

Fermentation of D-Xylose to Ethanol by *Bacillus macerans*

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Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

Xylose Fermentation, *Bacillus macerans*, Ethanol Formation, By-Products, Effect of Oxygen

In the absence of oxygen *Bacillus macerans* is able to ferment xylose. The principal products are ethanol, formate, CO₂, acetone and H₂. The yield of ethanol was 61% based on the theoretical value of 0.51 g per g xylose consumed. The cells could grow in the presence of up to 4% (v/v) of added ethanol but the growth rate was already reduced to about 50% by 1% (v/v) of alcohol. Glucose and the pentoses arabinose and xylose were sequentially utilized when initially present as a mixed substrate. Enzymatic studies indicate that xylose was metabolized via the pentosephosphate and Embden-Meyerhof pathways. At an oxygen concentration of 1–2% air saturation 42 g/l xylose were completely fermented within 160 h and 10 g/l ethanol were produced.

Introduction

The hydrolysis of cellulose and hemicellulose, the major constituents of plant materials, yields a mixture of sugars in which D-glucose and D-xylose are the major components [1]. The economic feasibility of ethanol production from these materials depends on the ability of microorganisms to fully convert the available carbon sources. However, neither any of the commonly used fermentative yeast strains nor the very potent ethanol forming bacterium *Zymomonas mobilis* can convert xylose to ethanol [2, 3].

Although many yeasts can grow with xylose as a substrate, so far, only a few yeasts have been found capable of xylose fermentation. Attention has been focused on *Pachysolen tannophilus* [4] and a number of *Candida* species [5, 6]. However, these yeasts appear to have limited value for ethanol production. Under anaerobic conditions, a large fraction of the xylose is converted to xylitol and the yield of ethanol is correspondingly low; oxygen is needed for optimal xylose fermentation [7]. Recently it was found that *Pichia stipitis* ferments xylose to ethanol also under anaerobic conditions quite well [8].

In addition to hexose, D-xylose and L-arabinose are also good substrates for the enteric group of facultative anaerobic bacteria forming alcohols, organic acids and gases [9]. Several species of the

genus *Clostridium* have been utilized for the conversion of hemicelluloses to mixtures of ethanol plus lactic and acetic acids [10]. The anaerobic fermentation of carbohydrates by *Bacillus macerans* was first described by Schardinger [11]. This bacterium carries out a modified mixed acid fermentation of glucose or xylose with the major products being ethanol, acetic acid, CO₂, H₂ and acetone [12]. The purpose of the present study was to examine in more detail the product formation during xylose fermentation, particularly with regard to the effect of oxygen and the xylose fermentation pathway.

Material and Methods

Microorganism and culture conditions

Bacillus macerans DSM 1574 was grown in a defined medium which contained per liter: 20 g xylose, 3 g NH₄Cl, 1 g KCl, 6.8 g KH₂PO₄, 0.2 g MgSO₄ × 7 H₂O, 30 mg MnSO₄ × H₂O, 30 mg Ethylenediaminetetraacetate, 10 mg CaCl₂ × 2 H₂O, 5 mg Na₂MoO₄, 5 mg FeSO₄ × 7 H₂O, 5 mg H₃BO₃, 3 mg CoCl₂ × 6 H₂O, 1 mg KJ, 1 mg CuSO₄ × 5 H₂O, 1 mg ZnSO₄ × 7 H₂O, 1 mg nicotinic acid, 2 mg biotin, 2 mg p-amino benzoic acid, 2 mg thiamine hydrochloride. The pH was adjusted to 7.0 with NaOH. The incubation was carried out at 37 °C in 18 × 150 mm anaerobic tubes (Bellco, USA) containing 10 ml medium or in a 270 ml glass bottle with 100 ml medium. For studying the influence of different oxygen concentrations the organism was grown in a 1.6 l fermenter (Bioengineering AG, Switzerland) equipped with an oxygen electrode. The pO₂ was maintained constant by automatic variation of the aeration rate.

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Preparation of cell-free extracts and enzyme assays

Cells were harvested in the end of the exponential growth phase, washed twice with 10 mM Na-citrate buffer, pH 6.0, and resuspended in the same buffer containing 2 mM $MgSO_4 \times 7 H_2O$ and 20 mM β -mercapto-1,2-propanediol. The organisms were disrupted in an X-Press (AB-Biox, Sweden) by passing the frozen cells through an orifice of 0.8 mm diameter. After removing the cell debris by centrifugation at $44,000 \times g$ for 30 min the supernatant was used as cell-free extract.

The enzymes were assayed according to the following methods: Transketolase activity (EC 2.2.1.1) was tested according to de la Haba *et al.* [13]. Transaldolase activity (EC 2.2.1.2) was assayed by the method of Tchola and Horecker [14]. Fructose diphosphate aldolase (EC 4.1.2.13) was determined according to Bechtler [15]. Pyruvatekinase (EC 2.7.1.40) was assayed according to Gutmann and Bernt [16]. Phosphoketolase activity (EC 4.1.2.9) was measured according to Goldberg *et al.* [17]. Lactate dehydrogenase activity (EC 1.1.1.27) was tested in the following reaction mixture: 95 mM citrate buffer, pH 6.0, 20 mM $MgSO_4 \times 7 H_2O$, 20 mM Na-pyruvate, 0.15 mM NADH and cell-free extract in a final volume of 1.0 ml. Alcohol dehydrogenase (EC 1.1.1.1) was determined according to Lamed and Zeikus [18] and aldehyde dehydrogenase (EC 1.2.1.3) was assayed according to Lundquist [19].

One unit of enzyme activity is defined as the amount of enzyme which consumes/produces 1 μ mol substrate/product per min at 30 °C under anaerobic conditions. Specific activity is expressed as units per

mg of protein. Protein concentration was determined according to Bradford [20] using bovine serum albumin as a standard.

Analytical methods

The growth of the bacteria was determined by measuring the optical density of the cultured broth at 600 nm with a Zeiss DM4 photometer or by determination of the dry weight of the cells after centrifugation. Broth samples were analyzed for ethanol, acetone and acetate by gas chromatography as described by Finn *et al.* [21]. The formation of formate and lactate were estimated by isotachophoresis [22]. Acetoin and 2,3-butanediol were determined by gas chromatography (Hewlett-Packard, Modell 5790A with automatic sampler and flame ionisation detector). The $\frac{1}{4}$ inch glass column was packed with 3 feet of 100–120 mesh Porapak P. Flow rate of the carrier gas, N_2 , was 30 ml/min and temperatures of the injection port, column and detector were 200 °C, 150 °C and 230 °C respectively. Xylose was determined with neocuproin reagent [23]. In the experiment with multiple substrates, individual sugars were assayed by low-pressure liquid chromatography (Biotronik, Munich) with borate buffer elution and photometric detection as furfural/orcinol complexes [24].

Results

Product formation during D-xylose fermentation

The time course of a typical batch fermentation of D-xylose by *Bacillus macerans* DSM 1574 is shown in Fig. 1. During the first 50 h there was an increasing

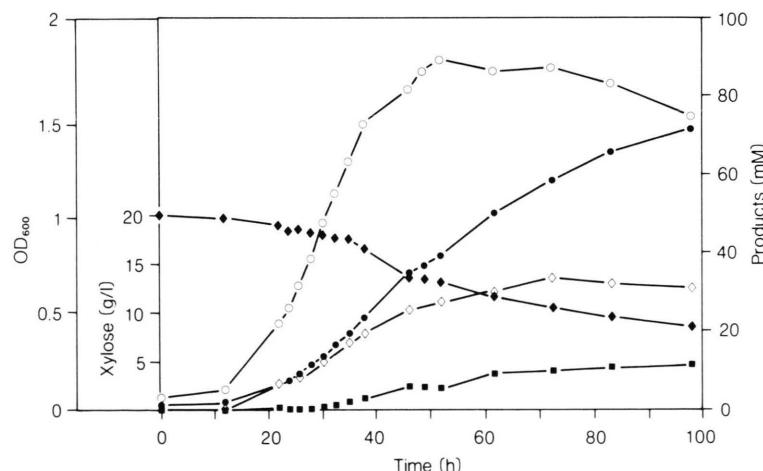


Fig. 1. Batch fermentation of D-xylose by *Bacillus macerans* at a controlled pH of 6.0 under anaerobic conditions. \blacklozenge , D-xylose; \circ , OD; \bullet , ethanol; \lozenge , formate; \blacksquare , acetone.

rate of sugar utilization and parallel increases in the concentrations of ethanol and formate as the main products of xylose fermentation, whereas acetone was detected as minor product. This growth phase was followed by an abrupt cessation in the growth, which indicates a nutritional deficiency or accumulation of growth inhibitory end products; more than 12 g/l of fermentable sugar were still present. The rate of sugar usage started to decline and the ethanol/formate ratio increased as the alcohol formation still continued but the acid production decreased. After an incubation time of 120 h the alcohol level was 75 mM and about 60% of the sugar added to the medium (19 g/l) was utilized. The yield of ethanol was 31% (w/w) based on xylose consumed, a value of 61% of that obtainable theoretically from a microbial fermentation.

In separate experiments at pH 6.5 it was found that ethanol and formate, along with acetone, CO₂ and H₂ accounted for most of the xylose fermented (Table I). Furthermore small amounts of acetate and lactate were formed but the following compounds were not detected as products of xylose fermentation: acetaldehyde, C₃-C₆ volatile acids or alcohols, glycerol, 2,3-butanediol, diacetyl, acetoin or xylitol.

A similar distribution of end products, with minor amounts of formate and lactate has been reported for glucose fermentation [25]. Since there was evidence that acetate served as an intermediate in acetone formation, different concentrations of acetate were added to the culture. As shown in Table II by this way a preferential increase in acetone accumulation but not in ethanol production took place.

Tests of the influence of added ethanol on growth demonstrated that the growth of this bacterium can continue up to a level of 4% (v/v) of added ethanol (Fig. 2). Since the growth rate was already reduced to about 50% by the addition of 1% (v/v) ethanol, it

Table II. Effect of acetate addition on xylose fermentation by *B. macerans*. Cultures were grown for 5 days in a medium containing 10 g/l xylose.

Acetate added [mM]	Product concentration [mM]	
	Ethanol	Acetone
0	48.25	6.64
10	55.05	11.44
15	59.01	14.35
20	57.43	15.73

was tried to select mutants with a higher ethanol tolerance but so far with no success.

Since a typical hydrolysate of hemicelluloses from various biomass would contain also other sugars besides xylose, it was of interest to know how *B. macerans* ferments a sugar mixture. Figure 3 shows a sequential utilization of glucose, arabinose and xylose. Glucose was metabolized at a faster rate than the pentoses which were used when the glucose concentration was lower than 1 g/l. The product spectrum was similar to that observed during growth on xylose alone.

Xylose fermentation pathway

Recently it was found that *B. macerans* ferments glucose via the Embden-Meyerhof pathway and in cell extracts of this bacterium pyruvate formate-lyase and formate dehydrogenase activities were detected

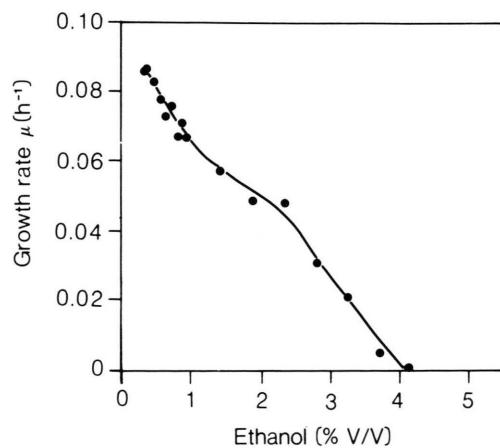


Fig. 2. Effect of added ethanol on the growth of *B. macerans*. The growth rate was measured by changes in OD within 36 h.

Table I. End products formed from xylose based on 100 mmol fermented.

Ethanol	102.2
Acetone	16.0
Acetate	6.2
Formate	46.0
Lactate	2.5
CO ₂	107.9
H ₂	18.2
Carbon accounted for	90.7%
O/R balance	1.0

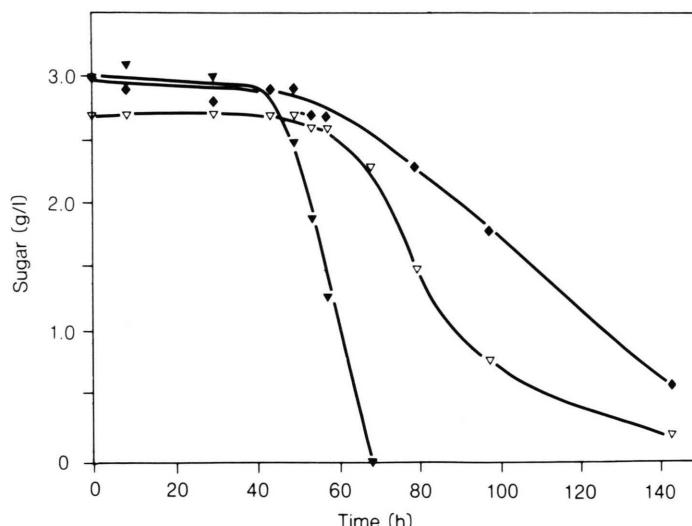


Fig. 3. Sequential utilization of mixed sugars by *B. macerans*. The inoculum was grown on glucose. ▼, glucose; ▽, arabinose; ◆, xylose.

(Weimer [25]). So far, there was no information available on the enzymes involved in pentose fermentation in *B. macerans*, therefore some catabolic key enzymes were assayed. As expected considerably high levels of transaldolase and transketolase activities could be detected in cell extracts (Table III). Therefore, it is suggested that the pentose phosphate pathway is utilized to channel the carbon atoms of xylose into the glycolytic Embden-Meyerhof enzyme sequence. An enzyme indicator of the heterolactic route of sugar degradation – phosphoketolase – was not detected. Besides the pyruvate formate lyase and the pyruvate kinase a third enzyme for the conversion of pyruvate was detected namely a lactate dehydrogenase. In contrast to the lactate dehydrogenase found in many other bacteria

this enzyme needs not fructose-1,6-diphosphate but fructose-6-phosphate as an activator. Furthermore, *B. macerans* has a NAD dependent acetaldehyde dehydrogenase which reduces acetyl-CoA to acetaldehyde, and finally this compound is converted to ethanol by a NAD dependent alcohol dehydrogenase. From these data it appears that *B. macerans*, like other pentose-fermenting bacteria [26], uses a combination of the pentose-phosphate and Embden-Meyerhof pathways for the conversion of xylose to pyruvate which is further metabolized by various enzymes to the different end products.

Effect of oxygen

As oxygen has a profound effect on ethanolic fermentations by yeasts, the influence of aeration on xylose fermentation by *B. macerans* was studied by comparing the end product formation in cultures in which the oxygen concentrations were varied. In the range between 80% and 20% of air saturation most of the xylose was oxidized to CO_2 and water, and only very small amounts of lactate and acetate could be detected at an oxygen concentration of 1.4 mg O_2/l . At about 10% of air saturation the aerobic metabolism of the facultatively anaerobic bacterium was partially changed to the anaerobic one and some more fermentation products were formed (Fig. 4). Further reduction of dissolved oxygen to about 5% of air saturation caused a decrease in the growth rate of *B. macerans* and an increase in the accumulation

Table III. Specific activities of enzymes of carbohydrate and pyruvate metabolism in cells of *B. macerans*.

Enzyme	Specific activity [U/mg protein]
Transaldolase	0.15
Transketolase	0.35
Fructose diphosphate aldolase	0.21
Pyruvate kinase	0.40
Phosphoketolase	0.0
Lactate dehydrogenase	0.07
Acetaldehyde dehydrogenase	0.02
Alcohol dehydrogenase	0.06

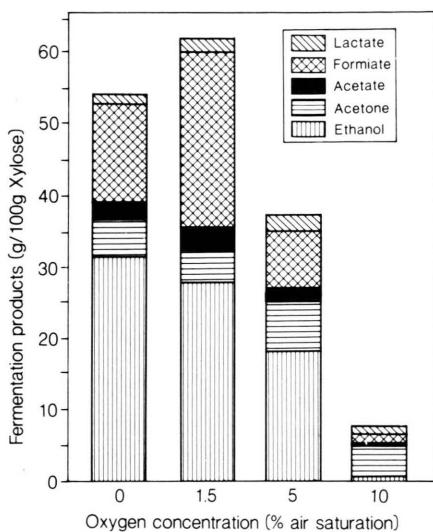


Fig. 4. Product formation during D-xylose fermentation by *B. macerans* at various oxygen concentrations.

mainly of ethanol, formate and acetone. The formation of these three fermentation products could be further increased by reducing the oxygen concentration to about 1–2% of air saturation. Under these conditions about the same amount of formate was formed as ethanol. As the growth rate was considerable higher at these microaerophilic conditions ($\mu = 0.25 \text{ h}^{-1}$), than under strict anaerobiosis ($\mu = 0.14 \text{ h}^{-1}$), 42 g/l xylose were completely fermented

within 160 h and about 10 g/l ethanol were formed in the presence of this small amount of oxygen (Fig. 5).

Discussion

Our data confirm the observation of Rosenberg *et al.* [26] and Weimer [25] that *B. macerans* can not only ferment various mono- and dihexoses but also xylose. The sequential utilization of multiple substrates, as shown in Fig. 3 is not uncommon, it is likely due to catabolite inhibition rather than repression [27, 28]. The product stoichiometry and enzymatic studies suggest a xylose fermentation pathway for this bacterium as shown in Fig. 6. Xylose is catabolized primarily by the pentose-phosphate and Embden-Meyerhof pathways to yield pyruvate, which may then be converted via acetyl-CoA to ethanol, acetate and acetone. A pyruvate decarboxylase could not be detected in cell extracts. The enhanced production of acetone observed upon addition of acetate indicates that acetate is an intermediate in acetone formation as reported also by Weimer [25].

The fermentation product ratios observed indicate that *B. macerans* produces under strict anaerobic conditions up to 1 mol ethanol per mol xylose fermented and converts more than 50% of acetyl-CoA formed to ethanol. These ratios suggest that reducing equivalents for ethanol synthesis are derived not only from oxidation of xylose to pyruvate, but also from

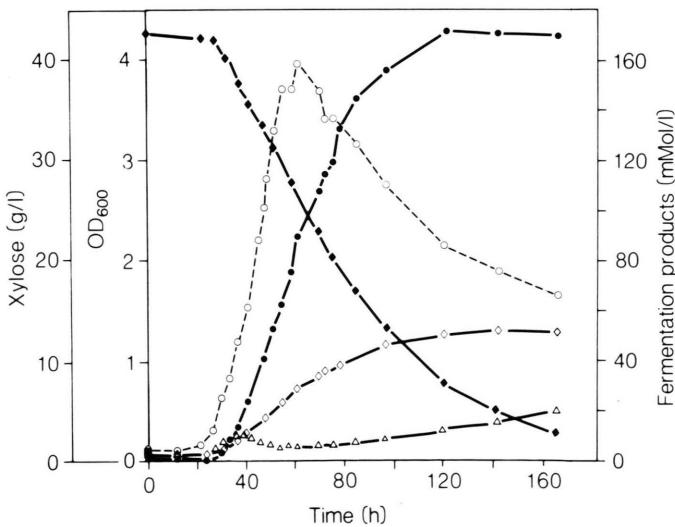


Fig. 5. Time course of D-xylose fermentation by *B. macerans* at an oxygen concentration of 2% air saturation. \blacklozenge , D-xylose; \circ , OD; \bullet , ethanol; \diamond , formate; \triangle , acetate.

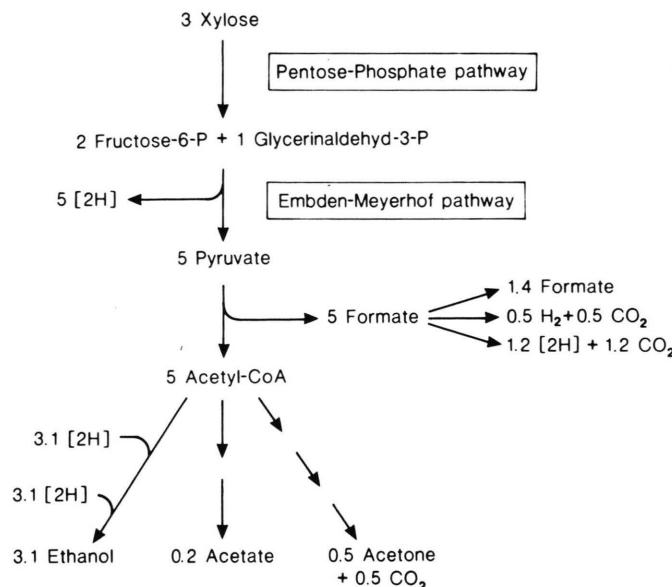


Fig. 6. Proposed scheme of xylose fermentation pathway in *B. macerans* under anaerobic conditions. The stoichiometries are estimates based on end products formed by cleavage of formate to (2H), *i.e.* reduced coenzymes, and CO₂ was calculated.

metabolism of pyruvate or its subsequent products, formate and H₂. Pyruvate as a source of NADH-generation would require an active pyruvate dehydrogenase activity, but Weimer [25] could detect this enzyme only in trace quantities. Reducing equivalents for ethanol formation could also be obtained from formate by the formate dehydrogenase reaction which is normally associated with the formic hydrogen lyase complex. Since pyruvate metabolism and H₂ production in *B. macerans* resembles that of other facultative anaerobes [29], it seems that most of the reducing equivalents generated by the formate dehydrogenase are used in H₂ production, probably by a hydrogenase associated with a formic hydrogen lyase complex. However, some of the reducing equivalents appear to be utilized in the reductive formation of ethanol.

It is well known that oxygen has a profound effect on ethanolic fermentations by microorganisms. In the presence of an oxygen concentration of 1.5% air saturation the yield of ethanol produced by *B. macerans* was only about 50% of theoretical maximal value. This means that only the reducing equivalents derived from the glycolytic pathway are used for

ethanol synthesis. In accordance with this hypothesis the amount of formate accumulated under these conditions was about twice as high as in the absence of oxygen. These results indicate that under these culture conditions the formate dehydrogenase is not active in *B. macerans* as reported for *E. coli* [30]. The stimulation of xylose fermentation by aeration may be attributed to the fact that NADH is partially also oxidized by the respiratory chain and not only by acetaldehyde dehydrogenase and alcohol dehydrogenase which have both rather low specific activities in *B. macerans*. To overcome these rate limiting steps the activities of these two final enzymes in the ethanol fermentation pathway should be increased by genetical engineering techniques. As pyruvate decarboxylase is considered to be the key enzyme for potent ethanol production in *Zymomonas mobilis* as well as in yeasts [31], the gene for this enzyme should also be transferred to *B. macerans* for increasing the rates and yields of ethanol fermentation from xylose.

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