Xylose Metabolism

in

Bacteroides xylanolyticus X5-1

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S. Biesterveld

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 22 april 1994
des namiddags te half twee in de aula
van de Landbouwuniversiteit te Wageningen.

STELLINGEN

1. Het verstrekken van het promotiereglement bij aanvang van het promotie onderzoek kan een hoop frustraties voorkomen.

2. Het niet vermelden van de fosfoketolase route als belangrijke route voor de fermentatie van xylose door gisten (Skoog en Hahn-Hägerdahl 1988) is zeer onvolledig en getuigd van gebrekkige literatuurkennis.
   Evans, C.T., and C. Ratledge. 1984. Arch Microbiol 139:48-52.
   Skoog, K., and B. Hahn-Hägerdal. 1988. Enzyme Microb Technol 1:66-80.

3. Er zijn mensen die denken dat hun waarde stijgt, evenredig met het aantal vakantiedagen dat zij per jaar overhouden.

4. Dat het NADP-afhankelijke alcohol dehydrogenase in *Thermoanaerobium brockii* verantwoordelijk is voor de *in vivo* reductie van aceton (Ben-Bassat et al. 1981) is een te hard getrokken conclusie, aangezien ook een alcohol dehydrogenase geïnduceerd kan worden.
   Ben-Bassat, A., R. Lamed and J.G. Zeikus. 1981. J Bacteriol 146:192-199.
   Dit proefschrift.

5. Het door de dierenbescherming gebruiken van "biotechnologie" en "genetische modificatie" als synoniemen, bemoeilijkt de maatschappelijke acceptatie van andere disciplines van de biotechnologie.

6. De conclusie "Xylose isomerase appeared to be produced constitutively in these ruminal bacteria, ..." in het artikel van Matte et al. (1992) is onbegrijpelijk als in hetzelfde artikel wordt aangetoond dat xylose isomerase niet actief is als een van de organismen op glucose gekweekt wordt.
   Matte, A., C.W. Forsberg, and A.M. Verrinder Gibbins. 1992. Can J Microbiol 38:370-376.

7. Koude thee is lekker als het warm is en hete chocola als het koud is.

8. De zin uit het artikel van Salyers (1984): "Glucose is fermented via the Embden-Meyerhof pathway (Macy and Probst, 1979)" is een stellige bewering gebaseerd op de zeer voorzichtige uitspraak gedaan door Macy en Probst (1979): " ...catabolism of sugars to pyruvate is possibly accomplished via the Embden-Meyerhof pathway, ...".
   Macy, J.M., and I. Probst. 1979. Ann Rev Microbiol 33:561-594.
   Salyers, A.A. 1984. Ann Rev Microbiol 38:293-313.

9. Een continuiteit binnen het universitair promotieonderzoek in tegenstelling tot het wisselende projectmatige promotieonderzoek, zal waarschijnlijk een hogere wetenschappelijke output hebben en een promotieduur die binnen de gestelde tijd valt.

10. Een hogere H_2-opbrengst in *Clostridium thermocellum* LQR1 door een "hogere hydrogenase activiteit" is strijdig met het katalytische karakter van enzymen.
    Lamed, R.J., and J.G. Zeikus. 1980. J Bacteriol 144:569-578.

11. "Universitair onderzoek is zien wat iedereen al gezien heeft, en bedenken wat nog niemand bedacht heeft".
    Prof. Dr. Szentgyorgyii, Chemisch weekblad, 2 september 1993.

12. De bewering dat de fermentatie van xylose en arabinose door *Prevotella ruminicola* subsp. *brevis* B_14 via "similar metabolic pathways" verloopt (Strobel 1993), is gebaseerd op verhoudingen in eindprodukten, maar is nooit echt bewezen.
    Turner, K.W., and A.M. Roberton. 1979. Appl Environ Microbiol 38:7-12.
    Strobel, H.J. 1993. Arch Microbiol 159:465-471.

13. Dat van de 4 basis elementen van het bestaan (aarde, lucht, vuur en water), "aarde" zo'n grote invloed kan hebben op wetenschappelijk onderzoek was mij niet bekend bij aanvang van het promotie onderzoek.

Stellingen behorende bij het proefschrift "Xylose metabolism in *Bacteroides xylanolyticus* X5-1".

Steef Biesterveld Wageningen, 22 april 1994

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Chapter 1

General introduction

Biomass as renewable resource.

The oil crisis of the mid 1970s lead to a renewed interest in the use of plant biomass for the production of chemical feedstock and fuels (Mishra and Singh 1993, Rosenberg 1980). It can be calculated that the total energy content of plant biomass presently available on earth is equivalent to about 640 billion tonnes of oil (Coughlan 1985; Gilbert and Hazlewood 1993). Due to the annual fixation of more than 100 billion tonnes of CO_2 by photosynthesis (Schlegel 1984) plant biomass is an abundantly available, inexpensive and virtually inexhaustible resource for industrial production processes to obtain alcohols and solvents. Several industrial waste streams are potential resources as well, as they are rich in (biomass derived) carbohydrates. These include agricultural and logging residues, pulp and paper mill effluent, food industry wastes, and molasses (Magee and Kosaric 1987). Of these, the agricultural and forestry waste streams are most abundant.

Plant cell walls consist of three groups of polymers; lignin, cellulose and hemicellulose (Gong et al. 1981, Wong et al. 1988). Cellulose molecules, forming highly ordered, crystalline fibrils, are surrounded by amorphous hemicellulose. These polymers are embedded in a matrix of lignin. Lignin has a complex polyphenolic structure, and is difficult to degrade biologically. Cellulose is a linear polymer composed of ß-1,4-glycosidically linked D-glucose units (500-1,000 units per molecule). Unlike cellulose, hemicelluloses are short branched-chain heteropolysaccharides of mixed hexosans and pentosans (Gong et al. 1981). The ratio of cellulose, hemicellulose and lignin depends on the plant source and the part of the plant (Table 1).

In particular cellulose and hemicellulose, which can make up more than 50% of plant biomass (Gilbert and Hazlewood 1993), are ideal sources to be used as renewable substrates for the chemical and food industries (Zeikus 1980). Hydrolysis of these polymers (either chemically or enzymatically) will yield

Table 1. Biomass constituents

Type of material	Hemicellulose %	Cellulose %	Lignins %
Monocotyledons			
Stems	25 ~ 50	25 ~ 40	10 ~ 30
Leaves	80 ~ 85	15 ~ 20	-
Fibers	5 ~ 20	80 ~ 95	-
Woods			
Hardwood (angiosperms)	24 ~ 40	40 ~ 55	18 ~ 25
Softwood (gymnosperms)	25 ~ 35	45 ~ 50	25 ~ 35
Papers			
Newspaper	25 ~ 40	40 ~ 55	18 ~ 30
Wastepaper	10 ~ 20	60 ~ 70	5 ~ 10
Waste fibers	20 ~ 30	60 ~ 80	2 ~ 10

data from Gong et al. 1981

high amounts of hexoses and pentoses. Microbial fermentation of these monomers offers the possibility to produce a variety of compounds. Aerobic microbial processes are important for the biotechnological production of antibiotics, amino acids and single cell protein (Zeikus 1980). However, aerobic microorganisms can not be used for large scale production of e.g. fuels and solvents, because CO_2 and H_2O are the main end-products. Anaerobic fermentation can yield organic acids and solvents (Jones and Woods 1986, Zeikus 1980). Furthermore, a complete anaerobic mineralization of carbohydrates will result in the formation of methane, which can be used as a combustible energy source. Less attention has been paid to the fermentative production of H_2. H_2 is a very important chemical used for industrial hydrogenation reactions (e.g. vegetable and animal oils) and H_2 is a clean and highly efficient energy carrier (Heyndrickx et al. 1991b).

Microbial degradation of cellulose and hemicellulose polymers will not only yield

free sugars that can be used for the fermentation processes. The (hemi)cellulolytic enzymes themselves, produced for (hemi)cellulose hydrolysis by the microorganisms, possess a great biotechnological value as well. Applications of cellulases and hemicellulases can be found in the food industry. They can be used as a flavour improver, for the production of sugar syrups for both human and animal consumption, or to obtain improved nutritional quality and digestibility of ruminant feeds (Gilbert and Hazlewood 1993). Purified hemicellulases can be used e.g. for structural analysis of polysaccharides and oligosaccharides, for the production of protoplasts in plant biotechnology (Zimmermann 1992), and for the bio-bleaching of paper pulps (Gilbert and Hazlewood 1993).

Hemicellulose composition and degradation.

For a long period of time, the potential use of hemicellulose has largely been ignored. At present it is recognized that hemicellulose, which can make up to 40% of plant biomass, has the same biotechnological potential as cellulose. According to the type of sugar present in the main chain of the polysaccharide, hemicelluloses can be classified in four groups: i) xylans (1→4 linked ß-D-xylopyranosyl residues), *ii) mannans* (1→4 linked ß-D-mannosyl and ß-D-glucopyranosyl residues), *iii) arabinogalactans* (1→3 linked galactopyranosyl residues), and *iv) arabinans* (1→5 linked α-L-arabinofuranosyl residues). Accessibility of hemicelluloses for microbial degradation may be obtained by a variety of methods. These include alkaline or acidic pretreatment, treatments with SO_2, Na_2SO_3, TFA (trifluoroacetic acid) and steam explosion, or pretreatment by enzymatic digestion. Combinations of these methods can be used as well (Saddler et al. 1983, Skoog and Hahn-Hägerdal 1988, Wong 1988). In contrast to e.g. acid hydrolysis (Skoog and Hahn-Hägerdal 1988, Yu et al. 1984), the enzymatic degradation is not accompanied with the formation of toxic compounds (e.g. (hydroxy methyl) furfural), which is preferred for further microbial fermentation.

For a complete enzymatic hydrolysis of hemicelluloses, the synergistic action of a variety of enzymes is required. Enzymes involved are endo-1,4-ß-xylanases, ß-xylosidases, α-L-arabinofuranosidases, acetylxylan esterases, α-glucuronidases, ferulic and p-coumaric acid esterases, mannanases, ß-mannosidases, α-galactosidases, galactanases and arabinases (Biely 1985, Gong et al. 1981, Zimmermann 1992). Xylans are the most abundant hemicelluloses and are present in large quantities in annual plants and deciduous trees (Gong et al. 1981). Five distinct enzymes are known to be involved in xylan depolymerization (Fig. 1).

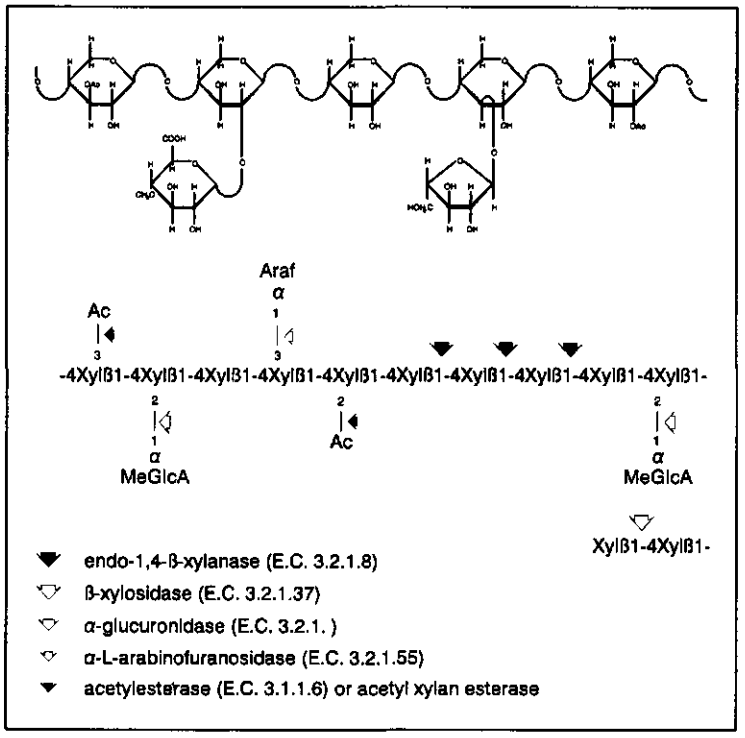

Fig.1. A hypothetical plant xylan and the sites of attack by microbial xylanolytic enzymes. A fragment comprising five D-xylose units is presented in the upper part of the figure. The sites of enzymatic attack are drawn schematically in the lower part of the figure. *Abbreviations:* Ac, acetyl group; Araf, L-arabinofuranose; MeGlcA, 4-O-methyl-D-glucuronic acid; Xyl, D-xylose. (Redrawn from Biely 1985).

Several xylan degrading enzyme systems have been studied, but in much less detail than the cellulose degrading enzymes (Biely 1985). The regulation of the synthesis of hemicellulolytic enzymes in microorganisms is not yet well understood.

A complete enzymatic hemicellulose digestion will yield a mixture of mainly monomeric sugars. The ratio of these sugars depends again on the plant source and the part of the plant. The main sugar components in hemicelluloses are xylose and glucose (Table 2).

Table 2. Hemicellulose neutral carbohydrate content of agricultural residues

Plant residues	% of total sugars			
	Xylose	Arabinose	Glucose	Others[a]
Corn residues				
Cobs	65.1	9.6	25.3	-
Leaves	59.0	9.4	29.1	2.5
Stalks	70.5	9.0	14.5	5.9
Husks	53.5	12.3	32.6	1.6
Pith	71.5	9.8	15.7	3.0
Fibers	63.8	6.6	26.8	2.8
Wheat straw	57.9	9.1	28.1	5.0
Soybean				
Stalks and leaves	59.9	6.6	6.1	27.4
Hulls	26.6	12.7	21.0	39.7
Sunflower				
Stalks	60.6	2.2	32.6	4.6
Pith	10.7	11.8	63.5	14.0
Flax straw	64.6	12.8	1.2	21.4
Sweet clover hays	49.3	21.9	8.9	9.9
Peanut hulls	46.3	5.0	46.6	2.1
Sugar cane bagasse	59.5	14.5	26.0	-

data from Gong et al. 1981
[a] mannose and galactose

Anaerobic sugar metabolism.

The anaerobic fermentation of (hemi)cellulose derived (mixtures of) sugars can yield organic acids (e.g. acetic, propionic, butyric acids, lactic acid), alcohols (ethanol, (iso)propanol, butanol) and acetone. The composition of the product mixtures formed, depends on the organism (Table 3).

Table 3. Representative fermentation products, microorganisms and growth substrates of selected acidogenic and solventogenic bacteria.

Product	Organism	Energy source
acetic	*C. formicoaceticum*	fructose
	C. thermoaceticum	hexose, pentose, lactic
	A. woodii	hexose, lactic, H_2/CO_2
lactic	*L. amylophilus*	starch, hexose
	L. casei	hexose, pentose, cellobiose
	L. brevis	hexose, pentose
	T. brockii	starch, hexose, cellobiose
butyric	*C. butyricum*	starch, hexose, pentose
	C. thermosaccharolyticum	hexose, pentose, cellobiose
	S. maxima	carbohydrates
propionic	*C. propionicum*	lactic
	P. arabinosum	hexose, pentose
succinic	*R. flavofaciens*	cellulose, hexose
	F. succinogenes	cellulose, hexose
caproic	*C. kluyveri*	ethanol/acetate
ethanol	*C. thermocellum* strain LQR1	cellulose, cellobiose
	C. thermohydrosulfuricum	starch, hexose, pentose, cellobiose
	srain 39E	starch, hexose, cellobiose
	T. brockii strain HTD4	hexose
	S. ventriculi	cellulose, hexose, pentose, cello-
	R. albus	biose
butanol	*C. acetobutylicum*	starch, hexose
	C. butylicum	starch, hexose
isopropanol	*C. butylicum*	starch, hexose
acetone	*C. acetobutylicum*	starch, hexose

data from Zeikus (1980)

Relatively little information is available on the utilization of pentoses and pentose/hexose mixtures by strictly anaerobic microorganisms. As a result, the efficiency of conversion of pentose oligosaccharides and pentoses still is a major obstacle in the fermentation processes of wood hydrolysates (Lacis and Lawford 1991). To be able to influence the fermentation and product formation from these sugars a detailed knowledge on the metabolic pathways of the organisms involved is required.

In strictly anaerobic microorganisms sugars are transported mainly via active transport systems. These include i) the PEP-dependent sugar:phosphotransferase system (PTS), ii) sugar transport linked to ATP hydrolysis, often in combination with a binding protein, and iii) ion gradient-linked translocation of sugars (Booth and Mitchell 1987). Most of the sugars are metabolized to pyruvate as the key intermediate of metabolism. Four main catabolic routes could be found in strict anaerobic microorganisms, namely the Embden-Meyerhof-Parnass pathway (=EMP =glycolysis), the pentose phosphate pathway (=PPP), the phosphoketolase pathway (PKP), and the Entner-Douderoff pathway (=EDP) (Table 4). In anaerobic microorganisms, hexoses are mainly converted *via* the EMP. Thus far, *Zymomonas mobilis* is the only strict anaerobic organism in which the EDP route has been demonstrated (Conway 1992). Pentoses are converted *via* sequential isomerization and phosphorylation reactions to xylulose-5-PO_4, which is a key intermediate in pentose metabolism (Fraenkel 1987). Xylulose-5-PO_4 is either rearranged *via* the PPP to intermediates of the EMP (fructose-6-PO_4 and glyceraldehyde-3-PO_4), or is cleaved in glyceraldehyde-3-PO_4 and a C_2-moiety (e.g. acetyl-PO_4) by means of the enzyme phosphoketolase (=PKP).

Concerning the 'known' metabolic pathways for pentoses in anaerobic microorganisms it should be mentioned that, especially in gastrointestinal *Bacteroides* species, the pathways involved in pentose metabolism were hardly investigated (Macy and Probst 1979, Salyers 1984, Caldwell and Newman 1986a

and b). Jones and Woods (1986) stated: 'The solvent-producing clostridia metabolize pentose sugars by way of the pentose phosphate pathway'. However, most pathways have been 'elucidated' by measuring one or two enzymes like transaldolase and transketolase in cell free extracts of organisms grown on only one substrate (e.g. Joyner and Baldwin 1966). However, transaldolase and transketolase also play a role in the anabolism in the production of pentose phosphates like ribose-5-PO_4, and probably are also present in microorganisms growing on e.g. glucose. Detailed research on the metabolism of D-arabinose has shown that pentoses can be fermented *via* three different metabolic pathways. These include i) the generally accepted pentose phosphate pathway in combination with the glycolysis as present in *B. ruminicola* subsp. *brevis* strain B_14 (Caldwell and Newman (1986b)), ii) conversion *via* the reaction sequence D-arabinose, D-ribulose, D-ribulose-1-PO_4, dihydroxy-acetone phosphate + glycolaldehyde as shown in *E. coli* strain K12 and *B. fragilis* strain 2044 (Leblanc and Mortlock (1971), Caldwell and Newman (1986a)), and iii) *via* the sequence D-arabinose, D-arabino-γ-lactone, D-arabonic acid, 2-keto-3-deoxy D-arabonic acid, pyruvic acid + glycolic acid as present in *Pseudomonas saccharophila* and probably in *B. vulgatus 8482* (Palleroni and Douderoff (1957), Leblanc and Mortlock (1971), Caldwell and Newman (1986a)). Different end-product ratios will be observed when pentoses are metabolized *via* one of these fermentation pathways, and an overestimation of product yields can occur when the pentose phosphate pathway is assumed to function, while one of the other routes mentioned above (item ii) or iii)) is operative. Therefor, when studying product formation from pentose sugars, one should be cautious *assuming* fermentation of a pentose *via* the generally accepted pentose phosphate pathway (Fraenkel 1987).

Table 4. Proposed fermentation pathways for several strict anaerobic fermentative bacteria.

Organism	Substrate	Route [a]	Reference(s)
F. succinogenes	D-glucose	EMP/PPP/PKP	Joyner and Baldwin 1966
	D-xylose	PPP, no transport system	Matte et al. 1992
P. ruminicola 23	D-glucose	EMP/PPP/PKP	Joyner and Baldwin 1966
P. ruminicola $B_1$4	D-glucose	EMP	Howlett et al. 1976
P. ruminicola $B_1$4	L-arabinose	EMP + PPP + some PKP	Turner and Roberton 1979
P. ruminicola $B_1$4	L-arabinose	EMP + PPP	Caldwell and Newman 1986a
P. ruminicola $B_1$4	D-arabinose	EMP + PPP	Caldwell and Newman 1986b
P. ruminicola $B_1$4	D-xylose	EMP + PPP	Matte et al. 1992
P. ruminicola S23	D-xylose	EMP + PPP	Matte et al. 1992
S. ruminantium HD4	D-glucose	EMP + PPP + some PKP	Joyner and Baldwin 1966
S. ruminantium HD4	D-xylose	EMP + PPP	Matte et al. 1992
R. flavefaciens C94	D-glucose	EMP + PPP + PKP	Joyner and Baldwin 1966
R. albus strain 7	D-glucose	EMP	Joyner and Baldwin 1966
B. fibrosolvens	D-glucose	EMP + PPP + PKP	Joyner and Baldwin 1966
B. fragilis 2044	D-arabinose	see text	Caldwell and Newman 1986a
B. vulgatus 8482	D-arabinose	see text	Caldwell and Newman 1986b
B. amylophilus 78	D-glucose	EMP + PPP + PKP	Joyner and Baldwin 1966
Z. mobilis	D-glucose	EDP	Conway 1992
C. perfringens	D-ribose	EMP + PPP	Cynkin and Gibbs 1958a and 1958b
	D-xylose	EMP + PPP	

Table 4. continued

Organism	Substrate	Route [a]	Reference(s)
C. beyerinckii/butylicum	D-ribose	EMP + PPP	Cynkin and Gibbs 1958a and 1958b
	D-xylose	EMP + PPP	Yan et al. 1988
	D-glucose	EMP	
C. acetobutylicum	D-glucose	EMP	Kim et al. 1984 Rao and Mutharasan 1987 and 1989
C. butyricum	D-xylose	EMP + PPP	Heyndrickx et al. 1991b
C. pasteurianum	D-glucose	EMP	Dabrock et al. 1992
C. sphenoides	D-glucose	EMP	Tran-Din and Gottschalk 1985
C. cellobioparum	D-glucose	EMP	Chung 1976
Thermoanaerobium brockii	D-glucose	EMP	Lamed and Zeikus 1980a
Thermoanaerobacter ethanolicus	D-glucose	EMP	Lacis and Lawford 1991
	D-xylose	EMP + PPP	
Thermobacteroides acetoethylicus	D-glucose	EMP	Ng and Zeikus 1982
C. thermocellum YS	D-glucose	EMP	Ng and Zeikus 1982, Lamed et al. 1988 Patni and Alexander 1971
C. thermocellum 651	D-glucose	EMP	
C. thermosaccharolyticum	D-glucose	EMP	Lee and Ordal 1967, Cameron and Cooney 1986
C. thermohydrosulfuricum	D-glucose	EMP	Ng and Zeikus 1982

[a] For abbreviations, see text.

Product formation.

Pyruvate is the central metabolite from which most of the fermentation end-products are formed (Figure 2).

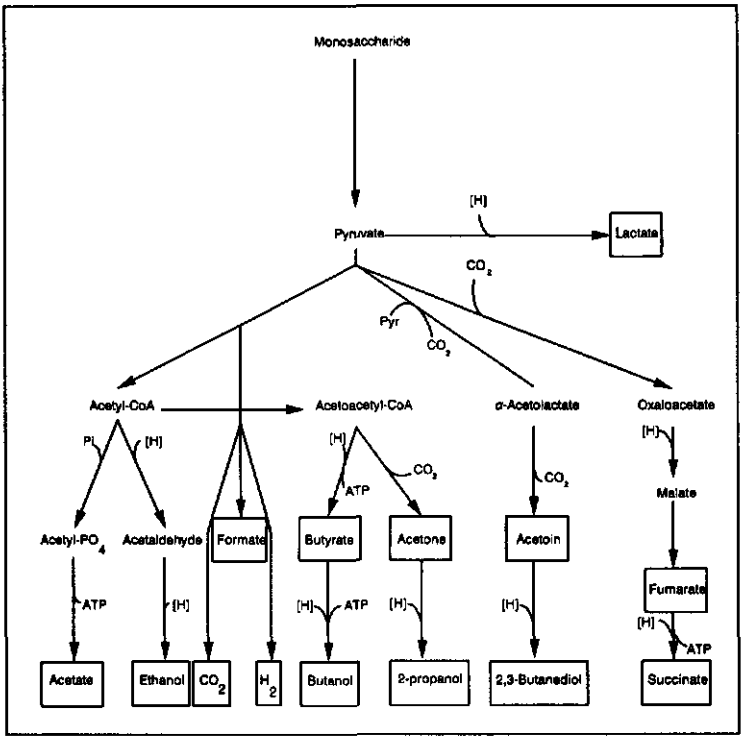

Fig.2. Fermentation pathways of the product formation from the most important sugar fermentations with pyruvate as central metabolite. (Adapted from Magee and Kosaric 1987, Schlegel 1984, and Wood 1961).

The amount of a product and the ratio between the end-products depends to a large extent on the pathway of the carbon and electron fluxes in microorganisms. These pathways can be affected by changing environmental parameters, like pH, temperature, sugar concentration, type of substrate, etc. In the following, an attempt is made to discuss the regulation of the pathways of carbon and electron fluxes, separately. However, it is often difficult to strictly discriminate between the two pathways, and regulation of one flux can affect the other.

Regulation of the carbon flow.

Fermentation of mixed substrates. Wood hydrolysates (both enzymatically and chemically obtained) consist of mixtures of sugars. Product formation from these hydrolysates may be affected adversely due to 'catabolite regulatory mechanisms' when small amounts of preferred sugars are present in these sugar mixtures (duPreez et al. 1986, Hsiao et al. 1982, Jeffries and Sreenath 1988, Kilian et al. 1983, Lee 1992, Lucas and van Uden 1986). Catabolite repression and inducer exclusion are the best known catabolite regulatory mechanisms. Both mechanisms are regulated by components of the phosphotransferase system (PTS) and have been studied extensively in *E. coli* and *S. typhimurium* (figure 3, Saier 1989).

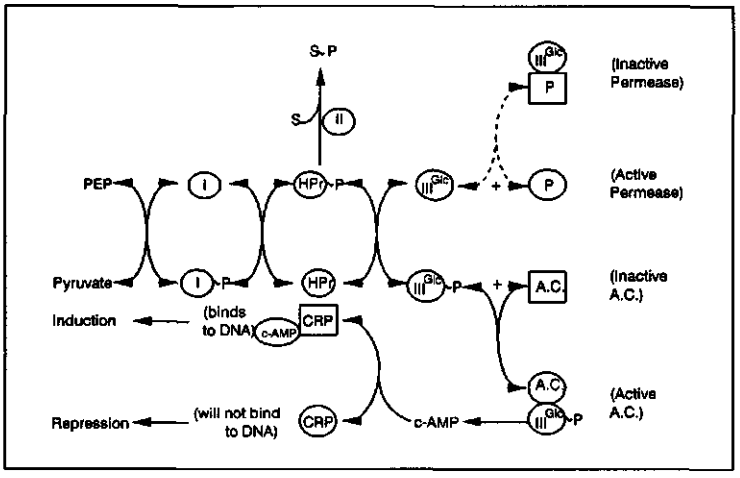

Fig.3. Mechanism of the regulation of non-PTS carbohydrate uptake systems and adenylate cyclase in *E. coli* and *S. typhimurium* by catabolite repression (solid lines) and inducer exclusion (solid and dashed lines). I, Enzyme I; II, an enzyme specific for a particular PTS-sugar (S); III^{Glc}, the central regulatory protein; HPr, heat-resistant protein; ~P, phosphate group; P, a non-PTS permease; A.C., adenylate cyclase; CRP, c-AMP receptor protein. (Adapted from Gottschalk 1985 and Saier 1989).

When a PTS-sugar is absent, the enzymes of the PTS are phosphorylated. E-III^{Glc} ~ P plays a key role in the regulation of catabolite repression and inducer

exclusion. In the case of *catabolite repression*, E-III^{Glc} ~ P will bind to adenylate cyclase (A.C.), which will become active. Active A.C. will produce cyclic-AMP (c-AMP) from ATP. c-AMP-receptor protein (CRP) is a regulator protein. In the presence of c-AMP, CRP will bind to the DNA and enzyme induction is possible. In the presence of a PTS sugar, E-III^{Glc} will not be phosphorylated and A.C. is not active. As a consequence, cyclic-AMP is not produced and CRP does not bind to the DNA, thereby preventing enzyme induction. In the case of *inducer exclusion*, E-III^{Glc} ~ P has the same function as described for catabolite repression. But also the non-phosphorylated E-III^{Glc} has a function. It will bind to the permease of a non-PTS sugar, thereby inactivating transport of the non-PTS sugar. Inducer exclusion is a much faster mechanism than catabolite repression.

Mixed substrate fermentation by strictly anaerobic rumen microorganisms has been studied extensively (Russell and Baldwin 1978, Russell and Baldwin 1979, Ounine et al. 1985, Patel et al. 1986, Cook et al. 1993, Strobel 1993a). Rumen microorganisms have a broad, overlapping range of substrates. Competition for these substrates will occur, especially because the substrate concentrations in the rumen are very low (Russell and Baldwin 1979). Different strategies to control the utilization of sugar mixtures have evolved in rumen microorganisms. These different strategies (e.g. high substrate affinities, catabolite repression, inducer exclusion) enable the various microorganisms to coexist in the rumen (Russell and Baldwin 1978 and 1979). To increase the efficiency of biotechnological product formation from sugar mixtures (e.g. wood hydrolysates), organisms without substrate preference have obtained special attention in the last decade (Patel 1984, Patel et al. 1986).

Fermentation by mixed cultures. Two major processes were investigated to get a combined polymer hydrolysis and fermentation. These are, i) sequential polymer hydrolysis and monomer fermentation (Yu et al. 1985a and b), and ii)

simultaneous saccharification and fermentation of the polymers (Asther and Khan 1984 and 1985, Cavedon and Canale-Parola 1992, Ng et al. 1981, Scheifinger and Wolin 1973, Yu et al. 1984, Yu and Saddler 1985). In the latter process an increased availability of fermentable carbohydrates is obtained and an improved product recovery is found because the (extracellular) hydrolytic enzymes are less inhibited by catabolite repression. Methanogenic cocultivation was used as well to improve polymer digestion and to produce methane from (hemi)cellulosic material (Khan et al. 1979, Latham and Wolin 1977, Pavlostathis et al. 1990, Weimer and Zeikus 1977). Most of these studies were performed with rumen microorganisms including several anaerobic fungi (Latham and Wolin 1977, Bauchop and Mountfort 1981, Mountfort et al. 1982, Joblin et al. 1990, Pavlostathis et al. 1990). These investigations showed that methanogenic cocultures are helpful in increasing the rate and the extent of the carbon flux in microorganisms from polymers to end-products (e.g. by relieving catabolite repression; Joblin et al. 1990).

Regulation by environmental parameters. In order to affect the carbon flow, much research has been done on the effect of environmental factors on the physiology of microorganisms. Much of the present knowledge in this field is obtained from research done on the acetone-butanol-ethanol fermentation by *C. acetobutylicum*. Especially the effect of pH was studied in detail, since the pH drops in a culture simultaneously with the onset of solventogenesis (e.g. Datta and Zeikus 1985, Jones and Woods 1986). The industrial strains for solvent production are grown at a pH of 6.0, indicating that pH itself is not the only trigger for solvent production (Gottschal and Morris 1981, Long et al. 1984). In continuous culture experiments with *C. acetobutylicum*, at several pH values and under limitation of either ammonia or glucose exclusively an acetate-butyrate fermentation was observed (Andersch et al. 1982, Bahl et al. 1982a and b). However, when grown under phosphate limitation and at a pH value of 4.3

almost solely acetone-butanol fermentation was obtained (Andersch et al. 1983). Enzyme activity measurements in cell free extracts of cells grown under phosphate limitation at low pH (4.3) or high pH (6.0) revealed that the catabolic carbon flow leading to solvent production was regulated at enzyme level (Andersch et al. 1983). Experiments with increased extracellular levels of acetate or butyrate at different pH values showed that during solvent production the intracellular CoA and PO_4 pools decreased drastically (Monot et al. 1984, Gottwald and Gottschalk 1985, Huang et al. 1986). Gottwald and Gottschalk (1985) pointed out that continuous cultures run under phosphate limitation also might result in decreased levels of CoA, suggesting a relation between solventogenesis and the CoA-pool. Jones and Woods (1986) formulated several parameters that are important factors for solvent production: i) low pH, ii) low growth rate, iii) an excess of the carbon substrate, iv) a definite threshold concentrations of acetate and butyrate, and v) an appropriate growth limiting factor.

Other factors affecting product formation in several microorganisms include growth a) under limiting concentrations of sulfate and magnesium (Bahl and Gottschalk 1985), b) at elevated temperatures (McNeil and Kristiansen 1985, Alexander et al. 1989, Barbosa et al. 1990), c) in the presence of metabolic inhibitors like sodium azide, dinitrophenol and polyethylene glycol (Lohmeier-Vogel and Hahn-Hägerdal 1985, Singh et al. 1991), d) under high or low concentrations of CO_2 (Caspari and Macy 1983, Parameswaran et al. 1988), e) in the presence of culture filtrate concentrate and cell-free extract concentrate (Soni et al. 1987), and f) at high sugar concentrations (Lacis and Lawford 1991). Of these studies only the use of sulfate limiting growth conditions (Bahl and Gottschalk 1985) and the use of metabolic inhibitors (Singh et al. 1991) resulted in an improved product formation. The other investigations did not result in a clear concept on how to increase product formation by microorganisms. In contrast to the findings by Bahl and Gottschalk (1985), Stephens and co-workers

(1985) reported that *C. acetobutylicum* grown under magnesium limitation displayed an increased solvent production. These conflicting results might indicate that more, thus far unknown, factors are involved in the regulation of the catabolic carbon flux.

Strain selection and strain improvement. Mutants and genetically modified microorganisms, with changed enzyme levels and metabolic pathways can help to direct the carbon flux in microorganisms. *Z. mobilis* is an interesting organism because of its high ethanol production from glucose and its high tolerance for ethanol. Pentoses are no growth substrates for this organism (Bringer-Meyer and Sahm 1988). Recombinant strains of *Z. mobilis* were constructed that contained *xylAB* genes of *Klebsiella pneumoniae* and a *tkt* gene of *E. coli*. This resulted in increased xylose isomerase, xylulose kinase and transketolase activities. However, growth on xylose as sole carbon source could still not be detected, which is probably due to the absence of a gene coding for transaldolase activity (Feldmann et al. 1992). In these recombinant cells sedoheptulose-7-PO_4 accumulated to a concentration of 9 mM, showing that the first part of the pentose phosphate pathway was present in *Z. mobilis*. *E. coli* mutants, containing the wild-type pentose catabolic pathway and an artificial operon (=*pet* operon), coding for pyruvate decarboxylase and alcohol dehydrogenase II genes of *Z. mobilis*, exhibited a change of the catabolic carbon flow towards an efficient ethanol production from xylose (Ohta et al. 1990).

Thus far only few results were obtained with genetically modified clostridia. This was due to the lack of gene transfer systems in these organisms. At present, two transfer systems have been developed (electroporation and conjugal plasmid immobilization). Problems of fundamental biological interest in clostridia can now be studied (Young et al. 1989). The two molecular techniques, combined with the knowledge of (wild-type) microbial physiology, will eventually lead to fermentation processes with the highest product recoveries.

Regulation of the electron flow.

Cofactor regeneration. The regeneration of oxidized cofactors is of vital importance for the continuation of sugar metabolism, because only limited amounts of cofactors are present in the cell. In anaerobic, fermentative bacteria re-oxidation of these cofactors occurs *via* either proton reduction or *via* the reduction of intermediates of the sugar metabolism (e.g. pyruvate, acetaldehyde, acetoacetate). Reduced ferredoxin is involved in low potential oxidation-reduction reactions, like e.g. the formation of H_2 (Glass et al. 1977, Ragsdale and Ljungdahl 1984). NAD(P)H is often observed as the electron donor in reactions leading to reduced end-products like e.g. ethanol and lactate (Petersen et al. 1991, Vancanneyt et al. 1990). However, it has been shown in clostridia that H_2 also could be produced from NAD(P)H (Thauer et al. 1969). The biochemistry of cofactor regeneration was studied extensively in clostridia (Jungermann et al. 1971, Jungermann et al. 1973, Petitdemange et al. 1976, Thauer et al. 1969, Thauer et al. 1971). Ferredoxin and Fd:NAD(P) oxidoreductases were shown to play pivotal roles in the distribution of reducing equivalents liberated during carbohydrate oxidation. The NADH:Fd oxidoreductase is obligatory activated by acetyl-CoA, whereas free CoA is a strong inhibitor of this activity. The reduction of NAD by FdH is strongly inhibited by NADH and will only proceed under conditions where the end-product (NADH) is efficiently removed (Figure 4A). NADPH:Fd oxidoreductase is not regulated by the acetyl-CoA/CoA couple, but NAD is an obligatory activator of this enzyme. NADH inhibits the oxidation of NADPH by Fd. The reduction of NADP is only inhibited to some extent by its end-product NADPH (Figure 4B).

Ferredoxin:NADP oxidoreductase was supposed to function as an anabolic enzyme to supply NADPH for biosynthesis (Jungermann et al. 1973). However, several NADPH-dependent alcohol dehydrogenases have been detected (Dürre et al. 1987, Hiu et al. 1987), suggesting that ferredoxin:NADP oxidoreductase plays

an essential role in the regulation of the catabolic electron flow.

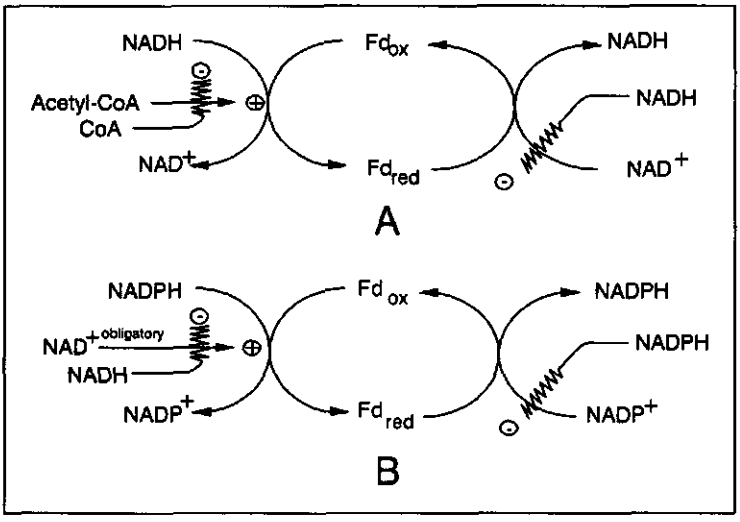

Fig.4. Scheme of the regulation of the reversible ferredoxin reduction by NADH (A) or NADPH (B). *Abbreviations:* Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin. (Redrawn from Thauer et al. 1977).

Apparently, the electron flow between these cofactors (NAD, NADP, FD) is strictly regulated. However, under appropriate physiological conditions, electrons can flow 'freely' from one reduced cofactor to the other. In the following several ways are described how the flow of electrons can be influenced.

Regulation by interspecies electron transfer. Hydrogen formation from NAD(P)H *via* the reaction :

$$NAD(P)H + H^+ \leftrightarrows NAD(P)^+ + H_2 \ (\Delta G^{0'} = +\ 18.8 \text{ kJ/reaction})$$

is under standard conditions a thermodynamically unfavourable reaction (Thauer et al. 1977, Gottschalk 1985). However, when H_2 is removed from the reaction mixture, the equilibrium will shift towards the right side of the reaction equation shown above. At a partial H_2 pressure of less than 10^{-3} atmospheres the $\Delta G'$ for this reaction will become negative (Figure 5, Gottschalk 1985) meaning that H_2

formation from NAD(P)H becomes thermodynamically possible. H_2 formation from reduced ferredoxin is much easier as can be deduced from figure 5.

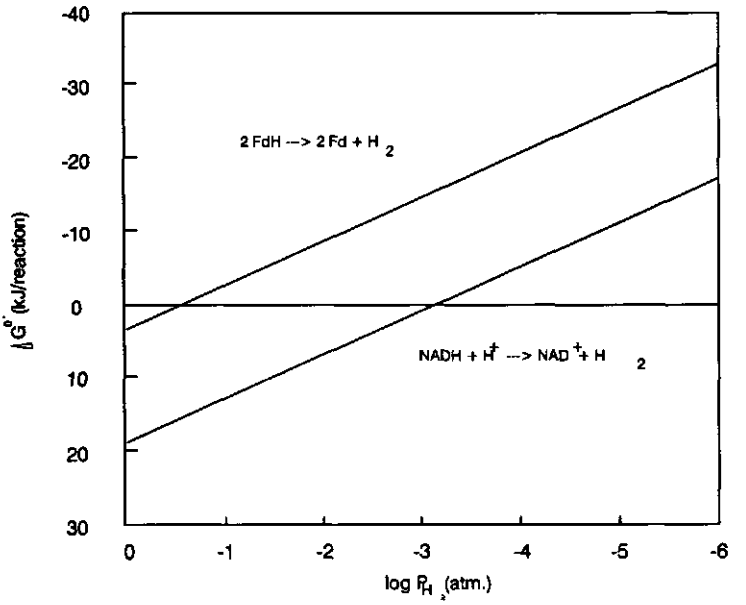

Fig.5. Effect of the partial pressure of hydrogen (P_{H_2}) on the change of free energy ($\Delta G'$) for the reactions involving H_2 formation from reduced ferredoxin (upper line) and NAD(P)H (lower line). The $\Delta G^{0'}$-values to calculate this figure were taken from Thauer et al. 1977.

Methanogenic organisms (among others) possess the ability to remove H_2 efficiently to a very low level (10^{-4}-10^{-5} atmospheres, i.e. 1-10 Pa). Therefore, when a hydrogen producing organism is cocultivated with a hydrogen consuming organism, reducing equivalents formed during substrate oxidation in the form of NAD(P)H can be used for the production of H_2. This process, i.e. the coupling of formation and consumption of H_2, is called interspecies hydrogen (or electron) transfer. Amino acid and fatty acid oxidation reactions are thermodynamically unfavourable reactions, like the H_2 formation from NAD(P)H. Nevertheless, several organisms are able to obtain energy for growth on e.g. propionate (Houwen et al. 1990), butyrate (Ahring and Westermann 1987a and b), ethanol

(Bryant et al. 1967), and glutamate (Stams and Hansen 1984) due to the process of interspecies electron transfer.

The effect of interspecies electron transfer on fermentative bacteria was first shown in a coculture of *Ruminococcus albus* with *Vibrio (Wolinella) succinogenes* growing on a glucose/fumarate mixture (Figure 6; Ianotti et al. 1973).

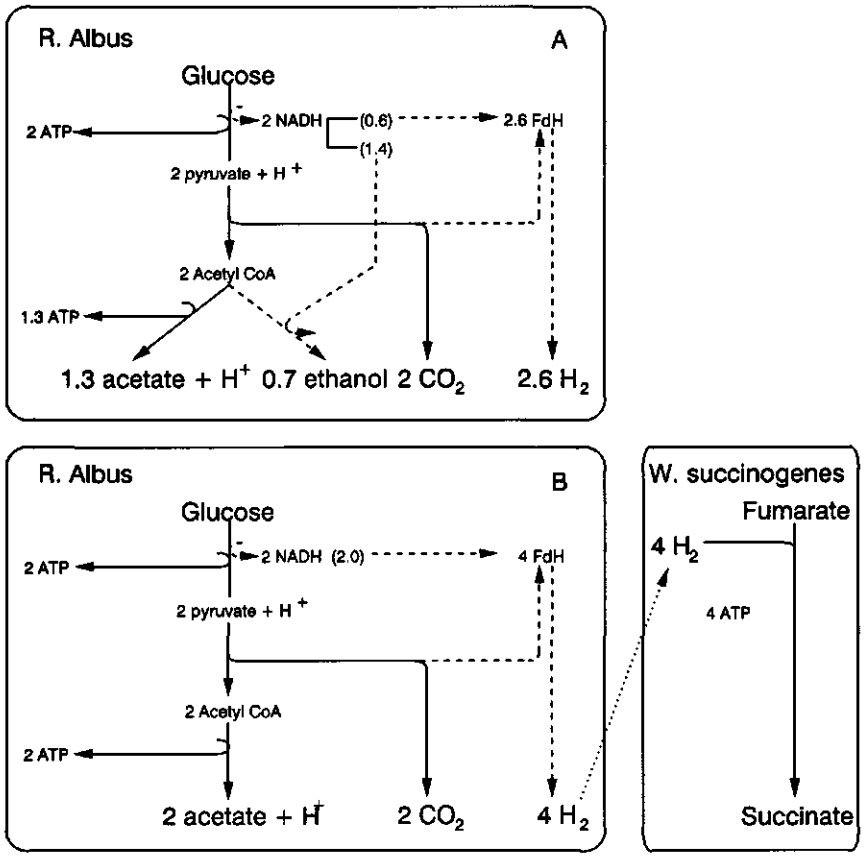

Fig. 6. The effect of interspecies electron transfer on the product formation and the ATP gain during glucose metabolism by *Ruminococcus albus*, when grown in the absence (A) and presence (B) of *Wolinella succinogenes*. (Redrawn from Thauer et al. 1977, after Ianotti et al. 1973).

In pure culture *R. albus* produced H_2, CO_2, ethanol and acetate as end-products. Due to the cocultivation a shift in product formation was observed to acetate and

CO_2 as the main end-products, whereas fumarate was stoichiometrically converted with hydrogen to succinate by *V. succinogenes*. Since the discovery of interspecies electron transfer the effects of methanogenic cocultivation on carbohydrate fermentation has been shown in many other studies (e.g. Chung 1976, Khan et al. 1979, Latham and Wolin 1977, Marvin-Sikkema et al. 1993, Mountfort et al. 1982, Pavlostathis et al. 1990, Scheifinger et al. 1975, Weimer and Zeikus 1977).

Regulation of hydrogen formation. As discussed above, H_2 is an important electron sink of reducing equivalents in anaerobic microorganisms. Inhibition of H_2 formation by the addition of high amounts of exogenous H_2, resulted in *C. saccharoperbutylacetonicum* and *C. thermocellum* in a shift in the fermentation products to more butanol and ethanol and less acetate and butyrate (Brosseau et al. 1986, Ben-Bassat et al. 1981, Freier et al. 1988, Lamed et al. 1988). A more effective method to affect H_2 formation is to inhibit the hydrogenase by carbon monoxide (CO), which is a competitive inhibitor of this enzyme (Legall et al. 1982). Modulation of electron flow using CO was studied thoroughly in *C. acetobutylicum* (Datta and Zeikus 1985, Kim et al. 1984, Meyer et al. 1986) and *C. pasteurianum* (Dabrock et al. 1992). A shift in the fermentation products to more solvents was observed. The effect of CO could be increased by the addition of acetic and butyric acids to the fermentation broth. These acids were used as alternative electron sinks by the organism, resulting in increased butanol yields. Remarkably, a complete inhibition of hydrogenase was not obtained; the H_2 production decreased maximally 50% (Datta and Zeikus 1985). Similar results were found using methyl and benzyl viologen in continuous cultures of *C. acetobutylicum* (Rao and Mutharasan 1987). Due to the addition of the viologen dye, a transient shift to the production of more reduced compounds and less H_2 was observed. It was concluded that the viologen dyes inhibited hydrogenase competitively, analogous to CO.

The trigger for the initiation of solvent formation remains still to be answered. Meyer et al. (1986) showed evidence that solvent formation in *C. acetobutylicum*

might be triggered by an *altered* electron flow. This hypothesis was further supported by the analysis of NADH levels in *C. acetobutylicum* grown under different physiological conditions (Rao and Mutharasan 1989). It was shown that not NADH levels, but $NADH/NAD^+$ turnover rates controlled the solvent formation. CO might be used as a modulator of the microbial electron flow in a fermentation process, though the optimization of this process still needs further investigations.

Cofactor regeneration with alternative electron acceptors. The value of the use of alternative electron acceptors to control the catabolic electron flow is probably underestimated. Growth of *Thermoanaerobium brockii* was shown to be inhibited completely by exogenous H_2 gas (1 atmosphere). The use of acetone not only relieved the growth inhibition, but also increased the specific growth rate and the growth yield of the organism. Furthermore, the product formation shifted to less ethanol and to more acetic acid, whereas the reducing equivalents were mainly used to reduce the acetone stoichiometrically to 2-propanol. Similar effects on growth and product formation were observed with other electron acceptors like acetate (Heyndrickx et al. 1989, Hino et al. 1991), glycerol (Talarico et al. 1990), acetoin (Delgenes et al. 1991) and pyruvate (Nuraida 1992). These results show, that several electron acceptors can be used to control the intraspecies electron flow during carbohydrate fermentation.

Regulation of catabolic electron flow at the level of enzyme activities. Lamed and Zeikus (1980b) determined the relationship between fermentation product yields and catabolic enzyme activities in *C. thermocellum* and *T. brockii*. They showed that the electron flow in these organisms was regulated by means of differences in kinetic properties of enzymes (i.e. substrate specificity, K_m and V_{max}) involved in electron transfer. Significant quantitative differences in end-product yields were observed for each organism when grown on cellobiose, due to the differences in the kinetic properties. pH-controlled enzyme induction was found with several microorganisms (Lowe and Zeikus 1991, Vancanneyt et al. 1990, Yan et al.

1988). At neutral pH, pyruvate dehydrogenase and acetaldehyde dehydrogenase were active in *Sarcina ventriculi*, with a concomitant production of acetate, ethanol and formate. When grown at acid pH (3.0), pyruvate decarboxylase activity increased significantly and acetaldehyde dehydrogenase decreased, leading primarily to ethanol production (Lowe and Zeikus 1991).
Regulation at enzyme level could also be obtained by genetical modification of an organism. As mentioned above, the *pet* operon was cloned in *E. coli*, which resulted in the expression of pyruvate decarboxylase and alcohol dehydrogenase II. As a result, the electron flow was affected and an efficient ethanol production was observed (Ohta et al. 1990). Thus far, little is known about mutants defective in ferredoxin and/or NAD(P)H ferredoxin oxidoreductases. Mutants defective in these enzymes could be of particular interest for product formation from sugars.

Bacteroides xylanolyticus X5-1 as a model organism.

As part of a study on the production of biogas from agricultural waste (cattle manure), several organisms were isolated at our department (Scholten-Koerselman et al. 1986). One of these organisms was *Bacteroides xylanolyticus* X5-1 (Figure 7).
This organism, a strictly anaerobic, non-sporeforming, motile, Gram-negative, rod-shaped bacterium, could ferment a wide range of monomeric and dimeric sugars. Furthermore, it could grow on the hemicellulose xylan, a polymer of mainly xylose molecules. Other hemicelluloses (e.g. gum xanthan, laminaran, gum arabic) and cellulose were not utilized. As xylan was the only hemicellulose degraded by this organism, it was an interesting model organism to study the biochemistry and the regulation of the xylanolytic system, as no interference could occur with cellulolytic enzymes. The research on the xylanolytic system was performed by Philippe Schyns. Endo-1,4-ß-xylanases (I and II), ß-xylosidase, acetyl esterase, and α-L-arabinofuranosidase could be detected when the organism was grown on xylan. These enzymes were inducible, as no activities were found when the organism was grown on glucose. The mode of action of these enzymes

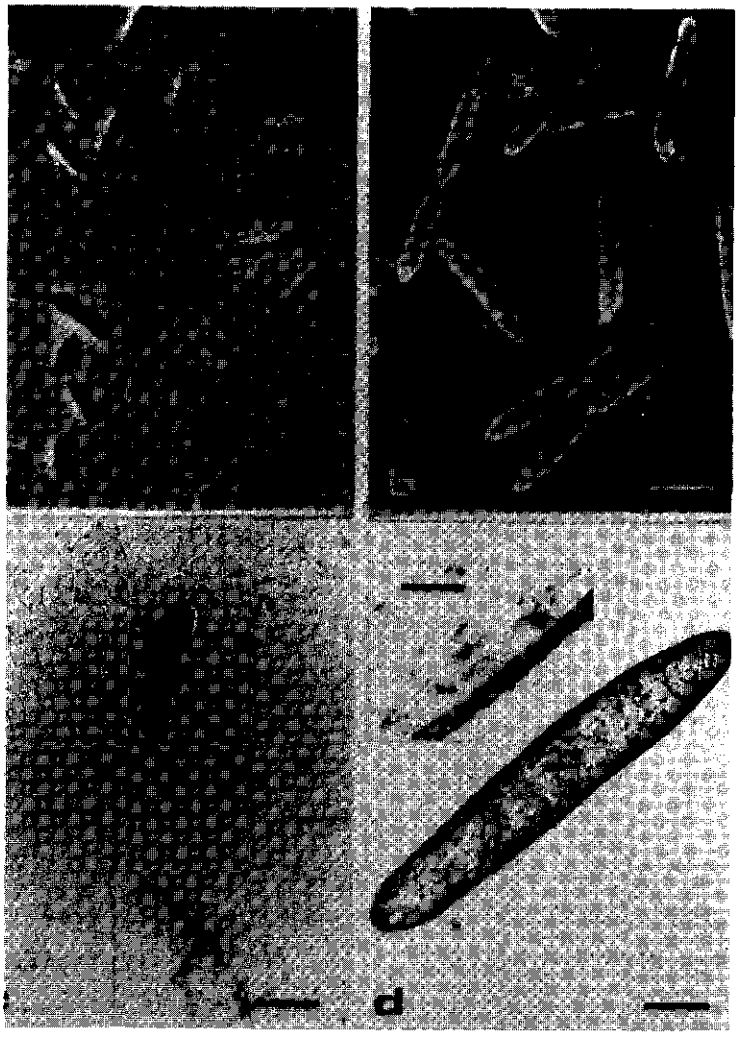

Fig. 7. a. Phase contrast photomicrograph of *B. xylanolyticus* X5-1, bar equals 4.3 μm. **b.** Scanning electron micrograph shows clearly the tapered ends, bar equals 1.4 μm. **c.** Electron micrograph depicting the length and the location of the flagella, bar equals 1.3 μm. **d.** Transmission electron micrograph indicates the tapered ends and the Gram negative cell wall (insert), bar equals 0.3 μm, in insert 7.7 nm. (Taken from Scholten-Koerselman et al. 1986).

was elucidated. Furthermore, the induction mechanisms of the xylanase and the ß-xylosidase were studied (Schyns and Stams 1992).
When grown on xylan or xylose the organism produced acetate, ethanol, H_2, CO_2, and formate as the main fermentation products. In addition, small amounts of 1,2-propanediol and lactate were produced. This suggested that the organism has a branched xylose fermentation pathway. As the organism was able to grow on a variety of sugars (including the pentose xylose) at a relatively high growth rate, and as it seems to possess a branched xylose catabolic pathway, it was an interesting model organism to study the regulation of xylose fermentation under anaerobic conditions in detail. The results of the study on regulation are described here.

Outline of the thesis.

The aim of the research presented in this thesis, was to study the biochemistry and the physiology of product formation from xylose in Bacteroides xylanolyticus X5-1. As only very little was known of this microorganism, first the xylose fermentative pathway had to be resolved. The results from substrate uptake studies, fermentation balance determination, and the elucidation of the xylose-degrading pathway by enzyme measurements and studies with ^{13}C-labelled xylose are described in chapter 2. Hemicellulose hydrolysates contain mixtures of sugars. Therefor, the conversion of glucose/xylose mixtures by *B. xylanolyticus* X5-1 and the induction mechanism of key enzymes of the xylose catabolism were studied (chapter 3). Chapter 4 describes the regulation of the anaerobic xylose metabolism by interspecies electron transfer, in particular, the regulation of the enzymes involved in ethanol production. Results obtained on the effect of alternative electron acceptors on the physiology and the biochemistry of the xylose metabolism are summarized in chapter 5. The references are given in chapter 6, and the thesis ends with the Summary (chapter 7) and the Samenvatting (chapter 8).

Chapter 2

D-Xylose catabolism in *Bacteroides xylanolyticus* X5-1

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Alexander J.B. Zehnder, and Alfons J.M. Stams
In press in Arch. Microbiol.

Abstract

The xylose metabolism of *Bacteroides xylanolyticus* X5-1 was studied by determining specific enzyme activities in cell free extracts, by following ^{13}C-label distribution patterns in growing cultures and by mass balance calculations. Enzyme activities of the pentose phosphate pathway and the Embden-Meyerhof-Parnas pathway were sufficiently high to account for *in vivo* xylose fermentation to pyruvate *via* a combination of these two pathways. Pyruvate was mainly oxidized to acetyl-CoA, CO_2 and a reduced cofactor (ferredoxin). Part of the pyruvate was converted to acetyl-CoA and formate by means of a pyruvate-formate lyase. Acetyl-CoA was either converted to acetate by a combined action of phosphotransacetylase and acetate kinase or reduced to ethanol by an acetaldehyde dehydrogenase and an ethanol dehydrogenase. The latter two enzymes displayed both a NADH- and a NADPH-linked activity. Cofactor regeneration proceeded *via* a reduction of intermediates of the metabolism (i.e. acetyl-CoA and acetaldehyde) and *via* proton reduction. According to the deduced pathway about 2.5 mol ATP are generated per mol of xylose degraded.

Introduction

Hemicellulose, a major component of plant cell walls, is a group of hetero polysaccharides consisting of hexoses and pentoses with xylose as the most abundant sugar (Gong et al. 1981). Bacteroides species and Clostridium species have been shown to degrade hemicellulose. The anaerobic product formation from hemicellulose and hemicellulose hydrolysates has been studied to some extend (Patel et al. 1986). For the genus *Bacteroides* anaerobic hexose metabolism has been documented abundantly (Joyner and Baldwin, 1966; Howlett et.al. 1976; Miller and Wolin 1979; Franklund and Glass 1987; Martin 1992).

However, relatively little attention has been paid to anaerobic pentose metabolism.

In most aerobic and anaerobic bacteria, pentoses are converted by a sequence of reactions to xylulose-5-PO_4. This is a central metabolite of the pentose phosphate pathway (PPP) and of the phosphoketolase pathway (PKP). The PPP is the main metabolic pathway for the degradation of pentose (Fraenkel 1987). It was found to be present in several anaerobic organisms like *Prevotella (Bacteroides) ruminicola brevis* B_14 (Turner and Robertson 1979; Caldwell and Newman 1986b), *Thermoanaerobacter ethanolicus* (Lacis and Lawford 1991), and *Clostridium butyricum* LMG $1213t_1$ (Heyndrickx et al. 1991a). However, also the PKP may be an important catabolic pathway in microorganisms. It was found in several Lactobacilli (Heath et al. 1958; Hurwitz 1958), *Acetobacter xylinum* (Racker 1962), *Leuconostoc mesenteroides* (Goldberg et al. 1966), *Bifidobacterium globosum* (Sgorbati et al. 1976), different yeast species (Evans and Ratledge 1984), and two Bacteroides species, *B. fragilis* strain 2044 and *B. vulgatus* strain 8482 (Caldwell and Newman 1986a). Pentose catabolism *via* the PKP or the PPP will result in a different substrate to product ratio. *Via* PKP per mol of pentose 2 C2-products (ethanol and acetate) will be formed whereas 1.67 C2-products are formed *via* the PPP. In addition, product ratios will differ because the PKP yields only 4 mol of reducing equivalents per mol of xylose, whereas the PPP will yield 6.7 mol of reducing equivalents per mol of xylose.

At our department the anaerobic degradation of the hemicellulose xylan is studied, using *Bacteroides xylanolyticus* X5-1 as a model organism. *B. xylanolyticus* X5-1 is able to grow on a variety of mono-, di- and tri-meric saccharides and on xylan, but no growth can be found on other hemicelluloses or cellulose (Scholten-Koerselman et al. 1986). The aim of the present study was to obtain detailed knowledge on the energy conservation and the cofactor regeneration during growth of *Bacteroides xylanolyticus* X5-1 on xylose.

Materials and methods

Microorganism and cultivation.

Bacteroides xylanolyticus X5-1 (DSM 3808) was isolated and described by Scholten-Koerselman et al. (1986). The organism was grown in a basal bicarbonate buffered medium with a composition as described by Huser et al. (1982). To one litre of medium 0.5 g of yeast extract, 1 ml of a trace elements solution (Zehnder et al. 1980) and 1 ml of a vitamin solution (Wolin et al. 1963) were added. The vitamin solution was filter sterilized separately. The gas phase above the medium was N_2/CO_2 (80%/20%) and the pH of the medium was 6.8-6.9. Xylose was added from a 2 M filter-sterilized stock solution. Experiments performed to determine CO_2 formation were done in a phosphate-buffered medium. Bicarbonate was replaced by 20 mM of sodium-potassium-phosphate (pH 6.8), and the gas phase was replaced by N_2.
Routinely, *B. xylanolyticus* X5-1 was cultivated at 37 °C in the dark in 120-ml serum vials with 30 ml of medium. Bottles were sealed with butyl rubber stoppers (Rubber BV, Hilversum, Holland) and aluminium caps. Mass cultivation was done at the same conditions in 1- or 3-l serum bottles or in 10-l carboys containing 0.3, 1 or 8 l of medium, respectively.

Analytical methods.

Sugars, organic acids and alcohols were quantified using a LKB high performance liquid chromatograph (HPLC) as described by Stams et al. (1993). Samples were diluted 1:1 with 20 mM xylitol in 50 mM of HCl; xylitol was used as an internal standard. 1,2-Propanediol was also measured by gas chromatography using a CP9000 gas chromatograph (Chrompack, Middelburg,

The Netherlands) as described by Stams et al. (1993). Hydrogen was determined by gas chromatography using a Packard-Becker 406 gas chromatograph equipped with a thermal conductivity detector and molecular sieve. The column temperature was 100°C and the carrier gas was argon at a flow rate of 20 ml/min. CO_2 was determined in a similar fashion as hydrogen, using a Poropak Q column. Bicarbonate was determined as CO_2; culture samples (5 ml) were injected into closed 36-ml serum bottles and 1 ml 5N HCl was added to purge the CO_2 from the liquid phase.

Growth was determined by measuring the increase in optical density at 660 nm (OD_{660}) in a LKB/Biochrom Ultraspec K spectrophotometer using cuvettes with a 1-cm light path. When necessary, samples were diluted 1:1 with water to obtain an OD_{660} below 0.4. Bacterial dry weight was quantified by centrifuging 100 ml culture samples at 20,000 × *g* for 15 min. Cells were washed once with 50 mM Tris-HCl (pH 7.8) and 2 mM of $MgCl_2$. Cell pellets were transferred quantitatively with demineralized water to preweighed aluminium trays and dried overnight at 100°C. The trays were placed in a vacuum exicator until a constant weight of the trays was obtained. Protein in cell free extracts was estimated with coomassie brilliant blue G250 as described by Bradford (1976). Total cell protein was determined after boiling of cell pellets in 1 N NaOH for 15 min according to the method described by Lowry et al. (1951). Bovine serum albumin was used as a standard for the protein determinations.

Xylose uptake.

Cells were harvested in the mid-exponential growth-phase by centrifugation under anaerobic conditions, and washed once with anaerobic medium without substrate. Cells were suspended in the same medium to an OD_{660} of 3. This value corresponded to about 0.6 mg of cell protein per ml. Unless stated otherwise,

uptake studies were performed under an atmosphere of N_2/CO_2 (80%/20%) in 13-ml vials closed with butyl rubber stoppers. The reaction mixtures (1.5 ml) were preincubated at 37°C for 10 min. The uptake study was started by addition of xylose (1 mM final concentration, 0.25 μCi/μmol). At various periods of time samples (0.2 ml) were taken using a 1-ml syringe equipped with an 18-gauge needle and filtered through prewashed membrane filters (Schleicher & Schuell BA85). Filters were washed with 4 ml of 100 mM LiCl and transferred to 4 ml aqualuma scintillation cocktail. The radioactivity was counted in a LKB Wallac liquid scintillation counter (Pharmacia/LKB, Woerden, The Netherlands). Metabolic inhibitors were dissolved either in demineralized water or in absolute ethanol (96%) and were added to the cells 10 min prior to the initiation of the uptake. Controls were incorporated to measure the effect of the solvent only.

Cell extract preparation and enzyme assays.

Unless stated otherwise, all operations were performed at room temperature under strict anaerobic conditions in an anaerobic glove box with N_2/H_2 (96%/4%) as gas phase. To remove traces of oxygen the gas phase was continuously circulated over a palladium catalyst (BASF, Arnhem, the Netherlands). Cells were harvested at the late log phase by centrifugation at 20,000 × *g* for 10 min, washed once in 50 mM Tris-HCl (pH 7.8) containing 5 mM $MgCl_2$ and 1 mM DTT, and resuspended in either the same buffer or in 20 mM triethanolamine-HCl (TEA) (pH 8.0) and 10 mM $MgSO_4$. The latter buffer was used when xylose isomerase and xylulose-5-PO_4 kinase were assayed. The cell suspension was kept on ice and disrupted by sonication (Sonics & Materials sonifier, CT, USA; 10 times 20 s with an intermittent cooling for 20 s). The cell debris was removed by centrifugation (8,000 × *g* for 15 min), and the supernatant was used as cell free extract.

Enzyme measurements and absorbance readings were made in a LKB/Biochrom Ultraspec K spectrophotometer using 1-ml cuvettes with a 1-cm light path. All enzymes were assayed anaerobically at 37°C, unless stated otherwise. Calculations of the specific activity were made in a range where linearity in time and in protein concentration was established. One unit of enzyme activity represents the amount of enzyme catalyzing the conversion of 1 μmol of substrate per min.

The following enzymes were assayed according to standard methods. Details of the individual assays are described in the references; *xylose isomerase* (Callens et al. 1986); *xylulose kinase* (Shamana and Sanderson 1979; KCN and NaF were omitted from the assay mixture); *transaldolase* (Levering et al. 1982; 50 mM Tris-HCl (pH 7.5) was used instead of 100 mM TEA); *hexokinase*, *glyceraldehyde-3-PO$_4$dehydrogenase* (assayed aerobically; 3 mM glyceraldehyde-3-PO$_4$ was used as a substrate), *lactate dehydrogenase* (100 mM KH_2PO_4 (pH 6.2) was used instead of imidazole-HCl), *hydrogenase*, *phosphotransacetylase*, *acetaldehyde dehydrogenase*, and *ethanol dehydrogenase* (Lamed and Zeikus 1980a); *phosphoglucose isomerase*, *phosphofructokinase* and *pyruvate kinase* (Wu and Racker 1959); *phosphogluconate dehydratase/6-phospho-2-keto-3-desoxygluconate aldolase* (these enzymes were assayed together), *glucose-6-PO$_4$ dehydrogenase* and *6-phosphogluconate dehydrogenase* (van Dijken and Quayle 1977); *pyruvate carboxylase* (Scrutton et al. 1969); *malate dehydrogenase* (Stams et al. 1984); *pyruvate decarboxylase* (Bringer-Meyer et al. 1986); *formate-H$_2$-lyase* (Houwen et al. 1990); *formate dehydrogenase* (Thauer et al. 1973); *acetate kinase* (assayed aerobically; Lamed and Zeikus 1980b); *methylglyoxal reductase* (assayed aerobically; Willets and Turner 1970). *Pyruvate-formate-lyase* was assayed according to the method described by Jungermann and Schön (1974). Pyruvate removal and formate production were analyzed by HPLC. The assay

was performed under an atmosphere of carbon monoxide to prevent H_2 formation (Thauer et al. 1972). *Acetol reductase* was assayed in the same fashion as ethanol dehydrogenase but with acetol (=hydroxy propanon) instead of acetaldehyde. *Phosphoketolase* activity was assayed by determining both the amount of acetyl-PO_4 and the amount of glyceraldehyde-3-PO_4 released from xylulose-5-PO_4. Glyceraldehyde-3-PO_4 was determined as described by Evans and Ratledge (1984). Acetyl-PO_4 was determined as ferric acetyl hydroxamate as described by Whitworth and Ratledge (1977). *Pyruvate:ferredoxine oxidoreductase* was assayed using methyl viologen (MV) as electron acceptor. The assay mixture contained 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 2 mM $MgCl_2$, 2 mM MV, 0.2 mM HSCoA, 20 mM sodium pyruvate. *Transketolase* was assayed in a mixture that contained 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 2 mM $MgCl_2$, 0.5 U of triose-phosphate isomerase, 0.5 U of α-glycero-phosphate dehydrogenase, 3 mM thiamine pyrophosphate, 0.15 mM NADH, 1 mM ribulose-5-PO_4 and 1 mM xylulose-5-PO_4. *Ferredoxine (MV):NAD(P) oxidoreductase* was assayed in 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 2 mM MV, gas phase 100% CO (1 atmosphere), 1 mM of either NAD or NADP. MV was prereduced with small amounts of sodium dithionite. Pyridine nucleotide oxidation or reduction was measured at 340 nm ($\epsilon_{340\ nm}$=6.22 $mM^{-1}cm^{-1}$). Methyl viologen reduction or oxidation was measured at 560 nm ($\epsilon_{560\ nm}$=8.0 $mM^{-1}cm^{-1}$)

Nuclear magnetic resonance experiments.

Media (10 ml) were prepared in 30-ml serum bottles. The media contained 5 mM D-[1-^{13}C]-xylose and 15 mM unlabelled xylose. To study xylose conversion in a growing culture of *B. xylanolyticus* X5-1 the bottles were inoculated (2.5 %) with a xylose fermenting culture and incubated at 37°C for 22 hours. Periodically 2.1 ml samples were taken and the cells were centrifuged. D_2O (25 % v/v) was

added to the supernatant to provide the NMR lock signal and 1 mM of [3-^{13}C]propionate was used as internal standard, resulting in a final sample volume of 3.0 ml. ^{13}C-labelled compounds were analyzed at 75.47 MHz with a Bruker AMX-300 Fourier-Transform spectrometer equipped with a 10 mm probe-head. The sample temperature was kept at 16°C. For all samples 7200 (2 hrs) free induction decay's were accumulated into 16k data points and stored on disk, using a puls angle of 45° (9 μs) and an interpulse delay of 1 s; a spectral range of 20,000 Hz was covered. ^{13}C-NMR spectra were obtained after zero-filling to 64k data points and subsequent Fourier transformation, using a Lorentzian line-broadening of 3 Hz. The analyzed products were quantified by integration of their resonance intensities. Resonance intensities were corrected for differences in Nuclear Overhauser Effect enhancement and relaxation by comparison with a calibration spectrum of an equimolar mixture (50 mM) of all measured compounds in the medium used and recorded under identical conditions as the fermentation samples.

Chemicals.

All chemicals were at least of analytical grade. Enzymes and biochemicals were obtained from Boehringer Mannheim (Almere, The Netherlands), from Sigma Chemical Co. (Amsterdam, the Netherlands), or from Merck (Darmstadt, F.R.G.). D-^{12}C-xylose was purchased from Janssen (Geel, Belgium), D-[1-^{13}C]-xylose from Campro Scientific (Elst, The Netherlands) and D-[U-^{14}C]-xylose from Amersham ('s Hertogenbosch, The Netherlands). Gases and gas mixtures were supplied by Hoekloos (Schiedam, The Netherlands). Aqualuma scintillation cocktail was obtained from Lumac (Landgraaf, The Netherlands)

Results

Xylose fermentation and product formation.

Growth and product formation during xylose conversion by *B. xylanolyticus* X5-1 are given in figure 1.

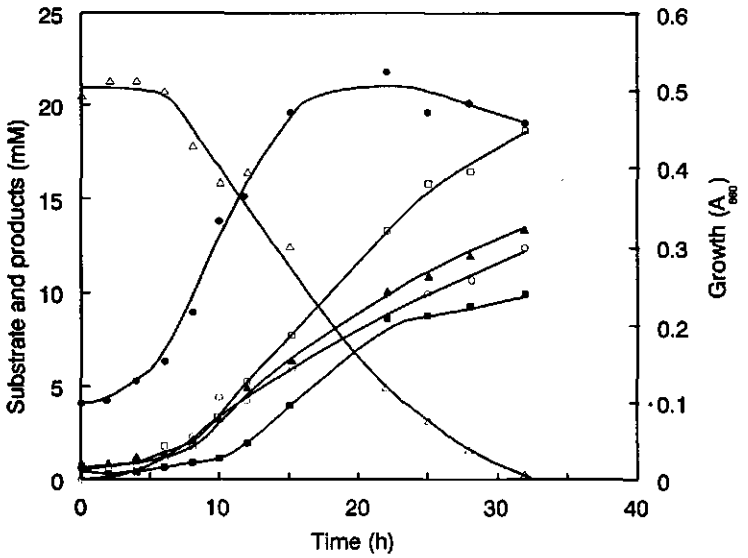

Fig. 1. Growth and product formation by *B. xylanolyticus* X5-1 during xylose catabolism under standard conditions on 20 mM of xylose at 37°C in the dark. A_{660} (•), xylose (△), acetate (▲), ethanol (□), formate (■), H_2 (○).

The short exponential growth phase is characteristic for the organism. In the medium used in this study its maximum specific growth rate was 0.11 ± 0.02 h^{-1}. Table 1 shows the fermentation balance after growth on 20 mM of xylose for 48 hours. A reasonable good carbon and electron recovery was found. The main fermentation products were acetate, ethanol, H_2, CO_2 and formate. Small amounts of lactate and 1,2-propanediol were also produced. During fermentation about 8.7 g of dry cells per mol of xylose were formed.

Table 1. Xylose fermentation products of *Bacteroides xylanolyticus* X5-1.

product	amount[a]
acetate	0.66
ethanol	0.61
H_2	1.32
CO_2	1.75
formate	0.07
lactate	0.01
1,2-propanediol	0.02
biomass (g/mol xylose)	8.7
e-recovery (%)[b]	87
C-recovery (%)[b]	96

[a] Unless otherwise stated the amount of the products is given in mmoles per mmoles of xylose converted by *Bacteroides xylanolyticus* X5-1 after 48 hours of growth at 37°C.
[b] For the calculation of carbon and electron recovery, biomass was estimated using $<C_5H_7O_2N>$ as the structural formula for biomass with a molecular weight of 113 g/mol.

Xylose uptake and the effect of metabolic inhibitors.

Xylose uptake by washed whole cells of *B. xylanolyticus* X5-1 was linear for at least 6 minutes. An uptake rate of 20 nmol/min.mg cell protein was found. The effects of several metabolic inhibitors on xylose uptake were examined (Table 2). Strong inhibitors (>50%) of xylose uptake were mercury chloride, sodium arsenate, molecular oxygen, molecular hydrogen and 2,4-dinitrophenol. To some extent the uptake was also inhibited by the ATPase inhibitor DCCD. Valinomycin/nigericine and CCCP had only little effect on xylose uptake. Neither menadione nor Antimycin A inhibited the xylose uptake significantly. Xylose uptake was not affected by D-glucose and hardly affected by D-arabinose.

Table 2. Effect of metabolic inhibitors on xylose uptake by whole cells of *Bacteroides xylanolyticus* X5-1

inhibitor[a,b]	% inhibition
none	0
O_2	100
$HgCl_2$* (0.5 mM)	85
Sodium Arsenate* (10 mM)	55
Antimycine A (50 μM)	5
Menadione (0.1 mM)	10
Valinomycine/Nigericine (30 μM each)	22
CCCP (50 μM)	21
2,4-DNP (1 mM)	78
Monensin (12 μM)	29
DCCD (0.1 mM)	41
H_2/CO_2 (gas phase 80%/20%)	60
D-arabinose (15 mM)	22
D-glucose (15 mM)	0

CCCP, carbonylcyanide m-chlorophenyl hydrazone
DCCD, N,N-dicyclohexylcarbodiimide
2,4-DNP, 2,4 dinitrophenol
[a] Control specific activity values were 19 nmol/min.mg for untreated cells and 13 nmol/min.mg for ethanol treated cells.
[b] Inhibitors marked with an asteriks were dissolved in water and compared to controls without addition. All other inhibitors were dissolved in absolute ethanol and compared to incubations with the same amount of ethanol (final concentration 1% (v/v)).

Enzymes involved in the conversion of xylose to pyruvate.

From experiments with cell suspensions it became clear that *B. xylanolyticus* X5-1 was able to convert xylose at a rate of about 90 nmol/min.mg cell protein. Therefore, the specific activities of the key enzymes of the xylose metabolism should be in this range or higher. Table 3 shows the specific enzyme activities measured in cell extracts of *B. xylanolyticus* X5-1 grown on 20 mM of xylose.

Table 3. Specific activities of enzymes found in cell free extracts of *Bacteroides xylanolyticus* X5-1 grown on 20 mM xylose.

Enzyme	Cofactor	Sp. act[a]
xylose isomerase		0.08
xylulose kinase		0.2
transketolase		0.1
transaldolase		0.27
phosphoketolase		0.01
hexokinase		0.09
phosphoglucose isomerase		0.2
phosphofructokinase		0.08
glyceraldehyde-3-PO_4 dehydrogenase	NADH	7.5
	NADPH	0
pyruvate kinase		0.075
pyruvate-ferredoxin (MV) oxidoreductase	MV	0.5
pyruvate-formate lyase		0.02
pyruvate carboxylase		0.6
malate dehydrogenase	NADH	0.75
phosphotransacetylase		0.18
acetate kinase		0.8
acetaldehyde dehydrogenase	NADH	0.08
	NADPH	0.005
ethanol dehydrogenase	NADH	0.75
	NADPH	0.2
hydrogenase	MV	7.3
MV:NAD oxidoreductase	MV	0.025
MV:NADP oxidoreductase	MV	3.1
methyl glyoxal reductase	NADH	0.01
	NADPH	0.09
acetol reductase	NADH	0.04
	NADPH	0.35

No detectable activities could be found for the following enzymes; glucose-6-PO_4 dehydrogenase, phosphogluconate dehydrogenase, glucose dehydratase/6-phospho-2-keto-3-desoxy aldolase, pyruvate decarboxylase, lactate dehydrogenase[b], formate-hydrogen lyase, formate dehydrogenase and NADH:NADP transhydrogenase.
MV = Methyl viologen.
[a] Values are expressed as units per milligram protein and are the average of at least three separate measurements.
[b] See text.

Xylose isomerase, xylulose-5-PO_4 kinase, transketolase and transaldolase were detected, suggesting conversion of xylose via the pentose phosphate pathway to

fructose-6-PO_4 and glyceraldehyde-3-PO_4. Phosphoketolase could be detected when glyceraldehyde-3-PO_4 formation was taken to calculate the activity. However, acetyl-PO_4 formation from xylulose-5-PO_4 could not be detected. The activities found for glucokinase, phosphoglucose isomerase, phosphofructokinase, glyceraldehyde-3-PO_4 dehydrogenase and pyruvate kinase suggested the conversion of fructose-6-PO_4 and glyceraldehyde-3-PO_4 to pyruvate via the Embden-Meyerhof-Parnas pathway. Activities of glucose-6-PO_4 dehydrogenase, phosphogluconate dehydrogenase and glucose-6-PO_4 dehydratase/6-phospho-2-keto-3-desoxy aldolase could not be detected, suggesting the absence of the hexose monophosphate shunt and the Entner Douderoff pathway.

Enzymes associated with product formation from pyruvate.

Pyruvate conversion could be subscribed to the action of the enzymes pyruvate:ferredoxin oxidoreductase and pyruvate-formate lyase. No activities were found for pyruvate decarboxylase and lactate dehydrogenase. Lactate formation when cells were grown on 20 mM xylose was relatively low. However, when cells were grown under an atmosphere of CO/CO_2 more lactate was formed and a NADH-dependent lactate dehydrogenase activity of 50 nmol/min.mg protein could be detected (data not shown). A methyl viologen-linked hydrogenase was detected. Formate dehydrogenase and formate-hydrogen lyase activities could not be detected in cell extracts of *B. xylanolyticus* X5-1. Acetyl-CoA forms a branching point in the xylose metabolism of *B. xylanolyticus* X5-1. It was either converted to acetate or reduced to ethanol. Acetate is probably formed by the action of phosphotransacetylase and acetate kinase. Ethanol formation proceeds apparently *via* a NAD(P)H-linked acetaldehyde dehydrogenase and a NAD(P)H-linked ethanol dehydrogenase.

Enzymes coupled to the production of 1,2-propanediol.

1,2-Propanediol may be synthesized via the methyl glyoxal bypass (Tran-Din and Gottschalk 1985; Cameron and Cooney 1986). In this bypass dihydroxyacetone phosphate is converted to methyl glyoxal, which is then reduced in two steps via acetol or lactaldehyde to 1,2-propanediol. We found activities for methylglyoxal reductase and acetol reductase in cell extracts of *B. xylanolyticus* X5-1. The reduction reactions showed higher NADPH-dependent than NADH-dependent activities. Methyl glyoxal synthase activity could also be detected, but the activity was not linear with the amount of cell extract added.

D-[1-^{13}C]-xylose fermentation by B. xylanolyticus X5-1.

Figure 2 shows a stack plot of ^{13}C-NMR spectra of culture supernatant taken periodically during growth of *B. xylanolyticus* X5-1 on a mixture of 5 mM D-[1-^{13}C]-xylose and 15 mM unlabelled xylose.

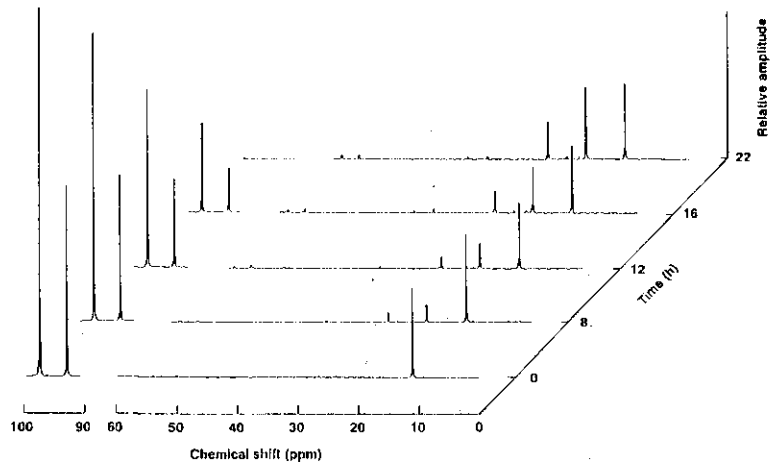

Fig. 2. Time course of the conversion of a mixture of 5 mM of D-[1-^{13}C]-xylose and 15 mM of unlabeled xylose in a growing culture of *B. xylanolyticus* X5-1 using the *in-vivo* NMR-technique. The resonances belonging to the products formed are enlarged by a factor of 4 in vertical sense. Resonances of the carbon atoms of xylose other than the C-1 position are not shown. Chemical shifts (ppm): 11.2, [3-^{13}C] propionate; 17.8, [2-^{13}C] ethanol; 24.2, [2-^{13}C] acetate; 58.5, [1-^{13}C] ethanol; 93.2, α-[1-^{13}C]-xylose; 97.6, ß-[1-^{13}C]-xylose.

All 10 resonances of the α- and the ß-form of xylose could be detected due to the natural abundance of ^{13}C-labelled carbon in the unlabelled substrate. A preferential utilization of the α- or the ß-form of the xylose was not observed. Concomitantly with the xylose utilization a production of both acetate and ethanol, labelled at the C-2 position was found. During degradation, also some [1-^{13}C]-ethanol was formed and small amounts of some unidentified products. From proton-carbon coupling spectra (data not shown) it could be deduced that about 10% scrambling of the label had occurred during xylose degradation. Assuming that 6 % of the xylose was used for biomass synthesis we could recover 63 % of the label as C-2 product.

Discussion

The data presented here are in accordance with a xylose fermentative pathway in *Bacteroides xylanolyticus* X5-1 as depicted in figure 3. Xylose uptake probably is an active process as can be deduced from the specificity of the uptake system (Gottschalk 1985) and the inhibition by 2,4-dinitrophenol, $HgCl_2$, arsenate and DCCD (Franklund and Glass 1987; Martin 1992; Williams and Martin 1990). However, the mechanism of xylose uptake in *B. xylanolyticus* X5-1 remains to be elucidated. After transport, xylose is isomerized, phosphorylated and converted *via* the enzymes of the pentose phosphate pathway and the Embden-Meyerhof-Parnas pathway to pyruvate. The activities of the key enzymes of both metabolic pathways are sufficiently high to account for the *in vivo* xylose conversion rates. Pyruvate is converted to acetyl-CoA, resulting in the formation of either formate or a low potential reduced cofactor (like ferredoxin) and CO_2. Pyruvate-formate lyase was the only formate producing enzyme which could be detected. Acetate is formed by the action of a phosphotransacetylase and an acetate kinase. Ethanol is produced in two steps from acetyl-CoA by a NAD(P)H dependent acetaldehyde dehydrogenase and a NAD(P)H-linked ethanol dehydrogenase.

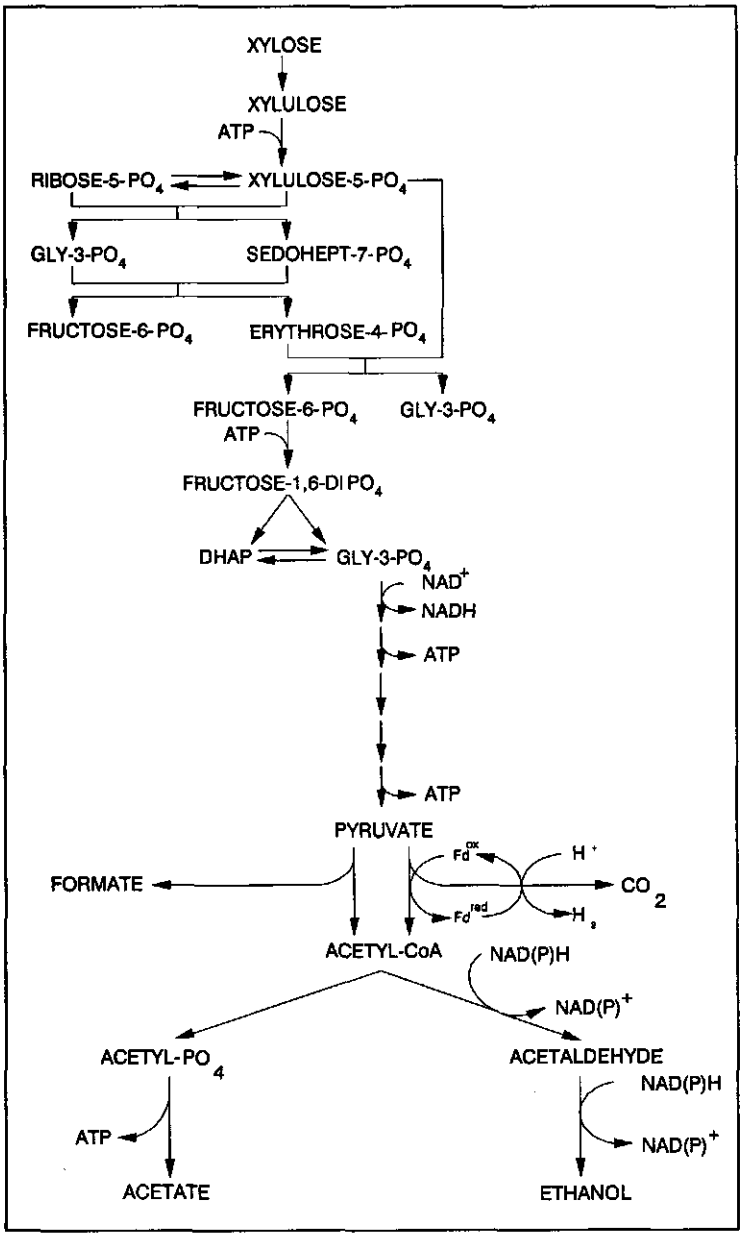

Fig. 3. Xylose fermentative pathway in *B. xylanolyticus* X5-1. (Sedohept = sedoheptulose; Gly-3-PO_4 = glyceraldehyde-3-PO_4; DHAP = dihydroxyacetone phosphate; Fd = Ferredoxin; CoA = coenzyme A).

The hexose monophosphate pathway and the Entner-Douderoff pathway can be excluded as metabolic pathways because the key enzyme glucose-6-PO_4 dehydrogenase could not be detected and because of the little scrambling of the label found in the ^{13}C-NMR experiments. Involvement of these pathways would have resulted in a significant decrease in the label present in the C2-products, as an increased amount of $^{13}CO_2$ would be formed. In the C2-products 63% of the label was recovered. This corresponds to 95% of the theoretical possible percentage (66%) when [1-^{13}C]-xylose is degraded *via* the PPP. The formation of [1-^{13}C] ethanol could be explained by scrambling of label *via* the reaction sequence pyruvate, oxaloacetate, malate, fumarate. Fumarate is a symmetric molecule resulting in a scrambling of label at the C-3 and the C-2 position in pyruvate. The C-2 position of pyruvate becomes the C-1 position in ethanol. To detect intermediates of the metabolism using the ^{13}C-NMR technique relatively high concentrations of compounds must be formed and be present during a longer period of time. Using cell suspensions of *B. xylanolyticus* X5-1 ($\pm$ 10^{12}cells/ml) we were able to detect the three characteristic resonances (the α-, the ß- and the open chain-form) of [1-^{13}C]-xylulose (data not shown). Other intermediates could not be detected, suggesting that the phosphorylation of xylulose is the rate limiting step in the xylose metabolism.

Using the growth yield and the product ratios as given in table 1, thereby neglecting the small amounts of 1,2-propanediol and lactate, a mass balance for the catabolism of xylose can be calculated (McCarty 1972). The following overall stoichiometric reaction equation was found:

$$C_5H_{10}O_5 + 0.913\ H_2O \rightarrow 0.86\ \text{Acetate} + 0.79\ \text{Ethanol} + 1.60\ CO_2 + 1.72\ H_2 + 0.10\ \text{Formate} + 0.96\ H^+ \quad (\Delta G^{0'} = -181.4\ \text{kJ/reaction})$$

The ratio substrate : C2-product = 1 : 1.64 and the ratio C1 : C2 product = 1.04 : 1. The amounts of ethanol, formate and H_2 can only be produced if 3.4 mol of reduced cofactors are available. These data fit very well with the expected

values for xylose fermentation *via* the PPP. The ratios should be 1 : 1.67 and 1 : 1, and the fermentation of one mol of xylose to acetyl-CoA should yield 3.4 moles of reduced cofactors. If the PKP had played a significant role in the xylose catabolism, both ratios would have been 1 : 2. These considerations strongly suggest the idea that xylose is solely fermented *via* the PPP. The activity found for the enzyme phosphoketolase is probably caused by an artifact as only glyceraldehyde-3-PO_4 formation but not acetyl-PO_4 formation could be detected from xylulose-5-PO_4. The glyceraldehyde-3-PO_4 formed in the assay for PKP could have been the result of a combined action of ribulose-5-PO_4-3-epimerase, ribose-5-PO_4 isomerase and transketolase. Studies suggesting the involvement of phosphoketolase as a major catabolic pathway in pentose metabolism (e.g. Evans and Ratledge 1984; Lachke and Jeffries 1986) in which only glyceraldehyde-3-PO_4 was determined as a product of the enzymatic reaction, should for this reason be interpreted with caution.

According to the proposed pathway 1.67 mol of NADH and 1.67 mol of reduced ferredoxin will be formed per mol of xylose converted to acetyl-CoA. Regeneration of the oxidized cofactors in this organism proceeds mainly *via* reduction of acetyl-CoA and acetaldehyde or *via* proton reduction, and to a minor extend *via* formation of 1,2-propanediol and lactate. NAD is regenerated by a NADH-dependent acetaldehyde dehydrogenase and a NADH linked ethanol dehydrogenase. Electrons from reduced ferredoxin are used for proton reduction to form H_2. Moreover, they can be transferred *via* the ferredoxin:NADP oxidoreductase to NADP, yielding NADPH. This can be used for reduction of acetaldehyde to ethanol and is needed for biosynthesis. Other reactions generating NADPH (e.g. NADH:NADP transhydrogenase, glucose-6-PO_4 dehydrogenase or 6-phosphogluconate dehydrogenase) could not be detected. The low activity found for the ferredoxin:NAD oxidoreductase suggests that under standard growth conditions no electron transfer occurs between ferredoxin and NADH.

Substrate level phosphorylation will yield 1.67 mol of ATP per mol of xylose degraded to acetyl-CoA and 1 additional mol of ATP per acetyl-CoA converted to acetate. On the basis of the data presented here it is not likely that additional ATP is formed *via* electron transport phosphorylation or decarboxylation linked Na^+ export. End-product-mediated proton extrusion, as described for lactic acid bacteria and *E. coli* (Konings 1985) might be an additional way of energy conservation for *B. xylanolyticus* X5-1. The amount of ATP equivalents used for xylose uptake or produced during end-product-mediated proton extrusion is not known. From the calculated mass balance we can deduce a nett ATP yield for *B. xylanolyticus* X5-1 of 2.53 moles of ATP per mol of xylose. This corresponds to a Y_{ATP} of about 3.5 g/mol, which is low when compared to the generally accepted value of 10.5 g/mol. However, it is known that this value can be strongly influenced by the growth conditions (Stouthamer 1978). The Gibbs free energy change for ATP formation in *B. xylanolyticus* X5-1 would be -72 kJ/mol. For *Clostridium pasteurianum* when grown on glucose a value of -70.6 kJ/mol ATP was found (Thauer et al. 1977). From other organisms it has been shown that the Gibbs free energy change for biological ATP formation was in the same range (Kröger 1980).

Chapter 3

Xylose and glucose utilization by batch and continuous culture cells of *Bacteroides xylanolyticus* X5-1

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Abstract

During cultivation on a mixture of xylose and glucose *B. xylanolyticus* X5-1 showed neither diauxic growth nor a substrate preference. Xylose limited, continuous culture cells were able to consume xylose and glucose both as single substrates and as mixed substrates without any lag phase. When glucose was the growth limiting substrate the microorganism was unable to consume xylose. However, in the presence of a small amount of glucose or pyruvate, xylose was utilized after a short lag phase. In glucose limited cells xylose isomerase was present in low activity, but xylulose kinase activity could not be detected. Upon addition of a mixture of xylose and glucose, xylose isomerase was induced immediately and xylulose kinase was induced after about 30 minutes. The induction of the two enzymes was sensitive to chloramphenicol showing *de novo* synthesis. Xylose uptake in glucose grown cells was very low, but the uptake rate could be increased when incubated with a xylose-glucose mixture. The increase in the uptake rate was not affected by chloramphenicol indicating that a constitutive uptake system had to be activated. The inability of *B. xylanolyticus* X5-1 to induce the xylose catabolic pathway in glucose limited continuous culture cells by addition of only xylose probably was caused by energy limitation.

Introduction

Hemicelluloses are short, branched-chain heteropolysaccharides of mixed pentosans and hexosans (Gong et al. 1981). Hydrolysis of these polymers yields mixtures of mainly xylose, arabinose, glucose, glucuronic acid, mannose and galactose (Gong et al. 1981, Rosenberg 1980). Fermentation of hemicellulose and hemicellulose hydrolysates was a major subject of research, mostly in connection with the production of ethanol (duPreez et al. 1986, Hsiao et al. 1982, Kilian et al. 1983, Patel 1984, Patel et al. 1986). One of the problems in this fermentation

was the inability of many microorganisms to efficiently metabolize all the carbohydrates derived from hemicellulose (Patel 1984). Moreover, the presence of mixtures of sugars may lead to a sequential utilization of substrates (Patel et al. 1986, Russell and Baldwin 1978). Because glucose often is the preferred substrate, xylose utilization was adversely affected (Lee 1992).

Sequential substrate fermentation is known to be under catabolite regulatory control mechanisms like catabolite inhibition, catabolite repression and inducer exclusion (Saier 1989). For ruminal bacteria both sequential and simultaneous substrate utilization was reported (Russell and Baldwin 1978, Russell and Baldwin 1979, Strobel 1993a). Different strategies for mixed substrate utilization are used in these organisms. These strategies include control of carbohydrate utilization by substrate affinities, catabolite regulatory mechanisms and phosphotransferase system mediated transport of carbohydrate (Russell and Baldwin 1979, Strobel 1993a). Much research has been conducted on mixed substrate utilization by anaerobic microorganisms with xylose as one of the constituent sugars (duPreez et al 1986, Hsiao et al. 1982, Lee 1992, Patel et al. 1986, Russell and Baldwin 1979, Standing et al. 1972). The regulation of key enzymes involved in the anaerobic xylose catabolism plays a crucial role. Xylose uptake, xylose isomerase and xylulose kinase are regulated differently in *Prevotella ruminicola* B_14 and S23 (Matte et al. 1992, Strobel 1993b), *Selenomonas ruminantium* strain HD4 and strain D (Matte et al. 1992, Strobel 1993a, Williams and Martin 1990), and in *Clostridium acetobutylicum* (ATCC 824) (Ounine et al. 1985).

Bacteroides xylanolyticus X5-1 is a strictly anaerobic hemicellulolytic organism. It can ferment several mono-, di-, and trimeric sugars and the hemicellulose xylan. Cellulose and hemicelluloses other than xylan are not utilized (Scholten-Koerselman et al. 1986). Xylose, the main constituent of xylan, is mainly fermented to acetate, ethanol, H_2, CO_2 and formate. Labelling studies and enzyme levels showed that the pentose phosphate pathway in conjunction with the glycolysis is involved in xylose catabolism. This route of xylose fermentation is

common in anaerobic bacteria growing on xylose (Caldwell and Newman 1986b, Heyndrickx et al. 1991a, Lacis and Lawford 1991, Turner and Roberton 1979). In this study, the utilization by *B. xylanolyticus* X5-1 of xylose as single and as mixed substrate was investigated in both batch and continuous cultures.

Materials and methods

Organism and cultivation.

Bacteroides xylanolyticus X5-1 (DSM 3808) was isolated and described by Scholten-Koerselman et al. (1986). The organism was grown in a basal bicarbonate buffered medium with a composition as described by Huser et al. (1982). To one litre of medium 0.5 g of yeast extract, 1 ml of a trace elements solution (Zehnder et al. 1980) and 1 ml of a vitamin solution (Wolin et al. 1963) were added. The vitamin solution was filter sterilized separately. The gas phase above the medium was N_2/CO_2 (80%/20%) and the pH of the medium was 6.8-6.9. Xylose and glucose were added from 2M filter-sterilized stock solutions. In batch cultures the organism was cultivated in 250-ml serum bottles containing 100 ml medium with 20 mM of substrate. For the continuous culture experiments the same medium was used except that 0.2% of yeast extract was added instead of 0.05%. In addition, 0.05% of cysteine was added. Continuous cultivation was performed at 37°C in 1L chemostats with a working volume of 500 ml. The cultures were grown at a dilution rate of 0.1 h^{-1}. The pH was maintained at 7.0 $\pm$ 0.1 with 2N NaOH. A continuous stream of N_2/CO_2 (80%/20%) at a flow rate of 130 ml per hour was led over the cultures. To guarantee steady state conditions cells were analyzed after at least 6 volume changes.

Mixed substrate utilization.

B. xylanolyticus X5-1 was adapted to glucose or xylose by subculturing the organism (1% inoculum) for three times on either substrate. Adapted log phase cells were used as inoculum (10%) to test growth in batch cultures on single substrates (20 mM glucose or 20 mM xylose) and on mixed substrates (10 mM glucose and 10 mM xylose). At different time intervals samples were taken to determine substrate consumption and growth.

Induction experiments.

The induction of xylose uptake, xylose isomerase and xylulose kinase was studied with glucose limited continuous culture cells. Steady state continuous culture cells were harvested anaerobically and divided up into serum bottles. The gas phase was changed to N_2/CO_2 (80%/20%) and the bottles were preincubated for 15 minutes at 37°C in the absence or presence of chloramphenicol (0.4 mg/ml). To the cells either 15 mM glucose, 15 mM xylose, 5 mM glucose + 15 mM xylose, 50 mM pyruvate or 50 mM pyruvate + 15 mM xylose were added. At different time intervals samples were taken and analyzed for xylose uptake, xylose isomerase and xylulose kinase activities. In addition, the substrate consumption was determined.

Preparation of cell free extracts and enzyme assays.

Cells were harvested by centrifugation at 20,000 × *g* for 10 min and washed once in 20 mM triethanolamine-HCl pH 8.0 and 10 mM $MgSO_4$. The cell pellets were resuspended in this buffer solution and disrupted by sonication (Sonics & Materials sonifier, CT, USA; 10 times 20 s with an intermittent cooling for 20 s). The cell debris was removed by centrifugation (8,000 × *g* for 15 min), and

the supernatant was used as cell free extract. Xylose isomerase and xylulose kinase activities were determined as described by Callens et al. (1986) and Shamanna and Sanderson (1979), respectively. Cell free extracts were prepared aerobically. No differences were found in enzyme activities when determined under either strict anaerobic conditions or aerobic conditions.

Xylose uptake.

Cells (10 ml) were harvested by centrifugation (8,000 × *g*) under anaerobic conditions, and washed once with anaerobic medium without substrate but with chloramphenicol (CAP, 0.4 mg/ml). Cells were suspended in the same medium to an OD_{660} of 3. Uptake studies were performed under an atmosphere of N_2/CO_2 (80%/20%) in 13-ml vials closed with butyl rubber stoppers. The reaction mixtures (1.5 ml) were preincubated at 37°C for 10 min. The uptake study was started by addition of xylose (1 mM final concentration, 0.25 μCi/μmol). At various periods of time, samples (0.2 ml) were taken using a 1-ml syringe equipped with an 18-gauge needle and filtered through prewashed membrane filters (Schleicher & Schuell BA85). Filters were washed with 4 ml of 100 mM LiCl and transferred to 4 ml aqualuma scintillation cocktail. The radioactivity was counted in a LKB Wallac liquid scintillation counter (Pharmacia/LKB, Woerden, The Netherlands).

Analytical methods.

Substrate consumption and product formation were measured by HPLC and GC as described by Stams et al. (1993). Samples for HPLC analysis were diluted 1:1 with 20 mM xylitol in 50 mM HCl; xylitol was used as an internal standard. Growth was determined by measuring the increase in optical density at 660 nm in a LKB/Biochrom Ultraspec K spectrophotometer, using cuvettes with a 1-cm

light path. When necessary samples were diluted 1:1 with water to obtain an OD_{660} below 0.4. Protein in cell free extracts was estimated with coomassie brilliant blue G250 (Bradford 1976). Total cell protein was determined after boiling of cell pellets in 1N NaOH for 15 min according to Lowry et al. (1951). Bovine serum albumin was used as a standard.

Chemicals.

All chemicals were at least of analytical grade. Enzymes and biochemicals were obtained from Boehringer Mannheim (Almere, The Netherlands), Sigma Chemical Co. (Amsterdam, the Netherlands), or Merck (Darmstadt, F.R.G.). D-[U-^{14}C]-xylose was purchased from Amersham ('s Hertogenbosch, The Netherlands). Gases and gas mixtures were supplied by Hoekloos (Schiedam, The Netherlands). Aqualuma scintillation cocktail was obtained from Lumac (Landgraaf, The Netherlands).

Results

Substrate utilization by batch grown cells.

B. xylanolyticus X5-1 grew well on D-xylose and D-glucose. The molar growth yields were 8.7 and 11.3 g dry cells and the maximum specific growth rates were $0.11 \pm 0.02\ h^{-1}$ and $0.24 \pm 0.01\ h^{-1}$, respectively. Metabolism of xylose, glucose and a xylose-glucose mixture by *B. xylanolyticus* X5-1 cells adapted to either one of these sugars is shown in Figure 1. With the mixed substrate diauxic growth was not observed. The maximum specific growth rate on a mixture of glucose and xylose ($0.23 \pm 0.01\ h^{-1}$) was almost the same as the maximum specific growth rate on glucose alone (Fig. 1A and B). A substrate preference was not observed, irrespectively of the substrate of pre-growth (Fig. 1C and D).

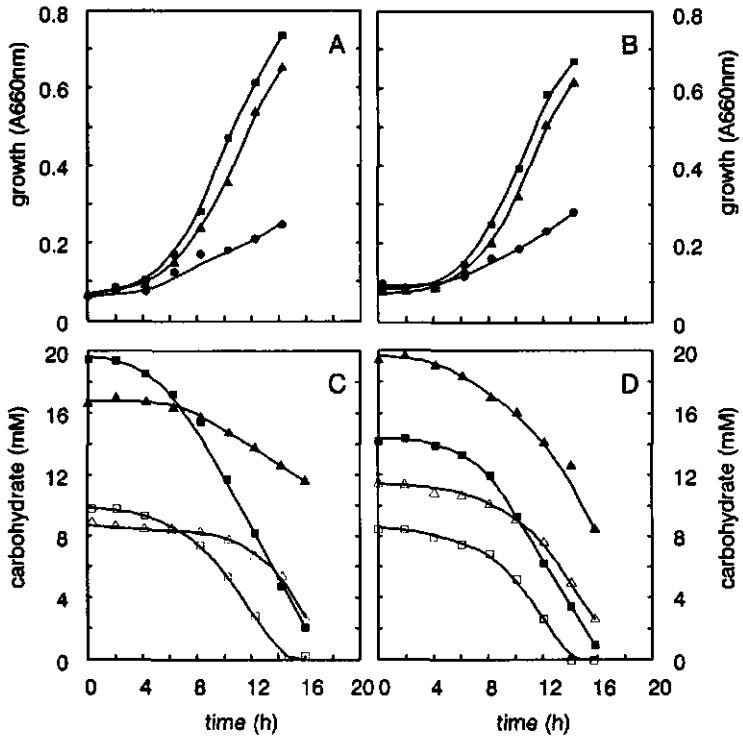

FIG. 1. Growth and substrate utilization by *B. xylanolyticus* X5-1 pregrown on glucose (panels A and C) or on xylose (panels B and D). Panels A and B show growth on glucose (■), xylose (●), or a mixture of glucose and xylose (▲). Substrate utilization in these cultures of glucose (■) or xylose (▲) as single substrates, and glucose (□) and xylose (△) as mixed substrates are shown in panels C and D.

Substrate utilization by continuous culture cells.

B. xylanolyticus X5-1 was grown in the chemostat with either xylose or glucose as the growth limiting substrate. Steady state cells of *B. xylanolyticus* X5-1, grown on xylose, were able to consume xylose, glucose or a mixture of xylose and glucose within 7 hours at a rate of approximately 6.3 mmol of carbohydrate/g dry cells per hour (Fig. 2A). No substrate preference was seen when a xylose-glucose mixture was given. When glucose was used as the growth

limiting substrate, steady state cells of *B. xylanolyticus* X5-1 consumed glucose at a rate of about 6.0 mmol/g dry cells per hour (Fig. 2B), but more than 24 hours were needed for a complete consumption of 15 mM of xylose. However, when glucose (4 mM) was added to these cells, xylose was consumed rapidly after a short lag phase (Fig. 2B).

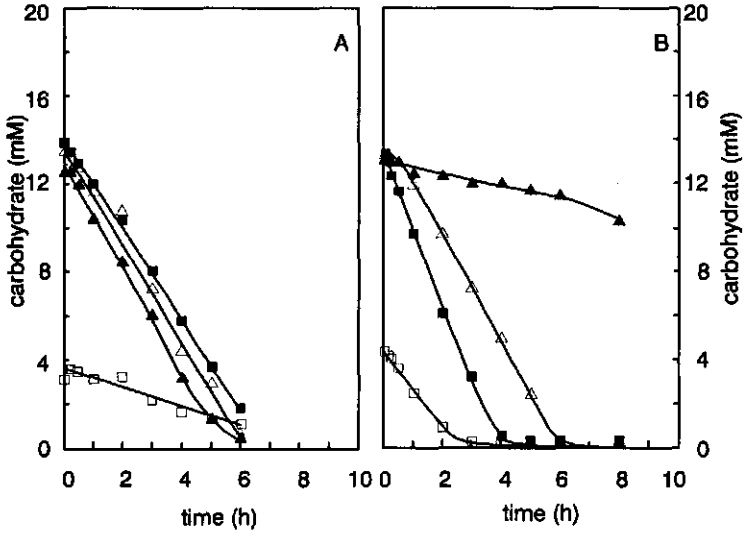

FIG. 2. Glucose (■,□) and xylose (▲,△) utilization by xylose limited (panel A) and glucose limited (panel B) steady state continuous culture cells of *B. xylanolyticus* X5-1. Closed symbols represent experiments with single substrates added, the open symbols with a mixture added.

The same phenomenon was found when pyruvate was used instead of glucose (data not shown). Chloramphenicol, an inhibitor of protein synthesis, did not affect glucose consumption in a xylose-glucose mixture, but the xylose fermentation was completely prevented (Fig. 3).

Activation of the xylose uptake system.

Xylose uptake was studied in *B. xylanolyticus* X5-1 cells grown in the chemostat. Glucose grown cells showed a relatively low ^{14}C-xylose uptake rate

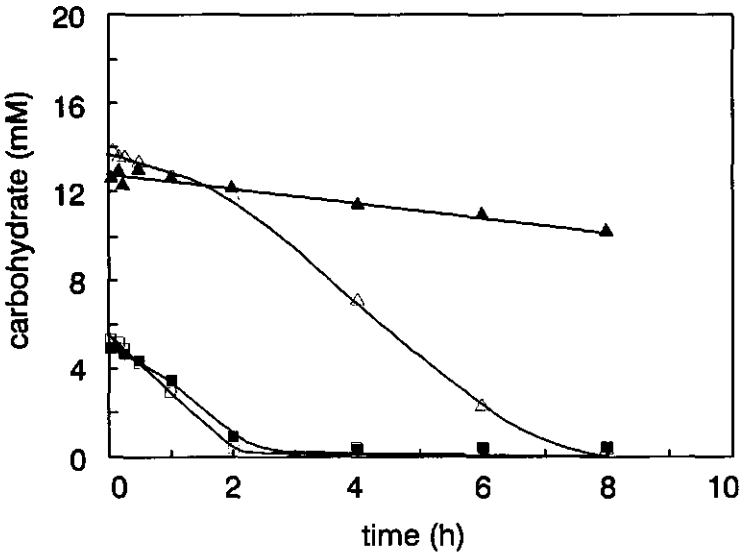

FIG. 3. Influence of chloramphenicol on the mixed substrate utilization by glucose limited, steady state continuous culture cells of *B. xylanolyticus* X5-1. Symbols: glucose (□,■), xylose (△,▲). Open symbols represent incubation without chloramphenicol, whereas closed symbols represent incubation with chloramphenicol.

(3 nmol/min.mg cell protein) compared to xylose grown cells (25 nmol/min.mg cell protein). However, incubation of the glucose grown cells with a xylose-glucose mixture increased the xylose uptake rate to 20 nmol/min.mg cell protein after 2 h (Fig. 4). This increase in uptake rate was not affected by chloramphenicol, indicating that protein synthesis is not required for this activation (Fig. 4).

Induction of xylose isomerase and xylulose kinase.

B. xylanolyticus X5-1 catabolizes glucose *via* the glycolysis, while xylose is catabolized *via* a combined pentose phosphate pathway and the glycolysis. Key enzymes of the pentose phosphate pathway are xylose isomerase and xylulose kinase. In extracts of xylose grown chemostat cells of *B. xylanolyticus* X5-1,

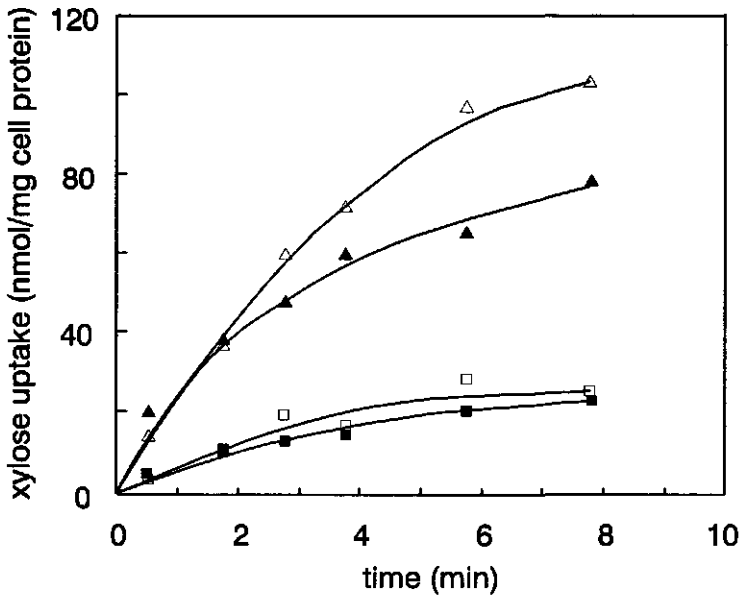

FIG. 4. Influence of chloramphenicol on the activation of the xylose uptake rate of glucose limited, steady state continuous culture cells of *B. xylanolyticus* X5-1. Induction was started at time zero by the addition of a xylose and glucose mixture in the absence (open symbols) and presence (closed symbols) of chloramphenicol. Uptake activity was determined at time 0 h (squares) and at time is 2 h (triangles) after the addition of the substrate mixture.

specific enzyme activities of 45 and 180 nmol/min.mg cell protein were found for xylose isomerase and xylulose kinase, respectively. Glucose grown cells showed a low xylose isomerase activity (5-10 nmol/min.mg protein) and no activity could be detected of xylulose kinase. When glucose, xylose or pyruvate were added to glucose grown cells, the enzymes were not induced. However, with a xylose-glucose mixture the activities of xylose isomerase and xylulose kinase increased rapidly. A short lag in the expression of xylulose kinase was observed (Fig. 5A). When the same experiment was performed with chloramphenicol in the medium, xylulose kinase and xylose isomerase were not induced (Fig. 5B).

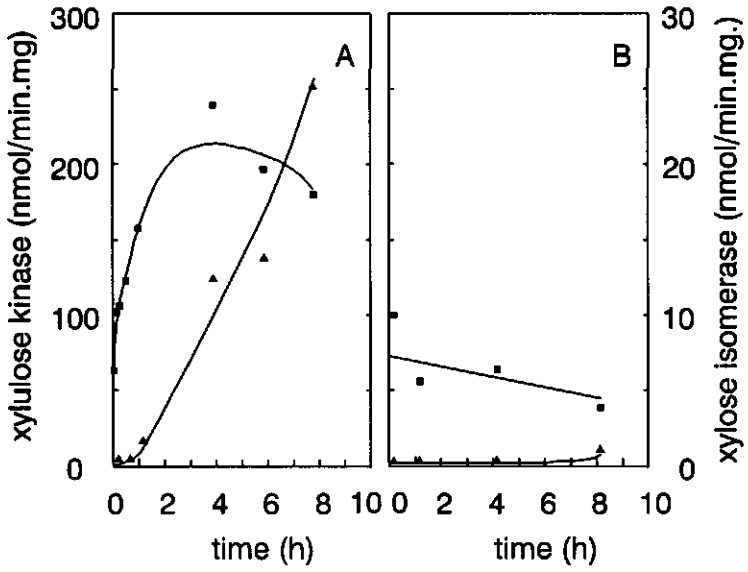

FIG. 5. Induction of xylose isomerase (■) and xylulose kinase (▲) in glucose limited continuous culture cells of *B. xylanolyticus* X5-1. Cells were induced at time zero with a mixture of xylose and glucose without (panel A) or with (panel B) chloramphenicol.

Discussion

B. xylanolyticus X5-1, when grown in batch cultures utilized mixtures of xylose and glucose simultaneously. A diauxic growth or a substrate preference was never observed, despite the fact that the maximum specific growth rates on glucose and xylose differed considerably. This indicates that the xylose metabolism in *B. xylanolyticus* X5-1 is not catabolite repressed, unlike the xylose metabolism in *S. ruminantium* strain D and in *C. acetobutylicum* (ATCC 824) (Ounine et al. 1985, Strobel 1993a). Other mixtures with xylose, glucose, arabinose and rhamnose were tested as well. With none of these mixtures we observed diauxic growth or substrate preference. Similar results were found with other *Bacteroides* strains, like *B. fragilis*, *B. fibrisolvens* A38 and *B.*

polypragmatus strain GP4 (Patel 1984, Patel et al. 1986, Russell and Baldwin 1978).

Glucose grown chemostat cells of *B. xylanolyticus* X5-1 hardly consumed xylose, whereas glucose was consumed immediately. However, when glucose was added together with xylose, the latter was metabolized after a short lag phase. The fact that chloramphenicol could reverse the glucose effect on the xylose fermentation showed that the xylose metabolism was regulated on enzyme level. The first three steps in xylose metabolism involve xylose uptake, and xylose conversion *via* the enzymes xylose isomerase and xylulose kinase. The xylose uptake rate was low in glucose grown continuous culture cells, but increased after 2 hours of incubation with a xylose-glucose mixture. Because this increase was not affected by the presence of chloramphenicol, it is likely that xylose uptake is not controlled on the level of enzyme synthesis. Induction with a xylose-pyruvate mixture instead of xylose-glucose mixture had the same effect on the xylose conversion. Probably the xylose uptake system is energized by a metabolic intermediate, like ATP, acetyl-PO_4 or another energy rich phosphate bond (Ames 1986). Xylose isomerase activity was present in glucose-grown cells, but the activity in xylose-grown cells was 4 to 5 times higher. Xylulose kinase activity could not be detected in glucose grown cells. Both enzymes were induced after the addition of a xylose-glucose mixture, whereas xylose or glucose alone did not induce the enzymes. The induction was repressed by chloramphenicol, showing *de novo* synthesis of these enzymes. *Prevotella (Bacteroides) ruminicola* $B_1$4 and S23 also possessed inducible xylose isomerases, but had constitutive xylulose kinases (Matte et al. 1992). Like *B. xylanolyticus* X5-1, strain $B_1$4 possessed a constitutive xylose permease (Strobel 1993b). *Selenomonas ruminantium* D showed a constitutive xylose isomerase and an active, but inducible xylulose kinase (Matte et al. 1992). Whether the inducible activities in *S. ruminantium* and *P. ruminicola* are controlled on the level of enzyme synthesis is not known.

B. xylanolyticus X5-1 grown in continuous culture on glucose is carbon and

energy limited. When the xylose catabolic pathway is induced with xylose as a single substrate, the cells need metabolic energy (i) to transport the substrate, (ii) to synthesize the xylose isomerase and the xylulose kinase and (iii) to phosphorylate xylulose. Due to the energy limiting conditions, xylose is poorly catabolized in glucose limited continuous culture cells. When some glucose is added as well, metabolic energy becomes available. Similar results were found by Standing et al. (1972), when a glucose fed chemostat of *E. coli* B/r was switched to xylose. About 50% of the cells was washed out in about 12 hours, before the cells were able to ferment the new substrate.

The regulation of the xylose catabolism in *B. xylanolyticus* X5-1 is different from the known xylose catabolisms in *E. coli*, *A. aerogenes* and *S. typhimurium*. In these organisms all three enzymes (permease, isomerase and kinase) are inducible enzymes (Shamanna and Sanderson 1979, Wilson and Mortlock 1973). Using xylose isomerase negative mutants it could be shown that xylose was the inducer of both the xylose isomerase and the xylulose kinase. Xylose concentrations as low as 0.66 mM were enough to induce the enzymes (Shamanna and Sanderson 1979). In *B. xylanolyticus* X5-1 xylulose probably is the inducer of the enzyme xylulose kinase, because the first response to the induction with the sugar mixture was an increase of xylose isomerase activity, followed by the induction of xylulose kinase. However, the lack of mutants and genetic data of *B. xylanolyticus* X5-1 precludes any conclusions regarding the inducer(s) involved in the xylose catabolic pathway.

Chapter 4

Regulation of product formation in *Bacteroides xylanolyticus* X5-1 by interspecies electron transfer

Steef Biesterveld, Alexander J.B. Zehnder, and Alfons J.M. Stams
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Abstract

Bacteroides xylanolyticus X5-1 was grown in pure culture and in mixed culture with *Methanospirillum hungatei* JF-1 under xylose limitation in the chemostat. In the pure culture ethanol, acetate, CO_2 and hydrogen were the products. In the mixed culture acetate, CO_2 and presumably hydrogen were the only products formed by *B. xylanolyticus* X5-1. The biomass yield of *B. xylanolyticus* X5-1 increased due to the cocultivation. In cell free extracts of the pure culture both NAD- and NADP-dependent acetaldehyde dehydrogenase and ethanol dehydrogenase activities were found. In cell free extracts of the mixed culture, activities of these enzymes were not detected. Inhibition of methanogenesis by the addition of bromo-ethano-sulfonic acid (BES), resulted in an accumulation of H_2, ethanol and formate. Immediately after the addition of BES, NAD-dependent acetaldehyde dehydrogenase and ethanol dehydrogenase activities were induced. After a short lag phase, a NADP-dependent ethanol dehydrogenase was also induced. The induction of acetaldehyde dehydrogenase and ethanol dehydrogenase was inhibited by chloramphenicol, showing *de novo* synthesis of these enzymes. These results clearly show that the shift in product formation caused by interspecies electron transfer is regulated at the level of enzyme synthesis.

Introduction

Interspecies electron transfer is an important process in methanogenic ecosystems. The influence of obligate and facultative electron transfer on the degradation of fatty acids, amino acids and carbohydrates has been studied in mesophilic and thermophilic microorganisms (Ahring and Westermann 1987a, Ahring and Westermann 1987b, Bonch-osmolovskaya and Stetter 1991, Boone et al. 1989, Dolfing 1992, Ianotti et al. 1973, Joblin et al. 1990, Marvin-Sikkema et al. 1993, Pavlostathis et al. 1990, Stams and Hansen 1984, Thiele and Zeikus 1988, Yang

and Tang 1991). These studies have indicated that H_2 and formate may function as electron carriers. Interspecies formate transfer was suggested to play an important role in the transfer of electrons during butyric acid, ethanol and lactic acid degradation (Boone et al. 1989, Thiele and Zeikus 1988). The influence of interspecies hydrogen transfer on carbohydrate fermentation is ascribed to a decrease in the partial hydrogen pressure. A role of formate transfer in sugar fermentation was not yet shown. Hydrogen is formed by proton reduction *via* the enzyme hydrogenase. The mid-point potentials for the redox couples $H_2/2H^+$ and reduced ferredoxin/oxidized ferredoxin (-414 mV and -398 mV, respectively (Thauer et al. 1977)) are in the same range. As a result H_2 can easily be formed from reduced ferredoxin, even at high hydrogen partial pressures (Gottschalk 1986). The mid-point potential of the redox coupple $NADH/NAD^+$ is much lower (-320 mV (Thauer et al. 1977)). Therefore, H_2 evolution from NADH cannot proceed, unless H_2 is removed efficiently by methanogens. It can be calculated that at a partial pressure of hydrogen below 10^{-3} atmosphere the Gibbs free energy of the reaction :

$$NADH + H^+ \rightarrow NAD^+ + H_2$$

will become negative (Gottschalk 1986). Enzymes involved in the electron flow from reduced adenine nucleotides (NAD(P)H) to ferredoxin and *vice versa* have been studied extensively (Jungermann et al. 1971, Jungermann et al. 1973, Petitdemange et al. 1976, Thauer et al. 1971, Thauer et al. 1974).

When grown in mixed culture with hydrogenotrophic organisms the carbon flux in fermentative organisms like *Clostridium thermocellum*, *C. pasteurianum*, *C. cellobioparum*, *Ruminococcus albus*, and *R. flavefaciens* shifts from reduced products, like ethanol, lactate, succinate and butyrate, to the more oxidized product acetate (Chung 1976, Ianotti et al. 1973, Latham and Wolin 1977, Thauer et al. 1977, Weimer and Zeikus 1977). Acetate formation is an important site of energy conservation in anaerobic metabolism as per acetate produced one

ATP is formed. Therefore, often more biomass is produced in cocultures. *B. xylanolyticus* X5-1 is a strictly anaerobic organism which is able to grow on the hemicellulose xylan and a variety of mono-, di- and tri-meric sugars (Scholten-Koerselman et al. 1986). It is not able to grow on other hemicelluloses or on cellulose. With labelling studies and enzyme measurements we could show that *B. xylanolyticus* X5-1 ferments xylose, which is the main constituent of xylan, *via* the pentose phosphate pathway in conjuction with the glycolysis (Biesterveld, unpublished results). This fermentation route is common in anaerobic bacteria growing on xylose (Caldwell and Newman 1986b, Heyndrickx et al. 1991a, Lacis and Lawford 1991, Turner and Roberton 1979). Acetate, ethanol, H_2, CO_2 and formate are the main fermentation products when grown on xylose (Scholten-Koerselman et al. 1986). Thus far, most research on the influence of interspecies electron transfer on fermentative organisms was focused on the regulation of product formation. The objective of the research presented here, was to investigate the regulation of product formation on enzyme level when *B. xylanolyticus* X5-1 is grown in pure culture and in mixed culture with methanogens. Preliminary results of this study have been published before (Biesterveld and Stams 1990).

Materials and methods

Organisms and cultivation.

Bacteroides xylanolyticus X5-1 (DSM 3808) was isolated at our laboratory (Scholten-Koerselman et al. 1986) and *Methanospirillum hungatei* JF1 (DSM 864) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). The organisms were grown in a basal bicarbonate buffered medium with a composition as described by Huser et al. (1982). To one litre of medium 0.5 g of yeast extract, 1 ml of a trace elements solution (Zehnder

et al. 1980) and 1 ml of a filter-sterilized vitamin solution (Wolin et al. 1963) were added. For the cultivation of *B. xylanolyticus* X5-1 the pH of the medium was 6.8-6.9 and the gas phase was N_2/CO_2 (80%/20%). The medium for *M. hungatei* JF-1 was supplemented with 0.05% biotrypticase and the gas phase was H_2/CO_2 (80%/20%). Xylose was added from a 2M filter-sterilized stock solution to a final concentration of 20 mM. In batch cultures the organisms were cultivated in 250-ml serum bottles containing 100 ml of medium. For the continuous culture experiments the same medium was used except that 0.2% of yeast extract was used instead of 0.05% yeast extract, and 0.05% of cysteine was added. Continuous cultivation was performed at 37°C in 1L-chemostats with a working volume of 500 ml. The cultures were grown at a dilution rate of 0.03 h^{-1}. The pH was maintained at 7.0 $\pm$ 0.1 with 2N NaOH. N_2/CO_2 (80%/20%) at a flow rate of 260 ml per hour was lead continuously over the cultures. Continuous cultivation of mixed cultures were started using 1% and 10% inocula of growing cultures of *B. xylanolyticus* X5-1 and *M. hungatei* JF1, respectively. Batch growth of the mixed culture was allowed for 12 hours before continuous feeding was started. When the partial pressure of H_2 in the gas phase had decreased to a value below 50 Pa, cultivation was continued during at least 6 volume changes to guarantee steady state conditions.

Preparation of cell free extracts and enzyme assays.

Cells were harvested by centrifugation at 20,000 $\times$ *g* for 10 min and washed once in 50 mM Tris-HCl pH 7.8 containing 5 mM $MgCl_2$ and 1 mM DTT. The cell pellets were resuspended in the same buffer and disrupted by sonication (Sonics & Materials sonifier, CT, USA; 10 times 20 s with an intermittent cooling on ice for 20 s). The cell debris was removed by centrifugation (8,000 $\times$ *g* for 15 min), and the supernatant was used as cell free extract.

Glyceraldehyde-3-PO_4 dehydrogenase, hydrogenase, phosphotransacetylase, acetaldehyde dehydrogenase, and ethanol dehydrogenase were assayed as described by Lamed and Zeikus (1980a). Acetate kinase was assayed as described by Lamed and Zeikus (1980b). Pyruvate:ferredoxine oxidoreductase was assayed using methyl viologen (MV) as electron acceptor. The assay mixture contained 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 2 mM $MgCl_2$, 2 mM MV, 0.2 mM HSCoA, 20 mM sodium pyruvate. Pyruvate-formate-lyase was assayed according to the method of Jungermann and Schön (1974); pyruvate conversion and formate production were analyzed by HPLC. The assay was performed under an atmosphere of carbon monoxide to prevent H_2 formation (Thauer et al. 1972). Formate dehydrogenase (NAD(P)-dependent) and formate:ferredoxin (MV) oxidoreductase were assayed according to the method of Spormann and Thauer (1988). Transhydrogenase was assayed according to the method as described by Höjeberg et al. (1976). Ferredoxine (MV):NAD(P) oxidoreductase was assayed in 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 2 mM MV, and 1 mM of NAD or NADP, under a gas phase of 100% CO (10^5 Pa). MV was prereduced with a small amount of sodium dithionite. NADH dehydrogenase and NADPH dehydrogenase were assayed as described by Kremer and Hansen (1987). However, 2 mM NADH was used for the NADH dehydrogenase assay instead of 0.2 mM, and for the NADPH dehydrogenase assay 0.2 mM NADPH was used instead of 2 mM. Pyridine nucleotide oxidation or reduction was measured at 340 nm ($\epsilon_{340\ nm}$ = 6.22 $mM^{-1}cm^{-1}$). Methyl viologen reduction or oxidation was measured at 560 nm ($\epsilon_{560\ nm}$ = 8.0 $mM^{-1}cm^{-1}$).

Induction experiments in mixed culture cells.

Enzyme induction studies were performed in steady state chemostat cultures after inhibition of methanogenic bacteria by bromo-ethano-sulfonic acid (BES; 5 mM

final concentration). The medium flow was kept at the same rate as before the addition of BES. At different time intervals liquid samples (60 ml) were taken to determine the cumulative amounts of substrate consumption, product formation and bacterial growth and to determine enzyme levels. Gas samples were taken to analyze for H_2 and CH_4 formation.

To study the effect of chloramphenicol (CAP; 0.4 mg/ml final concentration) on enzyme induction, steady state mixed cultures (90 ml) were transferred anaerobically to 250 ml serum bottles. BES (5 mM) and xylose (20 mM) were added to bottles with and to bottles without CAP. In these bottles product formation and substrate consumption were measured after 24 hours of incubation at 37°C.

Analytical methods.

Substrate consumption and product formation were measured by HPLC and GC as described by Stams et al. (1993). Samples for HPLC analysis were diluted 1:1 with 20 mM xylitol in 50 mM HCl; xylitol was used as internal standard. In the mixed culture experiments, the bacterial dry weight of the methanogen was estimated from the methane production, assuming that 3 g of dry weight was formed per mol of CH_4 produced (Pavlostathis et al. 1990, Schönheit et al. 1980). In the induction experiments, the yield of *B. xylanolyticus* X5-1 was calculated from the change in optical density at 660nm (OD_{660}). An $OD_{660}=1$ corresponded to about 600 mg of dry cells per litre. OD_{660} readings were done in a LKB/Biochrom Ultraspec K spectrophotometer using cuvettes with a 1-cm light path. When necessary, samples were diluted 1:1 with water to maintain an OD_{660} below 0.4. Protein in cell free extracts was estimated with coomassie brilliant blue G250 (Bradford 1976). Bovine serum albumin was used as a standard.

Chemicals.

All chemicals were at least of analytical grade. Enzymes and biochemicals were obtained from Boehringer (Almere, the Netherlands), Sigma Chemical Co. (Amsterdam, the Netherlands) or Merck (Darmstadt, F.R.G.). Xylose was purchased from Janssen (Geel, Belgium). Gases and gas mixtures were supplied by Hoekloos (Schiedam, the Netherlands).

Results

Xylose fermentation in chemostat cultures.

B. xylanolyticus X5-1 was grown in the chemostat in pure culture and in mixed culture with *M. hungatei* JF-1. The biomass yield and the specific rates of substrate consumption and product formation of steady state chemostat cultures are given in table 1. In batch cultures of *B. xylanolyticus* X5-1 low amounts of formate were produced (results not shown). However, in steady state continuous cultures formate production could not be detected neither in the pure culture nor in the mixed culture. Cocultivation with the methanogen led to a shift in product formation; the acetate production rate and the biomass yield increased, whereas the ethanol production rate decreased to a very low level. The H_2 partial pressure in the gas phase of the mixed culture was about 25-50 Pa, which was about 1 per cent of the concentration found in pure culture of *B. xylanolyticus* X5-1 measured under the same conditions. Reasonable carbon and electron recoveries were found in both the pure and the mixed culture.

Enzyme activities in cell extracts of pure and mixed cultures.

To investigate whether product formation was regulated at the level of enzyme synthesis, enzymes involved in product formation and electron transfer reactions

Table 1. Steady state values expressed in xylose limited chemostat cultures of *Bacteroides xylanolyticus* X5-1 in pure and mixed cultures (D=0.03 h^{-1}, 37°C, pH = 7.0 ± 0.1, xylose concentration influent = 20 mM, gas flow=260 ml per hour). In the mixed culture the methanogen *Methanospirillum hungatei* JF-1 was used.

	pure culture	mixed culture
Specific rates[a]		
xylose	1.9	1.6
acetate	1.2	2.3
ethanol	1.3	0.03
formate	ND	ND
H_2[b]	1.6	5.4
CO_2[c]	2.5	2.3
CH_4[d]	ND	8.8
dry weight (mg/l)[d]	316	372(X5-1) 56(JF-1)
Y_{xylose} (g/mol)[e]	15.8	19
Y_{ATP} (g/mol)[e]	6.6	6.0
C-recovery (%)[f]	93	105
e-recovery (%)[f]	89	109

ND means not detectable

[a] The specific rates are expressed as mmols consumed or produced per gram dry *B. xylanolyticus* X5-1 cells per hour, except for the methane production rate. The latter is expressed as mmols per gram dry *M. hungatei* JF-1 cells per hour.

[b] H_2 in the mixed culture was calculated from the methane formed, assuming that hydrogen was produced in stead of formate and that 4 mols of H_2 are used to form 1 mol of CH_4.

[c] CO_2 was calculated assuming that per acetate and per ethanol 1 CO_2 is formed.

[d] A yield of 3 g of dry *M. hungatei* JF-1 cells per mol of CH_4 formed was used (Pavlostathis et al. 1990, Schönheit et al. 1980) to estimate the dry weight of the methanogen in the mixed culture.

[e] The yields are given as g of dry *B. xylanolyticus* X5-1 cells per mol of either xylose or ATP. ATP was calculated assuming that per xylose consumed, 1.67 pyruvate + 1.67 ATP are formed. Furthermore, per pyruvate converted to acetate 1 additional ATP is produced, whereas per ethanol no extra ATP is gained.

[f] For the calculation of carbon and electron recovery, biomass was estimated using $<C_5H_7O_2N>$ as the structural formula for biomass with a molecular weight of 113 g/mol.

were measured. Table 2 shows enzyme levels measured in steady state cells of the pure and the mixed culture.

Table 2. Specific activities of enzymes of steady state cells of *B. xylanolyticus* X5-1 grown in pure and in mixed cultures, with *M. hungatei* JF-1. Xylose was the growth limiting substrate (D=0.03 h^{-1}, 37°C, pH = 7.0 ± 0.1). Values are expressed as μmol/min.mg protein. The specific activities measured in the mixed culture were not corrected for the protein content of the methanogen.

		specific activity	
enzyme	cofactor	pure culture	mixed culture
Fd(MV):NADP oxidoreductase	MV/NADP	3.35	1.89
NADH DH	MTT	0.03	0.06
NADPH DH	MTT	1.13	1.63
glyceraldehyde-3-PO_4 DH	NAD	3.82	1.78
pyruvate:Fd oxidoreductase	MV	1.39	0.34
hydrogenase	MV	17.4	3.5
formate DH	MV	<0.005	0.09
phosphotransacetylase		0.60	0.22
acetate kinase		1.01	0.82
acetaldehyde DH	NAD	0.16	<0.01
	NADP	0.01	<0.01
ethanol DH	NAD	0.34	<0.01
	NADP	0.69	<0.01

DH = dehydrogenase. No detectable activities could be found for the following enzymes: Fd(MV):NAD oxidoreductase, NADP dependent glyceraldehyde-3-PO_4 DH, pyruvate-formate lyase, NAD(P)-dependent formate DH and NADH:NADP transhydrogenase.

Assuming that 50 % of the dry weight consists of cell protein (Scholten-Koerselman et al. 1986) it can be calculated that the minimal activities of catabolic enzymes to account for the observed xylose conversion rates should be 57 and 53 nmol/min mg protein for the pure and the mixed culture, respectively. The activities found were high enough to account for the *in vivo* xylose conversion rates in both cultures. In the mixed culture, most of the enzymes showed lower activities when compared to the activities found in the pure culture.

The specific activities determined in the mixed culture were not corrected for the protein content of the methanogen. This can partly explain these differences. High activities were found in both cultures for Fd(MV):NADP oxidoreductase, whereas only low activities of Fd(MV):NAD oxidoreductase were detected. The most remarkable differences between pure and mixed culture cells were found for the activities of acetaldehyde dehydrogenase and ethanol dehydrogenase. These enzymes could not be detected in the mixed culture.

Inhibition of interspecies electron transfer.

Bromo-ethano-sulphonic acid (BES) is a specific inhibitor of methanogenesis, and was used in a steady state mixed culture. In a separate experiment it was assured that BES did not inhibit xylose catabolism by *B. xylanolyticus* X5-1 (data not shown). Methane formation stopped immediately after addition of BES and dilution of methane from the gas phase of the chemostat was observed (Fig. 1).

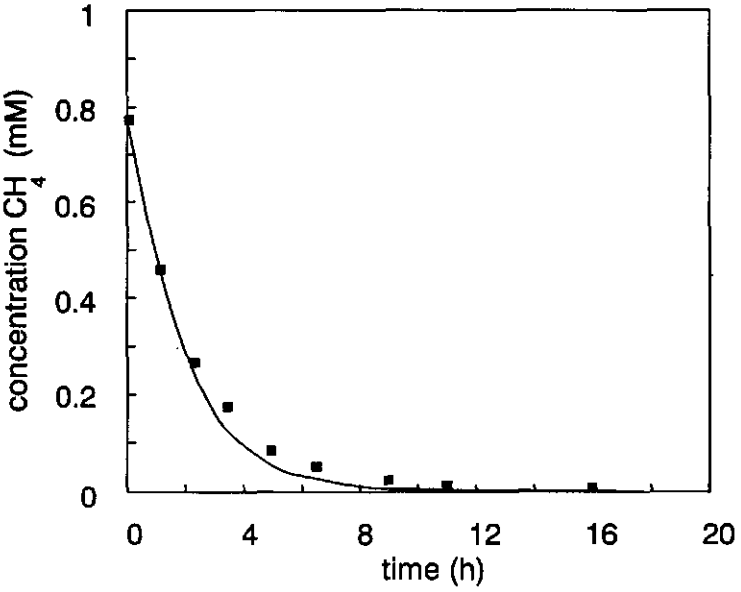

FIG. 1. Dilution of methane from the gas phase of the chemostat after addition of BES at t=0 to a steady state mixed culture of *B. xylanolyticus* X5-1 and *M. hungatei* JF-1. The squares show the measured methane concentration. The line shows the theoretical decrease in the methane concentration as could be expected at a gas flow rate of 260 ml per hour.

The formation of products and biomass after addition of BES is shown in figure 2.

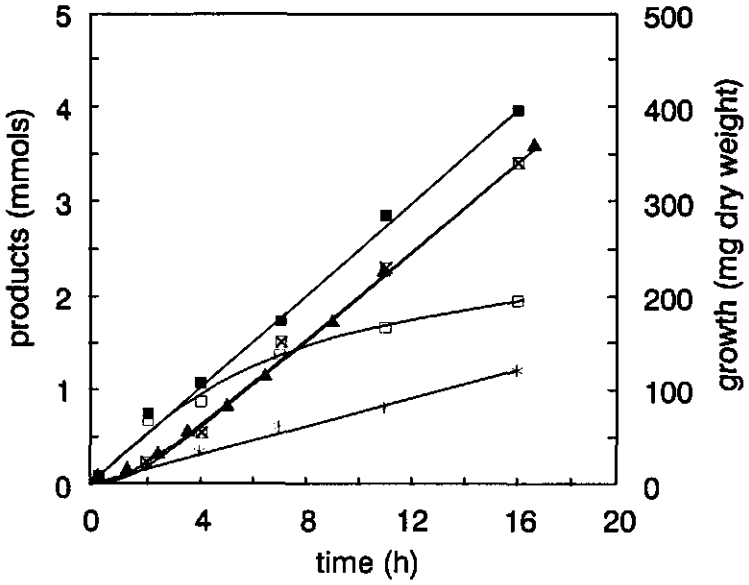

FIG. 2. Cumulative product formation and growth by *B. xylanolyticus* X5-1 after inhibition of methanogenesis in a steady state mixed culture of *B. xylanolyticus* X5-1 with *M. hungatei* JF-1. To a steady state mixed culture BES was added at t=0, while the medium flow was continued. At several time intervals, the cumulative amounts of the products and growth were determined. Symbols: ■ = acetate, ⊠ = ethanol, ▲ = H_2, □ = formate, * = biomass. Products are given in mmols, biomass is given in mg dry cells.

Formate and ethanol were additional products that could be measured in the liquid phase and H_2 was detected in the gas phase. Formate production stopped about 16 h after addition of BES, while a linear increase of the other products was measured for more than 30 h (data not shown).

Induction of acetaldehyde dehydrogenase and ethanol dehydrogenase.

Cocultivation with the methanogen influenced both product formation and enzyme levels in *B. xylanolyticus* X5-1. The induction of acetaldehyde dehydrogenase and

ethanol dehydrogenase was studied after inhibition of the methanogen in the mixed culture. Within two hours after addition of BES, NADH-dependent acetaldehyde dehydrogenase and NADH-dependent ethanol dehydrogenase were detected (Fig. 3).

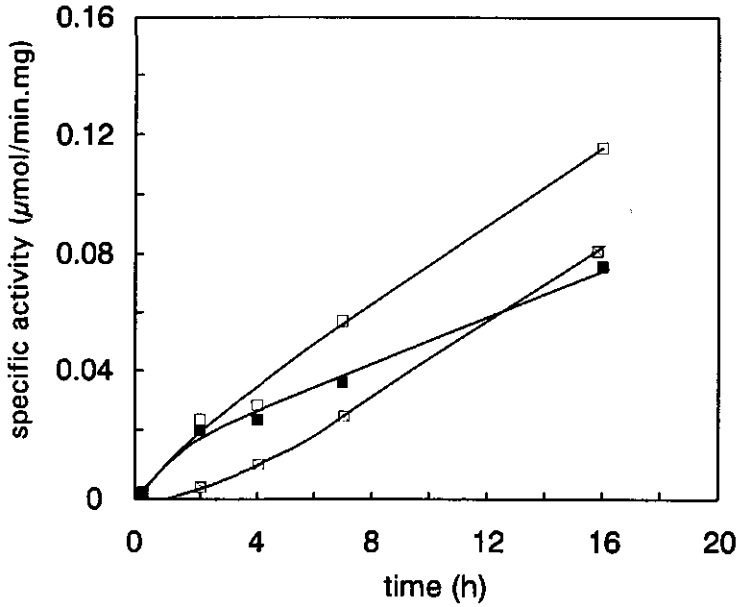

FIG. 3. Enzyme induction in cells of *B. xylanolyticus* X5-1 grown in mixed culture with *M. hungatei* JF-1. The experiment was performed under the same condition as mentioned in figure 2. Symbols: □ = ethanol dehydrogenase, NAD dependent; ▣ = ethanol dehydrogenase, NADP dependent; ■ = acetaldehyde dehydrogenase, NAD dependent.

Induction of the NADPH-dependent ethanol dehydrogenase was found after a short lag phase. Induction of a NADPH dependent acetaldehyde dehydrogenase, which was present in low activity in the pure culture of *B. xylanolyticus* X5-1, could not be detected over the period that enzyme activities had been measured.

Influence of chloramphenicol (CAP) on the enzyme induction.

To characterize the regulation of induction of acetaldehyde dehydrogenase and ethanol dehydrogenase, the influence of CAP on the enzyme induction was studied. Batch

incubation of steady state mixed culture cells with BES and xylose, showed xylose fermentation to mainly acetate, ethanol, H_2, formate and CO_2. In the presence of CAP, only an isomerization of xylose to xylulose occurred (Table 3). These data suggest *de novo* synthesis of the enzymes involved in ethanol formation in *B. xylanolyticus* X5-1.

Table 3. Influence of chloramphenicol on the xylose degradation by mixed culture cells of *B. xylanolyticus* X5-1 and *M. hungatei* JF-1. Steady state chemostat cells were harvested anaerobically and transferred to serum bottles. Incubations were done with xylose (20 mM) and BES (5 mM), and with or without chloramphenicol (CAP; 0.4 mg/ml). Samples were analyzed after 24 hours of incubation. Values are given in mmols.

	incubation	
	with CAP	without CAP
substrate degraded		
xylose	5.0	16.0
products formed		
xylulose	5.0	0.5
lactate	<0.1	0.3
formate	<0.2	7.8
acetate	<0.2	9.6
ethanol	<0.2	11.3
H_2	<0.05	6.2
CH_4	<0.2	0.3

Discussion

When cocultivated in continuous culture with *M. hungatei* JF-1, *B. xylanolyticus* X5-1 shifted its product formation to more acetate and less ethanol. Because acetate formation coincided with substrate level phosphorylation a biomass increase for *B. xylanolyticus* X5-1 of about 20% was observed. The same effect of interspecies electron transfer has been described for many other organisms in coculture with hydrogenotrophic anaerobes (Bryant et al. 1967, Ianotti et al. 1973, Pavlostathis et al. 1990, Scheifinger et al. 1975). Our results clearly show

that regulation by interspecies electron transfer is not only a control of metabolic fluxes, but that regulation of enzyme levels occurs as well. Activities of acetaldehyde dehydrogenase and ethanol dehydrogenase were repressed during cocultivation with the methanogen. Inhibition of methanogenesis by BES in a mixed culture of *B. xylanolyticus* X5-1 with *M. hungatei* JF-1 resulted in a rapid induction of NADH dependent acetaldehyde dehydrogenase and ethanol dehydrogenase activities. After a short lag phase a second, NADPH dependent ethanol dehydrogenase was induced. The enzyme induction was sensitive to chloramphenicol, showing *de novo* synthesis of these enzymes. Recently, a similar influence of cocultivation with methanogens on enzyme levels has been shown in the anaerobic fungus *Neocallimastix* sp. strain L2 (Marvin-Sikkema et al. 1993). However, in other studies with ethanol producing anaerobic bacteria like *Clostridium thermocellum* and *Ruminococcus albus* (Ben Bassat et al. 1981, Pavlostahis et al. 1990, Weimer and Zeikus 1977) this effect was never taken into account.

When grown in coculture with *M. hungatei* JF-1, *B. xylanolyticus* X5-1 has to couple the oxidation of NADH and reduced ferredoxin, which are formed during xylose degradation, to H_2 or formate formation. NADH-dependent hydrogenase and NADH-dependent formate dehydrogenase could not be detected, and NADH:ferredoxin oxidoreductase activity was too low to be involved in a catabolic route. In contrast, high activities of NADPH:ferredoxin oxidoreductase were present in both the pure and the mixed culture. After transhydrogenation, H_2 formation from NADH might occur *via* the enzymes NADPH:ferredoxin oxidoreductase and hydrogenase. However, NADH:NADP transhydrogenase could not be detected. Lamed and Zeikus (1980b) proposed transhydrogenation in *T. brockii* from NADH to NADP *via* the reaction sequence pyruvate, oxaloacetate, malate, pyruvate. In *B. xylanolyticus* X5-1 this is not very likely, as malic enzyme could not be detected. Therefore, it is not clear how reducing equivalents in *B. xylanolyticus* X5-1 are transformed to formate or H_2.

Formate was formed rapidly in mixed cultures when methanogens were inhibited by BES. However, we could not detect pyruvate-formate lyase activity in steady state mixed culture cells, and other formate producing enzymes were also not present. In *E. coli* pyruvate-formate lyase is constitutively present in an in-active form, as the active form of the enzyme contains a free radical. The activity is strictly regulated by two converter enzymes, an activating enzyme and a deactivating enzyme (Knappe and Sawers 1990). In strict anaerobes a similar mode of regulation may occur as well (Knappe and Sawers 1990). Inhibition of *M. hungatei* JF-1 in the mixed culture most probably will affect the intracellular concentrations of intermediates of the xylose catabolism (e.g reduced ferredoxin, pyruvate and acetyl-CoA), thereby activating pyruvate-formate lyase. An alternative explanation for the rapid formation of formate is that methanogens might be involved. Wu et al. (1993) showed that formate is produced by *Methanobacterium formicicum* and *Methanospirillum hungatei* when methanogenesis from H_2/CO_2 was inhibited by BES. In control experiments we were not able to detect formate production from H_2/CO_2 by a pure culture of *M. hungatei* JF-1. However, the time course of our induction experiment and the control experiment is much shorter, than the experiments performed by Wu et al. (1993), indicating that probably not the methanogens but *B. xylanolyticus* X5-1 itself is involved in formate production.

This study was done in xylose limited chemostat cultures in order to maintain the same constant growth rate (0.03 h^{-1}) for the hydrogen producing and the hydrogen oxidizing bacteria. High growth rates of fermentative organisms in batch mixed cultures caused problems in investigating the influence of interspecies electron transfer on carbohydrate fermentation (Chung 1976, Weimer and Zeikus 1977). The rapid enzyme induction as shown here after inhibition of methanogens shows that a precise control of cultivation is essential. Without the use of xylose-limited chemostats we would not have been able to demonstrate a regulation at the level of enzyme synthesis.

Chapter 5

Influence of external electron acceptors and of CO and H_2 on the xylose metabolism of *Bacteroides xylanolyticus* X5-1.

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Abstract

When *Bacteroides xylanolyticus* X5-1 was grown in batch culture on xylose, acetate, ethanol, H_2, CO_2 and formate were the main fermentation products. CO inhibited H_2 formation by *B. xylanolyticus* X5-1. As a result the product formation shifted to more ethanol and formate and less acetate. Furthermore, less biomass was produced. H_2 had almost no effect on the product formation from xylose. In batch cultures, dihydroxy acetone, acetone, acetoin and acetol could act as electron acceptors during xylose metabolism. The electron acceptors were reduced to their corresponding alcohols. The product formation from xylose by *B. xylanolyticus* X5-1 shifted to mainly acetate and CO_2, and an increased biomass yield was obtained. H_2, ethanol and formate were no longer produced. In continuous cultures not only 1,2-propanediol was formed from acetol, but also acetone. The NADP dependent ethanol dehydrogenase that was present in xylose grown continuous culture cells, was repressed when the organism was grown in the presence of acetol. However, another alcohol dehydrogenase was induced for the reduction of the external electron acceptor.

Introduction

Hydrogen formation is an important mechanism by which heterofermentative microorganisms dispose off reducing equivalents, formed during the oxidation of sugars (e.g. Chung 1976, Datta and Zeikus 1985, Caldwell and Newman 1986b, Dabrock et al. 1992). The product formation by these H_2 producing organisms has been studied extensively (Ben-Bassat et al. 1981, Datta and Zeikus 1985, Heyndrickx et al. 1989, Dabrock et al. 1992). Depending on the partial H_2 pressure, more reduced or oxidized products can be formed. Several methods have been used to influence the ratio of reduced and oxidized products in such H_2 forming organisms. Growth under H_2 and CO has been used to increase the ratio reduced/oxidized cofactors in the cell (Chung 1976, Kim et al. 1984, Datta

and Zeikus 1985, Meyer et al. 1986, Freier et al. 1988, Lamed et al. 1988), whereas electron acceptors and methanogenic cocultures were used to decrease this ratio (Chung 1976, Ben-Bassat et al. 1981, Rao and Mutharasan 1987, Heyndrickx et al. 1989, Pavlostathis et al. 1990, Hino et al. 1991). In these and other studies it was shown that ferredoxin:NAD(P) oxidoreductases play a key role in the regulation of the catabolic electron flow (Jungermann et al. 1973; Lamed and Zeikus 1980b; Ben-Bassat et al. 1981).

Bacteroides xylanolyticus X5-1 is a strict anaerobic hemicellulolytic organism, which can ferment several mono-, di-, and trimeric sugars and xylan, but not cellulose and hemicelluloses other than xylan (Scholten-Koerselman et al. 1986, Schyns and Stams 1992). With labelling studies and enzyme measurements we could show that xylose, the main constituent of xylan, is fermented *via* the pentose phosphate pathway and the glycolysis. This is a common pathway in anaerobic organisms growing on xylose (Turner and Roberton 1979; Caldwell and Newman 1986b; Heyndrickx et al. 1991a; Lacis and Lawford 1991). During xylose degradation, *B. xylanolyticus* X5-1 formed acetate, ethanol, H_2, CO_2 and formate as the main fermentation products. Small amounts of 1,2-propanediol and lactate were formed as additional products. The catabolic electron flow during xylose metabolism is influenced by interspecies electron transfer (Biesterveld and Stams 1990). In coculture with *Methanospirillum hungatei* JF-1, xylose is completely oxidized to acetate, CO_2 and presumably H_2, whereas ethanol and formate were no longer produced. Furthermore, the enzymes involved in ethanol production were completely repressed. The objective of this study was to investigate how product formation by *B. xylanolyticus* X5-1 can be affected. Alternative electron acceptors, and growth under H_2 and CO were used to prevent hydrogen formation. By cocultivation with *M. hungatei* JF-1, H_2 formation could be stimulated. The effect of the external electron acceptor acetol (=hydroxy propanon) on the catabolic electron flow during xylose catabolism was investigated in more detail.

Materials and methods

Organisms and cultivation.

Bacteroides xylanolyticus X5-1 (DSM 3808) was isolated and described by Scholten-Koerselman et al. (1986) and *Methanospirillum hungatei* JF-1 (DSM 864) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). The organisms were grown in a basal bicarbonate buffered medium with a composition as described by Huser et al. (1982). To one litre of medium 0.5 g of yeast extract, 1 ml of a trace elements solution (Zehnder et al. 1980) and 1 ml of a vitamin solution (Wolin et al. 1963) were added. The vitamin solution was filter sterilized separately. The gas phase above the medium was N_2/CO_2 (80%/20%) unless otherwise stated. The pH of the medium was 6.8-6.9. The medium for *M. hungatei* JF-1 was supplemented with 0.05% of biotrypticase and the gas phase was H_2/CO_2 (80%/20%). Xylose was added from 2M filter-sterilized stock solutions. External electron acceptors were added from 6M filter-sterilized stock solutions. In the batch culture experiments the organisms were cultivated in 250-ml serum bottles containing 100 ml medium. For *B. xylanolyticus* X5-1 20 mM of substrate was used. When CO was used as the gas phase, the serum bottles were first brought under vacuum. The proper amounts of CO and CO_2 were added and the bottles were pressurized with N_2 to 1.8 atmospheres. For the continuous culture experiments the same medium was used except that 0.2% of yeast extract was added instead of 0.05% yeast extract, and 0.05% of cysteine was added. Continuous cultivation was performed at 37°C in 1-L chemostats with a working volume of 500 ml. The cultures were grown at a dilution rate of 0.03 h^{-1}. The pH was maintained at 7.0 $\pm$ 0.1 with 2N NaOH. A continuous stream of N_2/CO_2 (80%/20%) at a flow rate of 130 ml per hour was led over the cultures. To guarantee steady state conditions cells were analyzed after at least 6 volume changes.

Preparation of cell free extracts and enzyme assays.

Cells were harvested by centrifugation at 20,000 × *g* for 10 min and washed once in 50 mM Tris-HCl pH 7.8 containing 5 mM $MgCl_2$ and 1 mM DTT. The cell pellets were resuspended in the same buffer and disrupted by sonication (Sonics & Materials sonifier, CT, USA; 10 times 20 s with an intermittent cooling on ice for 20 s). The cell debris was removed by centrifugation (8,000 × *g* for 15 min), and the supernatant was used as cell free extract. *Glyceraldehyde-3-PO_4 dehydrogenase*, *hydrogenase*, *phosphotransacetylase*, *acetaldehyde dehydrogenase*, and *ethanol dehydrogenase* were assayed as described by Lamed and Zeikus (1980a). *Acetate kinase* was assayed as described by Lamed and Zeikus (1980b). *1,2-propanediol dehydrogenase* was assayed in a similar fashion as ethanol dehydrogenase but with acetol (=hydroxy propanon) instead of acetaldehyde. *Pyruvate:ferredoxine oxidoreductase* was assayed using methyl viologen (MV) as electron acceptor. The assay mixture contained 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 2 mM $MgCl_2$, 2 mM MV, 0.2 mM HSCoA, 20 mM sodium pyruvate. *Pyruvate-formate lyase* was assayed according to the method described by Jungermann and Schön (1974). Pyruvate removal and formate production were analyzed by HPLC. The assay was performed under CO atmosphere to prevent H_2 formation (Thauer et al. 1972). *Formate dehydrogenase (NAD(P)-dependent)* and *formate:ferredoxin (MV) oxidoreductase* were assayed according to the method of Spormann and Thauer (1988). *Ferredoxine (MV):NAD(P) oxidoreductase* was assayed in 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 2 mM MV, gas phase 100% CO (1 atmosphere), 1 mM of either NAD or NADP. MV was prereduced with small amounts of sodium dithionite. *NADH:NADP transhydrogenase* was assayed according to the method as described by Höjeberg et al. (1976). *NADH dehydrogenase* and *NADPH dehydrogenase* were assayed as described by Kremer and Hansen (1987).

However, 2 mM NADH was used for the NADH dehydrogenase assay instead of 0.2 mM, and for the NADPH dehydrogenase assay 0.2 mM NADPH was used instead of 2 mM. Pyridine nucleotide oxidation or reduction was measured at 340 nm ($\epsilon_{340\ nm}$= 6.22 $mM^{-1}cm^{-1}$). Methyl viologen reduction or oxidation was measured at 560 nm ($\epsilon_{560\ nm}$= 8.0 $mM^{-1}cm^{-1}$)

Gel electrophoresis and activity staining.

Soluble alcohol dehydrogenase activities could be visualized in native polyacrylamide gels (10% acrylamide, pH 8.8). After ultra centrifugation (100,000 × *g*, 4°C, 2h) 25 μl of the cell free extracts (6 mg protein/ml) were applied on the gels. The gels were run at room temperature at 200 V (constant voltage) for about half an hour. Then, gels were stained for alcohol dehydrogenase activity with phenazine methosulfate and nitro blue tetrazolium chloride according to the method of Benoist and Schwencke (1990). NAD or NADP (1 mM each) and ethanol or 1,2-propanediol (100 mM each) were used as substrates. The activity staining was done by incubating the gels in the dark at room temperature for 10 to 30 minutes in an anaerobic glove box (gas phase N_2/H_2 (96%/4%)). A palladium catalyst (BASF, Arnhem, The Netherlands) was used to remove traces of oxygen from the gas phase. Control experiments were done under the same conditions in the absence of the alcohol in the incubation buffer.

Analytical methods.

Substrate consumption and product formation were measured by HPLC and GC as described by Stams et al. (1993). Samples for HPLC analysis were diluted 1:1 with 20 mM xylitol in 50 mM HCl; xylitol was used as internal standard. Growth was determined by measuring the increase in optical density at 660 nm in a

LKB/Biochrom Ultraspec K spectrophotometer, using cuvettes with a 1-cm light path. When necessary samples were diluted 1:1 with water to obtain an OD_{660} below 0.4. Protein in cell free extracts was estimated with Coomassie brilliant blue G250 (Bradford 1976). Bovine serum albumin was used as a standard.

Chemicals. All chemicals were at least of analytical grade. Enzymes and biochemicals were obtained from Boehringer Mannheim (Almere, The Netherlands), from Sigma Chemical Co. (Amsterdam, the Netherlands or from Merck (Darmstadt, Germany). Xylose was purchased from Janssen (Geel, Belgium). Gases and gas mixtures were supplied by Hoekloos (Schiedam, The Netherlands).

Results

Effect of CO and H_2 on product formation.

CO affected the product formation from xylose by *B. xylanolyticus* X5-1 (Table 1). A gas phase containing 20% CO or more, inhibited H_2 formation for more than 95%. The xylose conversion decreased in the presence of CO. However, even in the presence of 80% CO about 11 mM of xylose was degraded in 48 h, whereas 20 mM was used in the absence of CO. The amounts of ethanol and formate increased when cells were grown under CO. Both the biomass yield and the acetate production decreased. When *B. xylanolyticus* X5-1 was grown under an atmosphere of 80% H_2 only small changes in product formation were found. It could be calculated that a significant amount of H_2 still was formed under a gas atmosphere of 80% H_2. The calculated Y_{ATP} was constant for all gas phases used, and reasonably good carbon and electron recoveries were found.

Table 1. Xylose fermentation products by *B. xylanolyticus* X5-1 under an atmosphere of either N_2/CO_2, H_2/CO_2, or CO/CO_2 (80%/20%). Bottles were inoculated with 2% (v/v) of a xylose grown culture of *B. xylanolyticus* X5-1. Xylose conversion and product formation were analyzed after 48 hours of incubation. Unless otherwise stated the amount of the products is given in mmoles per mmol of xylose converted.

	gas phase (80%/20%)		
Products	N_2/CO_2	H_2/CO_2	CO/CO_2
acetate	0.66	0.65	0.33
ethanol	0.61	0.76	1.03
H_2	1.32	1.15[a]	0.002
formate	0.07	0.25	0.59
lactate	0.01	<0.01	0.03
1,2-propanediol	0.02	<0.01	0.06
biomass (g/mol xylose)	8.7	8.5	6.8
Y_{ATP}[b] (g/mol ATP)	3.7	3.7	3.4
carbon recovery (%)[c]	86	92	93
electron recovery (%)[c]	87	93	94

[a] H_2 production was estimated, assuming that per acetate, 2 (H_2 + formate) are formed.
[b] Y_{ATP} was calculated assuming that per xylose consumed, 1.67 pyruvate + 1.67 ATP are formed. Furthermore, per pyruvate converted to acetate 1 additional ATP is produced, whereas per ethanol formed no extra ATP is gained.
[c] For the calculation of carbon and electron recovery, biomass was estimated using $<C_5H_7O_2N>$ as the structural formula for biomass with a molecular weight of 113 g/mol, and CO_2 was estimated assuming that per acetate + ethanol, 1 (CO_2 + formate) were formed.

Effect of external electron acceptors.

Several alternative electron acceptors were used to influence the xylose metabolism by *B. xylanolyticus* X5-1 (Table 2). Only dihydroxy acetone (DHA), acetone, acetoin, and acetol were effective in withdrawing the reducing equivalents liberated during xylose metabolism. These electron acceptors were reduced to glycerol, 2-propanol, 2,3-butanediol, and 1,2-propanediol, respectively. Growth on these electron acceptors in the absence of xylose was

Table 2. Xylose fermentation products of *B. xylanolyticus* X5-1 when grown in the presence of several external electron acceptor (initial concentrations 60 mM) and with different gas phases. Substrate conversion and product formation were analyzed after 48 hours of incubation. Unless otherwise stated the amounts of substrates and products are given in mmols per mmol of xylose.

electron acceptor	DHA	acetoine	acetone	acetol	acetol	acetol
gas phase (80%/20%)	N_2/CO_2	N_2/CO_2	N_2/CO_2	N_2/CO_2	H_2/CO_2	CO/CO_2
Substrate used						
xylose	1	1	1	1	1	1
dihydroxy acetone	1.88	-	-	-	-	-
acetoin	-	1.98	-	-	-	-
acetone	-	-	1.56	-	-	-
acetol	-	-	-	2.49	2.50	2.72
Products formed						
glycerol[a]	1.73	-	-	-	-	-
2,3-butanediol	-	2.15	-	-	-	-
2-propanol	-	-	1.32	-	-	-
1,2-propanediol	-	-	-	2.60	2.77	2.89
acetate	0.98	0.99	1.01	1.20	1.25	1.43
ethanol	0.38	0.24	0.46	0.07	0.03	0.07
H_2	0.006	0.03	0.24	0.01	-0.08[b]	0.004
formate	<0.02	<0.02	0.13	<0.02	0.08	0.23
μ_{max} (h^{-1})[c]	0.08 ± 0.04	0.11 ± 0.02	ND	0.13 ± 0.02	ND	ND
biomass (g/mol)	12.9	11.1	ND	12.5	ND	ND
carbon recovery (%)[d]	92	99	86	98	97	99
electron recovery (%)[d]	91	101	85	99	100	102

DHA = dihydroxy acetone; ND = not determined; Other electron acceptors tested were acetaldehyde, acetate, fumarate, oxalate, pyruvate, glyoxylate, glyoxal, hydroxy acetate, methylglyoxal, and propanaldehyde. These electron acceptors were tested at different concentration (2 - 100 mM) and were either toxic or did not affect product formation during xylose metabolism.

[a] Glycerol formation from dihydroxy acetone reduction was assumed, analogous to the end products found for the other electron acceptors.

[b] H_2 formation was estimated assuming that 2 × acetate = H_2 + formate + amount of electron acceptor converted.

[c] The maximum specific growth rate of *B. xylanolyticus* X5-1 grown on xylose without electron acceptor was 0.11 ± 0.02 h^{-1}.

[d] For the calculation of carbon and electron recoveries, biomass was estimated using $<C_5H_7O_2N>$ as the structural formula for biomass with a molecular weight of 113 g/mol, and CO_2 was estimated assuming that per acetate + ethanol, 1 (CO_2 + formate) were formed. When biomass was not determined, it was not used in the recovery calculation.

never observed, irrespectively whether N_2/CO_2, H_2/CO_2 or CO/CO_2 was the gas phase. H_2 was hardly formed in the cultures containing the electron acceptors. A decreased ethanol production was observed when DHA, acetoin or acetone were used as electron acceptors, and very low amounts of ethanol were produced when acetol was used. Acetate and biomass yields increased significantly. No significant differences were observed for the maximum specific growth rate of *B. xylanolyticus* X5-1 when grown on xylose in the absence or presence of the electron acceptors. The effect of CO on acetate and ethanol production in a xylose grown culture (Table 1) was overcome by the addition of the external electron acceptor acetol (Table 2). It could be calculated that H_2 was not formed by *B. xylanolyticus* X5-1 in the culture containing H_2/CO_2 (80%/20%) and the electron acceptor acetol. This in contrast to the incubation in the absence of acetol (Table 1).

Xylose fermentation in continuous culture.

B. xylanolyticus X5-1 produced acetate, ethanol, H_2 and CO_2 as fermentation products when grown on xylose as the growth limiting substrate in continuous cultures. Formate, lactate, and 1,2-propanediol could not be detected (Table 3). In mixed culture with *M. hungatei* JF-1 a shift in product formation was observed due to interspecies electron transfer. Ethanol was no longer produced, and acetate, CO_2 and presumably H_2 were the only products formed during xylose catabolism. When grown in the presence of acetol, an increased biomass yield and acetate production rate were found. H_2 formation was hardly detectable and a substantial decrease in the ethanol production rate was measured. However, more ethanol was formed in the culture grown with acetol, than in the mixed culture. In contrast to the batch cultures, not only 1,2-propanediol was produced from acetol, but also high amounts of acetone.

Table 3. Steady state values expressed in xylose limited chemostat cultures of *B. xylanolyticus* X5-1 in a xylose culture and in a xylose + acetol culture ($D=0.03\ h^{-1}$, 37°C, pH = 7.0 ± 0.1, gas flow = 130 ml per hour, xylose influent concentration = 20 mM). The steady state values for the mixed culture were taken up in this table for a comparence with the xylose + acetol culture.

	pure culture	mixed culture[a]	xylose/acetol culture
Specific rates[b]			
xylose	1.9	1.6	1.3
acetol	-	-	3.1
1,2-propanediol	-	-	0.6
acetone	-	-	2.4
acetate	1.2	2.3	1.8
ethanol	1.3	0.03	0.2
H_2[c]	1.6	5.4	0.005
CO_2[d]	2.5	2.3	2.0
CH_4[e]	-	8.8	-
formate	<0.05	<0.05	<0.05
lactate	<0.01	<0.01	<0.01
dry weight (mg/L)[e]	316	372 (X5-1) 56 (JF-1)	426
Y_{xylose} (g/mol)[f]	15.8	19	22.8
Y_{ATP} (g/mol)[f]	6.6	6.0	7.1
C-recovery (%)[g]	93	105	101
e-recovery (%)[g]	89	109	99

[a] The methanogen *Methanospirillum hungatei* JF-1 was used in the mixed culture.
[b] The specific rates are expressed as mmols per gram dry *B. xylanolyticus* X5-1 cells per hour, except for the methane production rate. The latter is expressed as mmols per gram dry *M. hungatei* JF-1 cells per hour.
[c] H_2 in the mixed culture was calculated from the methane formed, assuming that hydrogen was produced in stead of formate and that 4 mols of H_2 are used to form 1 mol of CH_4
[d] CO_2 was calculated assuming that per acetate and per ethanol 1 CO_2 is formed.
[e] A yield of 3 g of dry *M. hungatei* JF-1 cells per mol of CH_4 formed was used (Schönheit et al. 1980, Pavlostathis et al. 1990) to estimate the dry weight of the methanogen in the mixed culture.
[f] The yields are given in g of dry *B. xylanolyticus* X5-1 cells per mol of either xylose used or ATP formed. ATP was calculated assuming that per xylose consumed, 1.67 pyruvate + 1.67 ATP are formed. Furthermore, per pyruvate converted to acetate 1 additional ATP is produced, whereas per ethanol formed no extra ATP is gained.
[g] For the calculation of carbon and electron recovery, biomass was estimated using $<C_5H_7O_2N>$ as the structural formula for biomass with a molecular weight of 113 g/mol.

Enzyme activities in extracts of continuous culture cells.

To investigate whether product formation in the different cultures was regulated at the level of enzyme synthesis, enzymes involved in product formation and electron transfer reactions were measured (Table 4).

Table 4. Specific activities of enzymes of steady state cells of *B. xylanolyticus* X5-1 grown in the absence and presence of the external electron acceptor acetol. The specific activities found in the mixed culture were taken up in this table for a comparence with the xylose + acetol culture. ($D=0.03\ h^{-1}$, 37°C, pH=7.0 ± 0.1). Values are expressed as μmol/min.mg protein. The specific activities measured in the mixed culture were not corrected for the protein content of the methanogen

		specific activity		
enzyme	cofactor	pure culture	mixed culture	xylose/acetol culture
Fd(MV):NADP oxidoreductase	MV/NADP	3.35	1.89	4.80
NADH DH	MTT	0.03	0.06	0.08
NADPH DH	MTT	1.13	1.63	1.79
glyc-3-PO_4 DH	NAD	3.82	1.78	0.95
pyruvate:Fd(MV) oxidoreductase	MV	1.39	0.34	0.18
hydrogenase	MV	17.4	3.5	3.0
formate DH	MV	<0.005	0.09	<0.005
phosphotransacetylase		0.60	0.22	0.16
acetate kinase		1.01	0.82	0.44
acetaldehyde DH	NAD	0.16	<0.01	1.21
	NADP	0.01	<0.01	0.17
ethanol DH	NAD	0.34	<0.01	0.25
	NADP	0.69	<0.01	0.01
1,2-propanediol DH	NAD	0.02	<0.01	0.03
	NADP	0.78	<0.01	0.16

Glyc-3-PO_4 = glyceraldehyde-3-PO_4, DH = dehydrogenase
No detectable activities could be found for the following enzymes: Fd(MV):NAD oxidoreductase, NADP-dependent glyceraldehyde-3-PO_4 dehydrogenase, pyruvate-formate lyase, NAD(P) dependent formate dehydrogenase, and NADH:NADP transhydrogenase.

Assuming that 50% of the cell dry weight consisted of cell protein (Scholten-Koerselman et al. 1986) it can be calculated that the minimal activities of catabolic enzymes needed to explain substrate conversion in the continuous cultures should be 57, 53 and 43 nmol/min.mg protein in the pure culture, the

mixed culture, and the xylose + acetol culture, respectively. In all cultures the activities were high enough to account for the *in vivo* xylose conversion rates. In the mixed culture no activities could be determined of the NAD(P)-dependent acetaldehyde and ethanol dehydrogenases, whereas these activities were present in the pure culture. Acetaldehyde dehydrogenase showed a much higher activity in the xylose + acetol culture than in the pure and the mixed culture. The NAD-dependent ethanol dehydrogenase activity was not significantly different in the pure and the xylose + acetol culture. However, as in the mixed culture, xylose + acetol grown cells did not contain NADP dependent ethanol dehydrogenase activity. Remarkably, cells from both the pure culture and the xylose + acetol culture exhibited NADP dependent 1,2-propanediol dehydrogenase activities, with the highest specific activity in the pure culture cells.

Activity staining of the alcohol dehydrogenase activities.

To characterize the alcohol dehydrogenases in more detail, proteins of ultracentrifuged cell free extracts were separated by native polyacrylamide gel electrophoresis and enzyme activities were stained using nitro blue tetrazolium chloride. The NAD-dependent ethanol dehydrogenase might be membrane associated, because the activity staining of the gel with NAD and ethanol as substrates revealed enzyme activity in the slot of the stacking gel (data not shown). This was not observed with the NADP-dependent alcohol dehydrogenases. After electrophoresis of ultra-centrifuged cell extracts, half of a gel was assayed for NADP dependent *ethanol* dehydrogenase (substrates: NADP and ethanol), whereas the other half was assayed for NADP dependent *1,2-propanediol* dehydrogenase (substrates: NADP and 1,2-propanediol) (Fig. 1). A prominent intensity was observed for the ethanol dehydrogenase in the pure culture cell free extract (Fig. 1, lane 2), whereas only a small intensity was

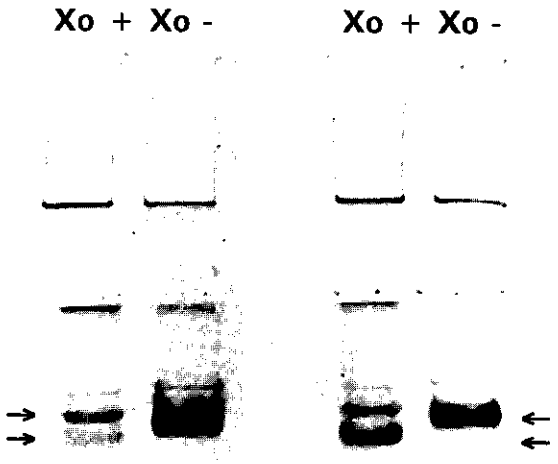

Fig. 1. Activity staining of NADP-dependent ethanol dehydrogenase (lanes 1 and 2) and NADP-dependent 1,2-propanediol dehydrogenase (lanes 3 and 4), after separation of ultra-centrifuged cell free extracts by native polyacrylamide gel electrophoresis. Lanes 1 and 3 contain cell free extract of the xylose + acetol (= Xo +) grown culture. Lane 2 and 4 contain cell free extract of the xylose grown culture (= Xo -). The arrows indicate the alcohol dehydrogenase activities, whereas the other bands with low intensity represent non-specific activities.

observed in the lane of the xylose + acetol culture (Fig. 1, lane 1). When the gel was incubated with 1,2-propanediol as the substrate the same protein band in the

pure culture cell free extract was stained as the one which was stained with ethanol as a substrate (Fig. 1, lanes 2 and 4). In contrast, another protein band appeared in the lanes of the xylose + acetol culture (Fig. 1, lane 3), suggesting another NADP dependent 1,2-propanediol dehydrogenase activity.

Discussion

Carbon monoxide (CO) is often used as an inhibitor of hydrogenases (Kim et al. 1984, Meyer et al. 1986, Rao and Mutharasan 1987, Marvin-Sikkema et al. 1993). However, CO did not completely inhibit H_2 formation in *Clostridium acetobutylicum* (ATCC 39236) (Datta and Zeikus 1985) and *C. pasteurianum* (DSM 525) (Dabrock et al. 1992). During xylose metabolism by *B. xylanolyticus* X5-1, H_2 production was completely inhibited by CO. Ethanol and formate production increased, and a decrease was observed in the acetate formation. In addition, a decreased biomass yield was found. This is in accordance with the decreased acetate production, as this is an important site of energy conservation for *B. xylanolyticus* X5-1. In batch cultures formate is only produced by pyruvate-formate lyase (data not shown) and not *via* a NAD(P)H dependent formate dehydrogenase. However, from the amount of formate produced, it can be deduced that part of the pyruvate formed during xylose catabolism is not converted *via* pyruvate-formate lyase, but *via* pyruvate:ferredoxin oxidoreductase, yielding acetyl-CoA, CO_2 and reduced ferredoxin. As the electrons of reduced ferredoxin cannot be transferred to the hydrogenase due to the inhibition by CO, they will be transferred to NAD(P) to form NAD(P)H. Re-oxidation of NAD(P)H is coupled to the production of ethanol, as no other products are known to function as electron sink in *B. xylanolyticus* X5-1.

Growth yields and product formation of *T. brockii*, *C. thermocellum* and *C. cellobioparum* were affected when the organisms were grown under an atmosphere of H_2 (Chung 1976; Ben-Bassat et al. 1981; Freier et al. 1988).

However, xylose metabolism and product formation by *B. xylanolyticus* X5-1 were hardly affected by high concentrations of molecular hydrogen. It could be calculated that during xylose catabolism by *B. xylanolyticus* X5-1 under an atmosphere of H_2/CO_2 (80%/20%) a significant amount of H_2 still was produced. The redox couples for $H_2/2H^+$ and reduced ferredoxin/oxidized ferredoxin (-414 mV and -398 mV, respectively (Thauer et al. 1977)) are in the same range. As a result, H_2 formation from reduced ferredoxin can occur and product formation is hardly affected.

During xylose metabolism in *B. xylanolyticus* X5-1 the electron flow was influenced by the external electron acceptors dihydroxy acetone, acetone, acetoin and acetol. The electron acceptors were reduced to their corresponding alcohols glycerol, 2-propanol, 2,3-butanediol and 1,2-propanediol, respectively. In continuous culture, acetol functioned as electron acceptor as well. However, not only 1,2-propanediol was a product of acetol reduction, but also acetone. It is not known whether acetone is formed *via* the classical pathway *via* acetoacetyl-CoA and acetoacetate as is found in *C. acetobutylicum* (Datta and Zeikus 1985) or *via* dehydration of 1,2-propanediol. Due to the presence of electron acceptors, a decrease was found in the H_2 and ethanol production, and an increased production of biomass and acetate. Similar results have been found by Ben-Bassat et al. (1981) who used acetone as electron acceptor to reverse the inhibition of the growth of *T. brockii* by H_2. In that organism, the presence of acetone even increased the specific growth rate on glucose. The growth rate of *B. xylanolyticus* X5-1 on xylose was not affected by the electron acceptors, suggesting that anabolic reactions were growth limiting. Acetoin has been used to overcome the redox imbalance in yeast cells grown anaerobically on xylose (Bruinenberg et al. 1983; Delgenes et al. 1991), though the concentrations used, inhibited the xylose conversion to some extent.

Enzyme levels in *B. xylanolyticus* X5-1 differed when the organism was grown on xylose, on xylose + acetol or on xylose in the presence of *M. hungatei* JF-1. However, the catabolic enzyme activities remained high enough to account for the *in vivo* xylose degradation. The reducing equivalents liberated during pyruvate

oxidation in the xylose + acetol grown cells were probably transferred to NADP *via* ferredoxin:NADP oxidoreductase. NADPH was used for the reduction of acetol by the NADPH-dependent 1,2-propanediol dehydrogenase. Glyceraldehyde-3-PO_4 dehydrogenase was strictly NAD-dependent. From the amount of ethanol produced by the xylose + acetol grown cells, it could be deduced that a large part of the NADH that is formed during xylose catabolism is used for the acetol reduction. However, NADH:NADP transhydrogenase and NADH:ferredoxin oxidoreductase could hardly be detected. In addition, NADP-dependent malic enzyme was not detectable either, thereby excluding the electron transfer from NADH to NADP *via* the reaction sequence pyruvate, oxaloacetate, malate, pyruvate. This sequence was proposed to act as a transhydrogenase activity in *T. brockii* (Lamed and Zeikus, 1980b). Thus far, it is not fully understood how the reducing equivalents are channelled from NADH to the NADP-dependent acetol reducing enzyme.

When grown on xylose, *B. xylanolyticus* X5-1 possesses a NAD-dependent alcohol dehydrogenase (ADH) and a NADP-dependent ADH. The latter likely has a non-specific activity with 1,2-propanediol, as suggested from the *in gel* activity staining. This explained the activity for the 1,2-propanediol dehydrogenase that was found in cell free extract of the xylose grown culture. A third ADH, also NADP dependent, was induced when *B. xylanolyticus* X5-1 was grown on xylose in the presence of the electron acceptor acetol. This enzyme is different from the NADP-dependent ADH that was found in the xylose grown culture, as could also be deduced from the *in gel* activity staining. Several NADP dependent ADH's have been described (De Moss 1955; Lamed and Zeikus 1980b; Dürre et al. 1987; Ismaiel et al. 1993). Lamed and Zeikus (1981) have purified and characterized the NADP dependent alcohol dehydrogenase of *T. brockii*. This enzyme is possibly of biotechnological value, since it has a broad substrate specificity. Primary and secondary alcohols, ketones and aldehydes were substrates for this enzyme (Lamed et al. 1981). From the data described in this paper, it is clear that *B. xylanolyticus* X5-1 is able to regulate the xylose metabolism by induction/repression mechanisms of several alcohol

dehydrogenases. A simplified scheme of the carbon and electron flow during xylose catabolism by *B. xylanolyticus* X5-1 grown under different physiological conditions is shown in figure 2.

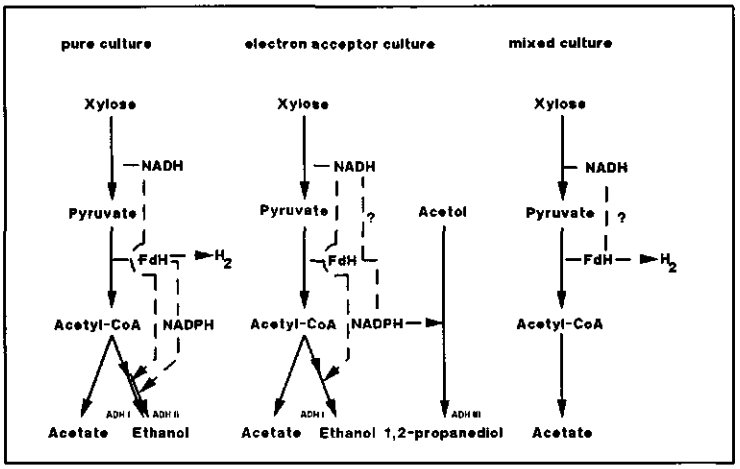

Fig. 2. Proposed pathway for the carbon and electron flow in *B. xylanolyticus* X5-1 during growth on either xylose as single substrate, on xylose in the presence of the electron acceptor acetol or on xylose in the presence of the methanogen *M. hungatei* JF-1. Solid and dashed lines represent the carbon and electron flow, respectively. The question mark indicates that the enzymes involved in these reactions could not be demonstrated.

Chiral products are important building blocks for the food, the agrochemical and pharmaceutical industry (Cameron and Cooney 1986, van den Tweel et al. 1992). R-(-)-1,2-propanediol can be used as emulsifier of foods (Cameron and Cooney, 1986), and is an important chiral building block in organic synthesis of for example optically active polymers. 1,2-Propanediol and 2,3-butanediol, formed by *B. xylanolyticus* X5-1 during growth on xylose in the presence of the electron acceptors acetol or acetoin, are optical active products. However, we did not investigate which of the enantiomers were formed. The data presented here can be used to evaluate the biotechnological potential of *B. xylanolyticus* X5-1 with respect to the production of optically active products, in particular the stability, the stereo specificity and the substrate range of its alcohol dehydrogenases.

Chapter 6

References

1. **Ahring, B.K., and P. Westermann.** 1987a. Thermophilic anaerobic degradation of butyrate by a butyrate-utilizing bacterium in coculture and triculture with methanogenic bacteria. Appl. Environ. Microbiol. **53**:429-433.
2. **Ahring, B.K., and P. Westermann.** 1987b. Kinetics of butyrate, acetate and hydrogen metabolism in a thermophilic, anaerobic butyrate degrading triculture. Appl. Environ. Microbiol. **53**:434-439.
3. **Alexander, M.A., T.W. Chapman, and T.W. Jeffries.** 1989. Continuous-culture responses of *Candida shehatae* to shifts in temperature and aeration: implications for ethanol inhibition. Appl. Environ. Microbiol. **55**:2152-2154.
4. **Ames, G. F-L.** 1986. Bacterial periplasmic transport systems: structure, mechanisms, and evolution. Ann. Rev. Biochem. **55**:397-425.
5. **Andersch, W., H. Bahl, and G. Gottschalk.** 1982. Acetone-butanol production by *Clostridium acetobutylicum* in an ammonium-limited chemostat at low pH values. Biotechnol. Lett. **4**:29-32.
6. **Andersch, W., H. Bahl, and G. Gottschalk.** 1983. Level of enzymes involved in acetate, butyrate, acetone and butanol formation by *Clostridium acetobutylicum*. Eur. J. Appl. Microbiol. Biotechnol. **18**:327-332.
7. **Asther, M., and A.W. Khan.** 1984. Influence of the presence of *Zymomonas anaerobia* on the conversion of cellobiose, glucose, and xylose to ethanol by *Clostridium saccharolyticum*. Biotechnol. Bioengin. **26**:970-972.
8. **Asther, M., and A.W. Khan.** 1985. Improved fermentation of cellobiose, glucose, and xylose to ethanol by *Zymomonas anaerobia* and a high ethanol tolerant strain of *Clostridium saccharolyticum*. Appl. Microbiol. Biotechnol. **21**:234-237.
9. **Bahl, H., W. Andersch, K. Braun, and G. Gottschalk.** 1982a. Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture. Eur. J. Appl. Microbiol. Biotechnol. **14**:17-20.
10. **Bahl, H., W. Andersch, and G. Gottschalk.** 1982b. Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. Eur. J. Appl. Microbiol. Biotechnol. **15**:201-205.
11. **Bahl, H., and G. Gottschalk.** 1985. Parameters affecting solvent production by *Clostridium acetobutylicum* in continuous culture. Biotechnol. Bioengin. **15**:217-223.
12. **Barbosa, M.D.S., H. Lee, H. Schneider, and C.W. Forsberg.** 1990. Temperature mediated changes of D-xylose metabolism in the yeast *Pachysolen tannophilus*. FEMS Microbiol. Lett. **72**:35-40.
13. **Bauchop, T., and D.O. Mountfort.** 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. Appl. Environ. Microbiol. **42**:1103-1110.
14. **Ben-Bassat, A., R. Lamed, and J.G. Zeikus.** 1981. Ethanol production by thermophilic bacteria: metabolic control of end product formation in *Thermoanaerobium brockii*. J. Bacteriol. **146**:192-199.
15. **Benoist, P., and J. Schwencke.** 1990. Native agarose-polyacrylamide gel electrophoresis allowing the detection of aminopeptidase, dehydrogenase, and esterase activities at the nanogram level: enzymatic patterns in some *Frankia* strains. Anal. Biochem. **187**:337-344.
16. **Biely, P.** 1985. Microbial xylanolytic systems. Trends in Biotechnology **3**:286-290.
17. **Biesterveld, S., and A.J.M. Stams.** 1990. Growth of *Bacteroides xylanolyticus* X5-1 in the presence and absence of a methanogen. In: Bélaich J-P, Bruschi M, García J-L (eds) Microbiology and biochemistry of strict anaerobes involved in interspecies hydrogen transfer. Plenum Press, New York, pp 365-368.
18. **Bonch-osmolovskaya, E.A., and K.O. Stetter.** 1991. Interspecies hydrogen transfer in cocultures of thermophilic archaea. System. Appl. Microbiol. **14**:205-208.
19. **Boone, D.R., R.L. Johnson, and Y. Liu.** 1989. Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems and its implications in the measurement of Km for H_2 and formate uptake. Appl. Environ. Microbiol. **55**:1735-1741.

20. **Booth, I.R., and W.J. Mitchell.** 1987. Sugar transport and metabolism in the clostridia. *In* Reizer J, Peterkofsky A (eds) Sugar transport and metabolism in gram-postive bacteria John wiley and Sons.
21. **Bradford, M.M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-254.
22. **Bringer-Meyer, S., K-L. Schimz, and H. Sahm.** 1986. Pyruvate decarboxylase from *Zymomonas mobilis*. Isolation and partial characterization. Arch. Microbiol. **146:**105-110.
23. **Bringer-Meyer, S., and H. Sahm.** 1988. Metabolic shifts in *Zymomonas mobilis* in response to growth conditions. FEMS Microbiol. Rev. **54:**131-142.
24. **Brosseau, J.D., J-W. Yan, and K.V. Lo.** 1986. The relationship between hydrogen gas and butanol production by *Clostridium saccharoperbutylacetonicum*. Biotechnol. Bioengin. **28:**305-310.
25. **Bruinenberg, P.M., P.H.M. deBot, J.P. vanDijken, and W.A. Scheffers.** 1983. The role of redox balances in the anaerobic fermentation of xylose by yeasts. Eur. J. Appl. Microbiol. Biotechnol. **18:**287-292.
26. **Bryant, M.P., E.A. Wolin, M.J. Wolin, and R.S. Wolfe.** 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch. Microbiol. **59:**20-31.
27. **Caldwell, D.R., and K. Newman.** 1986a. D-Arabinose metabolism by *Bacteroides fragilis* strain 2044. Curr. Microbiol. **14:**55-59.
28. **Caldwell, D.R., and K. Newman.** 1986b. Pentose metabolism by *Bacteroides ruminicola* subsp. *brevis* Strain B_14. Curr. Microbiol. **14:**149-155.
29. **Callens, M., H. Kersters-Hilderson, O. van Opstal, and C.K. de Bruyne.** 1986. Catalytic properties of D-xylose isomerase from *Streptomyces violaceoruber*. Enzyme Microb. Technol. **8:**696-700.
30. **Cameron, D.C., and C.L. Cooney.** 1986. A novel fermentation : the production of R(-)-1,2-propanediol and acetol by *Clostridium thermosaccharolyticum*. Bio/technology **4:**651-654.
31. **Caspari, D., and J.M. Macy.** 1983. The role of carbon dioxide in glucose metabolism of *Bacteroides fragilis*. Arch. Microbiol. **135:**16-24.
32. **Cavedon, K., and E. Canale-Parola.** 1992. Physiological interactions between a mesophilic cellulolytic clostridium and a non-cellulolytic bacterium. FEMS Microbiol. Ecol. **86:**237-245.
33. **Chung, K-T.** 1976. Inhibitory effects of H_2 on growth of *Clostridium cellobioparum*. Appl. Environ. Microbiol. **31:**342-348.
34. **Conway, T.** 1992. The Entner-Douderoff pathway: history, physiology and molecular biology. FEMS Microbiol. Rev. **103:**1-28.
35. **Cook, G.M., P.H. Janssen, and H.W. Morgan.** 1993. Simultaneous uptake and utilisation of glucose and xylose by *Clostridium thermohydrosulfuricum*. FEMS Microbiol. Lett. **109:**55-62.
36. **Coughlan, M.P., and L.G. Ljungdahl.** 1988. Comparative biochemistry of fungal and bacterial cellulolytic systems. In *Biochemistry and genetics of cellulose degradation*, FEMS symposium no. 43, Aubert J-P, Béguin P, Millet J (eds) London & San Diego: Academic Press p. 11-30.
37. **Cynkin, M.A., and M.J. Gibbs.** 1958a. Metabolism of pentoses by Clostridia. J. Bacteriol. **75:**335-338.
38. **Cynkin, M.A., and E.A. Delwiche.** 1958b. Metabolism of pentoses by Clostridia I. Enzymes of ribose dissimilation in extracts of *Clostridium perfringens*. J. Bacteriol. **75:**331-334.
39. **Dabrock, B., H. Bahl, and G. Gottschalk.** 1992. Parameters affecting solvent production by *Clostridium pasteurianum*. Appl. Environ. Microbiol. **58:**1233-1239.
40. **Datta, R., and J.G. Zeikus.** 1985. Modulation of acetone-butanol-ethanol fermentation by carbon monoxide and organic acids. Appl. Environ. Microbiol. **49:**522-529.
41. **Delgenes, J.P., R. Moletta, and J.M. Navarro.** 1991. Xylose metabolism by *Pichia stipitis* - the effect of ethanol. Appl. Microbiol. Biotechnol. **35:**656-661.
42. **DeMoss, R.D.** 1955. TPN-Alcohol dehydrogenase from *Leuconostoc mesenteroides*. Meth. Enzymol. **I:**504-508.
43. **Dolfing, J.** 1992. The energetic consequences of hydrogen gradients in methanogenic ecosystems. FEMS Microbiol. Ecol. **101:**183-187.

44. **du Preez, J.C., M. Bosch, and B.A. Prior.** 1986. The fermentation of hexose and pentose sugars by *Candida Shehatae* and *Pichia stipitis*. Appl. Microbiol. Biotechnol. **23**:228-233.
45. **Dürre, P., A. Kuhn, M. Gottwald, and G. Gottschalk.** 1987. Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. **26**:268-272.
46. **Evans, C.T., and C. Ratledge.** 1984. Induction of xylulose-5-PO_4 phosphoketolase in a variety of yeasts grown on D-xylose: the key to efficient xylose metabolism. Arch. Microbiol. **139**:48-52.
47. **Feldmann, S.D., H. Sahm, and G.A. Sprenger.** 1992. Pentose metabolism in *Zymomonas mobilis* wild-type and recombinant strains. Appl. Microbiol. Biotechnol. **38**:354-361.
48. **Fraenkel, D.G.** 1987. Glycolysis, pentose phosphate pathway, and Entner-Douderoff pathway. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella typhimurium* - cellular and molecular biology, American Society for Microbiology, Washington DC, pp. 142-150.
49. **Franklund, C.V., and T.L. Glass.** 1987. Glucose uptake by the cellulolytic ruminal anaerobe *Bacteroides succinogenes*. J. Bacteriol. **169**:500-506.
50. **Freier, D., C.P. Mothershed, and J. Wiegel.** 1988. Characterization of *Clostridium thermocellum* JW20. Appl. Environ. Microbiol. **54**:204-211.
51. **Gilbert, H.J., and G.P. Hazlewood.** 1993. Bacterial cellulases and xylanases. J. Gen. Microbiol. **139**:187-194.
52. **Glass, T.L., M.P. Bryant, and M.J. Wolin.** 1977. Partial purification of ferredoxin from *Ruminococcus albus* and its role in pyruvate metabolism and reduction of nicotinamide adenine dinucleotide by H_2. J. Bacteriol. **131**:463-472.
53. **Goldberg, M., J.M. Fessenden, and E. Racker.** 1966. Phosphoketolase. Methods Enzymol. **9**:515-520.
54. **Gong, C-S., L.F. Chen, M.C. Flickinger, and G.T. Tsao.** 1981. Conversion of hemicellulose carbohydrates. Adv. Biochem. Engin. **20**:93-118.
55. **Gottschal, J.C., and J.G. Morris.** 1981. The induction of acetone and butanol production in cultures of *Clostridium acetobutylicum* by elevated concentrations of acetate and butyrate. FEMS Microbiol. Lett. **12**:385-389.
56. **Gottschalk, G.** 1985. Bacterial metabolism, 2nd ed., pp. 104-114 and 225-226, Springer verlag, New york.
57. **Gottwald, M., and G. Gottschalk.** 1985. The internal pH of *Clostridium acetobutylicum* and its effect on the shift from acid to solvent formation Arch. Microbiol. **143**:42-46.
58. **Heath, E.C., J. Hurwitz, B.L. Horecker, and A. Ginsburg.** 1958. Pentose fermentation by *Lactobacillus plantarum*. J. Biol. Chem. **231**:1009-1029.
59. **Heyndrickx, M., P. deVos, A. Speybrouck, and J. deLey.** 1989. Fermentation of mannitol by *Clostridium butyricum*: role of acetate as an external electron acceptor. Appl. Microbiol. Biotechnol. **31**:323-328.
60. **Heyndrickx, H., P. De Vos, and J. De Ley.** 1991a. Fermentation of D-xylose by *Clostridium butyricum* LMG 1213t_1 in chemostats. Enzyme Microb. Technol. **13**:893-897.
61. **Heyndrickx, M., P. deVos, and J. deLey.** 1991b. Fermentation characteristics of *Clostridium pasteurianum* LMG 3285 grown on glucose and mannitol. J. Appl. Bacteriol. **70**:52-58.
62. **Hino, T., K. Miyazaki, and S. Kuroda.** 1991. Role of extracellular acetate in the fermentation of glucose by a ruminal bacterium, *Megasphaera elsdenii*. J. Gen. Appl. Microbiol. **37**:121-129.
63. **Hiu, S.F., C-X. Zhu, R-T. Yan, and J-S Chen.** 1987. Butanol-ethanol dehydrogenase and butanol-ethanol-isopropanol dehydrogenase: different alcohol dehydrogenase in two strains of *Clostridium beijerinckii* (*Clostridium butyricum*). Appl. Environ. Microbiol. **53**:697-703.
64. **Höjeberg, B., P. Brodelius, J. Rydström, and K. Mosbach.** 1976. Affinity chromatography and binding studies on immobilized adenosine 5'-monophosphate and 2',5'-bisphosphate of nicotinamide nucleotide transhydrogenase from *Pseudomonas aeruginosa*. Eur. J. Biochem. **66**:467-475.
65. **Houwen, F.P., J. Plokker, A.J.M. Stams, and A.J.B. Zehnder.** 1990. Enzymatic evidence for involvement of the methylmalonyl-CoA pathway in propionate oxidation by *Syntrophobacter wolinii*. Arch. Microbiol. **155**:52-55.

66. **Howlett, M.R., D.O. Mountfort, K.W. Turner, and A.M. Roberton.** 1976. Metabolism and growth yields in *Bacteroides ruminicola* strain B_14. Appl. Environ. Microbiol. **32**:274-283.
67. **Hsiao, H-Y., L-C. Chiang, P.P. Ueng, and G.T. Tsao.** 1982. Sequential utilization of mixed monosaccharides by yeasts. Appl. Environ. Microbiol. **43**:840-845.
68. **Huang, L., C.W. Forsberg, and L.N. Gibbins.** 1986. Influence of external pH and fermentation products on *Clostridium acetobutylicum* intracellular pH and cellular distribution of fermentation products. Appl. Environ. Microbiol. **51**:1230-1234.
69. **Hurwitz, J.** 1958. Pentose phosphate cleavage by *Leuconostoc mesenteroides*. Biochim. Biophys. Acta. **28**:599-602.
70. **Huser, B.A., K. Wuhrmann, and A.J.B. Zehnder.** 1982. *Methanothrix soehngenii* gen. nov. sp. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. Arch. Microbiol. **13**:1-9.
71. **Ianotti, E.L., D. Kafkewitz, M.J. Wolin, and M.P. Bryant.** 1973. Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*: changes caused by interspecies transfer of H_2. J. Bacteriol. **114**:1231-1240.
72. **Ismaiel, A.A., C-X. Zhu, G.D. Colby, and J-S. Chen.** 1993. Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of *Clostridium beijerinckii*. J. Bacteriol. **175**:5097-5105.
73. **Jeffries, T.W., and H.K. Sreenath.** 1988. Fermentation of hemicellulosic sugars and sugar mixtures by *Candida shehatae*. Biotechnol. Bioengin. **31**:502-506.
74. **Joblin, K.N., G.E. Naylor, and A.G. Williams.** 1990. Effect of *Methanobrevibacter smithii* on xylanolytic activity of anaerobic ruminal fungi. Appl. Environ. Microbiol. **56**:2287-2295.
75. **Jones, D.T., and D.R. Woods.** 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. **50**:484-524.
76. **Joyner, A.E., and R.L. Baldwin.** 1966. Enzymatic studies of pure cultures of rumen microorganisms. J. Bacteriol. **92**:1321-1329.
77. **Jungermann, K.A., E. Rupprecht, C. Ohrloff, R.K. Thauer, and K. Decker.** 1971. Regulation of the reduced nicotinamide adenine dinucleotide-ferredoxin reductase system in *Clostridium kluyveri*. J. Biol. Chem. **246**:960-963.
78. **Jungermann, K.A., R.K. Thauer, G. Leimenstoll, and K. Decker.** 1973. Function of reduced pyridine nucleotide-ferredoxin oxidoreductases in saccharolytic *Clostridia*. Biochim. Biophys. Acta **305**:268-280.
79. **Jungermann, K.A., and G. Schön.** 1974. Pyruvate formate lyase in *Rhodospirillum rubrum* Ha adapted to anaerobic dark conditions. Arch. Microbiol. **99**:109-116.
80. **Khan, A.W., T.M. Trottier, G.B. Patel, and S.M. Martin.** 1979. Nutrient requirement for the degradation of cellulose to methane by a mixed population of anaerobes. J. Gen. Microbiol. **112**:365-372.
81. **Kilian, S.G., B.A. Prior, and P.M. Lategan.** 1983. Diauxic utilization of glucosecellobiose mixtures by *Candida wickerhamii*. Eur. J. Appl. Microbiol. Biotechnol. **18**:369-373.
82. **Kim, B.H., P. Bellows, R. Datta, and J.G. Zeikus.** 1984. Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. Appl. Environ. Microbiol. **48**:764-770.
83. **Knappe, J., and G. Sawers.** 1990. A radical-chemical route to acetyl-CoA: the anaerobically induced pyruvate formate-lyase system of *Escherichia coli*. FEMS Microbiol. Rev. **75**:383-398.
84. **Konings, W.N.** 1985. Generation of metabolic energy by end-product efflux. Trends Biochem. Sci. **10**:317-319.
85. **Kremer, D.R., and T.A. Hansen.** 1987. Glycerol and dihydroxyacetone dissimilation in *Desulfovibrio* strains. Arch. Microbiol. **147**:249-256.
86. **Kröger, A.** 1980. Bacterial electron transport to fumarate. In: Knowles CJ (ed) Diversity of Bacterial Respiratory Systems, vol II, CRC Press Inc., Boca Raton, Florida pp 1-17.
87. **Lachke, A.H., and T.W. Jeffries.** 1986. Levels of enzymes of the pentose phosphate pathway in *Pachysolen tannophilus* Y-2460 and selected mutants. Enzyme Microb. Technol. **8**:353-359.

88. **Lacis, L.S., and H.G. Lawford.** 1991. *Thermoanaerobacter ethanolicus* growth and product yield from elevated levels of xylose or glucose in continuous cultures. Appl. Environ. Microbiol. **57**:579-585.

89. **Lamed, R., and J.G. Zeikus.** 1980a. Glucose fermentation pathway of *Thermoanaerobium brockii*. J. Bacteriol. **141**:1251-1257.

90. **Lamed, R.J., and J.G. Zeikus.** 1980b. Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. J. Bacteriol. **144**:569-578.

91. **Lamed, R.J., and J.G. Zeikus.** 1981. Novel NADP-linked alcohol-aldehyde/ketone oxidoreductase in thermophilic ethanologenic bacteria. Biochem. J. **195**:183-190.

92. **Lamed, R.J., E. Keinan, and J.G. Zeikus.** 1981. Potential application of an alcohol-aldehyde/ketone oxidoreductase from thermophilic bacteria. Enzyme Microb. Technol. **3**:144-148.

93. **Lamed, R.J., J.H. Lobos, and T.M. Su.** 1988. Effects of stirring and hydrogen on fermentation products of *Clostridium thermocellum*. Appl. Environ. Microbiol. **54**:1216-1221.

94. **Latham, M.J., and M.J. Wolin.** 1977. Fermentation of cellulose by *Ruminococcus flavefaciens* in the presence and absence of *Methanobacterium ruminantium*. Appl. Environ. Microbiol. **34**:297-301.

95. **LeBlanc, D.J., and R.P. Mortlock.** 1971. Metabolism of D-arabinose: a new pathway in *Escherichia coli*. J. Bacteriol. **106**:90-96.

96. **Lee, C.K., and Z.J. Ordal.** 1967. Regulatory effect of pyruvate on the glucose metabolism of *Clostridium thermosaccharolyticum*. J. Bacteriol. **94**:530-536.

97. **Lee, H.** 1992. Reversible inactivation of D-xylose utilization by D-glucose in the pentose fermenting yeast *Pachysolen tannophilus*. FEMS Microbiol. Lett. **92**:1-4.

98. **Legall, J., J.J.G. Moura, H.D. Peck Jr., and A.V. Xavier.** 1982. Hydrogenase and other iron-sulfur proteins from sulfate-reducing and methane-forming bacteria. In: Metal Ions Biology. **4**:176-248.

99. **Lohmeier-Vogel, E., and B. Hahn-Hägerdal.** 1985. The utilization of metabolic inhibitors for shifting product formation from xylitol to ethanol in pentose fermentations using *Candida tropicalis*. Appl. Microbiol. Biotechnol. **21**:167-172.

100. **Levering, P.R., L. Dijkhuizen, and W. Harder.** 1982. Enzymatic evidence for the operation of the FBP aldolase cleavage and the TK/TA rearrangement variant of the RuMP cycle in *Arthrobacter* P1. FEMS Microbiol. Lett. **14**:257-261.

101. **Long, S., D.T. Jones, and D.R. Woods.** 1984. Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P262. Appl. Microbiol. Biotechnol. **20**:256-261.

102. **Lowe, S.E., and J.G. Zeikus.** 1991. Metabolic regulation of carbon and electron flow as a function of pH during growth of *Sarcina ventriculi*. Arch. Microbiol. **155**:325-329.

103. **Lowry, O.H., N.J. Rosebrough, A. Farr, and R.J. Randall.** 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**:265-275.

104. **Lucas, C., and N. vanUden.** 1986. Transport of hemicellulose monomers in the xylose-fermenting yeast *Candida shehatae*. Appl. Microbiol. Biotechnol. **23**:491-495.

105. **Macy, J.M., and I. Probst.** 1979. The biology of gastrointestinal bacteroides. Ann. Rev. Microbiol. **33**:561-594.

106. **Magee, R.J., and N. Kosaric.** 1987. The microbial production of 2,3-butanediol. *In* Laskin AI (ed) Adv. Appl. Microbiol. vol. **32** Academic Press, Inc., New York p. 89-161.

107. **Martin, S.A.** 1992. Factors affecting glucose uptake by the ruminal bacterium *Bacteroides ruminicola*. Appl. Microbiol. Biotechnol. **37**:104-108.

108. **Marvin-Sikkema, F.D., E. Rees, M.N. Kraak, J.C. Gottschal, and R.A. Prins.** 1993. Influence of metronidazole, CO, CO2, and methanogens on the fermentative metabolism of the anaerobic fungus *Neocallimastix* sp. strain L2. Appl. Environ. Microbiol. **59**:2678-2683.

109. **Matte, A., C.W. Forsberg, and A.M. Verrinder-Gibbins.** 1992. Enzymes associated with metabolism of xylose and other pentoses by *Prevotella (Bacteroides) ruminicola* strains, *Selenomonas ruminantium* D, and *Fibrobacter succinogenes* S85. Can. J. Microbiol. **38**:370-376.
110. **McCarty, P.L.** 1972. Energetics of organic matter degradation. In: Mitchell (ed) Water Pollution Microbiology, John Wiley and Sons Inc., New York, pp 91-118.
111. **McNeil, B., and B. Kristiansen.** 1985. Effect of temperature upon growth rate and solvent production in batch cultures of *Clostridium acetobutylicum*. Biotechnol. Lett. **7**:499-502.
112. **Meyer, C.L., J.W. Roos, and E.T. Papoutsakis.** 1986. Carbon monoxide gasing leads to alcohol production and butyrate uptake without acetone formation in continuous cultures of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. **24**:159-167.
113. **Miller, T.L., and M.J. Wolin.** 1979. Fermentations by saccharolytic intestinal bacteria. Am. J. Clin. Nutr. **32**:164-172.
114. **Mishra, P., and A. Singh.** 1993. Microbial pentose utilization *In* Neidleman S, laskin AI (eds) Adv. Appl. Microbiol. vol 39 Academic Press, Inc., New York p. 91-151.
115. **Monot, F., J-M. Engasser, and H. Petitdemange.** 1984. Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. **19**:422-426.
116. **Mountfort, D.O., R.A. Asher, and T. Bauchop.** 1982. Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in a triculture with *Methanobrevibacter* sp. Strain ra1 and *Methanosarcina*. Appl. Environ. Microbiol. **44**:128-134.
117. **Ng, T.K., A. Ben-Bassat, and J.G. Zeikus.** 1981. Ethanol production by thermophilic bacteria: fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. Appl. Environ. Microbiol. **41**:1337-1343.
118. **Ng, T.K., and J.G. Zeikus.** 1982. Differential metabolism of cellobiose and glucose by *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. J. Bacteriol. **150**:1391-1399.
119. **Nuraida, L., I. Grigolava, J.D. Owens, and G. Campbell-Platt.** 1992. Oxygen and pyruvate as external electron acceptors for *Leuconostoc* spp. J. Appl. Bacteriol. **72**:517-522.
120. **Ohta, K., F. Alterthum, and L.O. Ingram.** 1990. Effects of environmental conditions on xylose fermentation by recombinant *Escherichia coli*. Appl. Environ. Microbiol. **56**:463-465.
121. **Ounine, K., H. Petitdemange, G. Raval, and R. Gay.** 1985. Regulation and butanol inhibition of D-xylose and D-glucose uptake in *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **49**:874-878.
122. **Palleroni, N.J., and M. Douderoff.** 1957. Metabolism of carbohydrates by *Pseudomonas saccharophila*. III. Oxidation of D-arabinose. J. Bacteriol. **74**:180-185.
123. **Parameswaran, A.K., R.N. Schicho, J.P. Soisson, R.M. Kel.** 1988. Effect of hydrogen and carbon dioxide partial pressures on growth and sulfide production of the extremely thermophilic archaebacterium *Pyrodictium brockii*. Biotechnol. Bioengin. **32**:438-443.
124. **Patel, G.B.** 1984. Ethanol production during D-xylose, L-arabinose, and D-ribose fermentation by *Bacteroides polypragmatus*. Appl. Microbiol. Biotechnol. **20**:111-117.
125. **Patel, G.B., C.R. MacKenzie, and B.J. Agnew.** 1986. Fermentation of xylose and hemicellulose hydrolysates by an ethanol-adapted culture of *Bacteroides polypragmatus*. Arch. Microbiol. **146**:68-73.
126. **Patni, N.J., and J.K. Alexander.** 1971. Utilization of glucose by *Clostridium thermocellum*: presence of glucokinase and other glycolytic enzymes in cell extracts. J. Bacteriol. **105**:220-225.
127. **Pavlostathis, S.G., T.L. Miller, and M.J. Wolin.** 1990. Cellulose fermentation by continuous cultures of *Ruminococcus albus* and *Methanobrevibacter smithii*. Appl. Microbiol. Biotechnol. **33**:109-116.
128. **Petersen, D.J., R.W. Welch, F.B. Rudolph, and G.N. Bennett.** 1991. Molecular cloning of an alcohol (butanol) dehydrogenase gene cluster from *Clostridium acetobutylicum* ATCC 824. J. Bacteriol. **173**:1831-1834.
129. **Petitdemange, H., C. Cherrier, G. Raval, and R. Gay.** 1976. Regulation of the NADH and NADPH-ferredoxin oxidoreductases in Clostridia of the Butyric Group. Biochim. Biophys. Acta **421**:334-347.

130. **Racker, E.** 1962. Fructose-6-phosphate phosphoketolase from *Acetobacter xylinum*. Methods Enzymol. **5**:276-280.
131. **Ragsdale, S.W., and L.G. Ljungdahl.** 1984. Characterization of ferredoxin, flavodoxin, and rubredoxin from *Clostridium formicoaceticum* grown in media with high and low iron content. J. Bacteriol. **157**:1-6.
132. **Rao, G., and R. Mutharasan.** 1987. Altered electron flow in continuous cultures of *Clostridium acetobutylicum* induced by viologen dyes. Appl. Environ. Microbiol. **53**:1232-1235.
133. **Rao, G., and R. Mutharasan.** 1989. NADH levels and solventogenesis in *Clostridium acetobutylicum*: new insights through culture fluorescence. Appl. Microbiol. Biotechnol. **30**:59-66.
134. **Rosenberg, S.L.** 1980. Fermentation of pentose sugars to ethanol and other neutral products by microorganisms. Enzyme Microbiol. Technol. **2**:185-193.
135. **Russell, J.B., and R.L. Baldwin.** 1978. Substrate preferences in rumen bacteria: evidence of catabolite regulatory mechanisms. Appl. Environ. Microbiol. **36**:319-329.
136. **Russell, J.B., and R.L. Baldwin.** 1979. Comparison of substrate affinities among several rumen bacteria: a possible determinant of rumen bacterial competition. Appl. Environ. Microbiol. **37**:531-536.
137. **Saddler, J.N., E.K.C. Yu, M. Mes-Hartree, N. Levitin, and H.H. Brownell.** 1983. Utilization of enzymatically hydrolyzed wood hemicelluloses by microorganisms for production of liquid fuel. Appl. Environ. Microbiol. **45**:153-160.
138. **Saier, jr. M.H.** 1989. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate: sugar phosphotransferase system. Microbiol.Rev. **53**:109-120.
139. **Salyers, A.A.** 1984. *Bacteroides* of the human lower intestinal tract. Ann. Rev. Microbiol. **38**:293-313.
140. **Scheifinger, C.C., and M.J. Wolin.** 1973. Propionate formation from cellulose and soluble sugars by combined cultures of *Bacteroides succinogenes* and *Selenomonas ruminantium*. Appl. Microbiol. **26**:789-795.
141. **Scheifinger, C.C., B. Linehan, and M.J. Wolin.** 1975. H_2 production by *Selenomonas ruminantium* in the absence and presence of methanogenic bacteria. Appl. Microbiol. **29**:480-483.
142. **Schlegel, H.G.** 1984. Allgemeine Mikrobiologie, 6th ed., Thieme Verlag, Stuttgart, New York pp. 1-12 .
143. **Scholten-Koerselman, I., F. Houwaard, P. Janssen, and A.J.B. Zehnder.** 1986. *Bacteroides xylanolyticus* sp. nov., a xylanolytic bacterium from methane producing cattle manure. Antonie van Leeuwenhoek **52**:543-554.
144. **Schönheit, P., J. Moll, and R.K. Thauer.** 1980. Growth parameters (K_s, μ_{max}, Y_s) of *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **127**:59-65.
145. **Schyns P.J.Y.M.J., and A.J.M. Stams.** 1992. Xylan degradation by the anaerobic bacterium *Bacteroides xylanolyticus* X5-1. In: Visser J, Beldman G, Kusters-Someren MA, Vorhagen AGJ (eds) Xylans and xylanases: progress in biotechnology, vol 7, Elsevier Science Publ. the Netherlands pp. 295-299.
146. **Scrutton, M.C., M.R. Olmsted, and M.F. Utter.** 1969. Pyruvate carboxylase from chicken liver. Methods Enzymol. **13**:235-249.
147. **Sgorbati, B., G. Lenaz, and F. Casalicchio.** 1976. Purification and properties of two fructose-6-phosphate phosphoketolases in *Bifidobacterium*. Antonie van Leeuwenhoek **42**:49-57.
148. **Shamanna, D.K., and K.E. Sanderson.** 1979. Uptake and catabolism of D-xylose in *Salmonella typhimurium* LT2. J. Bacteriol. **139**:64-70.
149. **Singh, A., P.K.R. Kumar, and K. Schugerl.** 1991. Shift in product formation from acetate to ethanol using metabolic inhibitors in *Fusarium oxysporum*. Biotechnol. Lett. **13**:527-532.
150. **Skoog, K., and B. Hahn-Hägerdal.** 1988. Xylose fermentation. Enzyme Microb. Technol. **10**:66-80.
151. **Soni, B.K., K. Das, and T.K. Ghose.** 1987. Inhibitory factors involved in acetone-butanol fermentation by *Clostridium saccharoperbutylacetonicum*. Curr. Microbiol. **16**:61-68.

152. **Spormann, A.M., and R.K. Thauer.** 1988. Anaerobic acetate oxidation to CO_2 by *Desulfotomaculum acetoxidans*. Demonstration of enzymes required for the operation of an oxidative acetyl-CoA/carbon monoxide dehydrogenase pathway. Arch. Microbiol. **150**:374-380.
153. **Stams, A.J.M., and T.A. Hansen.** 1984. Fermentation of glutamate and other compounds by *Acidaminobacter hydrogenoformans* gen.nov. sp.nov., an obligate anaerobe isolated from black mud. Studies with pure cultures and mixed cultures with sulfate-reducing and methanogenic bacteria. Arch. Microbiol. **137**:329-337.
154. **Stams, A.J.M., D.R. Kremer, K. Nicolay, G.H. Weenk, and T.A. Hansen.** 1984. Pathway of propionate formation in *Desulfobulbus propionicus*. Arch. Microbiol. **139**:167-173.
155. **Stams, A.J.M., J.B. van Dijk, C. Dijkema, and C.M. Plugge.** 1993. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl. Environ. Microbiol. **59**:1114-1119.
156. **Standing, C.N., A.G. Fredrickson, and H.M. Tsuchiya.** 1972. Batch- and continuous-culture transients for two substrate systems. Appl. Microbiol. **23**:354-359.
157. **Stephens, G.M., R.A. Holt, J.C. Gotschall, and J.G. Morris.** 1985. Studies on the stability of solvent production by *Clostridium acetobutylicum* in continuous culture. J. Appl. Bacteriol. **59**:597-605.
158. **Stouthamer, A.H.** 1978. Energy yielding pathways. In: Gunsalus IC (ed) The bacteria, a treatise on structure and function, vol VI, Academic Press Inc., New York, pp. 389-462.
159. **Strobel, H.J.** 1993a. Evidence for catabolite inhibition in regulation of pentose utilization and transport in the ruminal bacterium *Selenomonas ruminantium*. Appl. Environ. Microbiol. **59**:40-46.
160. **Strobel, H.J.** 1993b. Pentose utilization and transport by the ruminal bacterium *Prevotella ruminicola*. Arch. Microbiol. **159**:465-471
161. **Talarico, T.L., L.T. Axelsson, J. Novotny, and M. Fiuzat.** 1990. Utilization of glycerol as a hydrogen acceptor by *Lactobacillus reuteri*: purification of 1,3-propanediol:NAD^+ oxidoreductase. Appl. Environ. Microbiol. **56**:943-948.
162. **Thauer, R.K., K. Jungermann, E. Rupprecht, and K. Decker.** 1969. Hydrogen formation from NADH in cell free extracts of *Clostridium kluyveri*. Acetyl coenzyme A requirement and ferredoxin dependence. FEBS Lett. **4**:108-112.
163. **Thauer, R.K., E. Rupprecht, C. Ohrloff, K. Jungermann, and K. Decker.** 1971. Regulation of the reduced nicotinamide adenine dinucleotide phosphate-ferredoxin reductase system in *Clostridium kluyveri*. J. Biol. Chem. **246**:954-959.
164. **Thauer, R.K., F.H. Kirchniawy, and K.A. Jungermann.** 1972. Properties and function of the pyruvate formate lyase reaction in *Clostridiae*. Eur. J. Biochem. **27**:282-290.
165. **Thauer, R.K., G. Fuchs, U. Schnitker, and K. Jungermann.** 1973. CO_2-reductase from *Clostridium pasteurianum*: molybdenum dependence of synthesis and inactivation by cyanide. FEBS lett. **38**:45-48.
166. **Thauer, R.K., G. Fuchs, and K.A. Jungermann.** 1974. Reduced ferredoxin:CO_2 oxidoreductase from *Clostridium pasteurianum*: its role in formate metabolism. J. Bacteriol. **118**:758-760.
167. **Thauer, R.K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100-180.
168. **Thiele, J.H., and J.G. Zeikus.** 1988. Control of interspecies electron flow during anaerobic digestion: significance of formate versus hydrogen transfer during syntrophic methanogenesis in flocs. Appl. Environ. Microbiol. **54**:20-29.
169. **Tran-Din, K., and G. Gottschalk.** 1985. Formation of D(-)-1,2-propanediol and D(-)-lactate from glucose by *Clostridium sphenoides* under phosphate limitation. Arch. Microbiol. **142**:87-92.
170. **Turner, K.W., and A.M. Roberton.** 1979. Xylose, arabinose, and rhamnose fermentation by *Bacteroides ruminicola*. Appl. Environ. Microbiol. **38**:7-12.
171. **van den Tweel, W.J.J., T.J.G.M. vanDooren, and P.H. deJonge.** 1993. *Ochrobactrum antrophi* NCIMB 40321: a new biocatalyst with broad-spectrum L-specific amidase activity. Appl. Microbiol. Biotechnol. **39**:296-300.

172. **van Dijken, J.P., and J.R. Quayle.** 1977. Fructose metabolism in four *Pseudomonas* species. Arch. Microbiol. **114**:281-286.
173. **Vancanneyt, M., P. deVos, L. Vennens, and J. deLey.** 1990. Lactate and ethanol dehydrogenase activities in continuous cultures of *Clostridium thermosaccharolyticum* LMG-6564. J. Gen. Microbiol. **136**:1945-1951.
174. **Weimer, P.J., and J.G. Zeikus.** 1977. Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. Appl. Environ. Microbiol. **33**:289-297.
175. **Whitworth, D.A., and C. Ratledge.** 1977. Phosphoketolase in *Rhodotorula graminis* and other yeasts. J. Gen. Microbiol. **102**:397-401.
176. **Willets, A.J., and J.M. Turner.** 1970. Threonine metabolism in a strain of *Bacillus subtilis*: enzymes acting on methylglyoxal. Biochim. Biophys. Acta **222**:668-670.
177. **Williams, D.K., and S.A. Martin.** 1990. Xylose uptake by the ruminal bacterium *Selenomonas ruminantium*. Appl. Environ. Microbiol. **56**:1683-1688.
178. **Wilson, B.L., and R.P. Mortlock.** 1973. Regulation of D-xylose and D-arabitol catabolism by *Aerobacter aerogenes*. J. Bacteriol. **113**:1404-1411.
179. **Wolin, E.A., M.J. Wolin, and R.S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238**:2882-2886.
180. **Wong, K.K.Y., L.U.L. Tan, and J.N. Saddler.** 1988. Multiplicity of ß-1,4-xylanase in microorganisms: functions and applications. Microbiol. Rev. **52**:305-317.
181. **Wu, R., and E. Racker.** 1959. Regulatory mechanisms in carbohydrate metabolism III. Limiting factors in glycolysis of ascites tumor cells. J. Biol. Chem. **234**:1029-1035.
182. **Wu, W-M., R.F. Rickley, M.K. Jain, and J.G. Zeikus.** 1993. Energetics and regulations of formate and hydrogen metabolism by *Methanobacterium formicicum*. Arch. Microbiol. **159**:57-65.
183. **Yan, R-T., ZC-X. Zhu, C. Golemboski, and J-S. Chen.** 1988. Expression of solvent forming enzymes and onset of solvent production in batch cultures of *Clostridium beijerinckii* (*Clostridium butylicum*). Appl. Environ. Microbiol. **54**:642-648.
184. **Yang, S.T., and I.C. Tang.** 1991. Methanogenesis from lactate by a co-culture of *Clostridium formicoaceticum* and *Methanosarcina mazei*. Appl. Microbiol. Biotechnol. **35**:119-123.
185. **Young, M., W.L. Staudenbauer, and N.P. Minton.** 1989. Genetics of *Clostridium*. *In:* Minton NP, Clarke DJ (eds) Biotechnology Handbooks, vol 3, Clostridia. Plenum Press, New york pp. 63-103.
186. **Yu, E.K.C., L. Deschatelets, and J.N. Saddler.** 1984. The combined enzymatic hydrolysis and fermentation of hemicellulose to 2,3-butanediol. Appl. Microbiol. Biotechnol. **19**:365-372.
187. **Yu, E.K.C., L. Deschatelets, G. Louis-seize, and J.N. Saddler.** 1985a. Butanediol production from cellulose and hemicellulose by *Klebsiella pneumoniae* grown in sequential coculture with *Trichoderma harzianum*. Appl. Environ. Microbiol. **50**:924-929.
188. **Yu, E.K.C., MK-H. Chan, and J.N. Saddler.** 1985b. Butanediol production from lignocellulosic substrates by *Klebsiella pneumoniae* grown in sequential co-culture with *Clostridium thermocellum*. Appl. Microbiol. Biotechnol. **22**:399-404.
189. **Yu, E.K.C., and J.N. Saddler.** 1985. Biomass conversion to butanediol by simultameous saccharification and fermentation. Trends in Biotechnology **3**:100-104.
190. **Zehnder, A.J.B., B.A. Huser, T.D. Brock, and K. Wuhrmann.** 1980. Characterization of an acetate-decarboxylating, non-hydrogen-oxidizing methane bacterium. Arch. Microbiol. **124**:1-11.
191. **Zeikus, J.G.** (1980) Chemical and fuel production by anaerobic bacteria. Ann. Rev. Microbiol. **34**:423-464.
192. **Zimmermann, W.** 1992. Bacterial degradation of hemicelluloses. In: Sigel H, Sigel A (eds) Metal ions in biological systems. Vol **28**. Degradation of environmental pollutants. M Dekker, Inc. New York, New York pp. 357-398.

Chapter 7

Summary

Plant cell walls represent a major part of the available biomass on earth. They are mainly composed of the energy-rich polymers lignin, cellulose, and hemicellulose. For many decades, research is done to exploit agricultural and forestry wastes as renewable resources. Much research was focused on the degradation of cellulose. In contrast, hemicellulose has got less attention, though it can account for up to 40% of the total dry weight of plant cell walls. Fermentation by anaerobic bacteria offers the possibility to conserve most energy fixed in the energy-rich polymeric and monomeric sugars in the form of organic acids and solvents (e.g. acetic acid, butanol and acetone).

A project in which the anaerobic conversion of hemicellulose to potentially biotechnological interesting products was investigated, was divided into two parts. One part, performed by Philippe Schyns, concerned the microbial degradation of xylan, which was used as a model substrate for hemicellulose. Several xylanolytic enzymes (endo-xylanase, ß-xylosidase, acetylesterase, α-L-arabinofuranosidase) were purified and characterized. The mode of action of some of these enzymes was investigated. Furthermore, the induction mechanism of xylanase and ß-xylosidase was studied. The results of this research will be presented in a separate thesis. The other part of the project, of which the outcomes are given in this thesis, was focused mainly on the fermentation of xylose, a major constituent of hemicelluloses.

Bacteroides xylanolyticus X5-1 was used as a model organism. This organism had been isolated from fermenting cattle manure. *B. xylanolyticus* X5-1 can only grow on one specific hemicellulose, xylan. Cellulose and other hemicelluloses could not be utilized for growth. This fact made the organism interesting for studying the (regulation of the) xylanolytic enzyme synthesis, since interferences from other (hemi)cellulolytic enzymes could be excluded. In addition, the organism could ferment a wide variety of monomeric sugars, produced a mixture of end products, and showed a relatively high growth rate. These latter features

made *B. xylanolyticus* X5-1 a suitable microorganism for studying the regulation of the anaerobic xylose fermentation.

Information concerning the composition and degradation of biomass, and the (regulation of) product formation from biomass has been reviewed in a general context in **chapter 1.** Some biotechnological applications of biomass fermentation have been mentioned in this chapter as well.

Using ^{14}C-labelled xylose, the xylose uptake system of this organism was studied. It was shown that xylose transport occurs *via* an active uptake system, and probably a binding protein was involved. The exact mechanism of xylose uptake remains to be elucidated. Based on mass balance calculations, measuring specific enzyme activities of key enzymes of catabolic pathways, and determining label distribution patterns with ^{13}C-NMR, the pentose phosphate pathway in conjunction with the glycolysis was shown to be operative in xylose fermentation by *B. xylanolyticus* X5-1. Acetate, ethanol H_2, CO_2 and formate were the main end products formed during xylose metabolism. At higher xylose concentrations, lactate and 1,2-propanediol were produced in small amounts as additional products. Reducing equivalents formed during the oxidation of glyceraldehyde-3-PO_4 and pyruvate, were used for the production of H_2, formate, and ethanol. According to the proposed pathway about 2.5 mol of ATP, synthesized at substrate level, were generated per mol of xylose degraded. This part of the research is presented in **chapter 2.**

The degradation of mixtures of hexoses and pentoses by *B. xylanolyticus* X5-1 is described in **chapter 3**. Batch culture cells did not show diauxic growth or a substrate preference for either glucose, xylose, arabinose or rhamnose, independent of the substrate the organism was grown on. In contrast, glucose-limited continuous culture cells were not able to consume xylose, unless some glucose or pyruvate was present as additional substrate. Glucose-limited continuous culture cells exhibited low activities of xylose transport and of xylose isomerase. Xylulose kinase could not be detected at all. Upon addition of xylose

as single substrate to the glucose grown cells no increase in the transport rate and the isomerase and kinase activities was observed. However, when together with the xylose some glucose was added, all activities were induced. In the presence of chloramphenicol, an inhibitor of protein synthesis, xylose isomerase and xylulose kinase were not induced. The transport activity increased in a similar fashion as in the absence of chloramphenicol, suggesting that the transport system had to be activated and not induced. These experiments showed that i) xylose isomerase and xylulose kinase were regulated at the level of protein synthesis, ii) xylose transport was constitutively present, and iii) apparently, the glucose grown cells were carbon and energy limited. When grown under non-limiting conditions, as will probably happen in hemicellulose hydrolysates, *B. xylanolyticus* X5-1 can use sugar mixtures. This certainly is of biotechnological relevance, as conversion of the major substrate xylose will not be negatively affected by the minor, often preferred substrate glucose.

Chapter 4 describes the effect of a low partial hydrogen pressure on the xylose metabolism in *B. xylanolyticus* X5-1. When grown in pure culture in the chemostat with xylose as the growth limiting substrate, *B. xylanolyticus* X5-1 produced acetate, ethanol, H_2 and CO_2 as the only end products. When grown in the presence of the methanogen *Methanospirillum hungatei* JF-1, xylose was converted to mainly acetate and CO_2, and presumably H_2. Due to the cocultivation an increased biomass production was observed. H_2 could hardly be detected because it was efficiently converted to CH_4 by the methanogen. Ethanol was no longer produced. This type of regulation of product formation has been observed in many anaerobic microorganisms. However, xylose fermentation in *B. xylanolyticus* X5-1 was not only regulated at product level, but also on enzyme level. In cell free extracts of the pure culture of *B. xylanolyticus* X5-1 NAD and NADP-linked acetaldehyde and ethanol dehydrogenases could be detected. When grown in mixed culture with *M. hungatei* JF-1 these enzymes were no longer

observed. The NAD and NADP-linked dehydrogenases were induced sequentially, when the interspecies electron transfer was inhibited, unless chloramphenicol was present. These results showed that product formation at low partial hydrogen pressure in *B. xylanolyticus* X5-1 is regulated at the level of enzyme synthesis.

Several environmental conditions were used to affect xylose metabolism of *B. xylanolyticus* X5-1 (**chapter 5**). Growth under a hydrogen atmosphere did not affect the xylose metabolism significantly. CO inhibited H_2 production from xylose completely with formate and ethanol as major reduced products. An increased ethanol yield resulted in a reduced amount of acetate and biomass formation. Xylose metabolism could also be affected by using alternative electron acceptors such as acetol, acetone, acetoin, and dihydroxy acetone. They were reduced to their corresponding alcohols 1,2-propanediol, 2-propanol, 2,3-butanediol, and glycerol, respectively. With these electron acceptors mainly acetate and CO_2 were formed and hardly any H_2, formate and ethanol. As a result of more acetate formation, biomass production increased. In continuous culture with xylose as growth limiting substrate and acetol as electron acceptor, product formation from xylose shifted to mainly acetate and CO_2 as well. Acetol was not only reduced to 1,2-propanediol, but also converted to acetone. *In gel* activity staining of the alcohol dehydrogenases revealed that i) the NADP-linked ethanol dehydrogenase was repressed in the xylose + acetol grown culture, ii) the NADP-linked ethanol dehydrogenase in the xylose grown cells exhibited a non-specific activity for both ethanol and 1,2-propanediol, and iii) another, also NADP-linked, 1,2-propanediol dehydrogenase was induced in the xylose + acetol grown cells.

The data presented in this thesis show that it is possible to modulate the xylose metabolism of *B. xylanolyticus* X5-1 by several methods and at different levels during metabolism. The outcomes of this research might be applicable for other microorganisms of biotechnological value as well. Accordingly, the results can

be used for biotechnological production processes and the biotechnological formation of valuable products (e.g. microbiological reduction processes, optically active products, enzymes like (stereospecific) alcohol dehydrogenases).

Chapter 8

Samenvatting

Plantecelwanden vertegenwoordigen het grootste gedeelte van de op aarde aanwezige biomassa en bestaan uit energierijke polymeren zoals lignine, cellulose en hemicellulose. Gedurende tientallen jaren is er onderzoek gedaan om landbouw- en bos-afval te gebruiken als herbruikbare grondstof. Met name de afbraak van cellulose is veelvuldig onderzocht. Hemicellulose heeft veel minder aandacht gekregen, ondanks dat dit polymeer tot 40 % kan uitmaken van het totale drooggewicht van plantecelwanden. Fermentatie door anaërobe bacteriën biedt de mogelijkheid om de meeste energie die opgeslagen ligt in de polymere- en monomere-suikers te bewaren in de vorm van organische zuren en oplosmiddelen (bijvoorbeeld azijnzuur, alcohol en aceton).

Een project waarin de anaërobe omzetting van hemicellulose naar potentieel biotechnologisch interessante produkten werd onderzocht, werd in twee delen opgesplitst. Eén deel, uitgevoerd door Philippe Schyns, had betrekking op de microbiologische omzetting van xylaan, hetgeen als model substraat voor hemicellulose werd gebruikt. Verschillende xylaan splitsende enzymen (endo-xylanase, ß-xylosidase, acetylesterase, α-L-arabinofuranosidase) werden gezuiverd en gekarakteriseerd. Van enkele enzymen werd de werkwijze nader onderzocht. Bovendien werd het inductie mechanisme van xylanase en ß-xylosidase bestudeerd. De resultaten van dit deel van het onderzoek zullen in een apart proefschrift gepresenteerd worden. Het andere deel van het project, waarvan de uitkomsten in dit proefschrift gegeven worden, was gericht op de fermentatie van xylose, hetgeen één van de meest voorkomende suikers in hemicellulose is.

Bacteroides xylanolyticus X5-1 is gebruikt als model organisme. Het organisme was eerder geïsoleerd uit gefermenteerde koeiemest. *B. xylanolyticus* X5-1 kan alleen groeien op één specifiek hemicellulose, namelijk xylaan. Cellulose en andere soorten hemicellulose kunnen niet gebruikt worden door dit micro-organisme. Hierdoor was dit organisme interessant voor het bestuderen van de

(regulatie van) de synthese van xylanolytische enzymen, aangezien interferentie met andere (hemi)cellulolytische enzym systemen uitgesloten kon worden. Verder kan het organisme een grote verscheidenheid aan monomere suikers fermenteren, produceert het een mengsel aan eindprodukten en heeft het een relatief hoge groeisnelheid. Deze laatstgenoemde eigenschappen zorgden ervoor dat *B. xylanolyticus* X5-1 een geschikt micro-organisme was voor het bestuderen van de regulatie van de xylose fermentatie onder anaërobe omstandigheden.

De samenstelling en de afbraak van biomassa en de (regulatie van) produkt vorming uit biomassa, worden in algemene zin besproken in **hoofdstuk 1**. Enkele biotechnologische toepassingen van de fermentatie van biomassa worden ook in dit hoofdstuk genoemd.

Door gebruik te maken van ^{14}C-gelabeld xylose kon het xylose opname systeem bestudeerd worden. Er werd aangetoond dat de opname via een actief transport systeem plaatsvindt, en dat er waarschijnlijk een bindings eiwit bij betrokken is. Het exacte mechanisme van xylose opname moet nog verder opgehelderd worden. Gebaseerd op massa balans berekeningen, het bepalen van specifieke enzym activiteiten van sleutel enzymen uit katabole routes, en door het bepalen van het label-distributie patroon met behulp van ^{13}C-NMR kon aangetoond worden dat de pentose fosfaat weg in samenwerking met de glycolyse actief is tijdens de xylose fermentatie door *B. xylanolyticus* X5-1. Azijnzuur (acetaat), alcohol (ethanol), mierezuur (formiaat), waterstof-gas (H_2) en koolzuur-gas (CO_2) zijn de belangrijkste eindprodukten die gevormd worden tijdens de xylose fermentatie. Bij hogere xylose concentraties worden bovendien kleine hoeveelheden melkzuur (lactaat) en 1,2-propaandiol gevormd. Reductie equivalenten, gevormd tijdens de oxydatie van glyceraldehyde-3-fosfaat en pyruvaat, worden weer gebruikt bij de produktie van H_2, formiaat en ethanol. Volgens de voorgestelde route worden ongeveer 2,5 mol ATP op substraat niveau gevormd per mol omgezet xylose. Dit gedeelte van het onderzoek wordt gepresenteerd in **hoofdstuk 2**.

De afbraak van mengsels van C6-suikers en C5-suikers door *B. xylanolyticus* X5-1 wordt beschreven in **hoofdstuk 3**. Batch cultuur cellen vertoonden geen diauxie en geen substraat voorkeur voor glucose, xylose, arabinose of rhamnose, ongeacht het substraat waar het organisme op voorgekweekt was. In contrast hiermee, waren glucose gelimiteerd gekweekte continu cultuur cellen niet in staat om xylose om te zetten, tenzij een kleine hoeveelheid glucose of pyruvaat aanwezig was als extra substraat. Glucose gelimiteerd gekweekte continu cultuur cellen vertoonden lage activiteiten voor xylose transport en xylose isomerase. Xylulose kinase was helemaal niet meetbaar. Na toevoeging van xylose als enkelvoudig substraat aan de glucose gekweekte cellen, werd geen toename in transport snelheid, in xylose isomerase- en xylulose kinase- activiteit waargenomen. Echter, als tegelijkertijd met xylose ook een beetje glucose werd toegevoegd, namen alle activiteiten toe. In aanwezigheid van chlooramphenicol, een remmer van de eiwit synthese, werden xylose isomerase en xylulose kinase niet geïnduceerd. De transport activiteit nam in gelijke mate toe als in afwezigheid van chlooramphenicol, hetgeen suggereert dat het transport systeem geactiveerd moest worden en niet geïnduceerd. Deze experimenten toonden aan dat i) xylose isomerase en xylulose kinase werden gereguleerd op het niveau van enzym synthese, ii) dat het xylose transport constitutief aanwezig was, en iii) dat klaarblijkelijk de glucose gekweekte cellen zowel koolstof als ook energie gelimiteerd waren. Wanneer het organisme gekweekt wordt onder niet-limiterende omstandigheden, zoals waarschijnlijk plaatsvindt in hemicellulose hydrolysaat, kan *B. xylanolyticus* X5-1 suiker mengsels gebruiken. Dit is van biotechnologisch belang, aangezien de omzetting van het belangrijkste substraat xylose niet negatief beïnvloed zal worden door een lagere concentratie van het vaak geprefereerde substraat glucose.

Hoofdstuk 4 beschrijft het effect van een lage partiële waterstof spanning op het xylose metabolisme van *B. xylanolyticus* X5-1. Als het organisme gekweekt werd

in rein cultuur, met xylose als groei-limiterend substraat, produceerde *B. xylanolyticus* X5-1 acetaat, ethanol, H_2 en CO_2 als enige eindprodukten. Als het organisme gekweekt werd in de aanwezigheid van de methanogene bacterie *Methanospirillum hungatei* JF-1 werd xylose omgezet in acetaat, CO_2 en vermoedelijk H_2. Door het co-cultiveren (=interspecies elektron overdracht) werd een hogere biomassa produktie waargenomen. H_2 kon nauwelijks gemeten worden omdat het efficiënt werd omgezet in methaan (CH_4) door de methanogene bacterie. Ethanol werd niet meer geproduceerd. Deze soort regulatie van de produkt vorming is in meerdere anaërobe bacteriën waargenomen. Echter, de xylose fermentatie in *B. xylanolyticus* X5-1 werd niet alleen gereguleerd op produkt niveau, maar ook op enzym niveau. In cel vrije extracten van de rein cultuur van *B. xylanolyticus* X5-1 konden NAD- en NADP-afhankelijke acetaldehyde- en ethanol-dehydrogenase activiteiten gemeten worden. Als het organisme gekweekt werd in co-cultuur met *M. hungatei* JF-1 werden deze activiteiten niet meer waargenomen. De NAD- en NADP-afhankelijke dehydrogenases werden sequentieel geïnduceerd wanneer de interspecies elektron overdracht werd opgeheven, tenzij chlooramphenicol aanwezig was. Deze resultaten laten zien dat produkt vorming door *B. xylanolyticus* X5-1 bij lage partiële waterstof spanning wordt gereguleerd op het niveau van de eiwit synthese.

Verschillende groei condities werden gebruikt om het xylose metabolisme in *B. xylanolyticus* X5-1 te beïnvloeden (**hoofdstuk 5**). Het kweken onder een atmosfeer van H_2-gas beïnvloedde het xylose metabolisme niet significant. Koolmonoxyde (CO) gas remde de H_2 produktie uit xylose volledig, en formiaat en ethanol werden de belangrijkste gereduceerde produkten. De toegenomen ethanol opbrengst resulteerde in een gereduceerde hoeveelheid acetaat- en biomassa-vorming. Het xylose metabolisme kon verder beïnvloed worden door gebruik te maken van alternatieve elektronen acceptoren, zoals acetol, aceton,

acetoïne en dihydroxy aceton. Zij werden gereduceerd tot hun corresponderende alcoholen, respectievelijk 1,2-propaandiol, 2-propanol, 2,3-butaandiol en glycerol. Met deze elektronen acceptoren werden voornamelijk acetaat en CO_2 gevormd, en nauwelijks H_2, formiaat en ethanol. Door de verschuiving naar meer acetaat vorming, werd een hogere biomassa produktie bereikt. In continu cultuur met xylose als groei-limiterend substraat en acetol als elektronen acceptor verschoof de produkt vorming uit xylose ook naar acetaat en CO_2. Echter, acetol werd niet alleen gereduceerd naar 1,2-propaandiol, maar werd ook omgezet in aceton. De *in gel* activiteit kleuringen van de alcohol dehydrogenases liet zien dat i) het NADP-afhankelijke ethanol dehydrogenase gerepresseerd werd in xylose met acetol gekweekte cellen, ii) dat het NADP-afhankelijke ethanol dehydrogenase in xylose gekweekte cellen een niet specifieke activiteit vertoond voor zowel ethanol als 1,2-propaandiol, en iii) dat een ander, óók NADP-afhankelijk 1,2-propaandiol dehydrogenase werd geïnduceerd in xylose + acetol gekweekte cellen.

De meetgegevens die in dit proefschrift gepresenteerd zijn, laten zien dat het mogelijk is het xylose metabolisme van *B. xylanolyticus* X5-1 op verschillende manieren en op verschillende niveaus te beïnvloeden. De verkregen resultaten zijn mogelijk ook toepasbaar bij andere micro-organismen met een biotechnologische waarde. Daarom kunnen de resultaten gebruikt worden voor biotechnologische produktie processen en voor de biotechnologische produktie van waardevolle verbindingen (b.v. microbiologische reductie processen, optisch actieve produkten, enzymen zoals (stereospecifieke) alcohol dehydrogenases).

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Curriculum vitae

Op 15 juli 1964 werd ik in Amsterdam geboren, en kreeg daarbij de naam Steven Biesterveld mee. Na 4 jaar in Amsterdam te hebben gewoond heb ik in Leusden de lagere school gevolgd. Vervolgens heb ik op het "Baarnsch Lyceum" in Baarn het VWO gevolgd met o.a. wiskunde, natuurkunde, scheikunde en biologie als examen vakken. In mei 1983 ben ik voor het examen geslaagd. In september van datzelfde jaar ben ik begonnen met de studie scheikunde aan de Universiteit van Amsterdam. In het derde jaar van de studie is gekozen voor de biologische deelstroom. Deze is aangevuld met enkele radiochemische- en biochemische-vakken, als ook met een cursus research -management en -organisatie. Mijn afstudeervak heb ik verricht in de (toenmalige) werkgroep celwanden van de vakgroep microbiologie, waarbij onderzoek aan bacteriofaag-resistentie van melkzuur bacteriën gedaan is. Kort na het afstuderen in 1988 ben ik als assistent in opleiding begonnen in de "anaërobe groep" van de vakgroep microbiologie aan de Landbouwuniversiteit in Wageningen. Het onderzoek wat ik daar verricht heb aan de anaërobe omzetting van xylose is samengevat in het werk wat nu voor u ligt.