
Lactic Acid Bacteria in Hydrogen-Producing Consortia: On Purpose or by Coincidence?

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<http://dx.doi.org/10.5772/50364>

1. Introduction

Hydrogen is both a valuable energy carrier and a feedstock for various branches of the chemical industry. It is thought to be one of the most important energy carriers of the future, an alternative to conventional fossil fuels. Water vapor and heat energy are the sole products of hydrogen burning. Therefore, the use of hydrogen to generate energy does not contribute to ozone depletion, the greenhouse effect, climate changes or acid rains. Hydrogen is a highly efficient energy source; its specific energy equals 33 Wh/g, which is the highest among all fuels. For comparison, the specific energy of methane is 14.2 Wh/g and coal, 9.1 Wh/g. Hydrogen can be used as a fuel in hydrogen fuel cells or burn directly in internal combustion engines. In the chemical industry, hydrogen is used for syntheses of ammonia, alcohols, aldehydes, hydrogen chloride and for the hydrogenation of edible oils, heavy oils or ammonia, for removal of oxygen traces in prevention against metal oxidation and corrosion processes (Nath & Das, 2003; Logan, 2004; Antoni et al., 2007; Piela & Zelenay, 2004).

Conventional methods of hydrogen production, such as gasification of coal, steam reforming of natural gas and petroleum, and electrolysis of water, are based on fossil fuels. Therefore, these methods are regarded as energy expensive and cause environmental pollution (Nath & Das, 2003; Logan, 2004; Nath & Das, 2004).

Considering the limited reserves of fossil fuels, environmental pollution and global warming, there is great interest in biological methods of producing fuels, such as bio-hydrogen, biogas (methane), ethanol or diesel. Among the known biological processes leading to hydrogen production are dark fermentation, photofermentation, direct and indirect biophotolysis, as well as anaerobic respiration of sulphate-reducing bacteria under conditions of sulphate depletion. Taking under account potential applications, microbial hydrogen production has

been focused on: (i) photolysis of water using algae and *Cyanobacteria*, (ii) photofermentation of organic compounds by photosynthetic bacteria, and (iii) dark fermentation of organic compounds using anaerobic bacteria.

Members of the *Clostridiales* and *Enterobacteriaceae* are well-recognized hydrogen-producers during the process of dark fermentation. For future applications, dark fermentation seems to be the most promising concept. However, low hydrogen yields and generation of large quantities of non-gaseous organic products remain key problems of dark fermentation. The theoretical maximum hydrogen yield during dark fermentation is 4 moles of H_2 /mole of glucose (~33% substrate conversion), but the actual yield is only 2 moles of H_2 /mole of glucose (~17% conversion). Currently, many investigations are focused on improving the hydrogen yield during fermentation as an alternative method of hydrogen production and combining dark fermentation with other processes, like methanogenesis, photofermentation or microbial electrolysis of cells, to achieve more effective substrate utilization (Li & Fang, 2007; Das & Veziroglu, 2008; Hallenbeck & Ghosh, 2009; Lee et al., 2010; Hallenbeck, 2011). Biohydrogen fermentations may be carried out in different batch types, continuous or semi-continuous bioreactors, where mixed microbial consortia develop. In the most effective systems, consortia are selected for growth and dominance under non-sterile conditions and usually show high stability and resistance to transient unfavorable changes in the bioreactor environment. Depending on the bioreactor type and growth conditions, consortia form various structures which ensure retention and accumulation of the active biomass. These include microbial-based biofilms and macroscopic aggregates of microbial cells, such as flocs and granules (Campos et al., 2009; Hallenbeck & Ghosh, 2009). A good understanding of the structure of hydrogen-producing microbial communities, symbiotic relationships within the consortia as well as factors favoring hydrogen production is vital for optimizing the process.

Interestingly, lactic acid bacteria (LAB) are often detected in mesophilic hydrogen-producing consortia as bacteria that accompany hydrogen producers. In this chapter, we discuss the issue of whether LAB are bad or good (positive or negative) components of hydrogen-producing consortia. We present different opinions about the potential significance and the role of LAB in hydrogen-producing communities.

2. Hydrogen-producing bacteria

Fermentation is an anaerobic type of metabolic process of low energy gain in which organic compounds are degraded in the absence of external electron acceptors and a mixture of oxidized and reduced products are formed. Products, namely organic compounds and gasses (hydrogen and carbon dioxide), determine the type of fermentation. Main hydrogen yielding fermentations are butyric acid fermentation (saccharolytic clostridial-type fermentation) and mixed-acid fermentation (enterobacterial-type fermentation). The first step of both fermentations is the Embden-Meyerhof pathway or glycolysis in which glucose is converted into pyruvate and NADH is formed.

In the clostridial-type fermentation pyruvate is oxidized to acetyl-CoA by pyruvate:ferredoxin oxidoreductase (PFOR) in the presence of ferredoxin (Fd) (See Equation 1).

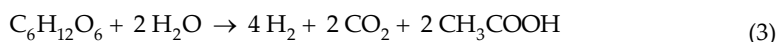


Reduced ferredoxin is also formed in the reaction with NADH catalyzed by NADH:ferredoxin oxidoreductase (NFOR) (See Equation 2).

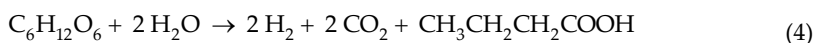


Hydrogen is released by hydrogenases that catalyze proton reduction using electrons from ferredoxin. The activity of PFOR and NFOR enzymes is thermodynamically regulated by the hydrogen concentration. Partial hydrogen pressure >60 Pa inhibits the NFOR activity and favors formation of non-gaseous end-products from acetyl-CoA including acetate, butyrate, ethanol, butanol and lactate. PFOR is active at hydrogen concentrations up to 3×10^4 Pa (Angenent et al., 2004; Girbal et al., 1995; Hallenbeck, 2005; Kraemer & Bagley, 2007; Lee et al., 2011).

The theoretical maximum hydrogen yield during clostridial-type fermentation is 4 moles of hydrogen per mole of glucose, when all of the substrate is converted to acetic acid (See Equation 3).



This gives the maximal possible level of hydrogen yield during dark fermentation. When the glucose is converted to butyrate the hydrogen yield drops to 2 moles (See Equation 4).



Formation of other non-gaseous end products of fermentation causes further decrease in hydrogen yields. The scheme of the clostridial-type fermentation is presented in Figure 1 (Papoutsakis, 1984; Saint-Amans et al., 2001).

The described type of fermentation is the most characteristic for spore-forming representatives of the *Clostridium* as well as *Bacillus* genera and others, such as the rumen bacteria e.g. *Ruminococcus albus*. Among the fermentative anaerobes, clostridia have been well known and extensively studied for their capability to produce hydrogen from various carbohydrates (Kalia & Purohit, 2008; Lee et al., 2011). The hydrogen yields of pure *Clostridium* cultures, including *C. acetobutylicum*, *C. bifermentans*, *C. butyricum*, *C. kluyveri*, *C. lentocellum*, *C. paraputrificum*, *C. pasteurianum*, *C. saccharoperbutylacetonicum*, *C. thermosuccinogenes*, and *C. thermolacticum* were examined. The optimum hydrogen yields observed for these bacteria varied between 1.1 moles of H_2 /mole of hexose and 2.6 moles of H_2 /mole of -hexose, dependent on the organism per se as well as environmental conditions (for review see Lee et al., 2011).

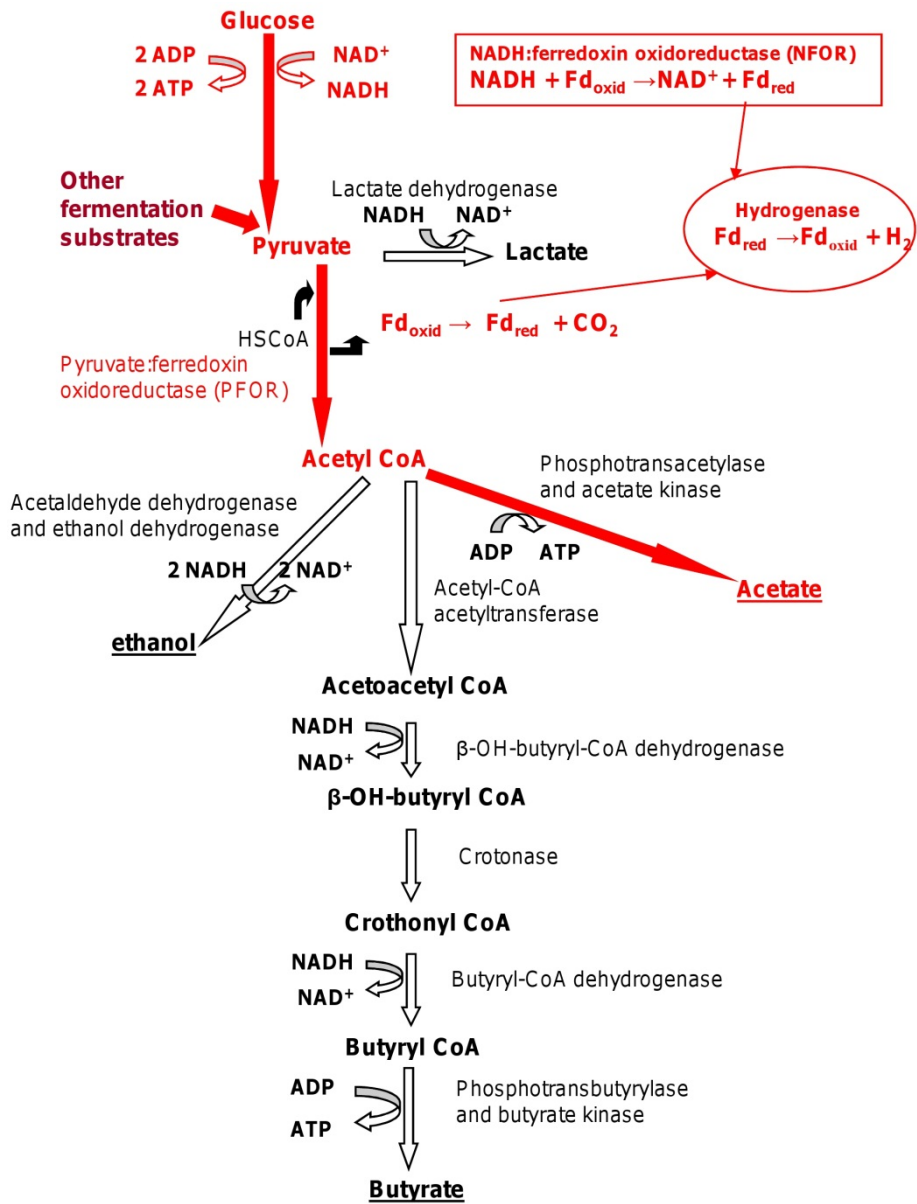
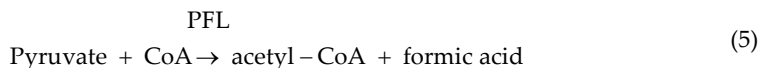
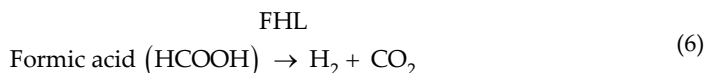


Figure 1. The scheme of clostridial-type fermentation. The pathway leading to the theoretical maximum hydrogen yield of 4 moles of hydrogen per 1 mole of glucose, when all of the substrate is converted to acetic acid is labeled in red.

In the mixed acid-fermentation (also known as formic acid fermentation) pyruvate formate-lyase (PFL) converts pyruvate to acetyl-CoA and formic acid (See Equation 5).



The formic acid can be degraded into hydrogen and carbon dioxide by formate hydrogen-lyase (FHL) (See Equation 6).



There are two types of mixed-acid fermentations. In the first type ethanol and a complex mixture of acids, particularly acetic, lactic, succinic and formic acids are produced. This pattern is seen in *Escherichia*, *Salmonella*, *Proteus* and other genera. The second type is characteristic for *Enterobacter*, *Serratia*, *Erwinia* and some species of *Bacillus*. In this type of fermentation, acetoin, 2,3-butanediol, ethanol and lower amount of acids are formed.

The theoretical hydrogen yields during mixed acid fermentation are lower than those described for the clostridial-type fermentation. Hydrogen yields of *Escherichia* spp., as obtained for the pure culture of *E. coli* NCIMB 11943, are in a range of 0.2–1.8 moles of H₂/mole of hexose, when glucose or starch hydrolysate are substrates, whereas hydrogen yields determined for pure *Enterobacter* spp. cultures are much higher, ranging from 1.1 moles of H₂/mole of hexose to ca. 3.0 moles of H₂/mole of hexose (Lee et al., 2011). It is known that in the *Enterobacter*-type fermentation hydrogen is also generated through oxidation of NADH by NFOR in reactions similar to those described for the clostridial-type fermentation (Nakashimada et al., 2002; Sawers, 2005; Maeda et al., 2007).

The pathway of the mixed-acid fermentation is presented in Figure 2.

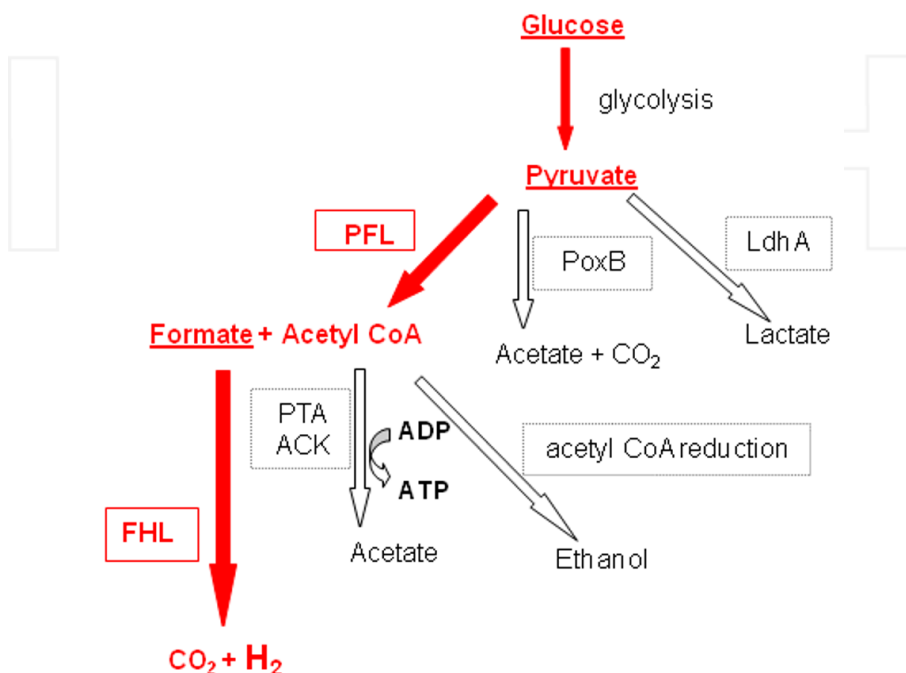
3. Lactic acid bacteria in hydrogen-producing consortia

3.1. Lactic acid bacteria – General information

Lactic acid bacteria are Gram-positive bacteria, producing lactic acid as the main product of carbohydrate fermentation. Two types of lactic acid fermentation are distinguished: homolactic and heterolactic fermentation. In homolactic acid fermentation, two molecules of pyruvate that are formed during glycolysis are converted to lactate. In heterolactic acid fermentation, one molecule of pyruvate is converted to lactate; the other is converted to ethanol and carbon dioxide.

At present, nearly 400 LAB species have been recognized. They include bacteria belonging to the order *Lactobacillales* classified into seven families: *Lactobacillaceae* (genera: *Lactobacillus* and *Pediococcus*); *Aerococcaceae* (genus *Aerococcus*); *Carnobacteriaceae* (genera: *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Granulicatella* and *Lactosphaera*); *Enterococcaceae* (genera: *Enterococcus*, *Tetragenococcus* and *Vagococcus*); *Leuconostocaceae* (genera: *Leuconostoc*, *Oenococcus* and *Weissella*); *Streptococcaceae* (genera: *Streptococcus*, *Lactococcus* and *Melissococcus*); *Microbacteriaceae* (genus *Microbacterium*). Extremely varied among lactic acid

bacteria is genus *Lactobacillus* which comprises over 145 species. Genera *Bifidobacterium* and *Propionibacterium* (class: *Actinobacteria*) as well as spore forming rods belonging to the order *Bacillales*, family *Sporolactobacillaceae*, genus *Sporolactobacillus* constitute further groups of LAB. With the exception of bacteria belonging to the genera *Lactobacillus*, *Carnabacterium*, *Weissella* and *Sporolactobacillus* which are rods, other species of lactic acid bacteria are cocci (de Vos et al., 2009).



LdhA – lactate dehydrogenase, PoxB – pyruvate oxydase, PTA – phosphotransacetylase, ACK – acetate kinase, PFL – pyruvate formate lyase, FHL – formate hydrogen lyase.

Figure 2. The scheme of mixed-acid fermentation (*Escherichia coli*-type). The pathway leading to hydrogen production is shown in red.

LAB are microorganisms ubiquitous in the environment. Due to their high nutritional requirements, they are usually found in environments rich in carbohydrates, amino acids and nucleotides. On the other hand, they show considerable adaptation to the harsh conditions, which allows them to inhabit a range of various niches (Korhonen, 2010).

The digestive tracts of man and animals are among the environments where LAB occur. They have been reported in saliva, the small intestine and colon (Korhonen, 2010). The development of the gastrointestinal microflora in infants is influenced by contact with diverse microflora of the mother and of the closest surrounding. The main species found in both infants and adults are *Lactobacillus ruminis*, *L. salivarius*, *L. gasseri*, *L. reuteri* as well as *Bifidobacterium longum* and *B. breve* (Salminen et al., 2005; Ishibashi et al., 1997). The diversity

of lactic acid bacteria colonizing the human digestive system is high; however, the species composition is constantly changing as most of the species colonize the gastrointestinal tract for only a short period (Korhonen, 2010). Microorganisms in the adult intestine outnumber by 10-fold cells constituting the human body. The microbial composition for each individual is unique, depending on age, diet, diseases and environmental factors (Qin J. et al., 2010). LAB have been widely used as probiotic bacteria in the human gastrointestinal tract, contributing to pathogen inhibition and immunomodulation (Zhang et al., 2011).

The natural occurrence of lactic acid bacteria on plants (fruits, vegetables and grains) as well as in milk permitted their use in biotechnology (Makarova et al., 2006). *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* which reside on grapes, enable fruit fermentation and wine production (de Nadra, 2007). Also, LAB can occur naturally or be intentionally added as starter cultures during plant, meat and dairy fermentation (Korhonen, 2010).

In marine environments LAB play a role in the breakdown of organic matter. In the last decade LAB belonging to the following genera: *Amphibacillus*, *Alkalibacterium*, *Marinilactibacillus*, *Paraliobacillus*, *Halolactibacillus* were isolated from the samples taken from the sea and oceanic as well as from animals that inhabit these ecosystems. These bacteria were named “marine LAB” (Ishikawa et al., 2005).

3.2. Lactic acid bacteria – Influence on hydrogen producers

Interestingly, lactic acid bacteria are often detected in mesophilic hydrogen-producing consortia as bacteria that accompany hydrogen producers. The technique most commonly used for analyzing the diversity of hydrogen-producing microbial communities is polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), followed by either direct sequencing or cloning and sequencing of DGGE bands. One of the disadvantages of this method is underestimation of the true bacterial diversity due to the fact that only the most prominent DGGE bands are analyzed. Various studies have shown that DGGE bands representing LAB are one of the most dominant bands (Fang et al., 2002; Kim et al., 2006; Li et al., 2006; Wu et al., 2006; Hung et al., 2007; Ren et al., 2007; Jo et al., 2007; Lo et al., 2008; Sreela-or et al., 2011). Another method of analyzing the biodiversity of hydrogen-producing consortia is cloning and sequencing of the 16S rDNA gene amplified on the total DNA isolated from the culture probes. Also with this method, sequences related to lactic acid bacteria have been detected (Yang et al., 2007). An alternative method used by our group for the first time to perform metagenomic analysis of hydrogen-producing microbial communities is 454-pyrosequencing. Our results showed that *Clostridiaceae*, *Enterobacteriaceae* and heterolactic fermentation bacteria, mainly *Leuconostocaceae*, were the most dominant bacteria in hydrogen-producing consortia under optimal condition for gas production (Chojnacka et al., 2011).

The aim of the chapter is a provocative discussion on the true role of LAB in hydrogen-producing bioreactors and their influence on hydrogen producers. Table 1 presents a set of selected studies which examine the possible influence of lactic acid bacteria on hydrogen production during dark fermentation.

Subject of examination	Results and suggested influence of LAB on hydrogen producers	References
A. Negative role of LAB		
Investigation of the effects of LAB on hydrogen fermentation of bean curd manufacturing waste in a series of co-cultures of <i>Clostridium butyricum</i> and two strains of <i>C. acetobutylicum</i> with <i>Lactobacillus paracasei</i> and <i>Enterococcus durans</i> .	Inhibition of hydrogen producers by LAB due to (i) substrate competition (replacement of hydrogen fermentation by lactic acid fermentation); (ii) excretion of bacteriocins.	Noike et al., 2002
Fermentative hydrogen production from molasses in continuous stirred-tank reactors and DG-DGGE (double gradient denaturing gradient gel electrophoresis) analysis of bacterial community structure.	<i>C. pasteurianum</i> , <i>Lactococcus</i> sp., <i>Desulfovibrio ferrireducens</i> , <i>Actinomyces</i> sp., <i>Klebsiella oxytoca</i> , <i>Acidovorax</i> sp., uncultured <i>Actinobacterium</i> and <i>Bacteroidetes</i> were detected in the bioreactor where the main non-gaseous end products were ethanol, butyric acid and acetic acid. Negative role of <i>Lactococcus</i> species: inhibition of hydrogen production by substrate competition (competitive ethanol production).	Ren et al., 2007
DGGE examination of microbial community during unstable hydrogen production from food waste of kimchi in a continuous culture.	Conversion of hydrogen fermentation to lactic acid fermentation due to shifts in the microbial community structure from <i>Clostridium</i> spp. to <i>Lactobacillus</i> spp. Negative role of LAB: substrate competition.	Jo et al., 2007
Investigation of hydrogen production from food waste in batch fermentation by anaerobic mixed cultures and DGGE analysis of microbial community.	<i>Clostridium</i> species (<i>C. butyricum</i> , <i>C. acetobutylicum</i> , <i>C. beijerinckii</i> , <i>Clostridium</i> sp.) were the dominant hydrogen producers. Negative role of LAB representatives (<i>Lactobacillus</i> sp., <i>Enterococcus</i> sp.): inhibition of hydrogen production by substrate competition (competitive ethanol and lactic acid production).	Sreela-or et al., 2011

Subject of examination	Results and suggested influence of LAB on hydrogen producers	References
B		
Role of LAB in hydrogen-producing consortia not discussed		
Fermentative hydrogen production from sucrose-containing wastewater in a well-mixed reactor and DGGE analysis of bacterial community structure of the granular sludge.	<i>Clostridium</i> species (<i>C. pasteurianum</i> , <i>C. tyrobutyricum</i> , <i>C. acidisoli</i>) and <i>Sporolactobacillus racemicus</i> were detected in the bioreactor. A high-rate fermentative hydrogen production was observed. The role of LAB (<i>Sporolactobacillus racemicus</i>) in the microbial community is not discussed.	Fang et al., 2002
Fermentative hydrogen production from sucrose in a continuously stirred anaerobic bioreactor seeded with silicone-immobilized sludge and DGGE analysis of bacterial community structure of the granular sludge.	DGGE analysis revealed the presence of representatives of the following genera and species: <i>Clostridium</i> (<i>C. intestinale</i> and <i>C. pasteurianum</i>), <i>Escherichia coli</i> , <i>Streptococcus</i> sp., <i>Klebsiella pneumoniae</i> . A high-rate fermentative hydrogen production was observed. The role of LAB (<i>Streptococcus</i> sp.) in the microbial community is not discussed.	Wu et al., 2006
Fermentative hydrogen production from sucrose in a continuous stirred tank reactor and DGGE analysis of bacterial community structure of the granular sludge.	<i>Clostridium cellulosi</i> , <i>Clostridium</i> sp., <i>Klebsiella ornithinolytica</i> , <i>Prevotella</i> sp. and <i>Leuconostoc pseudomesenteroides</i> were detected in the bioreactor. A high-rate fermentative hydrogen production was observed. The role of LAB (<i>L. pseudomesenteroides</i>) in the microbial hydrogen-producing community is not discussed.	Li et al., 2006
Fermentative hydrogen production from sucrose or xylose in a continuous dark fermentation bioreactor and DGGE analysis of the bacterial community structure.	<i>Clostridium</i> species (<i>C. butyricum</i> , <i>C. pasteurianum</i> on sucrose and <i>C. celerecrescens</i> on xylose), <i>Klebsiella pneumoniae</i> , <i>K. oxytoca</i> , <i>Streptococcus</i> sp., <i>Escherichia</i> sp., <i>Pseudomonas</i> sp. <i>Dialister</i> sp., <i>Bacillus</i> sp., <i>Bifidobacterium</i> sp. were detected in the bioreactor. The role of LAB (<i>Streptococcus</i> sp. and <i>Bifidobacterium</i> sp.) in the microbial community is not discussed.	Lo et al., 2008

Subject of examination	Results and suggested influence of LAB on hydrogen producers	References
C. Positive role of LAB		
Fermentative hydrogen production from glucose in anaerobic agitated granular sludge bed bioreactors and DGGE and FISH analyses of the granular sludge.	The DGGE analysis showed that the bacterial community was mainly composed of <i>Clostridium</i> sp., <i>Klebsiella oxytoca</i> and <i>Streptococcus</i> sp. A high-rate fermentative hydrogen production was observed. The FISH images suggested that <i>Streptococcus</i> cells acted as seeds for granule formation.	Hung et al., 2007
Fermentative hydrogen production from cheese whey wastewater by mixed continuous cultures and molecular analysis of the consortium by cloning and sequencing of the 16S rDNA gene amplified on the total DNA isolated from the culture probe.	The most prevalent bacteria, representing approximately 50% of the total sequences analyzed, were representatives of the genus <i>Lactobacillus</i> . Remaining sequences belonged to the genera <i>Olsenella</i> , <i>Clostridium</i> and <i>Prevotella</i> . Decrease in hydrogen production was accompanied by the reductions in the number of detected bacteria from the genus <i>Lactobacillus</i> . Authors declare isolation of <i>Lactobacillus</i> bacteria capable of hydrogen production in the process of lactose fermentation.	Yang et al., 2007
Fermentative hydrogen production from molasses in packed bed bioreactors and metagenomic analysis of bacterial biofilms and granules by 454-pyrosequencing.	Metagenomic analysis of microbial consortia by 454-pyrosequencing of amplified 16S rDNA fragments revealed that the most dominant bacteria were the representatives of the <i>Firmicutes</i> (<i>Clostridiaceae</i> and <i>Leuconostocaceae</i>) and <i>Gammaproteobacteria</i> (<i>Enterobacteriaceae</i>). Bacteria of heterolactic fermentation were one of the predominant microbes in hydrogen-producing consortia. The speculation that LAB may favor hydrogen production is discussed. For details see Tables 2-4, Figures 3-5 and description in the text.	Chojnacka et al., 2011

Table 1. A set of selected studies demonstrating the contribution of LAB in hydrogen-producing cultures and presenting their possible influence on hydrogen production.

Some studies argue that development of LAB in bioreactors may inhibit hydrogen production (Table 1, part A). Cessation of hydrogen generation by LAB was suggested to be due to (i) substrate competition and/or (ii) excretion of bacteriocins inhibiting growth of

other bacteria. These observations derive from examinations of both batch (Sreela-or et al., 2011) and continuous (Ren et al., 2007; Jo et al., 2007) mixed cultures as well as co-cultures where one component was a representative of clostridia and the second one of lactic acid bacteria (Noike et al., 2002). Heat treatment was proposed as a method of eliminating lactic acid bacteria (Noike et al., 2002; Baghchehsaraee et al., 2008).

Substrate competition includes changes in the type of fermentation occurring in the bioreactors during long-term continuous processes and replacement of hydrogen fermentation by lactic acid or ethanol fermentation (Noike et al., 2002; Jo et al., 2007; Ren et al., 2007; Sreela-or et al., 2011). In all of the studies decrease in hydrogen production was observed with simultaneous increase of lactic acid and ethanol concentrations in the effluents or fluid phase of the culture.

The hypothesis that bacteriocins may act as inhibitors of hydrogen production was postulated by Noike and co-workers (2002), who showed in a series of co-cultures experiments that cessation of hydrogen production by *C. acetobutylicum* and *C. butyricum* was caused by both the presence of *Enterococcus durans* and *Lactobacillus paracasei* as well as supernatants from their culture media. Moreover, treatment of the supernatants with trypsin recovered normal hydrogen production by selected clostridial strains.

Studies listed in part B of Table 1 determined the presence of lactic acid bacteria in hydrogen-producing consortia; yet, their role in these microbial communities is not discussed. It is noteworthy that (i) those papers discuss efficient systems of biohydrogen production and (ii) studies were performed under optimal conditions for hydrogen production (Fang et al., 2002; Kim et al., 2006; Li et al., 2006; Wu et al., 2006; Hung et al., 2007).

Part C of Table 1 presents the only so far available studies arguing that LAB could play a positive role in hydrogen-producing microbial communities and stimulate hydrogen production.

Hung and colleagues (2007) studied the efficiency of fermentative hydrogen production from glucose in anaerobic agitated granular sludge bed bioreactors under different substrate concentration and hydraulic retention times (HRT). PCR-DGGE and FISH methods were used to analyze the biohydrogen-producing microbial community of the granular sludge. The bacterial community was composed of *Clostridium* sp. (possibly *C. pasteurianum*), *Klebsiella oxytoca* and *Streptococcus* sp. The percentage of *Streptococcus* sp. contributing to the microbial community was dependent on the HRT. The shorter HRT, meaning the faster the flow of the medium and increased dilution rate, the higher the contribution of *Streptococcus* sp. in the bacterial consortium was observed. Formation of granular sludge enables biomass retention. FISH analysis revealed that *Streptococcus* cells are located inside granules surrounded by *Clostridium* cells. Authors postulate that *Streptococcus* cells may act as the seed for sludge granule formation.

According to Yang et al. (2007) some LAB are able to produce hydrogen. They declare isolation of strains from the genus *Lactobacillus* capable of hydrogen production during lactose fermentation.

3.3. Fermentative hydrogen production and microbial analysis of bacterial biofilms and granular sludge formed in packed bed bioreactors

We developed an effective system of bacterial hydrogen production based on long-term continuous cultures (from an inoculum of a lake bottom sediment) grown on sugar beet molasses in packed bed reactors filled with granitic stones (Chojnacka et al., 2011). In separate cultures, two consortia of anaerobic fermentative bacteria producing hydrogen-rich gas developed on the stones as biofilms. Furthermore, in one of the cultures a granular sludge was also observed (Figures 3 and 4). Cultures were named, respectively, (i) the culture with stone biofilm only and (ii) granular sludge culture. Both cultures were regularly renewed by removal of an excess of biomass.

Analysis of the surface topography of biofilms from both cultures revealed their porous, irregular structure with many cavities and channels. Bacteria appeared to be suspended in and surrounded by a matrix substance. The granules were white and light cream in color, with a diameter between 0.2 – 2 mm, and of hard structure, resistant to squashing or crumbling. Moreover, the granules were clustered in structures resembling bunches of grapes with a noticeable net of channels. Similar to the bacterial biofilm, the granules consisted of bacterial cells surrounded by a matrix.

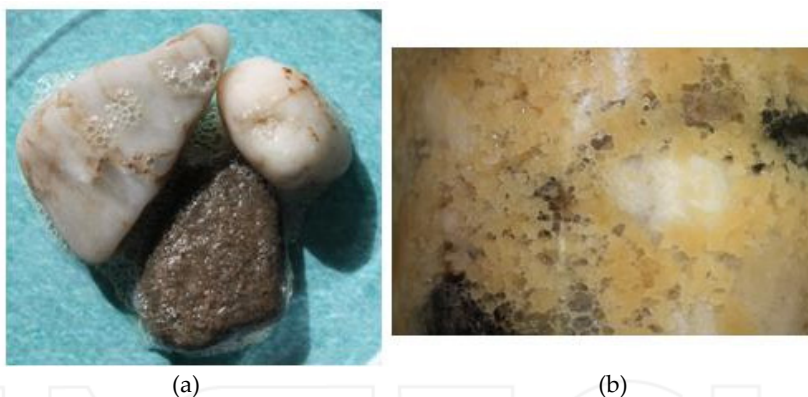


Figure 3. Images of the two structures formed by selected consortia of fermentative bacteria grown in a bioreactor on M9 medium containing molasses: (a) stones covered with bacterial biofilm (b) the granular sludge.

Metagenomic analysis of microbial communities by 454-pyrosequencing of amplified 16S rDNA fragments revealed that the overall biodiversity of hydrogen-producing cultures was quite small. Stone biofilm from the culture without the granular sludge was dominated by *Clostridiaceae* and heterolactic fermentation bacteria, mainly *Leuconostocaceae*. Representatives of *Leuconostocaceae* and *Enterobacteriaceae* were dominant in both the granules and the stone biofilm formed in the granular sludge culture. The granular sludge contained bacteria of heterolactic fermentation, dominated by *Leuconostoc* species as well as unclassified *Streptococcaceae* and unclassified *Enterobacteriaceae*. Surprisingly, sequences representing the *Clostridiaceae* were in a relative minority (Table 2).

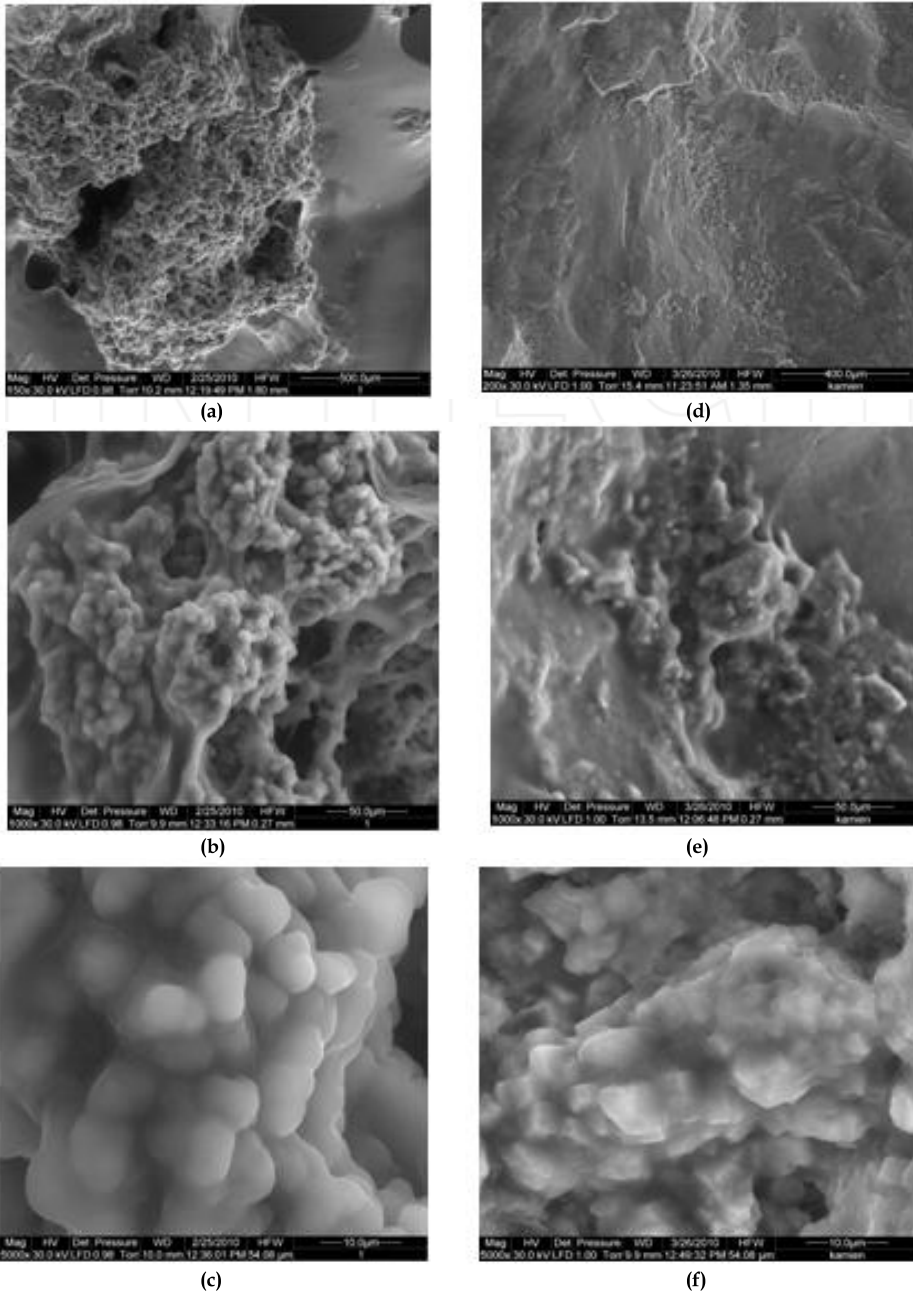


Figure 4. Scanning electron micrographs of structures formed by selected consortia of fermentative bacteria grown on M9 medium containing molasses: (a – c) granules; (d – f) bacterial biofilm formed on the granitic stones filling the bioreactor in the granular sludge culture.

taxon	B	G	Bg
Bacteria	4596	8578	26066
Firmicutes	4410	6473	10216
Bacilli	1564	5425	7586
Bacillales	119	17	96
Bacillaceae	1	0	11
Sporolactobacillaceae	94	6	44
Sporolactobacillus	94	6	44
Lactobacillales	1343	5302	7272
Enterococcaceae	5	16	417
Enterococcus	2	2	123
Lactobacillaceae	96	3	790
Lactobacillus	92	3	725
Leuconostocaceae	826	4661	4589
Leuconostoc	826	4634	4586
Streptococcaceae	4	37	95
Lactococcus	4	17	74
Clostridia	2627	579	2240
Clostridiales	2572	443	2023
Clostridiaceae	2131	134	1100
Clostridium	1182	66	593
Proteobacteria	168	1974	15755
Gammaproteobacteria	168	1970	15744
Enterobacteriales	156	1769	15528
Enterobacteriaceae	156	1769	15528
Enterobacter	61	319	2130
Raoultella	6	50	23
Pseudomonadales	6	8	137
Moraxellaceae	5	0	128
Acinetobacter	2	0	68
Pseudomonadaceae	1	8	9
Pseudomonas	1	7	9

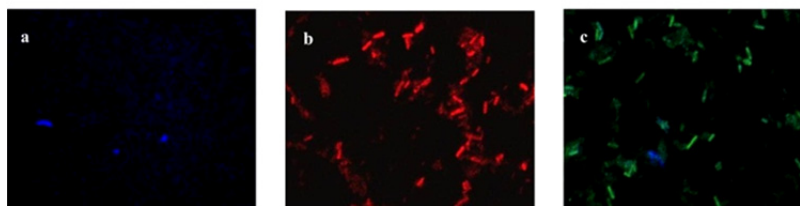
B – stone biofilm from the culture without granular sludge; Bg – stone biofilm from the granular sludge culture; G – granules from the granular sludge culture.

Table 2. Number of reads assigned to respective taxonomic branches of 16S rRNA gene fragments amplified from the total DNA pool from bacterial communities formed in bioreactors.

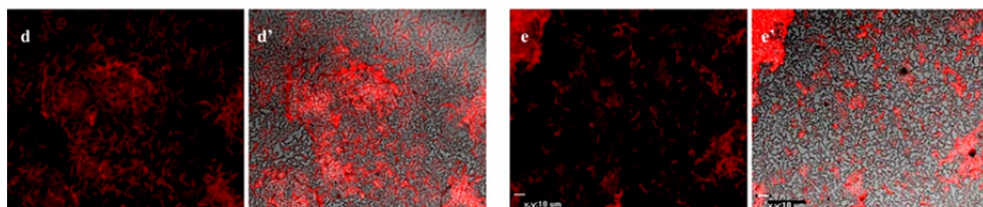
Results of the metagenomic analysis by 454-pyrosequencing were confirmed by FISH (Fluorescence-In-Situ-Hybridization) analysis (Fig. 5) as well as by isolating of lactic acid bacteria from the culture (Table 3).

Both, the stone biofilm and granules are composed of bacteria of many different shapes. As judged from fluorescence *in situ* hybridization, the relative abundance of selected bacterial groups varied during the rounds of bioreactor cycles. At the very beginning of biofilm development clostridial and lactobacilli cells were detected only sporadically among gammaproteobacteria (Fig. 5A a-c). In the growing biofilm systematic increase of Firmicutes (especially lactobacilli) cells was observed (Fig. 5Bd-e).

A



B



A: young biofilm; a-clostridia/dyLight405, b-lactobacilli/TAMRA; c-gammaproteobacteria/CY3, B: mature biofilm; d-firmicutes/CY5; e-lactobacilli/TAMRA; d', e'-in combination with phase contrast.

Figure 5. FISH image of the hydrogen-producing biofilm from the granular sludge culture described previously (Chojnacka et al., 2011) analyzed by confocal laser fluorescence microscopy. The sample was stained with fluorescently labeled specific probes.

A cultivable approach with the use of media promoting the growth of lactic acid bacteria (MRS, M17) revealed that the bioreactor was inhabited by a vast number of these bacteria. Similarly to the metagenomic data, the majority of growing colonies represented *Leuconostoc* or *Lactobacillus* genera. All in all, six different species listed in Tab. 3 were isolated. It was determined that heterofermentative species (*Leuconostoc*, *L. brevis*, *L. rhamnosus*) slightly outnumbered homofermentatives.

Isolate	Isolation ratio	Homofermenters:heterofermenters ratio
<i>Lactobacillus plantarum</i>	46,7%	0,89
<i>Enterococcus casseliflavus</i>	0,5%	
<i>Leuconostoc mesenteroides</i>	46,7%	
<i>Leuconostoc mesenteroides</i> ssp. <i>mesenteroides</i>	0,5%	
<i>Lactobacillus brevis</i>	5,1%	
<i>Lactobacillus rhamnosus</i>	0,5%	

Table 3. The species of LAB isolated from the hydrogen-producing bioreactor.

Samples were collected from both, stone (biofilm) and liquid phase of hydrogen-producing culture, and plated on selective media for lactic acid bacteria. Plates were incubated under anaerobic conditions. Obtained colonies were tested for Gram positivity and lack of catalase enzyme. For strains which gave positive results, the V3 fragment of the 16S rRNA gene was amplified. Subsequently, fragments were analyzed using MSCP technique. Strains with unique or representative gel patterns were chosen for further studies based on amplification and sequencing of 16S rRNA gene. Resulting sequences were identified by comparison to known sequences using the NCBI database. Names of homofermentative species are written in bold.

Formation of granular sludge rich in heterolactic bacteria significantly enhanced hydrogen production. Table 4 presents a list of parameters describing and comparing the two bacterial cultures that were the subject of the study of Chojnacka et al. (2011), under optimal conditions for hydrogen production. Significantly higher total gas production was observed for the culture containing granular sludge than for the biofilm-only culture (9.5 vs. 6.6 cm³/min/working volume of the bioreactor). Furthermore, the percentage contribution of hydrogen was almost 49 and 36 %, whereas of carbon dioxide 47 and 60%, in the former and latter cultures, respectively. The granular sludge culture produced hydrogen at the rate of 6649 cm³/day/working volume of the bioreactor, whereas the biofilm-only culture at the rate of 3393 cm³/day/working volume of the bioreactor. Fermentation gas produced by both cultures contained 0.0004% methane, meaning that it was practically methane-free. Consequently, under optimal conditions, the culture containing granular sludge rich in heterolactic bacteria was two-fold more effective in producing hydrogen than that containing biofilm only: 5.43 moles of H₂ vs. 2.8 moles of H₂/mole of sucrose from molasses, respectively.

It is known that butyrate is the predominant metabolite during butyric acid fermentation at pH 5.0 – 5.5 (Li and Fang, 2007). The analysis of the non-gaseous fermentation products in both cultures in the study of Chojnacka et al. (2011) revealed that butyric acid was the main metabolite with partial contribution of ethanol. Concentration of butyric acid was almost 1.8-fold higher in the culture containing granular sludge than in the biofilm-only culture. No net production of lactic and propionic acids was observed in the granular sludge culture, whereas these were the second and third most abundant fermentation products in the

cultures containing only biofilm. The formic and acetic acids present in the medium were utilized by both cultures. It is noteworthy that in the granular sludge culture rich in heterolactic bacteria showing very good performance in hydrogen production and a high content of butyric acid, the number of *Clostridiales* sequences was significantly lower than in the biofilm-only culture.

Based on our results presented in the study of Chojnacka et al. (2011) we speculate that LAB may possibly play a significant but not fully understood and perhaps underestimated role in the hydrogen producing communities. This hypothesis is based on two observations: (i) the higher the number of LAB in the hydrogen-producing community, the more efficiently hydrogen is produced; (ii) complete consumption of lactic acid, significantly increased concentration of butyric acid as well as larger hydrogen yield in the culture containing granular sludge than in that with just the biofilm.

Parameter	Culture without granular sludge	Culture containing granular sludge
Total gas production (cm³/min/working volume of the bioreactor)	6.6	9.5
Composition of fermentation gas (%):		
Hydrogen	35.7 %	48.6 %
Carbon dioxide	60%	47.1 %
Water vapor	~4.3%	~4.3 %
Methane	0.0004 %	0.0004%
Others (NH ₃ , H ₂ S, formic, acetic, propionic and butyric acids)	~1%	~1%
Hydrogen production (cm³/day/working volume of the bioreactor)	3393	6649
Yield of hydrogen (moles H₂/mole of sucrose)	2.8	5.43
Net production of the non-gaseous end products (mg/L):		
Lactic acid	2419 ± 42.6	0
Formic acid	0	0
Acetic acid	0	0
Propionic acid	248 ± 0.7	0
Butyric acid	4331 ± 60.0	7641 ± 33.1
Ethanol (%)	0.06 ± 0.002	0.1 ± 0.004

Table 4. Parameters describing two cultures of hydrogen-producing bacteria under optimal conditions for hydrogen production based on the study of Chojnacka et al. (2011).

3.4. Enhancement of hydrogen production by lactic acid

Based on the study of Chojnacka et al. (2011), for the culture containing granular sludge rich in heterolactic bacteria no net production of lactic acid was observed, indicating complete consumption of this metabolite, whereas its concentration in the biofilm-only culture was quite high (Table 4). Noticeable is the fact that molasses - a fermentative substrate in this study, also contains acetic and lactic acids at concentrations of about 800 mg/L each. Furthermore, a significantly higher concentration of butyric acid was detected in the culture containing granular sludge than in biofilm-only culture.

There are studies arguing that lactic acid and acetic acid mixed with the substrate stimulate biohydrogen production. Baghchehsaraee et al. (2009) showed that the addition of lactic acid to a mixed culture grown on starch-containing medium increased both hydrogen production and butyric acid formation. Furthermore, complete consumption of lactic acid produced by the culture was observed. When lactic acid was the only carbon source, the level of hydrogen production was very low (0.5% substrate conversion efficiency). Therefore, authors claimed that the addition of lactic acid to the medium probably alters the metabolic pathways in bacterial cells.

In the study of Kim et al. (2012), the effects of different lactate concentrations on hydrogen production from glucose in batch and continuous cultures were examined. Lactic acid was determined to be a factor increasing the efficiency of hydrogen production in a proper range of concentrations. The key issue was to establish the optimal lactic acid concentration. FISH analyses revealed that *Clostridium* sp. was the dominant hydrogen producer in the examined system.

Matsumoto and Nishimura (2007) examined fermentative hydrogen production from sweet potato sho-chu post-distillation slurry that contained large amounts of organic acids. Hydrogen production was accompanied by a decrease in the concentrations of acetic and lactic acids and co-production of butyric acids. The authors isolated a clostridial strain, *Clostridium diolis* JPCC H-3, capable of effective hydrogen production from the slurry solution and a mixture of acetic and lactic acids in an artificial medium.

The ability to produce hydrogen from lactic and acetic acids seems to be widely conserved in the genus *Clostridium* and other hydrogen-producing bacteria capable of butyric acid fermentation of carbohydrates. It was shown that the *Clostridium acetobutylicum* strain P262 and *Butyrivibrio methylotrophicum* utilized lactate and acetate and converted them to butyrate, carbon dioxide and hydrogen in the absence of carbohydrates in the medium. Cell extracts from bacteria grown on acetate and lactate showed a higher activity of NAD-independent lactate dehydrogenase than these from bacteria grown on carbohydrate-rich medium (Diez-Gonzales et al., 1995; Shen et al., 1996). The authors presented potential biochemical pathways leading to butyrate and hydrogen production from lactate and acetate. Conversion of lactate and acetate to butyrate and symbiotic interactions between LAB and clostridial species in animal intestinal tracts are intensively studied and discussed

in section 4. Therefore, also the biochemical routes leading to butyrate and hydrogen production from lactate and acetate are presented in the same section.

In the study of Matsumoto and Nishimura (2007) the process of hydrogen production by *C. diolis* from both the slurry solution and a mixture of acetic and lactic acids in an artificial medium occurred to be pH-dependent and was observed in a range of pH (~5.8 – 7.4). Juang et al. (2011) also observed utilization of lactate and acetate for biohydrogen and butyrate production during their studies on hydrogen and methane production from organic residues of ethanol fermentation from tapioca starch by mixed bacteria culture. Lactate and acetate came from maltose fermentation, the main carbohydrate of ethanol fermentation residues. The optimal hydrogen production was observed at pH 5.5 – 6.0. Jo et al. (2008) showed that conversion of lactate and acetate to butyrate and hydrogen by *Clostridium tyrobutyricum* was inhibited due to pH decrease from 5.5 to 4.6. The pH values were dependent on HRT and organic loading rate. At high organic loading rate accumulation of lactate, pH decrease and a lower efficiency of hydrogen production were observed.

Matsumoto and Nishimura (2007), Jo et al. (2008) and Juang et al. (2011) point to pH values as a critical factor for hydrogen production from lactate and acetate. Various optimal pH for hydrogen production are observed. The differences may depend on the microbial system applied for hydrogen production and the initial substrate. It is speculated that unfavorable changes in pH could be the main reason of inhibiting hydrogen production that could be incorrectly attributed to the presence of lactic acid bacteria in hydrogen-producing consortia. In the study of Chojnacka et al. (2011), the optimal pH was around 5.0. Any change in pH, a decrease below 4.5 or increase above 5.5, caused a significant decline in fermentative gas production. Changes in pH may either be the reason or the results of disturbing the “homeostasis” of hydrogen-producing microbial communities in bioreactors.

4. Interactions between LAB and clostridial species in the animal intestinal tract

Microflora of the mammalian intestine is composed of a diverse population of both aerobic and anaerobic bacteria. Symbiotic relationships occur between different intestinal species or groups of species, among which are interactions between LAB and clostridial species. Numerous observations arising from different models describe lactate conversion to butyrate by intestinal bacteria and enhancement of butyrate production by LAB (Hashizume et al., 2003; Duncan et al. 2004; Bourriaud, et al., 2005; Meimandipour et al., 2009; Abbas, 2010; Munoz-Tamayo, et al., 2011).

The microbial community of the human colon contains many bacteria that produce lactic acid including lactobacilli, bifidobacteria, enterococci and streptococci. However, lactate is normally detected only at very low concentration (<5 mM) in feces of healthy individuals due its rapid conversion to short chain fatty acids (SCFAs; acetate, propionate and butyrate) by acid-utilizing bacteria. Therefore, lactate is thought to be a precursor of the formation of

various SCFAs (Hashizume et al., 2003; Duncan et al., 2004; Bourriaud et al., 2005; Munoz-Tamayo et al., 2011). Bourriaud and colleagues (2005) performed convincing experiments exploring the lactate metabolism and short fatty acids production. They incubated three human microfloras with media containing ^{13}C -labelled lactate and detected the labeled products of fermentation by ^{13}C NMR spectrometry. Results revealed that butyrate was the major net product of lactate conversion by human fecal microflora. Other SCFAs produced were: propionate, acetate and valerate. Inter-individual differences between the three microfloras were observed. Similar studies performed using ^2H -labelled acetate and ^{13}C -labelled lactate and gas chromatography-mass spectrometry (GC-MS) analysis showed that acetic and lactic acids are important precursors of butyrate production in human fecal samples (Morrison et al., 2006).

The metabolic pathway of lactate and acetate utilization to produce butyrate proposed for *Eubacterium hallii* and *Anaerostipes caccae* is shown on Figure 6 (Duncan, et al., 2004; Munoz-Tamayo, et al., 2011). The butyrate produced (in moles) is approximately equal to the sum of half of the acetate and lactate coming from the medium. Lactate is converted to pyruvate by lactate dehydrogenase. The next steps are analogous to those ones presented on Figure 1. Pyruvate is oxidized to acetyl coenzyme A (acetyl-CoA), which is further routed to acetate and butyrate. Acetate is produced via acetate kinase, the pathway generating energy in the form of ATP. For butyrate formation, two molecules of acetyl-CoA are condensed to one molecule of acetoacetyl-CoA, and subsequently reduced to butyryl-CoA. Butyrate can be synthesized from two metabolic pathways: phosphotransbutyrylase and butyrate kinase as shown on Figure 1, and butyryl CoA:acetate CoA transferase as shown on Figure 6. The latter mechanism seems to be the dominant in the human colonic ecosystem. Butyryl-CoA:acetate CoA-transferase transports the CoA component to exterior of acetate releasing butyrate and acetyl-CoA (Duncan et al., 2004; Munoz-Tamayo, et al., 2011). Hydrogen can be produced by both PFOR and NFOR complexes and hydrogenases, as described in section 2. The reaction catalyzed by NFOR is assumed to be the main route for H_2 production by intestinal microflora (Bourriaud et al., 2005). Similar pathway is proposed for clostridial species (eg. *C. acetobutylicum*; Diez-Gonzales et al., 1995) and other hydrogen and butyrate producing bacteria (eg. *B. methylotrophicum*; Shen et al., 1996), as mentioned in section 3.4. Conversion of lactate and acetate to butyrate and hydrogen is an energetically favorable process (Duncan et al., 2004; Jo et al., 2008).

The known lactate-utilizing butyrate-producing bacteria belong to the *Firmicutes* phylum, which includes the following species: *Megasphaera elsdenii*, *Anaerostipes caccae*, *Anaerostipes coli*, *E. hallii* and species distantly related to *Clostridium indolis*. *A. coli* is a dominant member of the human colonic microbiota recognized for its importance in butyrate production. *M. elsdenii* is one of the main butyrate producers from lactate in ruminants as well as monogastric animals, such as pigs or rodents. *A. caccae*, *A. coli*, *E. hallii* and species distantly related to *Clostridium indolis* belong to the clostridial cluster XIVa (*Lachnospiraceae*), known butyrate-producing bacteria of gastrointestinal tracts in

mammals. However, only a few butyrate-producing species within the clostridial cluster XIVa are capable of converting lactate to butyrate (Duncan, 2004; Hashizume et al., 2003; Munoz-Tamayo, et al., 2011).

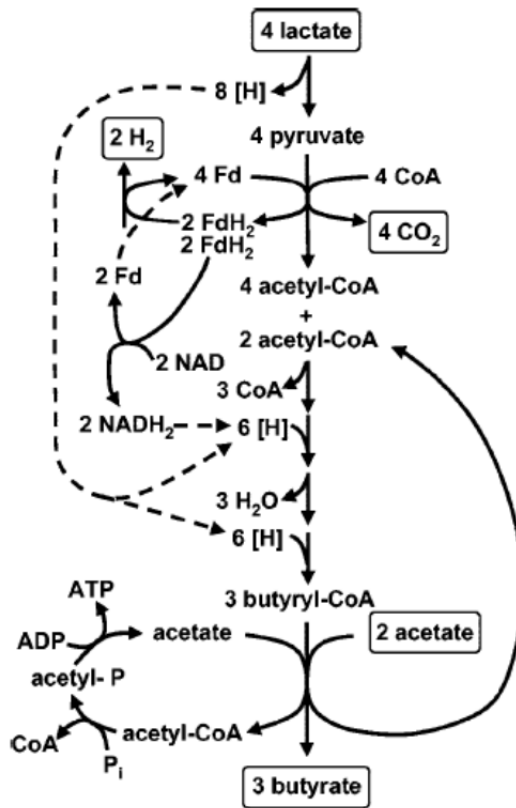


Figure 6. Scheme for butyrate production from lactate in *E. hallii* and *A. caccae*, adapted from Duncan et al., 2004.

The issue of stereospecificity of lactate utilization was addressed in the study of Duncan et al. (2004). Three *E. hallii*-related strains (SL6/1/1, SM6/1 and L2-7) and two *A. caccae* strains (L1-92 and P2) were able to use both D and L isomers of lactate during incubation on DL-lactate-containing medium. Interestingly, the addition of glucose to the medium almost

completely inhibited lactate utilization by the tested strains. Additional studies showed that *E. hallii* L2-7, when grown with DL-lactate, used all of the supplied lactic acid together with some acetate, producing more than 20 mM of butyrate. Less butyrate, but a noteworthy amount of formate, was produced during growth on glucose or on glucose plus lactate. Interestingly, the highest level of hydrogen production was observed when strains were grown on lactate and the lowest for growth on glucose plus lactate. However, the *Clostridium indolis*-related strain SS2/1 was able to use D-lactate, but not L-lactate, during growth on DL-lactate containing media, which suggests that it lacks both an L-lactate dehydrogenase, capable of producing pyruvate from L-lactate, and a racemase, capable of converting L-lactate into D-lactate. According to Bourriaud and colleagues (Bourriaud et al., 2005), both lactate enantiomers are equally utilized by human intestinal microflora, treated as a whole consortium, not as pure strains.

Conversion of lactate to butyrate is one of the important factors for maintaining homeostasis in gastrointestinal tracts. Accumulation of lactate leads to different intestinal disorders (Hashizume et al., 2003). A number of studies have been performed to confirm the symbiotic interaction between lactic acid bacteria and butyric acid bacteria, mainly the *Clostridiales* representatives isolated from animal gastrointestinal tracts. Co-culture experiments that simulated the relations occurring *in vivo* were carried out. Symbiotic interactions were described to rely on the phenomenon of cross-feeding of lactate and involve conversion of lactate to butyrate by butyrate-producing bacteria stimulated by LAB.

It is noteworthy that results from studies of the gastrointestinal microflora indicate that acidity seems to be a key regulatory factor in lactate metabolism. The pH values may influence both bacterial growth and development of specific groups of bacteria as well as fermentation processes affecting the relative proportions of SCFAs (Belenguer et al., 2006; Meimandipour, et al., 2009; Belenguer et al., 2011). These observations are in agreement with our position concerning the potentially important role of pH in hydrogen-producing consortia discussed in section 3.4.

We postulate that the phenomenon analogous to cross-feeding observed in the gastrointestinal tract might take place in hydrogen-producing bioreactors. Although LAB may seem to be undesirable in such processes as they use some of H₂ to produce lactate, their stimulatory effects on hydrogen producers seem to exceed the potentially unbeneficial features. In many studies, it has been explicitly proven that the presence of LAB positively affects the production of butyrate. Most probably, hydrogen producers, mainly species belonging to the *Clostridiales* order, are capable of utilizing lactate as the main precursor of butyrate formation. Further investigations are required.

5. Conclusions

Lactic acid bacteria are detected in almost all biohydrogen-producing microbial communities of dark fermentation. Many studies indicate that LAB inhibit hydrogen

production due to substrate competition and replacement of hydrogen fermentation by lactic acid and ethanol fermentations, and/or excretion of bacteriocines. On the other hand, some positive interactions between LAB and clostridial species have also been noted. They include hydrogen production from lactate by many clostridial species and symbiotic interactions, called lactate cross-feeding, occurring between LAB and clostridia.

These phenomena rely on the conversion of lactate and acetate to butyrate and hydrogen. Symbiotic interactions between LAB and butyrate-producing bacteria involving clostridia have been described in the gastrointestinal tract. We postulate that similar relations exist in biohydrogen-producing bioreactors. According to our hypothesis, pH may be a critical factor affecting bacterial growth, development of specific groups building hydrogen-producing microbial communities and fermentation processes. Acidity changes in bioreactors might be either the reason or the results of disturbances in the balance between microorganisms constituting hydrogen-producing microbial communities in bioreactors. Still, there are no data on symbiotic interactions between LAB and enterobacteria in hydrogen-producing microbial consortia. All these issues require further investigations.

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Acknowledgement

We would like to thank Dominika Brodowska for performance of FISH analyses and Dr. Agnieszka Szczepankowska for editorial assistance.

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