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The Role of Redox Balances in the Anaerobic Fermentation of Xylose by Yeasts

Peter M. Bruinenberg, Peter H. M. de Bot, Johannes P. van Dijken, and W. Alexander Scheffers

Delft University of Technology, Laboratory of Microbiology, Julianalaan 67A, 2628 BC Delft, The Netherlands

Summary. The kinetics of glucose and xylose utilization by batch cultures of *Candida utilis* were studied under aerobic and anaerobic conditions during growth in complex media. Rapid ethanol formation occurred during growth on glucose when aerobic cultures were shifted to anaerobic conditions. However, with xylose as a substrate, transfer to anaerobiosis resulted in an immediate cessation of metabolic activity, as evidenced by the absence of both ethanol formation and xylose utilization. The inability of the yeast to ferment xylose anaerobically was not due to the absence of key enzymes of the fermentation pathway, since the addition of glucose to such cultures resulted in the immediate conversion of glucose to ethanol. Furthermore, when the enzyme xylose isomerase was added to an anaerobic xylose culture, immediate conversion of xylose to ethanol was observed. This indicates that the inability of the yeast to form ethanol from xylose under anaerobic conditions is caused by metabolic events associated with the conversion of xylose to xylulose. A hypothesis is put forward which explains that ethanol production from xylose by yeast under anaerobic conditions is negligible. It is suggested that the failure to ferment xylose anaerobically is due to a discrepancy between production and consumption of NADH in the overall conversion of xylose to ethanol. When a hydrogen acceptor (i.e. acetoin) was added to anaerobic cultures of *C. utilis*, xylose utilization resumed, and ethanol and acetate were produced with the concomitant stoicheiometric reduction of acetoin to 2,3-butanediol.

Introduction

The most abundant resource of carbohydrate is cellulosic biomass which is presently considered as a possible raw material for the production of fermentation ethanol. Up to 35% of the total carbohydrate of plant material may consist of the pentose xylose (Rosenberg 1980). Economic fermentation of cellulosic biomass therefore requires the exploitation of xylose-fermenting organisms.

The ability of facultatively fermentative yeasts to ferment xylose to ethanol does not seem to be widespread. In his thesis, Kluyver (1914) already remarked: "Only a small amount of xylose was available. Therefore I did not routinely include xylose in my fermentation studies. However, although many fungi can assimilate this sugar, there are so many reports on their inability to ferment it that omission of xylose was not considered to be serious". The small amount of xylose available was used in a few experiments with selected yeast strains: "All results were negative". Thus, although many yeasts can assimilate xylose aerobically (Barnett et al. 1979) it appears that the conversion of xylose to ethanol in the absence of oxygen is limited to only a small number of yeasts (Jeffries 1981; Schneider et al. 1981; Dekker 1982; Margaritis and Bajpaj 1982; Suihko and Dražić 1983). However, the rate of xylose fermentation by these organisms under strictly anaerobic conditions is always very low and, apart from a small amount of ethanol, the major end product of fermentation is xylitol (Dellweg et al. 1982). The fermentation rate is strongly enhanced, and a higher ethanol yield is obtained, when oxygen is supplied to the fermentation broth. So far, no satisfactory explanation is known for this oxygen effect and for the inability of the majority of xylose-assimilating facultatively fermentative yeasts to ferment xylose under anaerobic conditions.

In this paper it is attempted to clarify the above problems. Our studies show that in *Candida utilis*, and probably in other yeasts as well, anaerobic utilisation of xylose intrinsically leads to a deregulation of the redox balance, prohibiting rapid alcoholic fermentation.

Offprint requests to: W. Alexander Scheffers

Materials and Methods

Micro-Organisms and Growth Conditions. *Candida utilis* CBS 621 was obtained from the Centraalbureau voor Schimmelcultures, Yeast Divison, Delft, The Netherlands. The organism was grown in a laboratory fermentor with a working volume of 1.5 l at 30 °C and pH 5.0. The medium contained per litre: yeast extract (Difco) 10 g; xylose or glucose 20 g; both components were heat-sterilized separately at 120° C. The pH was controlled at 5.0 by automatic addition of 1 M NaOH unless mentioned otherwise. The fermentors were fitted with butyl rubber tubings to avoid access of oxygen during anaerobic experiments. Anaerobic conditions were maintained by gassing with ultra-pure nitrogen (containing less than 25 ppm oxygen). Glucose, xylose isomerase and acetoin, when added in the anaerobic phase, were also gassed with N_2 prior to their addition. Immobilized xylose isomerase (Maxazyme) was washed with sterile 50 mM potassium phosphate buffer, pH 6.0, prior to addition (final concentration Maxazyme 50 $g \cdot l^{-1}$). Regular microscopic examination of cultures during experiments with this enzyme preparation showed that contamination with foreign organisms was negligible. In these experiments the culture pH was maintained at 6.0, since xylose isomerase is virtually inactive at pH 5.0 (van Tilburg 1983).

Preparation of Cell-Free Extracts. Cells were harvested in the mid-exponential phase in aerobic batch cultures just prior to the shift to anaerobiosis, and washed twice with 10 mM potassium phosphate buffer of pH 7.5, containing 2 mM EDTA. Cell-free extracts were prepared by sonication as described earlier (Bruinenberg et al. 1983b).

Enzyme Assays. Acetaldehyde dehydrogenase, xylose reductase, and xylitol dehydrogenase were determined according to Bruinenberg et al. (1983b). The assay mixture for acetoin reductase contained: potassium phosphate buffer, pH 6.5, 50 mM; NADH or NADPH, 0.15 mM. The reaction was started by the addition of acetoin to a final concentration of 10 mM. Protein was determined following the Lowry method with bovine serum albumin as a standard.

Analytical Methods. Growth was measured as optical density at 660 nm. Glucose, acetic acid and glycerol were determined by the GOD-Perid method, and the test combinations for acetic acid and glycerol from Boehringer. Xylose was determined according to Herbert et al. (1971). Ethanol was determined by gas chromatography on a Porapak Q column at 170° C. 2,3-Butanediol was determined via gas chromatography on a column of 5% Carbowax 20 M on Chromosorb-W at 200° C. Xylitol was determined by HPLC on an Aminex HPX 87-C column.

Chemicals. Xylose isomerase (immobilized glucose isomerase, trade name Maxazyme) was a gift from Gist-Brocades N.V., Delft, The Netherlands. Yeast extract was obtained from Difco, glucose, xylose, and acetoin from Merck. Other biochemicals were from Boehringer.

Results

In batch cultures on a medium containing glucose and yeast extract *Candida utilis* showed the typical behaviour of a glucose-insensitive yeast (Fiechter et al. 1981). As long as aerobic conditions were maintained the sugar was utilised but not fermented. After a shift to anaerobic conditions the culture rapidly adopted a fermentative metabolism. Within 4 h the remaining glucose had been consumed and biomass, ethanol, and glycerol were the major products formed (Fig. 1). With xylose as a substrate, however, the shift to anaerobic conditions stopped all metabolic activity: no ethanol was formed and the xylose concentration remained at a constant level (Fig. 2). The culture could be kept in this state for more than 1 week with only a slight decrease in optical density as a result of cell lysis. Growth immediately resumed when the culture was aerated again (results not shown). The absence of ethanol production from xylose under anaerobic conditions does not seem to be due to the absence of key enzymes of the fermentative route in cells pregrown on xylose. When glucose was added to an anaerobic xylose culture an almost immediate ethanol production was observed (Fig. 2). The rate of glucose fermentation in xylose-grown cells was comparable to that in glucose-grown cells (compare Fig. 1, Fig. 2).

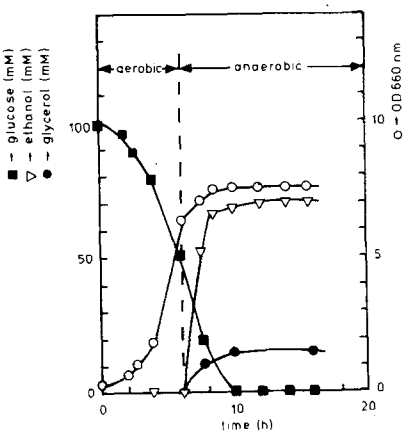

Fig. 1. Effect of aeration on alcoholic fermentation of glucose by *Candida utilis* CBS 621 in batch culture during growth on a complex medium. After 6 h of aerobic growth the culture was shifted to anaerobiosis by gassing with pure nitrogen

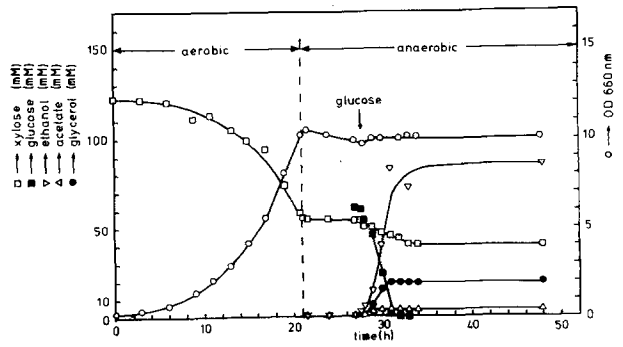

Fig. 2. Inability of *Candida utilis* CBS 621 to ferment xylose under anaerobic conditions. After 7 h of anaerobiosis, during which metabolic activity was absent, glucose was added to the culture and this resulted in an immediate ethanol formation

Ethanol, glycerol and some acetate were produced. In the course of the glucose fermentation also some consumption of xylose occurred (Fig. 2).

The blockade in xylose metabolism under anaerobic conditons was relieved upon addition of xylose isomerase to the culture (Fig. 3). In the presence of this enzyme xylose was fermented to ethanol and glycerol. The results indicate that the inability of *C. utilis* to ferment xylose under anaerobic conditions is caused by metabolic events associated with the conversion of xylose to xylulose. Since conversion of xylose to xylulose by *C. utilis* involves pyridine nucleotide-dependent oxidation–reduction reactions (Chakravorty et al. 1962; Horecker 1962; Table 1) it seems likely that a deregulation of the redox balance as a result of these two steps causes the metabolic block in xylose metabolism under anaerobic conditions. Indeed, addition of the hydrogen acceptor acetoin resulted in the fermentation of xylose to ethanol and acetate (Fig. 4). The acetoin added was stoicheiometrically converted to 2,3-butanediol. Since acetoin reduction by cell-free extracts of *C.*

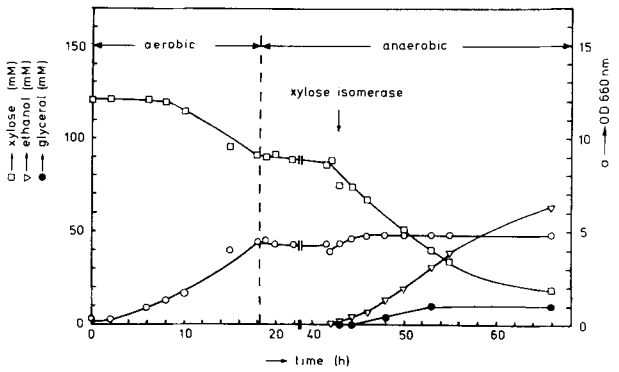

Fig. 3. Effect of xylose isomerase addition on anaerobic xylose metabolism by *Candida utilis* CBS 621. The enzyme was added 15 h after the shift of an aerobic culture to anaerobiosis

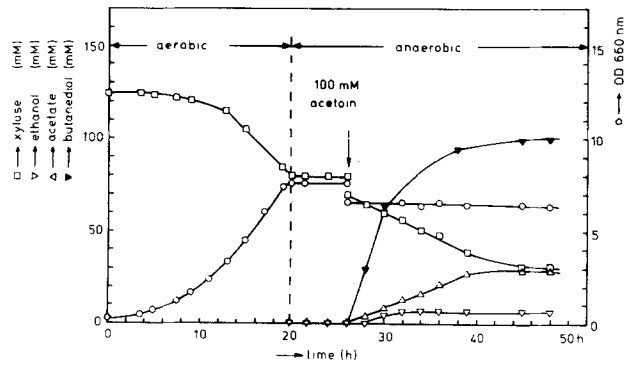

Fig. 4. Restoration of metabolic activity as a result of the addition of acetoin to an anaerobic xylose culture. Note that xylose consumption as well as ethanol and acetate production cease after exhaustion of acetoin by its reduction to butanediol

Table 1. Enzyme activities in cell-free extract of *Candida utilis* CBS 621 grown on xylose. Cells were harvested just prior to the shift to anaerobiosis. Activities are expressed as nmol $\cdot$ min^{-1} $\cdot$ (mg protein)$^{-1}$

Xylose reductase	
NADH	0
NADPH	75
Xylitol dehydrogenase	
NAD^+	280
$NADP^+$	0
Acetaldehyde dehydrogenase	
NAD^+	30
$NADP^+$	0
Acetoin reductase	
NADH	850
NADPH	10

utilis is strictly dependent on NADH (Table 1) it must be concluded that the metabolic inactivity under anaerobic conditions results from an imbalance in production and consumption of NADH in the overall conversion of xylose to ethanol.

Discussion

Of the 439 type strains of yeasts, listed by Barnett et al. (1979), 172 are able to metabolize xylose aerobically and to ferment glucose under anaerobic conditions. A recent study performed in our laboratory on the ability of these yeasts to ferment xylose established that, with few exceptions, these organisms cannot ferment xylose (A. Toivola et al., unpublished). *Pachysolen tannophilus* is one of the rare examples of a yeast which gives a fermentation, albeit it very slow, of xylose to ethanol under anaerobic conditions (Dellweg et al. 1982). *Candida utilis,* like the majority of facultatively fermentative xylose-assimilating yeasts, does not show any fermentative activity with xylose under anaerobic conditions. When aerobic xylose-grown cultures were shifted to anaerobic conditions, ethanol formation was below the detection limit (i.e. less than 20 mg $\cdot$ l^{-1}; Figs. 2–4).

Phenomenologically, the inability of facultatively fermentative xylose-assimilating yeasts to ferment xylose under anaerobic conditions can be regarded as a Kluyver effect. Sims and Barnett (1978) suggested that the Kluyver effect – the inability of a facultatively fermentative yeast to metabolize a sugar anaerobically although it can be metabolised aerobically – may be explained by an oxygen requirement for transport of the sugar. The inability of *C. utilis* to ferment xylose can, however, not be due to an oxygen requirement for transport, since the sugar was

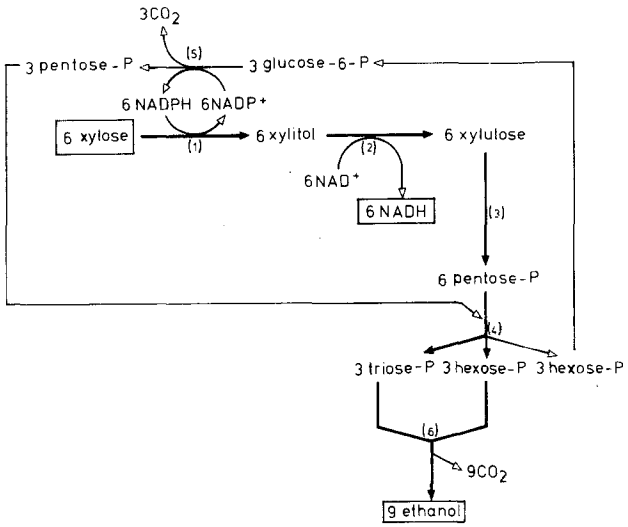

Fig. 5. Schematic representation of xylose metabolism in case of alcoholic fermentation. The various reactions, indicated by numbers, are catalyzed by the following enzymes: 1 xylose reductase; 2 xylitol dehydrogenase; 3 xylulose kinase + ribulose phosphate epimerase + ribose phosphate isomerase; 4 transaldolase + transketolase; 5 glucose-6-phosphate dehydrogenase + 6-phosphogluconate dehydrogenase; 6 enzymes of the glycolytic pathway + pyruvate decarboxylase + alcohol dehydrogenase

metabolized anaerobically in the presence of acetoin or glucose (Figs. 3, 4).

The effect of the addition of xylose isomerase to anaerobic cultures (Fig. 3) indicates that the metabolic block for anaerobic conversion of xylose resides in the conversion of xylose to xylulose. This is a two-step process catalyzed by NADPH-dependent xylose reductase and NAD^+-dependent xylitol dehydrogenase (Table 1). Xylose reductase and xylitol dehydrogenase are probably obligatory enzymes for xylose metabolism in yeasts. So far, direct conversion of xylose into xylulose has been reported only in *C. utilis* (Tomoyeda and Horitsu 1964), but the activity of the isomerase was extremely low (0.5 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$).

It seems unlikely that the metabolic inactivity of yeasts towards xylose under anaerobic conditions is due to a shortage of NADPH as proposed by Jeffries (1981) and Smiley and Bolen (1982). These authors argued that the NADPH required for the xylose reductase reaction must be generated in the TCA cycle via $NADP^+$-dependent isocitrate dehydrogenase. Since oxygen is required for a cyclic operation of the TCA cycle, this would explain the observed oxygen requirement for xylose fermentation by yeasts. However, it can be calculated that in yeasts the isocitrate dehydrogenase reaction can only be a minor source of NADPH, especially during growth on xylose (Bruinenberg et al. 1983a). Our calculations, which are supported by enzymic data (Bruinenberg et al. 1983b), demonstrate that the major source of NADPH in yeasts must be the hexose monophosphate (HMP) pathway. In addition, the stimulatory effect of acetoin on xylose fermentation (Fig. 4) indicates that anaerobic xylose metabolism does not lead to a shortage of reduction equivalents but rather to an excess. Since the acetoin reductase is strictly dependent on NADH (Table 1), anaerobic xylose metabolism is likely to result in a surplus of this reduction equivalent. This is also evident from Fig. 5 which summarizes the metabolic events required for the anaerobic conversion of xylose to ethanol.

Metabolism of xylose in *C. utilis* (Fig. 5) proceeds via NADPH-dependent xylose reductase and NAD^+-dependent xylitol dehydrogenase (Chakravorty et al. 1962; Table 1). The NADPH required in the first step is generated in the HMP pathway by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. After phosphorylation, xylulose is isomerized to pentose phosphates which are rearranged to hexose phosphates and triose phosphates by transaldolase and transketolase according to the overall equation:

$$9 \text{ pentose phosphate} \rightarrow 6 \text{ hexose phosphate} + 3 \text{ triose phosphate} \quad (1)$$

Under anaerobic conditions alcoholic fermentation of these sugar phosphates can serve as a source of ATP. This process does not involve a net production or consumption of reduction equivalents. The overall equation for the anaerobic conversion of xylose to ethanol would be:

$$6 \text{ xylose} + 6\ NAD^+ + 9\ ADP + 9\ P_i \rightarrow 9 \text{ ethanol} + 6\ NADH + 9\ ATP + 12\ CO_2 \quad (2)$$

Thus, although potentially energy-yielding, anaerobic fermentation of xylose would not be possible as a result of an imbalance in production and consumption of NADH. This is due to the fact that different pyridine nucleotides are required in the first two steps of xylose metabolism. The NADH generated by the xylitol dehydrogenase reaction cannot be used to produce NADPH for xylose reduction since *C. utilis*, and probably other yeasts as well, lack transhydrogenase (Bruinenberg et al. 1983b). Therefore, only exogenous hydrogen acceptors which can be reduced with NADH can restore the redox balance under anaerobic conditions. In this respect acetoin is a suitable compound for *C. utilis* since cell-free extracts contained high levels of an NADH-dependent acetoin reductase (Table 1). This yeast showed an absolute requirement for a hydrogen acceptor since anaerobic xylose utilization and metabolite production stopped after all acetoin had been reduced to

2,3-butanediol (Fig. 4). Acetate was the major product formed. Since formation of acetate via NAD^+-linked acetaldehyde dehydrogenase (Table 1) results in the formation of additional NADH, the production of this metabolite is seemingly in contrast with the proposed excessive formation of reduction equivalents as suggested above. It must be realized, however, that the intracellular rate of reduction of acetoin may be so fast that the NAD^+/NADH ratio becomes sufficiently high to allow a competition between acetaldehyde dehydrogenase and alcohol dehydrogenase for acetaldehyde. Although kinetic parameters, rather than overall redox balances may determine the nature of the fermentation products, it is evident that under anaerobic conditions metabolism of xylose to intermediates of the glycolytic pathway results in a net surplus of NADH.

Overproduction of NADH during anaerobic metabolism of sugars by yeasts is a general phenomenon. During anaerobic growth of *Saccharomyces cerevisiae* on glucose, a surplus of NADH occurs as a result of the formation of biomass which is more oxidized than glucose. This NADH can be reoxidized via formation of glycerol (Oura 1977). Also in *C. utilis* the glycerol shunt is operating during anaerobic metabolism of glucose, since apart from ethanol substantial amounts of glycerol were produced after a shift of an aerobic culture to anaerobiosis (Fig. 1). It could therefore be argued that glycerol formation during anaerobic xylose metabolism by this yeast would offer a possibility to neutralize the overproduction of NADH. However, the excess of NADH with xylose is much higher than with glucose. Fermentation of xylose to ethanol with a fitting redox balance would require the conversion of one molecule triose phosphate to glycerol per molecule of xylose metabolized. As a result, not enough triose phosphate can be metabolized to ethanol and the result is a shortage of ATP. This is evident from Eq. (3) which represents the fermentation of xylose with a fitting redox balance as a result of glycerol formation:

$$6 \text{ xylose} + 3 \text{ ATP} \rightarrow 3 \text{ ethanol} + 6 \text{ glycerol} + 6 \text{ CO}_2 + 3 \text{ ADP} + 3 P_i \quad (3)$$

Thus, glycerol formation cannot function as a redox valve during anaerobic xylose metabolism in *C. utilis*.

In *Pachysolen tannophilus* and various other yeasts alcoholic fermentation of xylose, which is very slow under anaerobic conditions, is considerably enhanced by the presence of oxygen (Dellweg et al. 1982; Slininger et al. 1982; Suikho and Dražić 1983). This stimulation of alcoholic fermentation by oxygen is known as the Custers effect (Scheffers 1966; Scheffers and Wikén 1969). As has been shown (Scheffers and Nanninga 1977; Scheffers et al. 1982), this effect is the consequence of a disturbed NADH/NAD^+ balance.

Also during anaerobic xylose fermentation by *P. tannophilus,* production of a surplus of reduction equivalents may be the basis for the very low rate of ethanol formation, thus explaining the stimulatory effect of oxygen. Nevertheless, *P. tannophilus,* in contrast to *C. utilis,* produces small amounts of ethanol under anaerobic conditions (Dellweg et al. 1982). Preliminary results from enzyme studies carried out in our laboratory with *P. tannophilus* have established an important difference in xylose metabolism as compared to *C. utilis.* In *P. tannophilus,* apart from an NADPH-linked enzyme, also NADH-dependent xylose reductase activity is present. The latter probably allows a certain degree of alcoholic fermentation of xylose without overproduction of reduction equivalents. The physiological consequenses of this difference, especially with regard to the observed xylitol formation by *P. tannophilus,* which was not observed in our experiments with *C. utilis,* will be the subject of a forthcoming paper.

From a fundamental point of view, xylose fermentation offers an intriguing model system to study the important role or redox balances in yeasts. From this paper it will be clear, however, that when applied aspects are to be considered, the mode of xylose metabolism by yeasts has unfortunate traits. Efficient anaerobic fermentation of xylose to ethanol by yeasts apparently requires that the first two reactions of its metabolism be circumvented. Implantation of a gene coding for xylose isomerase would offer a possibility. This might as well be performed in a yeast species with a high fermentation capacity and a high ethanol tolerance such as certain *Saccharomyces* strains, since *S. cerevisiae* and various other yeasts which cannot metabolize xylose are able to ferment xylulose anaerobically to ethanol (Gong et al. 1981). However, another option would be to select for a yeast possessing a xylose reductase and a xylitol dehydrogenase which are linked to the same coenzyme system, thus eliminating the formation of excess NADH in the process of ethanol production. Even the introduction of transhydrogenase activity by genetic manipulation might be considered. Our results show that research in these directions is more promising than empirical studies on the optimization of *aerobic* xylose fermentation by yeasts.

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