Microbiology and biochemistry of methanogens

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The topic has recently been reviewed by the author:
R.S. Wolfe, Methanogens: a surprising microbial group. Antonie van Leeuwenhoek *45*, 353 (1979).
R.S. Wolfe and I.J. Higgins, Microbial biochemistry of methane – a study in contrasts. Int. Rev. Biochem. (Microb. Biochem.) *21*, 268 (1979).
W.E. Balch, G.E. Fox, L.J. Magrum, C.R. Woese and R.S. Wolfe, Methanogens: reevaluation of a unique biological group. Microbiol. Rev. *43*, 260 (1979).

Formation of ethanol by bacteria. A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes

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Summary. Many anaerobic, facultatively anaerobic and even some strictly aerobic microorganisms form various amounts of ethanol from glucose. Only few mesophilic and extreme thermophilic bacteria ferment glucose stoichiometrically to 2 ethanol and 2 carbondioxid. Biotechnological processes at elevated temperatures seem advantageous in many respects. Hence, thermophiles and extreme thermophiles can potentially substitute for yeast in ethanol production on an industrial scale. Advantages and disadvantages of thermophilic mixed cultures and of potentially useful organisms are discussed.

All over the world numerous symposia and conventions are held to discuss 'the energy crisis'. Many new ideas and concepts to improve the energy conversion processes have been published. Highly industrialized countries and those approaching this status try to become independent from oil. Due to the raised oil prices old processes are becoming interesting and feasible again. One of these old processes is the production of ethanol from biological material by fermentation. Ethanol as a fuel and feedstock chemical can be produced from renewable, biological resources. Ethanol fermentation is one of the hopes for solving the energy problems of the future.

The production of ethanol from biological sources is mainly done with yeast of the type *Saccharomyces cerevisiae*[1]. The fermentation of glucose to ethanol is well-known[2–4]. The conversion of sugar to alcohol is one of the simplest and oldest applications of microorganisms by humans (e.g. Genesis 1.21). But the demands of today's industrialization with its increasing shortage of energy and with its economical considerations, lead to problems in the technical production of increasing amounts of ethanol. The main problems in yeast fermentations are a) aeration, since yeast needs some O_2 for cell wall synthesis, but aerobic conditions lower the ethanol yield (Pasteur effect) and concomitantly lead to an increased production of biomass, b) the cooling of large fermenters below 39.6 °C, since yeast is killed above that temperature, and c) the limited spectrum of resources, since the ethanol-producing yeasts can only use a restricted number of substrates. For a detailed discussion of these problems the reader is referred to the excellent overview by Bu'Lock[5].

Many possibilities have been evaluated to overcome these problems or to optimize the process (some are listed in table 1). New fermenters, like the rotor fermenter[6] with promising properties, have been introduced. Recently an unorthodox approach is made possible by the findings of Wümpelmann and Kjaergaard[7]. They reported that *S. cerevisiae* strains under potassium limitation are able to convert 78% of the glucose to ethanol under aerobic conditions.

But one of the main disadvantages of yeasts, their limited substrate spectrum, has not been overcome. The problem might be solved in the future, using

genetic engineering techniques; desired properties for *Saccharomyces* strains would be the ability to convert pentoses to ethanol and to hydrolyze cellulose to glucose. The necessary genetic material may possibly be transferred to *Saccharomyces* from other yeasts or fungi.

Alternative processes for the production of ethanol and feedstock chemicals with thermophilic and extreme thermophilic anaerobic bacteria are worth studying. Thermophilic bacteria are best defined by their temperature characteristics for growth (table 2). These bacteria ferment a wide range of substrates, including those which already cause or might cause in the near future disposal problems, e.g. low-quality starch, lactose and pentoses. They are also relatively resistant to heavy metal ions[8,9] as well as to other toxic substances and pollutants. These anaerobes are not killed by the contact with air, although they do not grow in the presence of oxygen. These and other properties are advantageous for their industrial application.

This article will first give a survey of the distribution of ethanol as a fermentation product among bacteria and then summarize some of the properties of those thermophilic and extreme thermophilic anaerobes which are of interest for application in industrial processes.

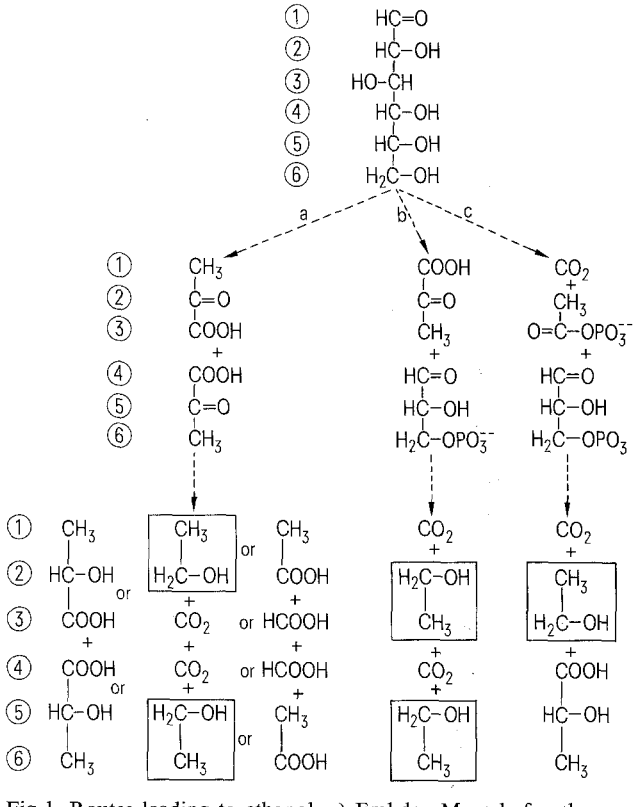

Fig. 1. Routes leading to ethanol. a) Embden-Meyerhof pathway; used by yeasts, many mesophilic and thermophilic clostridia; Enterobacteriaceae, etc. b) Entner-Doudoroff pathway; used by *Zymomonas* and pseudomonads. c) Heterofermentative pathway; used by heterolactics.

Pathways leading to ethanol

In figure 1 three major types of ethanol fermentation are schematically illustrated by the distribution of the different glucose carbon atoms among the products. It is not intended to review the biochemistry involved in the formation of ethanol (see e.g. Dawes[10]) or to give the distribution of the different pathways among ethanol-producing organisms. In several cases bacteria which produce unusually high amounts of ethanol from glucose or other substrates (e.g. *C. sphenoides* or *C. sporogenes;* table 4) have not yet been studied in detail. Thus, only general comments will be made, to outline some problems in a search for efficient ethanol-producing bacteria and in manipulations to increase the ethanol yield through changes in the carbon and electron flow to the products.

Yeasts degrade glucose via the Embden-Meyerhof pathway (route a in fig. 1), forming 2 moles of pyruvate from 1 mole of glucose. Pyruvate is decarboxylated in an irreversible reaction via the thiamine pyrophosphate-dependent reaction of pyruvate decarboxylase to acetaldehyde, which is than reduced to ethanol (fig. 2, enzymes 1 and 2). Although the Embden-Meyerhof pathway is used by many other bacteria (Enterobacteriaceae, clostridia, *Spirochaeta, Bacteroides,* etc.), pyruvate decarboxylase is only found in few organisms: *Acetobacter suboxydans* (respiratory metabolism[11]), *Sarcina ventriculi*[12], *Erwinia amylovorans*[13]. *Zymomonas mobilis*[14] also contains this enzyme, but it uses the Entner-Doudoroff (route b, fig. 1) instead of the Embden-Meyerhof pathway. The majority of bacteria which lack pyruvate decarboxylase produce ethanol from pyruvate via acetyl coenzyme A (fig. 2; enzymes 3–5). Acetyl coenzyme A is first reduced to acetaldehyde and then to ethanol. If ethanol is formed, the free energy embodied in acetyl

Table 1. New approaches for the industrial production of ethanol with yeast

Continuous culture including the use of
vacuum fermentation
cell recycling
immobilized yeast cells
Fermentation process at
39.0 °C (to avoid high biomass production)
under K-deficiency (aerobic)
New fermenter construction: e.g. Uplift-fermenter, Roto-fermenter, 2-step fermentation and
Mixed yeast cultures to ferment pentoses and unconventional resources

Table 2. Thermophilic organisms

	T_{min} (°C)	T_{opt} (°C)	T_{max} (°C)
Thermotolerant	-	< 40	≥ 43
Thermophilic	≥ 25	≥ 40	≥ 50
Extreme thermophilic	≥ 35	≥ 65	≥ 70

coenzyme A cannot be conserved to form ATP. In order to increase the energy yield a part of the acetyl coenzyme A is often converted via acetyl phosphate to acetate and ATP (fig. 2; enzymes 6 and 7). In many bacteria the fermentation of sugar is therefore accompanied by the formation of considerable amounts of acetate.

Many organisms which use the Embden-Meyerhof pathway and all the heterolactics form significant amounts of lactate in addition to acetate. Most of them contain highly active lactate dehydrogenase (EC 1.1.1.27). The heterolactic bacteria use a different pathway for glucose degradation (path c in fig. 1): glyceraldehyde 3-phosphate and acetyl phosphate are formed via xylulose 5-phosphate. Acetyl phosphate is converted to acetyl coenzyme A and finally to ethanol as described above.

According to the pathway utilized and the amount of ethanol formed there are significant differences with respect to energy yields: The yeasts, forming 2 moles of ethanol from 1 mole of glucose, gain 2 ATP via the Embden-Meyerhof pathway only; *Zymomonas* gains one ATP using the Entner-Doudoroff pathway; and the heterolactics, if they convert *all* acetyl coenzyme A to ethanol, obtain 1 ATP per glucose utilized. The majority of bacteria which use the Embden-Meyerhof pathway, gain 2 ATP plus the ATP from the production of acetate via acetyl phosphate. The extreme thermophile *Clostridium thermohydrosulfuricum* obtains about 2.5 ATP per mole of glucose[9], although the Y_{ATP}-value applied (10–11 g cellular dry weight/mole) might not be correct[15,16].

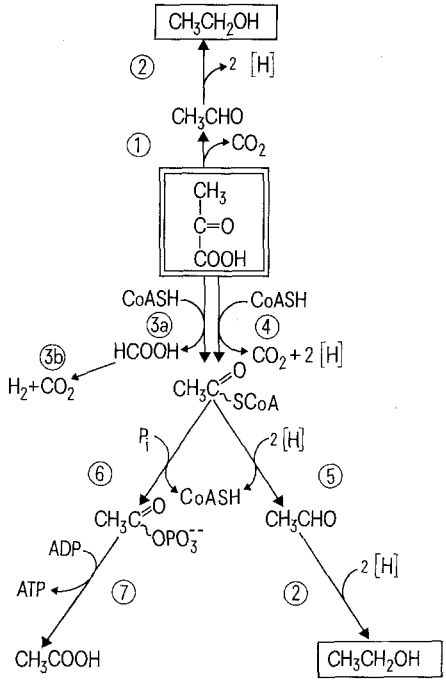

Fig. 2. Enzymatic steps in the degradation of pyruvate to ethanol. (1) Pyruvate decarboxylase (EC 4.1.1.1). (2) Alcohol dehydrogenase (EC 1.1.1.1). (3a) Pyruvate-formate lyase. (3b) Formate-hydrogen lyase reaction. (4) Pyruvate-ferredoxin oxidoreductase (EC 1.2.7.1). (5) Acetaldehyde dehydrogenase. (6) Phosphotransacetylase (EC 2.3.1.8). (7) Acetate kinase (EC 2.7.2.1). ~SCoA; CoASH: coenzyme A.

There is not much hope to find an autotrophic 'ethanol bacterium', which produces ethanol from carbondioxide and hydrogen gas. Such a bacterium would be similar to the recently discovered *Acetobacterium woodii*[17] or to the rediscovered *C. aceticum*[18] and to a newly isolated thermophilic clostridium (Wiegel et al., in prep.) which does grow autotrophically and which exclusively forms acetate during growth on H_2/CO_2 or on glucose, fructose, etc. Theoretical and thermodynamic calculations do not support any hope to find a 'homoethanol' bacterium.

Substrates leading to ethanol

As stated above, bacteria can use a wide variety of substrates other than glucose. However, one can not expect, a priori, that bacteria which form ethanol from glucose, produce ethanol from other substrates as well. The products have to be analyzed in each case. Well-known examples for substrate-specific fermentation products are the heterolactic *Leuconostoc mesenteroides* and the homolactic *Lactobacillus casei.* Both ferment glucose as expected: *L. mesenteroides* ferments glucose to lactate, CO_2 and ethanol in almost stoichiometric amounts. Ribose, however, is converted to lactate, CO_2 and acetate. *L. casei* degrades glucose exclusively to lactic acid, while ribose is converted to lactate, ethanol and carbondioxide by heterofermentative fermentation. After growth on ribose, resting cells of *L. casei* ferment glucose also via the heterofermentative pathway[19,20]. The fermentation of pentoses to ethanol is reviewed by S. L. Rosenberg[93]. *Bacillus macerans* produced about 0.9 moles of ethanol per mole of xylose.

Not only the nature of the substrates, but also the fermentation conditions may influence product formation. Two examples *(B. stearothermophilus* and *C. thermosaccharolyticum)* will be discussed later. Another example is *Streptococcus lactis.* It is regarded to carry out a homolactic fermentation with lactose as the substrate. If the C-source is growth-limiting, formate, acetate and ethanol are formed from lactose as well as from glucose[20].

There are bacteria which form ethanol as a main fermentation product from all utilizable hexoses, di- and polysaccharides, as well as from various pentoses like ribose and xylose. Two examples are the extreme thermophiles, *Clostridium thermohydrosulfuricum* and *Thermoanaerobacter ethanolicus*[9,21].

Besides sugars, other substances can serve as substrates for ethanol formation. The more they are reduced, the more efficiently they are converted to ethanol and vice versa[22]. All substrates which are metabolized to acetyl phosphate, acetyl coenzyme A

or pyruvate and yield high proportions of reducing equivalents, can theoretically be converted to ethanol. Substrates which require the presence of H_2-consuming bacteria (e.g. methanogens) for a reasonable degradation will not give high ethanol values. In contrast, an increase in the pressure of H_2, may result in the formation of ethanol at the cost of acid production. This was demonstrated by von Hugo with *Clostridium roseum* and *Clostridium rubrum*[23,24]. Under an H_2-atmosphere these bacteria produce ethanol and butanol from pyruvate instead of acetate and butyrate under an N_2-atmosphere. With the extreme thermophilic *C. thermohydrosulfuricum* and *T. ethanolicus* this effect was not observed. H_2 was inhibitory without causing a shift to higher yields of ethanol from the glucose utilized[9,21].

Corry[25] mentioned several Enterobacteriaceae and clostridia which produce up to about 0.9 moles of ethanol per mole of glycerol, and up to 1 mole of ethanol per mole of mannitol. The formation of ethanol from amino acids has been shown for many clostridia and other bacteria, e.g. *E. coli, Proteus vulgaris* and *Fusobacterium nucleatum* (0.2 moles per mole of serine[26]). The reported yields of ethanol from amino acids are relatively low. The formation of 0.57 moles per mole of serine by *C. botulinum* is one of the highest[25]. Instead of ethanol many peptolytic clostridia produce acetate and most likely ATP from those amino acids which lead to pyruvate (serine, leucine, tryptophan, cysteine). Threonine is degraded to ethanol via acetaldehyde, as was shown for *E. coli, C. pasteurianum* and the group N streptococci[27]. However, many other bacteria are able to form traces of ethanol from amino acids during growth on peptone, yeast extract, casamino acids and tryptone.

There are further nitrogen compounds which can be catabolized to ethanol. *Desulfovibrio desulfuricans* (syn. *Vibrio cholinicus*)[28,29] metabolizes choline to trimethylamine, acetate and ethanol. The intermediate acetaldehyde is dismutated to acetate and ethanol. Yields of about 0.6 moles of ethanol per mole of choline have been obtained. Similar results were obtained with ethanolamine[30].

Ethanol is also a product of some bacteria during growth on different di- and tricarboxylic acids. One example is *C. sphenoides*, growing on malate and citrate. It produces 0.36 and 0.13 moles of ethanol per mole of citrate and fumarate, respectively[31,32]. Other examples are given by Bogusz[33]. The conversion of acetate by *C. thermosaccharolyticum* and by *C. acetobutylicum* will be discussed later.

An usual product of glycolytic and cellulolytic anaerobic thermophiles is lactate. The conversion of lactate to ethanol was reported by Bogusz et al.[34] for mesophiles. Apparently none of the thermophiles carries out this conversion to a significant extent. It seems that there are no substrates other than sugars, their oligomers, sugar acids and sugar alcohols, which give the high yields required for ethanol production on an industrial scale. However, there is plenty of waste material from slaughter houses and ethanol fermentation plants, etc., consisting of protein, various amines and similar compounds.

Ethanol-forming microorganisms

There are several yeasts known, which in different quantities, form ethanol from glucose and also from lactose, pentoses and other hexoses[3,35]. Except for *Saccharomyces* and those species involved in the production of alcoholic beverages[1-4], there is little information available about ethanol formation. Among the eucaryotic organisms besides the yeasts, several fungi produce ethanol. Corry[25] listed 24 fungal species which, under restricted aeration in Czapek-Dox synthetic medium, form ethanol from glucose. 2 *Aspergillus,* 3 *Fusarium* and 3 *Penicillum* species formed more than 1 mole of ethanol per mole of glucose consumed; the highest valueof 1.6 mole of ethanol per mole of glucose was found for *Fusarium avenaceum.* However, the mesophilic fungi do not grow or metabolize as fast as yeasts or bacteria. They are therefore not promising for industrial ethanol fermentations and no straightforward research has been done with these organisms. One must assume that more species can produce ethanol. Recently the cellulose degrading thermophilic fungi attracted attention for the possible production of stable cellulases[36]. These fungi may have a chance to be used together with other fungi and/or bacteria in the fermentation of lignin-containing cellulose to ethanol and feedstock chemicals.

The presence of ethanol in the fermentation products is widespread among anaerobic and facultatively anaerobic bacteria, as can be judged from table 3. The list does not contain those bacteria which merely produce trace amounts of ethanol or produce it only from unusual substrates. The organisms are listed according to the parts in Bergey's Manual of Determinative Bacteriology[37] to demonstrate the diversity. Many diverse groups are represented: The strictly anaerobic *Bacteroides* and the facultatively anaerobic Enterobacteriaceae carry out a mixed acid fermentation thus forming only small amounts of ethanol. Lactic acid bacteria, which carry out a heterolactic fermentation, produce up to 1 mole of ethanol per mole glucose metabolized. The sporeforming clostridia produce ethanol from traces to nearly 2 moles of ethanol per mole of glucose. *Zymomonas* forms almost solely ethanol from glucose via the Entner-Doudoroff pathway. *Sarcina* produces ethanol by a mechanism resembling that of yeast, but forms also acetate from pyruvate and H_2 plus CO_2 via formate (fig. 2; enzymes 3a and b).

Formation of ethanol by aerobic bacteria

In addition to be anaerobic and facultatively anaerobic bacteria, even aerobes like *Pseudomonas* and *Alcaligenes* (table 3) can produce ethanol if they are incubated anaerobically or if they grow under O_2-limitation[38]. These bacteria are regarded as strict aerobes, e.g. they depend on respiratory energy conversion for growth[37]. The formation of ethanol by strict aerobes must be distinguished from ethanol formation by facultative anaerobes under microaerobic conditions. Such an example is the formation of 0.3 moles of ethanol per mole of glucose by the facultative anaerobe *Serratia marcescens* under aerobic conditions[39]. In several papers Vollbrecht and coworkers showed that at highly decreased aeration rates true fermentation products such as lactate, butanediol and ethanol were excreted. The excretion was due to the derepression of fermentation enzymes, which are not formed during growth under ordinary cultural conditions[40]. *Alcaligenes eutrophus, Pseudomonas acidovorans, P. delafieldii* and *Paracoccus denitrificans* have been tested; they produced up to 0.6, 0.8, 0.9 and 1.0 g of ethanol, respectively, per 1 of medium containing 1.5% gluconate. The excretion depended on the relative respiration rate of the cells. Under similar conditions other *Pseudomonas* and *Bacillus subtilis* excrete incompletely oxidized metabolites[41]. The distinction between strictly aerobic and facultatively anaerobic bacilli seems very week. In some species[37] the behaviour towards oxygen varies from strain to strain. The highest ethanol concentration formed by a bacillus was reported for *Bacillus stearothermophilus* (table 5). It is listed in Bergey's Manual[37] as an aerobic bacillus, but according to the experiments of Atkinson and coworkers[42,43], it is a facultative anaerobe. High amounts of alcohol dehydrogenase are formed under anaerobic conditions at 55 °C and above. In cells grown at 37 °C alcohol dehydrogenase is not detectable. Batch as well as continuous cultures have been investigated[42,44,45]. According to Atkinson et al.[43] the maximum ethanol concentration reached in continuous culture is too low, to be suitable for industrial ethanol production.

Table 3. Production of ethanol from hexoses by bacteria

Genus (part of Bergey's Manual)		Number of species*
Anaerobic and facultative anaerobic bacteria		
Part 5:	*Spirochaeta*	3
	Treponema	1
Part 8:	Enterobacteriaceae (10 genera)	16
	Vibrio	2
	Aeromonas	1
	Photobacterium	1
	Zymomonas	2
	Pasteurella	2
Part 9:	*Bacteroides*	4
	Fusobacterium	2
	Lachnospira	1
	Desulfovibrio	1
Part 14:	*Staphylococcus*	1
	Streptococcus	2
	Leuconostoc	2
	Peptococcus	2
	Peptostreptococcus	2
	Ruminococcus	2
	Sarcina	2
Part 15:	*Clostridium*	30
	Bacillus (facultative anaerobes)	15
Part 16:	*Lactobacillus*	6
Part 17:	*Eubacterium*	6
	Bifidobacterium	18 (all species)
	Actinomyces	1
Aerobic bacteria		
Part 7:	*Pseudomonas*	5
	Alcaligenes	1
Part 10:	*Paracoccus*	1
Part 15:	*Bacillus* (strict aerobes)	1

* Data were collected from several sources, in addition to those specified in the text or in tables 4 and 5, mainly from Bergey's Manual[37], Corry[25], Gottschalk et al.[51] and VPI manual[52].

Mesophilic bacteria with ethanol as the main fermentation product

As already indicated, many of the bacteria listed in table 3 produce ethanol only as a minor product. For the mixed acid fermentation carried out by Enterobacteriaceae, a theoretical maximum yield of 1 mole of ethanol per mole of glucose fermented was predicted[10]. However, the highest value found for *E. coli* is about 0.8[46]. There are several species or genera which excrete ethanol as the major fermentation product. The mesophilic bacteria producing more than 1 mole of ethanol per mole of glucose utilized are listed in table 4; the thermophilic species, in the order of their maximum growth temperature, in table 5.

Although some of the mesophilic clostridia produce higher amounts of ethanol, only the fermentation by *Zymomonas mobilis*[47] can be regarded as a 'pure'

Table 4. Bacteria with ethanol as a main fermentation product

Organism Mesophilic bacteria	Ethanol produced mmole/mmole glucose metabolized	References
Clostridium sporogenes	up to 4.15*	25***
Clostridium indolis (pathogenic)	1.96*	25
Clostridium sphenoides	1.8* (1.8)**	25, 31
Clostridium sordelli (pathogenic)	1.7	25
Zymomonas mobilis (syn. *anaerobica*)	1.9 (anaerobe)	47
Zymomonas mobilis ssp. *pomaceae*	1.7	47
Spirochaeta aurantia	1.5 (0.8)	91
Spirochaeta stenostrepta	0.84 (1.46)	91
Spirochaeta litoralis	1.1 (1.4)	91
Erwinia amylovora	1.2	92
Leuconostoc mesenteroides	1.1	19, 25
Streptococcus lactis	1.0	19
Sarcina ventriculi (syn. *Zymosarcina*)	1.0	25

* In the presence of high amounts of yeast extract. ** Values in brackets were obtained with resting cells. *** The values of Corry[25] are 48-h culture values.

Table 5. Thermophilic and extreme thermophilic, ethanol-producing bacteria

Organism	T_{max}	Ethanol produced mmole/mmole glucose utilized	References
Thermoanaerobacter ethanolicus (gen. nov.)	78	1.9	8, 21
Clostridium thermohydrosulfuricum	78	1.6	9
Bacillus stearothermophilus	78	1.0 (anaerobic above 55 °C)	43
Thermoanaerobium brockii	78	0.95	60
Clostridium sp. (cellulolytic)	75	0.8	*
Clostridium thermosaccharolyticum (syn. *tartarivorum*)	68	1.1	75, 76
Clostridium thermocellum (thermocellulaseum)	68	1.0	78

* Unpublished results.

ethanol fermentation. Glucose is metabolized solely to ethanol and CO_2 with the exception that traces of acetate are formed. This organism is successfully used in tropical countries to make palm wines[48].

As shown recently[49,50] at least some strains of *Zymomonas mobilis* can ferment, like yeast, high concentrations of glucose to ethanol, in batch as well as in continuous culture. The specific uptake rates for glucose reported and thus the rates of ethanol production were even higher than for yeast.

The high amounts of ethanol formed by some of the mesophilic clostridia listed in table 4, must be due to growth in complex media; mainly the pathogenic representatives require supplements. *Clostridium sporogenes* is regarded as a saccharolytic and peptolytic representative. Its fermentation products from glucose also include acetate and butyrate in significant amounts[51,52]. Similar to *C. spenoides, C. sporogenes* also produces significant amounts of ethanol from yeast extract without glucose.

The preferred substrates of *C. sporogenes* are amino acids. They are fermented by the 'Stickland reaction' to products which depend on the amino acids utilized. However, high amounts of ethanol as a product were not reported for these reactions[51].

According to the VPI-manual[52] the pathogenic *C. indolis* produces high amounts of acetate and succinate. *C. sordellii* forms acetate, formate, and low amounts of various fatty acids besides ethanol. The high ethanol yields reported in table 4 may reflect the involvement of different metabolic pathways under different conditions.

Walther[31] found that *C. sphenoides* produces 1.8 moles of ethanol, 0.4 moles of acetate and 0.8 moles of H_2 from 1 mole of glucose in growing cultures as well as in resting cell suspensions (buffer without significant amounts of yeast extract). Holdeman and Moore[52] found under their conditions also some lactate.

The advantages and disadvantages of mesophilic bacteria

The use of anaerobic bacteria in industrial fermentation processes has several advantages: They utilize a large variety of substrates, they cause no aeration problems, and they form little biomass. Although the clostridia mentioned in table 4 show high yields of ethanol from glucose, the formation of significant amounts of other products during fermentation excludes *C. sporogenes* and *C. sphenoides* as well as the pathogenic species *C. indolis* and *C. sordellii* from an industrial application. *C. sphenoides* may have a chance, if it is possible to suppress the formation of acetate.

The most promising mesophilic bacteria are the strains of *Zymomonas mobilis*. However, as far as known, they are not used for industrial ethanol production. Major reasons are the production of acetate (plus acetaldehyde) at a ratio of 100 ethanol: 2–5 acetate, the nonutilization of starch, maltose or xylose and the dependence on advanced fermentation techniques. Acetate causes problems, since it is not separated from ethanol by the presently applied distillation techniques. Contrary to yeast fermentation, which is carried out at pH 3.0–4.0, the bacteria require pH-values above 5.0; this pH-range is suitable for other microorganisms which withstand high ethanol concentrations. The fermentation thus requires sterilization of the medium and fermentation vessels. The ethanol concentrations at the beginning of the process are too low to prevent overgrowth by other bacteria. The advantages of the anaerobic *Zymomonas* species do not seem sufficient for substituting for yeast (H. Dellweg, pers. comm.).

Although thermophilic bacteria also produce lactate and acetate as minor products, their use in ethanol fermentation is more likely.

Advantages of thermophilic processes

The advantages of a fermentation process using thermophilic and extremely thermophilic anaerobic bacteria are summarized in table 6; obviously high-temperature fermentation has some important benefits:

1. The production of cell mass by anaerobic bacteria is low compared to yeast or aerobes; at the elevated temperature required by the thermophilic and extremely thermophilic anaerobes for their optimal growth even less biomass is produced[53]. The lower growth efficiencies (biomass produced per mole substrate utilized) of thermophiles can be explained by the increasing energy requirement for maintenance reactions at elevated temperatures. It is not necessarily an indication of a lower ATP yield of the thermophilic metabolism (e.g. uncoupling). The maintenance of the intracellular pH and ionic strength, the stabil-

ization of membrane and lipid structures and also higher turnover rates of some proteins apparently require more energy than that, which is gained by the net increase of higher catalytic activity at elevated temperatures (for theory of thermophily see Ljungdahl[53], Amelunxen and Murdock[54], Friedman[55] and Zeikus[56]).

2. As indicated before, thermophiles exhibit high catabolic activities at temperatures optimal for growth. These result in short fermentation times and high productivity[53] and thus in a high efficiency of industrial plants.

3. High temperature causes a decrease in the solubility of O_2 and other gases, which supports the establishment and the maintenance of anaerobic conditions. At the optimum temperature of *T. ethanolicus* and other extreme thermophiles (66–69 °C) the solubility of O_2 in the media is less than 20% of that at 30 °C. The elevated temperature could also allow the use of substrates which have a low solubility at ambient temperatures; at the higher temperature the substrate availability may not be any longer the rate limiting reaction.

4. The viscosity of the medium decreases with the increase of the temperature. This leads to a decrease in the energy required to maintain homogeneous conditions in the fermenting broth and to remove volatile substances like ethanol. This is an important point in the use of large industrial systems.

5. The use of extreme thermophiles favours the continuous recovery of the product (ethanol) during fermentation. A very mild vacuum or an anoxid gas stream is necessary to remove the ethanol or other volatiles like acetone and butanole.

6. The metabolic activity of microorganisms, especially the degradation of biopolymers, results in heat production. A well-known example is the spontaneous combustion of hay and grain, initiated by microbial activity[57]. Heat is also produced by stirring the culture. Hence, not much additional energy is required to keep the vessels at the desired elevated temperature. Expenditures for cooling, those after a sterilization included, can be kept to a minimum. For the preparation of enzymes or other metabolites one does not need to cool to 4 °C. Most of the enzymes of thermophiles are stable, but do not exhibit significant activity at room temperature. For example, the extracellular proteases of some *C. thermohydrosulfuricum* strains do not degrade the extracellular cellulase of *C. thermocellum* at room temperature but at 40 °C and above (J. Wiegel, unpublished results).

7. Compared to the use of mesophilic bacteria, sterile conditions are less essential. There are no obligate thermophilic pathogens known, thus pathogens do not grow at 60 °C. However, in many instances resources and fermentation vessels have to be sterilized. Organisms like the rapidly growing *B. stearothermophilus* are widespread. *Clostridium thermohydrosulfuricum* for instance was isolated from the dust collected in a fermentation plant. The organism had never been cultivated in that plant before. *C. thermohydrosulfuricum* was also isolated from several cultures of *Methanobacterium thermoautotrophicum*, which may not have been carefully handled. It was stated that no other bacteria could grow at high temperature under the anaerobic, autotrophic conditions used for the cultivation of *M. thermoantotrophicum*[58]. However, later it was found (J. Wiegel, unpublished results), that *C. thermohydrosulfuricum* can grow and multiply in the autotrophic culture of *M. thermoautotrophicum*. The clostridium cannot grow under the autotrophic conditions in the absence of the methanogen. It is assumed that *C. thermohydrosulfuricum* utilizes traces of not yet identified products and lysed cells of the methanogen.

Table 6. Advantages using anaerobic thermophilic fermentation conditions

1. High yields due to the fermentation of a wide range of sugars; direct fermentation of starch possible
2. High product yields and less biomass production
3. Fast fermentation due to high metabolic activity
4. No or little danger of contamination by undesired microorganisms including pathogens
5. No aeration problems; easy to maintain anaerobic conditions
6. It is easier to heat than to cool fermentation vessels (possibility to couple to other processes)
7. Possibility of continuous destillation of volatile products (such as ethanol) directly from fermentation vessels (continuous process) during fermentation

Disadvantage: Advanced biotechnology.

Summarizing these points: The thermophiles and extreme thermophiles generally have advantages over the mesophilic bacteria and yeasts. They are of principal interest for industrial application. Processes with thermophilic anaerobes should prove feasible for the production of fuel and feedstock chemicals. Whether they can compete with the conventional alcohol fermentation depends on the specific characteristics of the individual bacteria. An ethanol producing yeast with an optimal fermentation temperature above 50 °C is not known.

Properties of some thermophilic and extreme thermophilic, anaerobic bacteria for a possible industrial ethanol production

Although *C. thermohydrosulfuricum* has been known since 1965[59], its physiological characterization was started only few years ago[9]. The extreme thermophilic *Thermoanaerobium brockii*[60] and *Thermoanaerobacter ethanolicus*[21] have just recently been isolated. The recent interest in recycling of renewable resources and the increasing research on thermophiles will lead to the characterization of more new species. Additional ethanol producers might be discovered in the near future. So far *T. ethanolicus* exhibits the highest etha-

nol yield among the thermophiles. Ethanol formation by the thermophilic cellulose degrader *C. thermocellum* is of special interest. Its coculture offers an alternative for producing of ethanol directly from cellulose (table 7).

It is striking that a high percentage of the known anaerobic thermophiles and extreme thermophiles produce ethanol from sugars as a main product. In addition, *B. stearothermophilus* produces ethanol only at temperatures above 55 °C. Ethanol is a neutral, volatile compound, causing at elevated temperature no problems due to product accumulation. This may be a reason for its formation by thermophiles. The non-ethanol producers are specialists, like the methane bacteria, the homoacetate fermenters and the sulfate reducers. They produce solely the volatile methane and acetate, respectively. Among the thermophiles, acetate utilization is a rare property. Only the recently described methanogens, *Methanosarcina* sp., are known. They were isolated by Mah and coworkers[61] and by Schoberth[62]. They do not seem to be very numerous in soil. Thus, the acetate produced must be metabolized in nature by unknown thermophilic or by mesophilic bacteria.

Ethanol utilization is also a rare property among the thermophilic anaerobes. *Desulfotomaculum nigrificans* converts ethanol to acetate and H_2 in the presence of H_2-utilizing organisms[63].

Clostridium thermohydrosulfuricum

This organism was first isolated and described by Klaushofer and Parkinen[59]. The author isolated this clostridium from many different sources and locations in the United States[9, 64] and recently also from locations around Göttingen (West Germany), from mud from the bay of Naples (Italy) and from mud and soil collected in the forest around the solfatara in Hawaii (Wiegel, unpublished results). Zeikus and coworkers[56,65] isolated further strains from the hot springs in Yellowstone National Park. Apparently, this organism seems to be as ubiquitous as *B. stearothermophilus,* the facultatively anaerobic counterpart to this clostridium. The taxonomy and the fine structure of the outer cell-wall layer[9, 66] have been described. In view of the ethanol production, some interesting properties of *C. thermohydrosulfuricum* are summarized below: Optimal growth (T_{opt}) occurs between 67 and 70 °C with doubling times from 70 to 90 min; the highest (T_{max}) and the lowest temperature range (T_{min}) at which significant growth is observed under laboratory conditions is 75–78 and 36–39 °C, respectively. This bacterium thus belongs in the lower range of extreme thermophiles. Although vegetative cells still grow at 78 °C, they are rapidly killed at 80 °C. The D_{10}-time is less than 30 sec at 80 °C. However, the spores are very heat stable; the D_{10}-time at 100 °C is about 20 h. The incubation of spores for 10 min at 100 °C causes a typical spore activation[8,9]. Spores are frequently formed, even in liquid media under normal growth conditions, although in low numbers. Thus, an accidental heat treatment at temperatures above 78.2 °C, the boiling point of the ethanol-water azeotrope, would only temporarily interrupt the fermentation. Unfortunately, the spores of *B. stearothermophilus* are similarly heat stable; thus these contamination problems can not be solved by a heat treatment. A high sporulation ratio of about 30% can be obtained by cooling a growing culture slowly from above 60 °C to below 55 °C.

Growth occurs between pH-values of 5.5 and 9.3 (after adaptation), with a pH-optimum between pH 6.9 and 7.5. Although variations in the stoichiometry of the fermentation were observed, the fermentation of glucose may be described as follows:

$$\text{Glucose} + 0.5\ H_2O \rightarrow$$
$$\text{ethanol} + 1.5\ CO_2 + 0.5\ \text{acetate} + 0.5\ \text{lactate} + H_2.$$

Ethanol production varies from 0.5 to over 1.6 moles per mole of glucose utilized. Corresponding changes in lactate (98% L(+)-isomer) production were observed. The starting pH and the pH during growth were important parameters. Surprisingly, a low pH-value of 5.2 or less did not promote the formation of pH-neutral compounds as ethanol, as was found for heterolactics and *C. acetobutylicum* (shift from butyrate to butanol)[67]. High yields of ethanol were only obtained when the pH was allowed to drop from between 7.2 and 7.5 to pH 6.9 or less during fermentation. Fermentations with growing and resting cells at constant pH-values resulted in the low ethanol values; the tested range was pH 5.0–8.5. Contrary to *B. stearothermophilus,* ethanol was produced at all growth temperatures without dramatic changes in the ratio to the other products. The pH effect has yet to be explained. However, the fact that the ethanol fermentation is somehow regulated, points to the possibility that further manipulation of growth conditions or mutants with altered regulation could lead to higher ethanol yields. *C. thermohydrosulfuricum* needs yeast extract for growth. The final optical density of a culture at a given glucose concentration depends on the amount of yeast extract added. Resting cells also require yeast extract for fermentation with high ethanol yields. Experiments with radioactive glucose (Wiegel and Ljungdahl, unpublished results) showed that the ethanol was not formed from the yeast extract. The specific activity of the ethanol recovered was nearly the same as that of the glucose. The requirement for yeast extract could be fulfilled in industrial fermentations by using the destillation residues of the alcoholic yeast fermentations as additives. In this way, the pentoses, etc. not used by the yeast, could also be converted to ethanol since this clostridium ferments ribose, arabinose, xylose, starch, etc. to ethanol as a main product.

C. thermohydrosulfuricum is relatively tolerant to high heavy metal ion concentrations, to vacuum, O_2, and acid or base shocks[9] (Wiegel, unpublished results). These properties provide further evidence of its advantage for use in fermentation processes with unconventional resources and waste material.

Thermoanaerobium brockii

This extreme thermophilic bacterium was recently isolated from water and edge sediments of hot springs in Yellowstone National Park, USA, and described by Zeikus et al.[60]. It is the or, at least, one of the predominant anaerobic thermophiles in these volcanic environments[65]. Its temperature characteristics are similar to *C. thermohydrosulfuricum:* T_{min}, 34 °C; T_{opt}, 65–68 °C; T_{max}, 76 °C (Kohring and Wiegel, unpublished results). The doubling time is about 60 min at T_{opt}. In contrast to the *Clostridium, Thermoanaerobium* forms neither spores nor does it exhibit the characteristic structures of the outer cell-wall layer (unpublished results). In micrographs of ultrathin sections of this bacterium a gram-positive type cell wall is visible. The pH-range for growth is about 5.5–9.5 with the optimum at pH 7.5. *Thermoanaerobium* requires yeast extract for growth. Glucose is fermented to ethanol, acetate, lactate, CO_2 and H_2. The ethanol values reported[60] are below one. This bacterium seems therefore to be less promising for an industrial ethanol fermentation than the other 2 extreme thermophilic anaerobes described. Further experiments are required to show whether the ethanol yield can be increased by manipulating the carbon flow, either by varying the fermentation conditions or by genetic techniques. The metabolism of *Thermoanaerobium* is under the investigation by the group of Zeikus.

Thermoanaerobacter ethanolicus spec. nov.

This newly discovered extreme thermophilic, chemoorganotrophic anaerobe[8,21] was isolated from slightly acidic as well as from slightly alkaline hot springs of Yellowstone National Park, USA. With respect to its temperature characteristics it is very similar to *C. thermohydrosulfuricum,* but its doubling time is slightly longer ($t_d = 90$–100 min at T_{opt}, 69 °C). This new organism does not produce spores (at least, so far). The heat stability of the vegetative cell is higher than that of the *Clostridium;* the D_{10}-time at 80 is about 5 min, compared to 5 sec. *T. ethanolicus* differs from the *Clostridium* and from *Thermoanaerobium* in the morphology and cell-wall structure, although all 3 organisms have a cell wall of the meso-diaminopimelic acid direct type (Kandler, pers. comm.). *Thermoanaerobium* and *Thermoanaerobacter* form mini-cells during growth (for details see Wiegel and Ljungdahl[21], Zeikus et al.[60]).

T. ethanolicus has 2 advantages over the other 2 organisms: 1. It exhibits a very broad pH-optimum between 5.5 and 8.5 (growth from pH 4.5–9.5). 2. It ferments glucose and other sugars nearly quantitatively to ethanol as do yeast and *Zymomonas.* The glucose fermentation is summarized by the following equation:

$$\text{Glucose} + 0.2\ H_2O \rightarrow 1.9\ CO_2 + 1.8\ \text{ethanol} + 0.1\ \text{acetate} + 0.1\ \text{lactate} + 0.2\ H_2.$$

The formation of ethanol was not as pH-dependent as it was with the *Clostridium.* Ethanol was the main fermentation product during growth over the whole pH-range and also in the temperature range from 40 to 77 °C. *T. ethanolicus* grows with up to 10% glucose in the medium, however, fermentation balances have not been determined with this concentration. *T. ethanolicus* has also a wide substrate spectrum and produces ethanol as the sole main fermentation product from all substrates tested: starch, on which it grows as rapidly as on glucose; cellobiose, an intermediate in bacterial cellulose degradation; lactose, which has become a serious pollution problem as the main component of deproteinized whey ultrafiltrate; xylose, a component of hemicelluloses, fructose, ribose, maltose, mannose, galactose and pyruvate. Esculin and gelatin are hydrolyzed. Growth on yeast extract and casamino acids is very limited. Since *T. ethanolicus* does not produce spores and has the tendency to lyse in the stationary growth phase, an active culture of this bacterium is best maintained in a glycerol-medium mixture (50–60 v/v% glycerol under an N_2-atmosphere at −18 °C). The mixture does not freeze, so that an aliquot can always be taken without altering the temperature of the stock culture. After transfer into fresh media *Thermoanaerobacter* starts to grow immediately or only with a minor lag phase. The stock culture should be made with cells in the exponential growth phase. Such cultures have been stored for more than 1.5 years without any loss of viability. Similar results were also achieved with the other extreme thermophiles and with *C. thermocellum.* This method of storage might be a convenient alternative to the use of liquid N_2. *T. ethanolicus* contains, similar to *C. thermohydrosulfuricum,* 2 different ferredoxins and a rubredoxin[68]. Ferredoxin I contains 2 [4 FE-4 S]-clusters. The first 10 amino acids of this ferredoxin are similar to those of *C. thermosaccharolyticum* and its biotype *tartarivorum*[69,70]. Ferredoxin II contains only 1 cluster; it differs from Ferredoxin I in its amino-acid composition and in its greater heat stability. The first 10 amino acids in the sequence are homologous to those of other [4 FE-4 S]-ferredoxins *(B. stearothermophilus, B. polymyxin, C. thermoaceticum, Desulfovibrio gigas)*[71]. The heat stability is high and comparable to that of *C. thermoaceticum*[72]. Both ferredoxins catalyze at 60 °C a pyruvate-ferredoxin oxidoreductase reaction. However, further studies are necessary to elucidate the involvement of ferredoxin I and II in the formation of ethanol.

Clostridium thermosaccharolyticum

Its marginal growth temperatures characterize this bacterium as a thermophile. The temperature optimum is between 55 and 60 °C, the temperature maximum between 62 and 68 °C, depending on the strain investigated. Growing cells ferment a large number of carbohydrates including glycogen and starch[73] to acetate, butyrate, lactate, CO_2 and H_2[8,74]. However, resting cells and cells in the sporulation phase produce appreciable amounts of ethanol from glucose[75,76]. Spore-formation and the heat stability of the spores were studied thoroughly[76,77]. *C. thermosaccharolyticum* was not regarded as a potential producer of industrial ethanol. However, the findings of Wang and coworkers[78] indicate a possible application as a coculture with *C. thermocellum*. *Clostridium tartarivorum*[69] has to be regarded as tartrate-fermenting biotype of *C. thermosaccharolyticum*[70]. This finding was based on DNA/DNA homology studies.

Clostridium thermocellum

This is the well-known thermophilic cellulose degrading clostridium. Its taxonomical relationship to the other anaerobic thermophiles described is unclear. The author believes that it is to date the only valid species: The differentiation between *C. thermocellum*[79] and *C. thermocellulaseum*[80] on the basis of sugar utilization does not seem reliable. *C. thermocellum* should not be able to grow on glucose in contrast to *C. thermocellulaseum*. However, many strains of *C. thermocellum* do grow very well on glucose; at the beginning only in the presence of over 0.1% yeast extract but after a few serial transfers with decreasing yeast extract concentrations, with 0.02% yeast extract (Wiegel and Ljungdahl, in prep.). Presumably there occurs a spontaneous mutation in the transport system during the adaption process. This assumption is in good agreement with the previous findings of Patni and Alexander[81,82], who demonstrated that *C. thermocellum* contains all the enzymes for glucose metabolism. The wild type and the 'adapted' strains exhibited lag periods of up to 14 days after transfer from glucose to cellulose containing media. It is unlikely that the effects observed were due to contaminations with *C. thermosaccharolyticum* or *C. thermohydrosulfuricum:* no growth on glucose at 70 °C, no formation of butyrate, and a correspondence of colony counts in agar roll tubes with glucose and cellobiose, respectively, were observed after the adaption.

C. thermocellum can be isolated quite easily from nearly all decaying organic material (e.g. Wiegel and Ljungdahl[68]). However, after establishment of mixed cultures, it is often difficult to obtain pure cellulolytic cultures, since the 2 glycolytic clostridia mentioned above form closely associated, syntrophic, and very stable mixed cultures with *C. thermocellum* (e.g. Wang et al.[78] and own cultures). As far as is known, all strains examined produce ethanol from cellulose from 0.3 up to nearly 1 mole of ethanol per mole of glucose equivalent contained in the cellulose utilized[79,83–86]. At the M.I.T. (Cambridge, Mass., USA) a program has been started to achieve higher ethanol production while maintaining low acetate and lactate concentrations[87]. Highly ethanol resistant strains were isolated, which can degrade cellulose in the presence of up to 8% ethanol. The methods applied included serial transfers and very small increases in the concentration of ethanol added to the media. Fortunately, the mutants also exhibited an increase in the ethanol:acetate ratio from 1:1 with the wild type to 10:1 with the adapted strains. The mutants still formed only 0.77 moles of ethanol per mole of glucose equivalent. With 5% ethanol in the media the growth of the mutant was still inhibited to 50%. Another approach to reduce the formation of acetate, was the addition of acetate (about 6%) to the culture. Although this led to an increase of the ethanol:acetate ratio, the growth rate was decreased drastically by the addition of acetate[87].

The cellulolytic system of *C. thermocellum* is different from that of the fungi (e.g. *Trichoderma viride*) or aerobic bacteria (e.g. *Cellulomonas*). *C. thermocellum* produces much more oligoglucosides[56], which is of importance for the use of mixed cultures in the ethanol fermentation of cellulose.

Although the mutants and adapted strains would prove very valuable for an industrial application, the production of ethanol by a pure culture of *C. thermocellum* does not seem very promising at this time. Even under not yet optimized conditions, much higher ethanol yields were obtained with cocultures of *C. thermocellum* and *T. ethanolicus* (table 7).

The advantages of mixed cultures

As indicated under the description of *C. thermocellum*, the application of mixed cultures would diminish some of the disadvantages of the bacteria discussed or combine the advantages of 2 or more species.

The inhibition by fermentation products, a problem in yeast fermentation, is an even greater problem during fermentation with bacteria. Most of the bacteria described are more sensitive to ethanol than yeast (ethanol tolerance of 3–8% ethanol for bacteria compared to 12–20% for yeast). For some of the thermophiles and extreme thermophiles, the inhibition by products has been studied[8]. All strains tested adapted easily to higher concentrations by serial transfers with increasing concentrations of the products[8]. *T. ethanolicus* was the bacterium with the highest tolerance to the products studied, or the quickest with respect to adaptation to the higher product concentrations. More resistant cultures were obtained by subculturing twice at the same concentration of the product added to the media, before they

were transferred into media with higher concentrations. An adaptation to tolerance for one of the products did not result in an adaptation to tolerance for the other products. A mixture of differently adapted strains of *C. thermohydrosulfuricum* (for ethanol, acetate and lactate, respectively) in a medium containing all 3 products at the highest concentration tolerated, failet to grow. Thus, in order to obtain suitable cultures, an adaptation to all products has to be achieved by increasing the concentration of each product in small steps, together or one by one. Another approach is to maintain low concentrations of the products other than the desired end product. This can be achieved by adding other organisms which use the inhibitory products as substrates. From the inhibition studies with externally added products and other observations, a foodchain from cellulose to methane was postulated for a mixed culture of thermophiles (fig. 3).

The reassociated culture, corresponding to this scheme, was as effective in cellulose degradation as the best mixed culture isolated from nature (above 5 g cellulose per day and 1 of culture). Since there was no thermophile available which could efficiently convert acetate to ethanol during growth only the coculture of *C. thermocellum* and *C. thermohydrosulfuricum* was used. However, it should be possible to find such an organism. Stevens and Emilian[67] reported for *C. acetobutylicum,* that pyruvic acid and acetic acid were converted with 85–90% yield into ethanol, if the acids were continually added to the culture during the solvent-producing phase, thus keeping the pH between 4.0 and 4.7. We did not observe any significant conversion of acetate by *C. thermohydrosulfuricum* or *C. thermocellum* under normal conditions. Hsu and Ordal[76] reported that *C. thermosaccharolyticum* converts acetate to ethanol during its sporulation phase.

The use of the lactate utilizers in an ethanol-producing culture might be of less importance since *T. ethanolicus* produces only traces of lactate and is not inhibited by lactate below 200 mM. A mixture of *C. thermocellum* JW 20 and *T. ethanolicus* JW 200 yielded over 1.4 moles ethanol per mole glucose equivalent of the cellulose utilized (table 7). This is the highest yield from cellulose reported so far. In the future, optimized conditions or *C. thermocellum* strains which produce less acetate and lactate (e.g. strains of the M.I.T., Wang et al.[78]) should give much higher yields, such as 1.8 moles ethanol per glucose equivalent. The group at M.I.T., USA, also started to use mixed cultures. They used a strain of *C. thermosaccharolyticum,* which was isolated from a cellulolytic culture. *C. thermosaccharolyticum* normally forms butyrate and low concentrations of ethanol from glucose[8,74]. Cells of the sporulating phase produce only very low amounts of butyrate and up to 1 mole of ethanol per mole of glucose[75,76]. It is possible that the interactions and the low actual concentration of glucose in a mixed culture keep *C. thermosaccharolyticum* in the ethanol-forming stage. In addition, acetate, which is formed by the cellulolytic bacteria, might be converted to ethanol. Wang et al.[78] did not report whether butyrate was formed or not; the C-recovery reported (lactate, acetate plus ethanol) was about 68%. They also adapted the strain of *C. thermosaccharolyticum* to higher resistance against ethanol. However, the reported yields in cocultures with *C. thermocellum* were not much higher than 1 mole

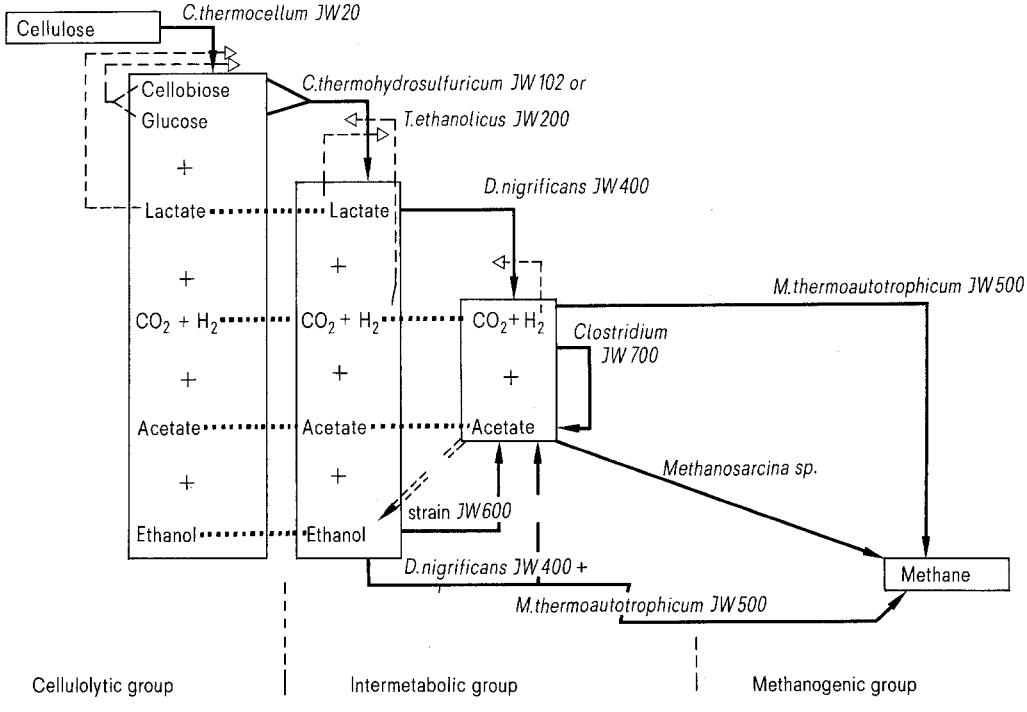

Fig. 3. Scheme for optimal fermentation of cellulose in a mixed culture containing thermophilic and extreme thermophilic anaerobes.---→ Inhibition.

Table 7. Mixed fermentation of cellulose using *Clostridium thermocellum* and *Thermoanaerobacter ethanolicus*

	C. thermocellum	*T. ethanolicus*	*C. thermocellum* and *T. ethanolicus*
Fermented cellulose	351	4	500
mg	2.13	0.024	3.05
as glucose equivalent	1.79	0.05	4.45
Ethanol produced (mmole)			
Ethanol (mole) per glucose residue	0.84		1.46

ethanol per mole glucose equivalent of cellulose degraded, although cellulose degradation was much better than in the cellulolytic monoculture[78].

Another mixed culture on cellulose was reported by Weimar and Zeikus[88]. They studied the influence of *M. thermoautotrophicum* on cellulose degradation by *C. thermocellum*. In the coculture, compared to the monoculture of *C. thermocellum*, ethanol production decreased from about 25 to 2–4 mM while, the concentration of acetate increased from about 13 to about 40 mM. At a later stage of the cultures with *C. thermocellum* significant amounts of butyrate were found (7–8 mM). *C. thermocellum* should not form butyrate[79] at least in the absence of high concentrations (2%) of yeast extract (unpublished results). Possibly a butyrate-forming *C. thermosaccharolyticum* was present in the mixed culture.

As indicated above, the glycolytic bacteria ferment xylose to ethanol, whereas *C. thermocellum* does not grow on this pentose, although it hydrolyzes hemicelluloses. Since *C. thermocellum* produces relatively high amounts of oligoglucosides[56] from cellulose, a bacterium like *C. thermohydrosulfuricum* should be suitable for a mixed culture. It utilizes not only different pentoses, starch and cellobiose, but it should also utilize different oligoglucosides. This is concluded from good growth of *C. thermohydrosulfuricum* JW 102 on the supernatants of *C. thermocellum* cultures following cellulose degradation.

The results reported show, that the concept of mixed, thermophilic and extremely thermophilic cultures is quite useful and promising. Mixed cultures have not only proved valuable for methane production from waste and manure[90], but may also prove advantageous for the production of ethanol from cellulose- and hemicellulose-containing material. However, more data and new species need to be collected. The final selection of the species and strains for industrial applications should be based on the results of pilot fermentations utilizing the available biomass and waste materials as carbon sources.

Conclusions

Many mesophilic and thermophilic anaerobes or facultative anaerobes produce ethanol as a fermentation product, but reasonable yields are only obtained with a minority of strains. However, from the data presented, the conclusion can be drawn, that thermophilic and extremely thermophilic bacteria possesses several advantages for application in the industrial production of ethanol from various biological resources. It is conceivable that the most efficient organism for ethanol production is still hidden in nature, occupying a so far unknown ecological niche. This 'superbacterium' certainly is an extreme thermophilic anaerobe. Without doubts, more efforts are required to investigate the ethanol-producing anaerobes before a final evaluation with respect to their applicability for industrial production of ethanol can be made. Some of the studies concern the diversity of thermophilic and extreme thermophilic anaerobes, especially the search for new isolates with desirable properties; the physiology of new as well as of 'well-known' species; the accessibility to genetic manipulations (genetic engineering); the pattern of regulation, i.e. how to manipulate the carbon flow to the desired products; and the application on an industrial scale.

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