that, contrary to the specific ethanol-production rate, q_{Xy} diminishes throughout each experiment. Values for the specific production rate of xylitol at certain times the cultures are set out in Table 3, where it can be seen that these are generally lower than the mean values for the specific production rate of ethanol.

3.4. Ethanol and xylitol yields

In accordance with the definitions of instantaneous ethanol and xylitol yields

$$Y_{E/s} = \frac{dE}{d(s_0 - s)} \tag{14}$$

$$Y_{Xy/s} = \frac{dXy}{d(s_0 - s)} \tag{15}$$

if these yields remain constant throughout the culture period, the plots of E and Xy versus (s_0−s) should result in straight lines with slopes $Y^G_{E/s}$ and $Y^G_{Xy/s}$ and in fact this constancy in ethanol and xylitol yields corresponds on the whole to our experimental observations (Fig. 3).

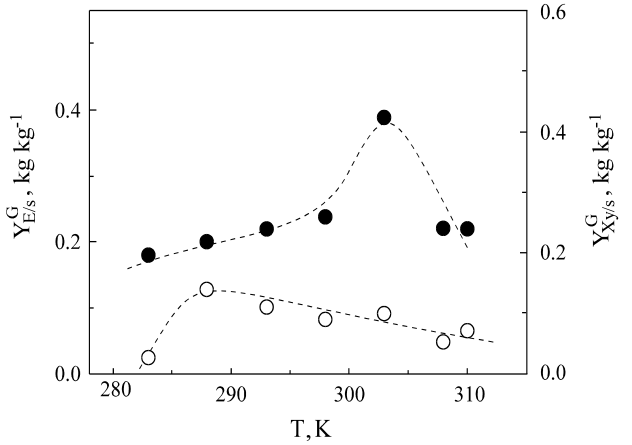

Fig. 3. Temperature dependence of ethanol (●) and xylitol (○) yields.

As far as $Y^G_{E/s}$ is concerned, there was a slight increase in concomitant with a rise in temperature until about 300 K, after which the increase became quite pronounced to reach a maximum value of 0.39 kg/kg at 303 K and then decline again rapidly at higher temperatures. This decrease may be put down in part to the fact that at higher temperatures the solubility of the oxygen dissolved in the medium is reduced and also that there tends to be more evaporation of ethanol. These results agree with those of other authors [6,8] who, although working under somewhat different conditions, determined the optimum temperature at between 303 and 305 K.

With regard to overall xylitol yield, the maximum value for this parameter, 0.14 kg/kg, was obtained at 288 K, and it diminished slowly at higher temperatures. $Y^G_{Xy/s}$, on the other hand, decreased by sixfold at temperatures lower than 288 K.

In conclusion, we can deduce from our results that temperature does indeed have a considerable effect upon the overall yield in ethanol to the extent that even a small change around the optimum value results in a great change in the values of $Y^G_{E/s}$. The overall yield in xylitol, on the other hand, is less affected by variations in temperature. Nevertheless, the optimum temperatures for the generation of both these bioproducts is not the same, which agrees with the observations made by Barbosa et al. [7]. This fact should be of considerable interest to industry when either ethanol or xylitol is the preferred product.

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