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NADH-linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose by yeasts

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Summary. The kinetics and enzymology of D-xylose utilization were studied in aerobic and anaerobic batch cultures of the facultatively fermentative yeasts *Candida utilis, Pachysolen tannophilus*, and *Pichia stipitis*. These yeasts did not produce ethanol under aerobic conditions. When shifted to anaerobiosis cultures of *C. utilis* did not show fermentation of xylose; in *Pa. tannophilus* a very low rate of ethanol formation was apparent, whereas with *Pi. stipitis* rapid fermentation of xylose occurred. The different behaviour of these yeasts ist most probably explained by differences in the nature of the initial steps of xylose metabolism: in *C. utilis* xylose is metabolized via an NADPH-dependent xylose reductase and an NAD^+-linked xylitol dehydrogenase. As a consequence, conversion of xylose to ethanol by *C. utilis* leads to an overproduction of NADH which blocks metabolic activity in the absence of oxygen. In *Pa. tannophilus* and *Pi. stipitis,* however, apart from an NADPH-linked xylose reductase also an NADH-linked xylose reductase was present. Apparently xylose metabolism via the NADH-dependent reductase circumvents the imbalance of the NAD^+/NADH redox system, thus allowing fermentation of xylose to ethanol under anaerobic conditions. The finding that the rate of xylose fermentation in *Pa. tannophilus* and *Pi. stipitis* corresponds with the activity of the NADH-linked xylose reductase activity is in line with this hypothesis. Furthermore, a comparative study with various xylose-assimilating yeasts showed that significant alcoholic fermentation of xylose only occurred in those organisms which possessed NADH-linked aldose reductase.

Introduction

Alcoholic fermentation of xylose-containing agricultural waste materials is presently considered to be of potential economic value. For such a process yeasts may offer a number of advantages over bacteria. For example, the latter organisms nearly always produce undesirable byproducts and frequently have a low ethanol tolerance. Although many facultatively fermentative yeasts can grow with xylose as a substrate (Barnett et al. 1979), only a few yeasts have been reported to ferment xylose to ethanol (Jeffries 1981; Schneider et al. 1981; Dekker 1982; Margaritis and Bajpaj 1982; Suihko and Dražić 1983; du Preez and van der Walt 1983). Indeed, an extensive screening of the CBS yeast collection (Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands), performed in our laboratory, confirmed that anaerobic fermentation of xylose is rare amongst yeasts (Toivola et al. 1983).

Yeasts which have been studied so far were shown to require oxygen for fermentation of xylose to ethanol. Under strictly anaerobic conditions no or very low amounts of ethanol were formed. Only with a limited supply of oxygen high ethanol yields could be obtained (Dellweg et al. 1982; Jeffries 1983). The explanation for this oxygen effect most probably resides in the coenzyme specificity of the enzymes specific for the xylose metabolism. Bruinenberg et al. (1983c) demonstrated that metabolism of D-xylose via NADPH-linked xylose reductase and NAD^+-dependent xylitol dehydrogenase under anaerobic conditions results in an overproduction of NADH, which blocks metabolic activity in the facultatively fermentative yeast *Candida utilis*. Although the behaviour of *C. utilis* is typical for the majority of xylose-assimilating facultatively fermentative yeasts, a slow anaerobic fermentation of xylose can be observed with some organisms, amongst others the yeast *Pachysolen tannophilus* (Dellweg et al. 1982; Debus et al. 1983).

In this paper an explanation will be presented for the different behaviour of *C. utilis* and *Pa. tannophilus* with respect to the anaerobic fermentation of

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xylose. The kinetics and enzymology of xylose fermentation of these organisms will be compared with that of *Pichia stipitis*, a yeast which exhibits a rapid fermentation of xylose under anaerobic conditions[1] (Toivola et al. 1984).

Materials and methods

Micro-organisms and growth. *Candida shehatae* CBS 5813; *Candida tenuis* CBS 615, 2226, 2885, 4113, 4285, 4434, 4435, 4604; *Candida utilis* CBS 621; *Pachysolen tannophilus* CBS 4044; *Pichia segobiensis* CBS 6857 and *Pichia stipitis* CBS 5773 were obtained from Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands. *C. utilis*, *Pa. tannophilus* and *Pi. stipitis* were grown in a laboratory fermentor as described earlier (Bruinenberg et al. 1983c). For enzyme assays *C. shehatae*, *C. tenuis* and *Pi. segobiensis* were grown in shake cultures at 30° C on a mineral medium (Bruinenberg et al. 1983b) containing 1% D-xylose. The pH of this medium was adjusted to 6.0 prior to sterilization.

Test for fermentation of D-xylose. The standard procedure of the Centraalbureau voor Schimmelcultures was adopted. Tubes containing 5 ml medium, consisting of 1% yeast extract + 2% xylose (Merck) and a Durham vial were inoculated with cell material from agar slants (1% yeast extract; 2% glucose; 2% agar). Cultures were incubated statically at 25° C. Gas formation was followed during a period of 10 days. In addition, after this period ethanol was determined by gas chromatography.

Preparation of cell-free extracts and enzyme assays. The preparation of cell-free extracts and the assays of xylose reductase and xylitol dehydrogenase were performed as described by Bruinenberg et al. (1983b).

Analytical methods. Growth was monitored by following optical density of the culture at 660 nm. At certain points in the growth curve dry-weight determinations of cell suspensions were performed by applying 10-ml samples to vacuum-dried filters (Sartorius, pore size 0.45 µm), washed twice with two volumes of distilled water and dried to constant weight at 70° C. Ethanol was determined by gas chromatography as described by Bruinenberg et al. (1983c). Xylose was determined according to Herbert et al. (1971). Protein concentrations in cell-free extracts were determined by the Lowry method with bovine serum albumin as a standard.

Results

When aerobic batch cultures of *Candida utilis*, growing on a medium containing xylose and yeast extract, were shifted to anaerobic conditions, xylose consumption stopped and no ethanol was formed (Fig. 1). Even after prolonged incubation under anaerobic conditions up to 1 week no fermentation of xylose to ethanol was detected (results not shown).

Aerobic batch cultures of *Pachysolen tannophilus* also did not produce ethanol from xylose. However, with *Pa. tannophilus*, in contrast to *C. utilis*, a shift to anaerobiosis resulted in the formation of ethanol. Xylose consumption and ethanol formation under these conditions were very slow (Fig. 2).

Alcoholic fermentation of xylose was also absent in aerobic batch cultures of *Pichia stipitis*. This yeast exhibited a higher growth rate than *C. utilis* and *Pa. tannophilus* (doubling times of 5.2, 4.3, and 1.6 h, respectively; Figs. 1, 2, 3).

Upon the shift to anaerobic conditions *Pi. stipitis* fermented xylose to ethanol. The fermentation rate of this organism was much higher than that of *Pa. tannophilus* (see Table 1). Neither *Pa. tannophilus* nor *Pi. stipitis*, however, showed any growth with xylose under anaerobic conditions. The slight decrease in optical density in the anaerobic period (Figs. 2, 3) did not correspond to a decrease in cell dry-weight.

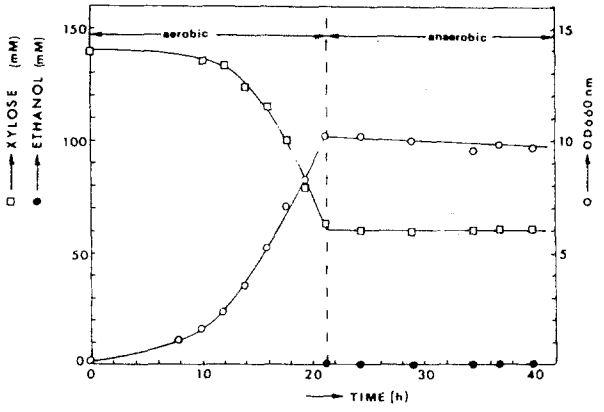

Fig. 1. Batch culture of *Candida utilis* CBS 621 on D-xylose. A shift from aerobic to anaerobic conditions leads to cessation of metabolic activity

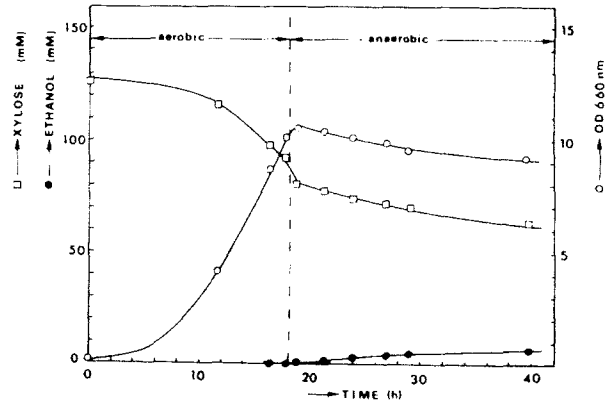

Fig. 2. Slow alcoholic fermentation of D-xylose under anaerobic conditions in a batch culture of *Pachysolen tannophilus* CBS 4044 pregrown aerobically on xylose

1 Patent application submitted by Alfa-Laval, Tumba, Sweden

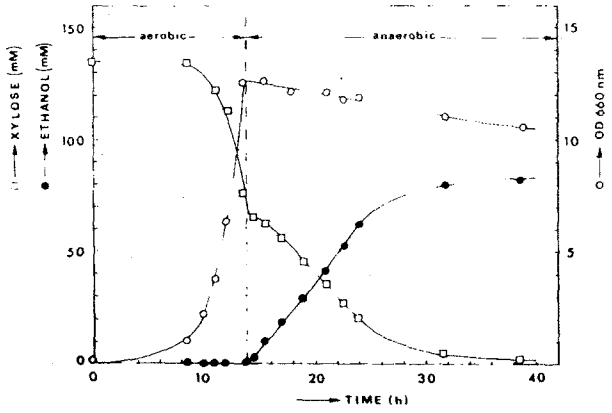

Fig. 3. Shift from aerobic to anaerobic conditions in a batch culture of *Pichia stipitis* CBS 5773 with D-xylose as the substrate, resulting in the rapid formation of ethanol

Table 1. Activities of xylose reductase and xylitol dehydrogenase and rates of ethanol production from D-xylose under anaerobic conditions (Ø EtOH), expressed as mg ethanol · g cells^{-1} · h^{-1}. Enzyme activities were determined in cells harvested just prior to the shift from aerobic to anaerobic conditions (Figs. 1, 2, 3) and are expressed as mmol · min^{-1} · (mg protein)$^{-1}$

	C. utilis	*Pa. tannophilus*	*Pi. stipitis*
Ø EtOH	0	8	54
Xylose reductase			
NADH	0	9	310
NADPH	75	220	600
Ratio	0	0.04	0.5
Xylitol dehydrogenase			
NAD^+	280	910	720
$NADP^+$	0	70	75
Ratio	∞	13	10

Since the observed anaerobic fermentation of xylose by *Pa. tannophilus* and *Pi. stipitis* is at variance with the enzymology of xylose metabolism as occurring in *C. utilis* (Bruinenberg et al. 1983c), it was investigated whether the xylose-metabolizing enzymes in these two organisms are different from those in *C. utilis*. Activities of enzymes specific for xylose metabolism in xylose-grown *C. utilis*, *Pa. tannophilus* and *Pi. stipitis* are listed in Table 1. In *C. utilis* the xylose reductase activity was specific for NADPH, whereas the xylitol dehydrogenase was strictly NAD^+-dependent. In *Pa. tannophilus* and *Pi. stipitis*, however, the two enzymes exhibited a dual coenzyme specificity. The ratio of the NADH-linked and the NADPH-linked activities of xylose reductase in *Pi. stipitis* was much higher than in *Pa. tannophilus*. On the other hand, the ratio between NAD^+-linked and $NADP^+$-linked xylitol dehydrogenase activities was similar in both organisms (Table 1).

In order to test whether the apparent correlation between the activity of NADH-linked xylose reductase and the rate of anaerobic xylose fermentation is a general phenomenon, various xylose-assimilating yeasts were assayed for NADH-dependent xylose reductase during growth on xylose. For this purpose *Pichia segobiensis* and *Candida shehatae* were selected, as these yeasts had given positive results in the standard fermentation test. Also various strains of *Candida tenuis* were included, since within this species marked differences were observed with respect to xylose fermentation (Toivola et al. 1984). The results listed in Table 2 confirm that a correlation between the fermentative ability of a yeast with xylose and the activity of NADH-linked xylose reductase exists: those yeasts which were capable of alcoholic fermentation of xylose also possessed

Table 2. NADPH-linked and NADH-linked xylose reductase activities in batch cultures of various D-xylose-assimilating yeasts. Cells were harvested at the mid-exponential growth phase. Enzyme activities are expressed as nmol · min^{-1} · (mg protein)$^{-1}$

Organism	CBS no.	Specific activity			D-xylose fermentation[a]
		NADH	NADPH	Ratio	
Candida tenuis	615	2	130	0.02	−
	2226	7	320	0.02	−
	2885	0[b]	100	0	−
	4113	60	120	0.5	+
	4285	305	670	0.5	+
	4434	0[b]	485	0	−
	4435	340	670	0.5	+
	4604	0[b]	365	0	−
Candida shehatae	5813	210	480	0.4	+
Pichia segobiensis	6857	365	640	0.6	+

[a] Results obtained in the standard fermentation test: −: no gas production, ethanol less than 0.3 g · l^{-1}; +: gas production, ethanol higher than 5.0 g · l^{-1}

[b] 0: Not detectable

NADH-linked xylose reductase during growth on xylose.

Discussion

In a previous paper (Bruinenberg et al. 1983c) we demonstrated that anaerobic fermentation of D-xylose in yeasts is incompatible with metabolism of this pentose via NADPH-linked xylose reductase and NAD^+-linked xylitol dehydrogenase, as illustrated in *Candida utilis*. This is due to the fact that conversion of xylose to intermediates of the glycolytic pathway via these enzymes results in an overproduction of reducing equivalents in the form of NADH (Fig. 4A), which cannot be reoxidized in the absence of oxygen. Since *C. utilis*, and probably other yeasts as well, lack transhydrogenase activity (Bruinenberg et al. 1983a, b), the reducing equivalents produced in the second step of xylose metabolism (i.e., NADH) cannot be used for the initial reaction. The majority of xylose-assimilating, facultatively fermentative yeasts, like *C. utilis*, is unable to ferment xylose anaerobically (Toivola et al. 1984) and hence it seems likely that this mode of xylose metabolism is wide-spread amongst yeasts.

The results presented in Table 1 reveal a marked difference between coenzyme specificities of xylose reductase and xylitol dehydrogenase in *C. utilis*, *Pa. tannophilus* and *Pi. stipitis*, respectively. Whereas in *C. utilis* the xylose reductase and xylitol dehydrogenase activities were specifically dependent on one coenzyme, in the other two yeasts the reactions proceeded with both pyridine nucleotides. Therefore, contrary to the situation in *C. utilis*, in *Pa. tannophilus* and *Pi. stipitis* reducing equivalents produced in the second reaction can be used for the initial reaction of xylose metabolism (Fig. 4B). It is evident that such a mechanism eliminates the overproduction of NADH under anaerobic conditions which would occur when different coenzymes are involved in the reduction of xylose and the oxidation of xylitol (Fig. 4A).

Theoretically, in *Pa. tannophilus* and *Pi. stipitis* both NAD(H) or NADP(H) may be used for the conversion of xylose to xylulose. However, it is noteworthy that the activity of NADH-linked xylose reductase in *Pi. stipitis* was higher than in *Pa. tannophilus*. Since also the rate of anaerobic xylose fermentation in *Pi. stipitis* was much higher than in *Pa. tannophilus* (Figs. 2, 3) it seems likely that in *Pi. stipitis* predominantly NADH rather than NADPH serves as the reductant for the conversion of xylose to xylitol. The correlation between NADH-linked xylose reductase activity and the xylose-fermenting capacity of yeasts is further suggested by the results

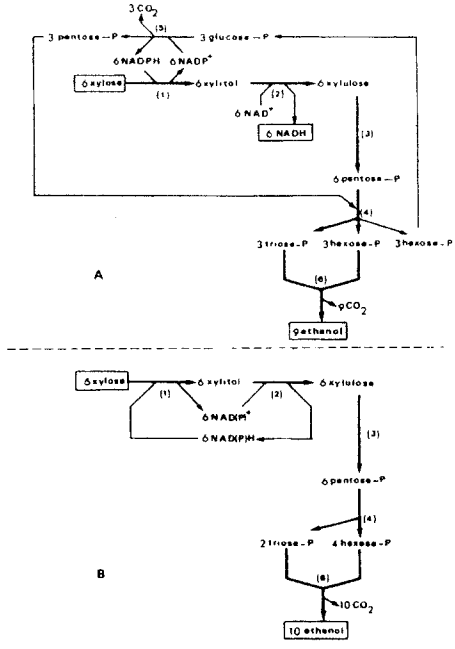

Fig. 4A, B. Metabolic scheme for the conversion of xylose to ethanol. **A** Via NADPH-dependent xylose reductase and NADH-dependent xylitol dehydrogenase 1.5 mol of ethanol are formed per mol of xylose with concomitant production of 1 NADH. **B** Alcoholic fermentation of xylose with a closed redox balance leads to the formation of 10 mol of ethanol from 6 mol of xylose. Enzymes are indicated by numbers: (*1*) xylose reductase; (*2*) xylitol dehydrogenase; (*3*) xylulose kinase + ribulose phosphate epimerase + ribose phosphate isomerase; (*4*) transaldolase + transketolase; (*5*) glucose 6-phosphate dehydrogenase + 6-phosphogluconate dehydrogenase; (*6*) enzymes of the glycolytic pathway + pyruvate decarboxylase and alcohol dehydrogenase

listed in Table 2. Those yeasts which showed a positive result in the standard fermentation test had a high ratio of NADH-linked over NADPH-linked xylose reductase activity. No correlation was observed between the ability to ferment xylose and the activity of $NADP^+$-linked xylitol dehydrogenase. Similar to *Pa. tannophilus* and *Pi. stipitis*, also *C. shehatae*, *C. tenuis* and *Pi. segobiensis* had high ratios of NAD^+-dependent over $NADP^+$-dependent xylitol dehydrogenase activities (results not shown).

At present it remains to be established whether the observed NADH-dependent and NADPH-dependent xylose reductase activities in xylose-grown *Pa. tannophilus* and *Pi. stipitis* result from the presence of more than one enzyme. It should be noted here that xylose reductases as observed in these yeasts are enzymes exhibiting broad substrate specificities and should be named aldose reductases (Scher and Horecker 1966).

The results of our fermentor studies make clear that for alcoholic fermentation of xylose the yeast *Pi. stipitis* is to be preferred above *Pa. tannophilus*. *Pi. stipitis* not only exhibited a much higher rate of

anaerobic xylose fermentation but also had a higher overall conversion efficiency. Assuming a theoretical maximum of 1.66 mol ethanol per mol xylose (Fig. 4B), the conversion efficiency for *Pi. stipitis* was 75% (Fig. 3) whereas the conversion of xylose to ethanol by *Pa. tannophilus* proceeded with only 25% efficiency. These different efficiencies and the deviation from the theoretical maximum must probably be ascribed to the formation of byproducts. It is well established, for example, that *Pa. tannophilus* produces large amounts of xylitol from xylose under anaerobic conditions (Dellweg et al. 1982; Debus et al. 1983). This phenomenon has been explained by Debus et al. (1983) in terms of an electron sink for NADPH, generated in the hexose monophosphate pathway. However, for this purpose xylose first of all has to be converted to pentose phosphates and this, under anaerobic conditions, implies the occurrence of a closed redox balance. As pointed out above, this requires that xylose reductase and xylitol dehydrogenase use the same coenzyme. This prerequisite for anaerobic xylose metabolism has so far been neglected.

The occurrence of NADH-linked xylose reductase activity in xylose-grown *Pa. tannophilus* has also been noted by Maleszka et al. (1983). However, these authors did not pay attention to the possible relationship between the presence of this enzyme and the capacity of *Pa. tannophilus* to perform a slow anaerobic metabolism of xylose.

D-Xylose-fermenting yeasts which have been studied so far require oxygen for optimal conversion efficiency and for a high rate of ethanol production from xylose. This property, as pointed out above, is a reflection of a disturbed redox balance. It necessitates a careful control of the oxygen supply in xylose fermentation with these yeasts. Both high and very low concentrations of oxygen inhibit the alcoholic fermentation by organisms like *Pa. tannophilus*. Like *Pa. tannophilus*, also *Pi. stipitis* requires oxygen for growth (Figs. 2, 3). However, *Pi. stipitis* seems better equipped for anaerobic alcoholic fermentation of xylose. Whereas *Pa. tannophilus* requires oxygen both for growth and for alcoholic fermentation, *Pi. stipitis* only needs oxygen for growth. This is an advantage in processes using cell recycling or immobilized organisms. The basis for the oxygen requirement for growth on xylose is not clear and demands further study, particularly since these yeasts do not need oxygen for growth on glucose in complex media.

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