Biotechnology Letters **21:** 719–723, 1999.

Microaerophilic metabolism of *Pachysolen tannophilus* at different pH values

Attilio Converti[1,*], Patrizia Perego[1] & José Manuel Domínguez[2]
[1]*Department of Chemical and Process Engineering 'G.B. Bonino', University of Genoa, Via Opera Pia 15, 16145 Genoa, Italy*
[2]*Departamento de Ingenieria Química, Universidad de Vigo, Campus de Ourense, As Lagoas s/n, 32004 Ourense, Spain*
[*]*Author for correspondence (Fax: +39 0103532586; E-mail: converti@unige.it)*

Received 28 May 1999; Revisions requested 18 June 1999; Revisions received 2 July 1999; Accepted 2 July 1999

Key words: microaerophilic conditions, *Pachysolen tannophilus*, pH variation, xylitol production

Abstract

Microaerophilic production of xylitol by *Pachysolen tannophilus* from detoxified hemicellulose hydrolyzate was optimal between pH values 6.0 to 7.5 when about 90% of xylose was utilized for xylitol production, the rest being fermented to ethanol. At pH values of 3.0 and 12.0, respiration became important, consuming up to 30% of available xylose. A graphic procedure suggests that histamine and cysteine are at the active site of xylose reductase in this yeast.

Introduction

Xylose reductase, the enzyme responsible for xylose reduction to xylitol, can use either NADH or NADPH as reducing cofactors in *Pachysolen tannophilus*, with the latter always being preferred. Hahn-Hägerdal *et al.* (1994) showed that xylose reductase activity becomes progressively more NADPH-dependent with increasing dissolved O_2 concentrations in the medium. This means that phosphate could become a growth-limiting factor under strictly aerobic conditions.

P. tannophilus proved a particularly versatile microorganism, whose metabolism can be oriented towards xylitol or ethanol production, according to both operating conditions and hydrolyzate pretreatment techniques (Converti & Del Borghi 1996). In particular, three different metabolic routes are possible according to the O_2 availability.

Under aerobic conditions, cell growth is favoured, whereas xylitol production and ethanol fermentation, which require a reducing environment, are both decreased.

Under strictly anaerobic conditions, which can be ensured by replacing O_2 with N_2 supply, xylitol formation should be stimulated. In fact, NAD^+ (or $NADP^+$) formed by xylose reductase is reduced to NADH (or NADPH) and is then not available for the successive reaction catalyzed by xylitol dehydrogenase, which is necessary for growth sustained by the pentose phosphate pathway. So, it is possible to accelerate the formation of xylitol using high starting biomass levels. Although this effect has been shown in *P. tannophilus*, the fermentation enhancement is not sufficient as to justify the use of strictly anaerobic conditions.

Lastly, microaerophilic conditions proved the best solution for xylitol production (Converti & Del Borghi 1996), mainly at low cell concentrations, because the scarce oxygen available is completely consumed for the growth, thus increasing productivity.

The combined effects of pH on *P. tannophilus* metabolism as well as on xylose reductase activity are investigated. For this purpose, the data of batch fermentations carried out at different pH on adequately pretreated hemicellulose hydrolyzates are used to carry out a material balance of carbon consumed by this yeast under microaerophilic conditions. Finally, the values of maximum xylitol specific pro-

ductivity are worked out with the graphic method described by Villadsen *et al.* (1990) to get information on the mechanism of xylose reductase catalysis.

Materials and methods

Substrate preparation

The hemicellulose acid-hydrolyzate used in this work, prepared by Tennessee Valley Authority by a final counter-current washing scheme, contained about 106 g xylose l^{-1}, 13.2 g glucose l^{-1}, 26.6 g acetic acid l^{-1}, 8.6 g galactose l^{-1}, 6.8 g mannose l^{-1}, 4.2 g furfural l^{-1}, and other minor components. After adequate dilution with water 1:1, it was detoxified by overliming (Perego *et al.* 1990). In order to remove acetic acid and furfural as much as possible (Parajó *et al.* 1997), the hydrolyzate was then 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}.

Microorganism

P. tannophilus (NRRL Y-2460) was maintained on agar slants containing 0.1% yeast extract and 50% detoxified hemicellulose hydrolyzate through periodic transfers and subcultures. The cells used for inocula were grown for 72 h on the same medium and then centrifuged.

Conditions

Batch fermentations were performed in an orbital shaker at 200 rpm and 30 $\pm$ 0.1 °C under microaerophilic conditions, using 100-ml Erlenmeyer flasks with 50 ml of fermentation broth.

Analytical procedures

Sugar components of the hydrolyzate, as well as 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^{-1}; IR and UV detectors). Biomass concentration was determined by dry weight filtering known volumes of samples on 0.45 μm membrane filters.

Carbon material balance

Carbon material balance has been performed on the basis of the productions of xylitol, ethanol, and CO_2, on the consumption of xylose, and biomass determinations.

Results and discussion

Carbon material balance

As shown in Figure 1, three different possible metabolic pathways are considered to be responsible for xylose consumption: reductive production of xylitol, ethanol fermentation and respiration. The cell growth has not been considered because it was always negligible under the conditions selected for this study (high starting biomass level and microaerophilic conditions).

These results indicate pH = 6.0–7.5 as the optimum pH range for xylitol production by *Pachysolen tannophilus*, which is slightly different from the value (pH = 8.0) reported for the optimal growth of this yeast (Parajó *et al.* 1998). This suggests that the optimum pH for xylose reductase activity in *P. tannophilus* is likely included in this range, outside of which the reaction rate decreases and alternative metabolic routes (respiration and alcohol fermentation) become relatively more important.

Another outstanding fact is the growing significance of alcohol fermentation with increasing pH up to 9.0. This result is quite surprising if one considers that Beck & Strickland (1984) observed an optimum pH of 5.5–6.0 for this metabolic pathway in *P. tannophilus* using hemicellulose hydrolyzate. It is likely that, with substrates adequately pretreated for xylitol production under microaerophilic conditions, alcohol fermentation always keeps a secondary alternative, as demonstrated by the relatively low percentages of carbon source (<20%) consumed by this yeast through this way.

As earlier said, a progressive increase in pH, however, affects xylitol production, thus allowing alternative metabolic routes to be followed. So, a pH increase over 6.0–7.5 tends to decelerate xylitol production and favours simultaneously the alcohol fermentation, which should anyhow occur at a rate decreasing with increasing pH. This means that, although both processes decelerate with increasing pH, ethanol production, which competes with xylitol formation, gains relative significance.

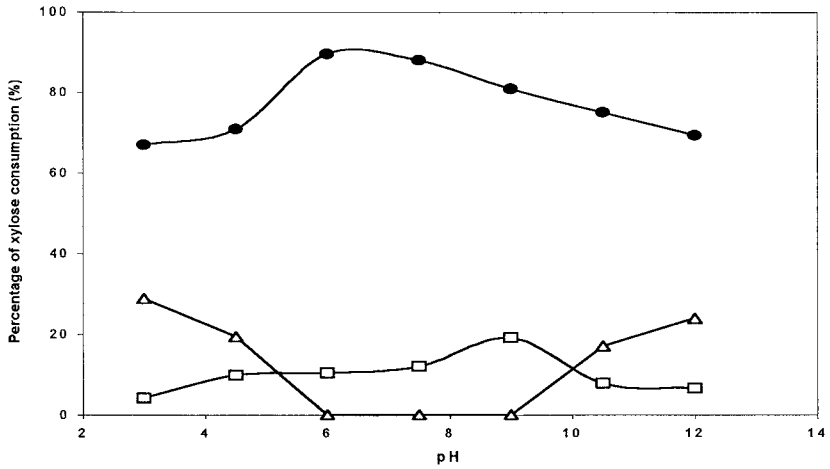

Fig. 1. Material balance of carbon in microaerophilic metabolism of *P. tannophilus*. (●) xylitol production; (△) alcohol fermentation; (□) respiration.

It should finally be worth noticing that the biomass growth is practically negligible, because the process is carried out under conditions able to stimulate xylitol production and to prevent, at the same time, the cell growth (microaerophilic conditions in the presence of high cell concentration).

Finally, at extreme pH values (3.0 and 12.0) both these processes are affected to such an extent that a further metabolic pathway, the respiration, becomes progressively more important, consuming up to 30% of the carbon source. In fact, although strongly limited by oxygen availability, the aerobic respiration is always possible even under microaerophilic conditions.

Effect of pH on xylose reductase activity

As is well known, the pH of the microenvironment close to the active site of an enzyme can influence the ionization state of the functional groups of amino acids involved in the catalysis and so also the reaction rate strongly depends on pH.

Villadsen *et al.* (1990) described a simple graphic technique that allows making reasonable hypotheses on the mechanism of the enzymatic catalysis, using the experimental data of the specific reaction rate at different pH values. This model, based on the Michaelis-Menten equation, hypothesizes the formation of an enzyme-substrate (ES) complex (Figure 2), which can derive only from the active form of the enzyme (HX-E-Y^-). This is in equilibrium with two alternative (inactive) forms, which result from an acidic ionization (at pH lower than optimum) and a basic ionization (at pH higher than optimum), respectively. A pH variation in the reaction environment and/or the active site can lead to a modification of the ionization state of amino acidic R groups and then to the deactivation of a fraction of the enzyme, with consequent decrease of the global reaction rate.

Table 1 lists the experimental data of batch fermentations of hemicellulose hydrolyzate carried out at variable pH with *P. tannophilus*. These data show that, at comparable starting xylose level (69–70 g l^{-1}), the final xylitol concentration gradually increases from 22 to 44 g l^{-1} with increasing pH from 3.0 to 7.5, whereas an evident drop of xylitol production takes place over this threshold. From the kinetic results of these tests listed in Table 2, it can be supposed that the pH dependence of maximum xylitol specific productivity (ν_m), defined as the ratio of volumetric productivity, Q_p, to biomass concentration, is the result of the variation of xylitol formation rate through the reaction catalyzed by xylose reductase.

Considering that HX-E-$Y^-$is the active form of this enzyme, the following expression can be derived to relate the maximum specific productivity (ν_m) to the concentration of H^+ ions:

$$\nu_m = \bar{\nu}_m/(1 + [H^+]/K_a + K_b/[H^+]), \qquad (1)$$

where $\bar{\nu}_m$ is the maximum specific productivity under optimal pH conditions for xylitol production, while K_a and K_b are the acidic and basic ionization constants of the two main ionizable groups which are more likely involved in the xylose reductase catalysis.

As Figure 3 shows, the typical bell-shaped behavior of $\log_{10}(\nu_m)$ versus pH can approximately be described by a set of three straight lines. The intercep-

Table 1. Experimental data of batch fermentations of hemicellulose hydrolyzate carried out at variable pH with *P. tannophilus*.

pH	S_o (g l^{-1})[a]	S_f (g l^{-1})[b]	P_f (g l^{-1})[c]	X_o (g l^{-1})[d]
3.0	76.1	39.4	22.0	16
4.5	72.8	24.2	28.8	17
6.0	70.7	18.9	41.2	19
7.5	79.7	23.5	43.8	20
9.0	76.6	25.7	36.5	20
10.5	69.3	14.8	36.2	21
12.0	71.5	20.9	27.4	19

[a]Starting xylose concentration.
[b]Final xylose concentration.
[c]Final xylitol concentration.
[d]Starting biomass concentration.

Table 2. Kinetic results of batch fermentations of hemicellulose hydrolyzate carried out at variable pH with *P. tannophilus*.

pH	Q_p (g lh^{-1})[a]	$Y_{p/s}$ (g g^{-1})[b]	ν_m ($g_p/g_x h$)[c]	log ν_m
3.0	0.16	0.60	0.010	−2.00
4.5	0.21	0.59	0.013	−1.90
6.0	0.30	0.79	0.016	−1.79
7.5	0.32	0.78	0.016	−1.79
9.0	0.27	0.72	0.014	−1.87
10.5	0.27	0.67	0.013	−1.89
12.0	0.21	0.54	0.011	−1.96

[a]Volumetric xylitol productivity.
[b]Yield of xylitol on consumed xylose.
[c]Maximum specific productivity.

tion points of the two oblique straight lines (referring to pH values outside the optimum range) with the straight line parallel to the abscissae axis (concerning on the contrary to the optimum pH range) give the values of pK_a and pK_b. In fact, for pH values lower than the optimum, the contribution of the

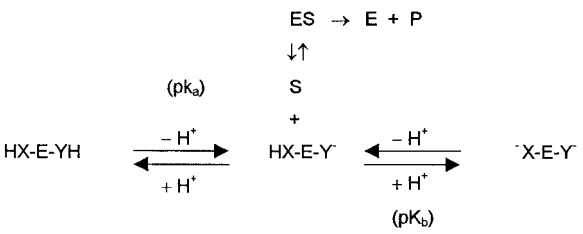

Fig. 2. Mechanism proposed by Villadsen *et al.* (1990) to describe the pH influence on enzymatic activity. HX-E-YH = inactive acidic form of the enzyme; HX-E-Y$^-$ or E = active form of the enzyme; $^-$X-E-Y$^-$ = inactive basic form of the enzyme; S = substrate; ES = enzyme-substrate complex; P = product.

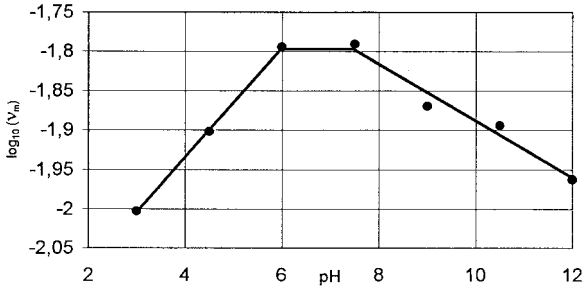

Fig. 3. Estimation of pK_a and pK_b of ionizable functional groups likely responsible for xylose reductase catalysis in *P. tannophilus*.

term $[H^+]/K_a$ at the denominator of Equation (1) becomes predominant. Then, neglecting the other terms, it simplifies:

$$\nu_m = \bar{\nu}_m/([H^+]/K_a) \tag{2}$$

or:

$$\log_{10} \bar{\nu}_m = \log_{10} K_a + \log_{10} \nu_m - \log_{10}[H^+].$$

At every point lying on the horizontal line of maximum productivity, ν_m equals $\bar{\nu}_m$ and then pK_a = pH. Analogously pK_b can be calculated.

The values of pK_a and pK_b that can be estimated from Figure 3 are 6.0 and 7.5, respectively. The former (6.0) could correspond to the imidazole ionizable *R* group of a histidine residue, while, among the values reported in the literature for pK_b of amino acids, the one of the lateral group of cysteine (8.33) is the closest to the latter (7.5). The remarkable difference between these values (about 0.8 pH units) could be due to the influence of a particular microenvironment in the active site on the dissociation constant of this group, in addition to the uncertainty due to the limited number of tests.

Since, however, there are no other ionizable amino acidic *R* groups with pK values within the range 6.0–8.33, it is reasonable to suppose that two residues of histidine and cysteine could be actually involved in the enzymatic catalysis of xylose reductase in *P. tannophilus*.

References

Beck MJ, Strickland RC (1984) Production of ethanol by bioconversion of wood sugars derived from two-stage dilute acid hydrolysis of hardwood. *Biomass* **6**: 101–110.

Converti A, Del Borghi M (1996) Selection of hemicellulosic hydrolysate pretreatments and fermentation conditions to stimulate xylitol production by ethanol-producing yeasts. *Acta Biotechnol.* **16**: 133–144.

Hahn-Hägerdal B, Jeppsson H, Skoog K, Prior BA (1994) Biochemistry and physiology of xylose fermentation by yeasts. *Enz. Microbiol. Technol.* **16**: 933–943.

Parajó JC, Domínguez H, Domínguez JM (1997) Improved xylitol production with *Debaryomyces hansenii* Y-7426 from raw or detoxified wood hydrolyzates. *Enz. Microbiol. Technol.* **21**: 18–24.

Parajó JC, Domínguez H, Domínguez JM (1998) Biotechnological production of xylitol. Part 2: Operation in culture media made with commercial sugars. *Bioresource Technol.* **65**: 203–212.

Perego P, Converti A, Palazzi E, Del Borghi M, Ferraiolo G (1990) Fermentation of hardwood hemicellulose hydrolysate by *Pachysolen tannophilus*, *Candida shehatae* and *Pichia stipitis*. *J. Ind. Microbiol.* **6**: 157–164.

Villadsen J, Chand S, Bisaria VS, Ramachandran KB (1990) Enzyme reactions and technology. In: Ghose TK, ed., *Bioprocess Computations in Biotechnology*, Vol. 1, New York: Ellis Horwood, pp. 117–120.