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Effect of temperature on the microaerophilic metabolism of *Pachysolen tannophilus*

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Abstract

Xylitol production by *Pachysolen tannophilus* from detoxified hemicellulose hydrolysate was investigated under microaerophilic conditions at temperature ranging from 20 to 40°C. A carbon balance previously proposed to study the influence of pH was used in this work to evaluate the amounts of carbon source (xylose) utilised in competitive metabolic ways: reductive production of xylitol, ethanol fermentation and respiration. At pH = 5.5 more than 83% of xylose was reduced to xylitol at $25 < T < 30$°C, whereas respiration became the main process at low temperature (71.1% at 20°C). At high temperature, on the other hand, all three processes took place at comparable rate, consuming at 40°C nearly the same percentage of carbon source (33–35%). Finally, the maximum values of volumetric productivity calculated at variable temperature were used to estimate the main thermodynamic parameters of both xylitol production ($\Delta h^* = 105.4$ kJ mol^{-1}; $\Delta s^* = -13.2$ J mol^{-1} K^{-1}) and thermal deactivation ($\Delta h^*_D = 210.5$ kJ mol^{-1}; $\Delta s^*_D = 3.63$ J mol^{-1} K^{-1}).

Keywords: xylitol production; *Pachysolen tannophilus*; carbon material balance; kinetic results; thermodynamic parameters

1. Introduction

An interesting alternative to the traditional chemical methods for the production of xylitol is the enzymatic reduction of xylose contained in hemicellulose hydrolysates by xylose reductase (XR).

Amongst the best xylitol producers, the yeast *Pachysolen tannophilus* proved a particularly versatile microorganism, whose metabolism can be oriented towards xylitol or ethanol productions, according either to operating conditions or to hydrolysate pretreatment techniques [1], but little is known about the reasons of its interesting dual behaviour.

In particular, xylose can be metabolised in different ways depending on the oxygen availability in the medium. Under aerobic conditions, cell growth is preferred to xylitol production and ethanol fermentation, both requiring a reducing environment. Although the shortage of NAD^+ (or $NADP^+$) should favour xylitol formation under strictly anaerobic conditions, high starting biomass levels were necessary to accelerate this process to acceptable levels [2]. Lastly, microaerophilic conditions proved the best solution for xylitol production [1], mainly at low cell concentrations, because the poor oxygen available under these conditions was completely consumed for the growth, thus increasing the productivity.

The reason of this versatile behaviour could be searched in the specificity of the cofactor (NADH or NADPH) of XR in *Pachysolen tannophilus*. Hahn-Hägerdal et al. [3] demonstrated that xylose reductase activity in some yeasts becomes progressively more NADPH-dependent with increasing dissolved oxygen level in the medium. Thus, NADPH could be more efficient than NADH for XR activity in *P. tannophilus* under microaerophilic conditions.

The effect of pH on *P. tannophilus* XR activity was investigated in a previous study [2] with the aim of providing further information on the metabolism of this yeast. In that work, using the standard procedure of Villadsen et al. [4], it was proposed that two residues of histidine and cysteine could be involved in its catalysis [2].

As direct carrying on of that work, the effect of temperature on the microaerophilic metabolism of *P. tannophilus* was investigated in this study. The data of batch fermenta-

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List of symbols

A Arrhenius pre-exponential factor of xylitol production, h^{-1}
B Arrhenius pre-exponential factor of thermal deactivation, h^{-1}
Δh^* activation enthalpy of xylitol production, kJ mol^{-1}
$\Delta h^{*\prime}$ thermodynamic quantity defined in Eq. (3), kJ mol^{-1}
Δh^*_D activation enthalpy of thermal deactivation, kJ mol^{-1}
P product (xylitol) concentration, $g_{xyt} l^{-1}$
Q_P xylitol volumetric productivity, $g_{xyt}\ l^{-1}\ h^{-1}$
Q_E ethanol volumetric productivity, $g_E\ l^{-1}\ h^{-1}$
r correlation coefficient
R ideal gas constant, kJ $mol^{-1}\ K^{-1}$
S substrate (xylose) concentration, $g_{xyl}\ l^{-1}$
Δs^* activation entropy of xylitol production, J $mol^{-1}\ K^{-1}$
Δs^*_D activation entropy of thermal deactivation, J $mol^{-1}\ K^{-1}$
T temperature, °C or K
X biomass concentration, $g_X\ l^{-1}$,
Y_E yield of ethanol on consumed xylose (selectivity), $g_E\ g_{xyl}^{-1}$
$Y_{P/X}$ yield of xylitol on biomass, $g_{xyt}\ g_X^{-1}$,
Y_P yield of xylitol on consumed xylose (selectivity), $g_{xyt}\ g_{xyl}^{-1}$

Greek symbols

θ_E yield of ethanol on starting xylose, $g_E\ g_{xyl}^{-1}$
θ_P yield of xylitol on starting xylose, $g_{xyt}\ g_{xyl}^{-1}$
ν_E ethanol specific productivity, $g_E\ g_X^{-1}\ h^{-1}$
ν_P xylitol specific productivity, $g_{xyt}\ g_X^{-1}\ h^{-1}$

Subscripts

E ethanol
max maximum value
o starting value
opt optimum value
P product (xylitol)
X biomass
xyl xylose
xyt xylitol

tions of hardwood hemicellulose acid-hydrolysate carried out at variable temperature were used to perform material balances of carbon consumed by this yeast through different metabolic ways under conditions of negligible cell growth: xylitol production, alcohol fermentation, and respiration. The related kinetic results were compared with those recently presented for *Debaryomyces hansenii* in synthetic solutions [5]. Finally, the Arrhenius model, which proved in a previous study the best tool for the thermodynamic study of fermentation systems [6], was used to estimate the activation enthalpies and entropies of both the reductive production of xylitol by this yeast and the related phenomenon of thermal deactivation.

2. Materials and methods

2.1. Microorganism

Fermentations were performed using the yeast strain *Pachysolen tannophilus* NRRL Y-2460, which was maintained in agar slants containing 0.1% yeast extract and 50% detoxified hemicellulose hydrolysate through periodic transfers and subcultures. The yeast was grown in the same medium for 72 hours at 30°C using an incubator shaker at 200 rpm (New Brunswick). The cells were then centrifuged and harvested for the inoculum.

2.2. Fermentations

Batch experiments at variable temperature were carried out at pH 5.5 in 250-mL Erlenmeyer flasks containing 100 ml of culture medium, placed in a gyratory shaker at 200 rpm.

The hemicellulose acid-hydrolysate used in this work, kindly supplied by Tennessee Valley Authority, was diluted with water 1:1 and detoxified by overliming [7]. In order to remove acetic acid and furfural [8], the hydrolysate was boiled for 160 min at 105°C and finally treated with activated charcoal for 1 h at room temperature in the ratio 1/10 $g\ g^{-1}$.

2.3. Analytical methods

Xylose, xylitol, acetic acid and ethanol were determined before and during each fermentation by HPLC using two Shodex SH columns (mobile phase, H_2SO_4 0.01 M; flow rate, 0.7 ml/min; IR and UV detectors). Biomass concentration was determined by dry weight at 85°C for 48 h filtering known volumes of samples on 0.45 μm membrane filters.

2.4. Carbon material balance

Carbon material balance was performed on the basis of the productions of xylitol, ethanol, and CO_2, on the consumption of xylose and biomass determinations. Biomass growth was minimized to negligible levels by using very high starting biomass levels ($19 < X_o < 29\ g_X\ l^{-1}$). The sum of every contributions to xylose consumption oscillated between 96.7 and 103.8%, indicating that the error intro-

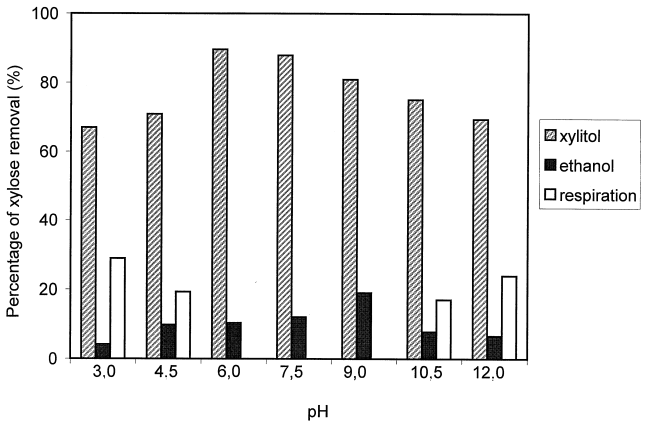

Fig. 1. Percentage of xylose consumed for xylitol production, alcohol fermentation, and respiration by *P. tannophilus* at different pH values in detoxified hemicellulose hydrolysate. $T = 30°C$.

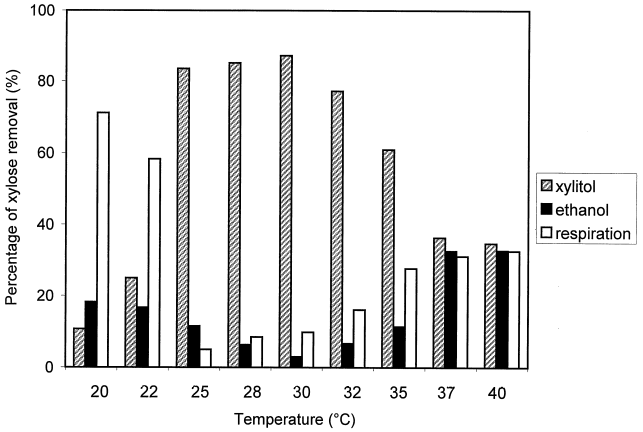

Fig. 2. Percentage of xylose consumed for xylitol production, alcohol fermentation, and respiration by *P. tannophilus* at different temperatures in detoxified hemicellulose hydrolysate. pH = 5.5.

duced by this calculation was always less than the experimental error.

3. Results and discussion

3.1. Carbon material balance

3.1.1. Previous tests at variable pH

The results of preliminary tests at variable pH, discussed in more detail in a previous paper [2], demonstrated that xylose was preferentially utilised for xylitol production under all tested conditions (Fig. 1). However, the carbon source consumption by this way reached a maximum (about 90%) close to pH 6.0–7.5. This datum, which slightly differs from the pH value (pH = 8.0) reported for optimal growth [9], suggested that the optimum pH for XR activity in *P. tannophilus* could be included in this range, outside of which the reaction rate decreased [2].

A progressive increase or decrease in pH affected xylitol production favouring respiration and alcohol fermentation, that notoriously compete with xylitol formation. In particular, the contribution of alcohol fermentation to the carbon source consumption grew with increasing the pH up to a maximum percentage only of 20% at pH 9.0, which means that this metabolic pathway always keeps a secondary alternative in this yeast when well detoxified hemicellulose hydrolysate is used as carbon source.

At extreme pH values (3.0 and 12.0) xylitol production was affected to such an extent that even the respiration became progressively more important, consuming up to 30% of the carbon source.

3.1.2. Batch tests at variable temperature

A set of batch tests was carried out in this work to estimate the influence of the temperature on the xylitol production from detoxified hemicellulose hydrolysate by *P. tannophilus* under conditions intentionally selected to favour xylitol production, that is at pH = 5.5 and using a very high starting biomass level ($19 < X_o < 29\ g_X\ l^{-1}$). As expected, no relevant cell growth was observed under these conditions, therefore xylose consumption was only ascribed to xylitol production, ethanol fermentation and respiration.

Fig. 2 shows the percentage of xylose consumed by *P. tannophilus* through each one of these ways varying the temperature from 20 to 40°C. Between 25 and 30°C, more than 83% of xylose was used to produce xylitol, which means that this process can effectively be carried out over a relatively wide range of temperature; besides, when the temperature was lowered to 20°C, xylitol was no more the main product, its formation being responsible for less than 20% of consumed xylose. A less marked decrease in xylitol production was observed at higher temperatures.

Opposite behaviours were observed, on the other hand, for alcohol fermentation and respiration, which showed minimum activities at 30°C and 25°C, respectively, and became progressively more significant with either increasing or decreasing the temperature outside this optimal range. In particular, the respiratory activity was maximum at 20°C, consuming most of xylose at this temperature. On the other hand, nearly the same percentage of carbon source was consumed by all three routes (32.6–34.7%) at 40°C, which means that respiration, alcohol fermentation, and xylose reduction took place at about the same rate at high temperature. In other word, the high temperature could be responsible for a sort of loss of the microorganism capacity to select the best metabolic pathway for its energetic requirements.

3.2. Kinetic study

Tables 1 and 2 list the main kinetic results obtained from the experimental data of xylitol and ethanol productions at different temperatures. These results on the whole confirm what is known from the literature on the microaerophilic

Table 1
Kinetic results of xylitol production obtained from batch fermentations of detoxified hemicellulose hydrolysate by *Pachysolen tannophilus*

T (°C)	Q_P ($g_{xyt}\ l^{-1}\ h^{-1}$)	$\nu_P \cdot 10^3$ ($g_{xyt}\ g_X^{-1}\ h^{-1}$)	θ_P ($g_{xyt}\ g_{xyl}^{-1}$)	Y_P ($g_{xyt}\ g_{xyl}^{-1}$)
20	0.072	2.89	0.050	0.095
22	0.108	4.68	0.165	0.222
25	0.206	8.23	0.631	0.740
28	0.253	11.5	0.595	0.755
30	0.304	16.0	0.583	0.773
32	0.249	10.8	0.535	0.684
35	0.198	6.8	0.491	0.540
37	0.127	5.3	0.277	0.322
40	0.061	2.1	0.146	0.307

Q_P = xylitol volumetric productivity
ν_P = xylitol specific productivity
θ_P = yield of xylitol on starting xylose
Y_P = yield of xylitol on consumed xylose

metabolism of this yeast. The yield of xylitol on consumed xylose (xylitol selectivity) reached a maximum value (Y_P = 0.773 $g_{xyt}\ g_{xyl}^{-1}$) at 30°C, whilst the highest yield of xylitol on starting xylose (θ_P = 0.631 $g_{xyt}\ g_{xyl}^{-1}$) was observed at 25°C. These results are also confirmed by the behaviours of both xylitol volumetric productivity and specific productivities, which achieved their maximum values (Q_P = 0.304 $g_{xyt}\ l^{-1}\ h^{-1}$; ν_P = 0.0160 $g_{xyt}\ g_X^{-1}\ h^{-1}$) at 30°C.

A completely different situation can be observed in Table 2 for ethanol fermentation, which exhibited the highest selectivity (Y_E = 0.196 $g_E\ g_{xyl}^{-1}$) at the highest temperature (40°C). In addition, although it consumed the carbon source nearly at the same rate as xylose reduction and respiration at 40°C, its maximum volumetric productivity was obtained at the lowest temperature (20°C), which suggests that this process could be kinetically favoured at low temperature and thermodynamically favoured at high temperature.

It should be noticed, however, that all the kinetic parameters of xylitol production by this yeast are about one order of magnitude less than those observed for *D. hansenii* [2], thus requiring further optimisation and metabolic engineering studies to guarantee interesting xylitol productions.

3.3. Thermodynamic study

The kinetic results listed in Tables 1 and 2 were used to estimate the thermodynamic parameters of xylitol production from detoxified hemicellulose hydrolysate by *P. tannophilus*.

A previous study clearly demonstrated that maximum values of either volumetric or specific productivities could be used to this purpose [10]. Standard procedures based on the Arrhenius model [11] or the so-called "kinetic" and "thermodynamic" approaches [12] were used by several authors to estimate the thermodynamic parameters of different enzymatic [11–14] or microbial systems [6,15–20], with concern not only to the transition state of the bioprocess under consideration but also to that of biocatalyst thermal deactivation. The Arrhenius model proved the best tool for the thermodynamic study of bioprocesses [6], therefore it was used also in this work for the estimation of the activation enthalpy and entropy of both xylitol production and thermal deactivation.

The Arrhenius equation, which describes the general

Table 2
Kinetic results of ethanol production obtained from batch fermentations of detoxified hemicellulose hydrolysate by *Pachysolen tannophilus*

T (°C)	Q_E ($g_{EtOH}\ l^{-1}\ h^{-1}$)	$\nu_E \cdot 10^3$ ($g_{EtOH}\ g_X^{-1}\ h^{-1}$)	θ_E ($g_{EtOH}\ g_{xyl}^{-1}$)	Y_E ($g_{EtOH}\ g_{xyl}^{-1}$)
20	0.072	2.89	0.050	0.095
22	0.044	1.91	0.067	0.090
25	0.021	0.83	0.064	0.075
28	0.016	0.74	0.038	0.049
30	0.012	0.66	0.024	0.032
32	0.018	0.77	0.038	0.049
35	0.026	0.88	0.064	0.070
37	0.068	2.86	0.149	0.174
40	0.039	1.34	0.093	0.196

Q_E = ethanol volumetric productivity
ν_E = ethanol specific productivity
θ_E = yield of ethanol on starting xylose
Y_E = yield of ethanol on consumed xylose

dependence of the reaction rate constant on the temperature, can be written for microbial processes in the form [6]:

$$Q_P = AXY_{P/X} \exp(-\Delta h^*/RT) \quad (1)$$

where Q_P is the maximum volumetric productivity, A the Arrhenius pre-exponential factor linked to the activation entropy, X the cell mass concentration, $Y_{P/X}$ the yield of product on biomass, Δh^* the activation enthalpy, R the ideal gas constant, and T the absolute temperature.

The values of Q_P usually describe curves including a first tract (consistent with the activated complex theory) where the rate constant increases with temperature up to an optimal value (T_{opt}) and a second one where the rate constant decreases.

This behaviour can then be described by two Arrhenius-type straight lines:

$$\ln Q_P = \ln(AXY_{P/X}) - \Delta h^*/RT \qquad \text{For } T < T_{opt} \quad (2)$$

$$\ln Q_P = \ln(BXY_{P/X}) - \Delta h^{*\prime}/RT \qquad \text{For } T > T_{opt} \quad (3)$$

where:

$$\Delta h^*_D = \Delta h^* + |\Delta h^{*\prime}| \quad (4)$$

and B are the activation enthalpy and a sort of pre-exponential factor for thermal deactivation, respectively.

As the values listed in Table 1 show, the maximum productivity regularly increased with temperature up to a temperature threshold (30°C), beyond which a sharp fall took place likely due to the progressive denaturation of the enzyme controlling xylitol formation (xylose reductase) or, at least, to a decrease in its activity.

Following the same approach used for alcohol fermentation [6], the product yield on biomass ($Y_{P/X}$) was inserted into the Arrhenius-type Equation (2) in order to estimate the values of the activation enthalpy (Δh^*) and entropy (Δs^*) of xylitol production. This made the estimation of the thermodynamic parameters easier, implying the use of the volumetric rather than the specific productivity. The value of $Y_{P/X}$ used in this work (4.67 $g_{xyt}\ g_X^{-1}$) is the average value calculated from literature data for this yeast in synthetic xylose solutions [9].

A similar approach (Eq. 3) was used to estimate the same thermodynamic parameters for thermal deactivation (Δh^*_D and Δs^*_D), which became significant at $T > 30$°C.

Starting from the experimental values of Q_P plotted in Fig. 3, enthalpies and entropies of xylitol production and thermal deactivation were estimated at a reference temperature of 25°C (Table 3).

The typical behaviour shown in Fig. 3 is qualitatively similar to those observed for most bioprocesses: fermentations [1,6], microbial growth [16], and enzymatic catalysis [11,21]. The activation enthalpy (105.4 kJ mol^{-1}) is higher than that estimated for growth (54–71 kJ mol^{-1}) and alcohol fermentation (32–51 kJ mol^{-1}), but nearly twice that obtained for xylitol production by *D. hansenii* in synthetic solution (57.7 kJ mol^{-1}) [5], because of the presence of a lot of inhibitors in the acid hemicellulose hydrolysate (furfural, hydroxymethylfurfural, metals, etc.). A wider variability exists, on the other hand, for enzyme activation, with values ranging from 18 to 83 kJ mol^{-1} [22], according to the enzyme, the substrate, etc. Immobilisation proved to remarkably reduce activation enthalpy of enzymatic systems [13,21], thus suggesting that xylitol production by whole cells could be favoured if compared with the use of isolated and purified xylose reductase.

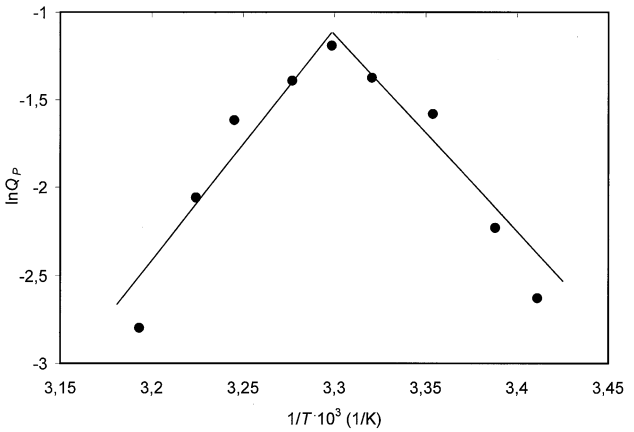

Fig. 3. Graphical estimation of the thermodynamic parameters of both xylitol production and thermal deactivation according to the Arrhenius model.

As far as the thermal deactivation is concerned, the productivity fall observed in fermentation processes beyond the optimum temperature could be justified by an increased denaturation rate of one controlling enzyme (XR in this specific case) or, at least, a decrease in its activity.

The enzyme denaturation is usually characterised by higher activation enthalpies (60–100 kJ mol^{-1}) [6], which means that the thermal deactivation of an enzyme requires to cross an energy barrier higher than that of enzymatic catalysis. Just for this reason, according to Arrenhius equation, the thermal denaturation rate grows more rapidly with temperature than product formation rate, therefore the overall productivity declines above its maximum value. In this specific case, the activation enthalpy of thermal deactivation was much higher (210.5 kJ mol^{-1}) than that observed for *D. hansenii* in synthetic solution (130.4 kJ mol^{-1}) [5]. Since this latter yeast showed an optimal temperature for xylitol

Table 3
Thermodynamic parameters estimated with the Arrhenius model for xylitol production from detoxified hemicellulose hydrolysate by *P. tannophilus*

Parameter	Xylitol production	Thermal deactivation
Activation enthalpy (kJ mol^{-1})	105.4	210.5
Activation entropy (J mol^{-1} K^{-1})	−13.2	3.63
r^2	0.953	0.960

production (35°C) slightly higher than *P. tannophilus* (30°C) and productivity values one order of magnitude higher (1.21–4.67 $g_{xyt}\ l^{-1}\ h^{-1}$), it is reasonable to suppose that, although more sensitive to thermal denaturation, *D. hansenii* XR exhibits a much higher activity than *P. tannophilus* XR. Nevertheless, both activation enthalpies are obviously lower than those reported for microbial death associated to heat supply (290–380 kJ mol^{-1}) [16].

The activation enthalpy estimated in this study for thermal deactivation is comparable with the highest values found in the literature for a variety of enzymatic systems [11–14,22] and for xylitol production by *D. hansenii* [5]. This not only confirms the consistency of considering thermal deactivation as a phenomenon related to the effectiveness decrease of enzymatic catalysis, but also suggests the occurrence of a sort of enzyme protection against denaturation due to its natural confinement and immobilisation within the cell.

The activation entropy of xylitol production (−13.2 J $mol^{-1}\ K^{-1}$) has the same negative sign as those estimated by Sizer for some specific reactions catalysed by enzymes [22]. As previously outlined also for *D. hansenii* [5], these values are consistent with the formation of an enzyme-substrate complex implying a reduction of the system randomness during the complex formation.

On the other hand, the positive value of the activation entropy of thermal deactivation (3.63 J $mol^{-1}\ K^{-1}$), which can be associated to an increase in the randomness degree of the activated complex, seems to provide a further confirmation of the enzymatic nature of thermal deactivation of whole cell systems. Finally, the value of Δs^*_D close to zero suggests that thermal deactivation did not imply any relevant variation in the enzyme tertiary structure. In other words, most of the hydrogen bonds responsible for the active structure of XR catalytic site were still present in the activated complex.

4. Conclusions

In order to get further knowledge on the microaerophilic metabolism of *Pachysolen tannophilus*, the production of xylitol from detoxified hemicellulose hydrolysate by this yeast was studied at different temperatures.

A preliminary study on the material balance of consumed carbon source, carried out at variable pH, showed that about 90% of xylose was utilised for xylitol production close to the neutrality, the rest having been nearly completely fermented to ethanol. The same approach extended to the effect of temperature on the metabolism of this yeast revealed that more than 83% of xylose was utilised to produce xylitol at temperatures ranging from 25 to 30°C, whereas both respiration and alcohol fermentation became significant outside this temperature range.

Finally, the main thermodynamic parameters of this process were estimated with the Arrhenius approach: activation enthalpy and entropy were 105.4 kJ mol^{-1} and −13.2 J $mol^{-1}\ K^{-1}$ for xylitol production and 210.5 kJ mol^{-1} and 3.63 J $mol^{-1}\ K^{-1}$ for thermal deactivation, respectively.

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