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# The Past, Present, and Future of Biofuels – Biobutanol as Promising Alternative

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## 1. Introduction

While the worldwide energy consumption is projected to grow by 57 % until 2030 (U. S. Energy Information Administration [EIA], 2007) fossil sources are limited and it is questionable how long they will last. Meanwhile, even unconventional sources such as tar sands and oil shales become economically producible since crude oil prices have reached sustained highs and even surpassed a historic mark of US\$ 145 per barrel in June 2008 (EIA, 2011).

Besides concerns about availability and prices of fossil fuels as well as the quest for energy independence, there is also an intense discussion about environmental impacts. Burning of fossil fuels leads to a massive increase of the greenhouse gas CO<sub>2</sub> in the atmosphere and is thus contributing to global warming (Intergovernmental Panel on Climate Change [IPCC], 2007). This could be counterbalanced by using alternative energy sources. Biofuels are the most promising alternative energy for the transportation sector, which is most rapidly growing (an annual average of 3 % is projected until 2030, especially due to increasing mobility in China and India) and accounts for over 20 % of the worldwide primary energy (EIA, 2010).

Biofuels can either be produced microbially or chemically from renewable biomass and are therefore CO<sub>2</sub> neutral. However, only few compounds such as alcohols (ethanol, butanol), alkyl esters of fatty acids (biodiesel), and alkanes (renewable diesel) have the required properties. Today, only bioethanol, biodiesel, and renewable diesel are produced at industrial scale, but several second generation technologies are on path to commercialization. Especially biobutanol fermentation seems to be a promising alternative.

## 2. The past: History of biofuels

The use of biofuels is no novel invention. Fueling up with vegetable oils or ethanol was popular long before the development of the combustion engine. Vegetable and animal oil lamps have been used since the dawn of civilization. Already in 1834, the first US patent for alcohol as a lamp fuel was awarded to S. Casey (Kovarik, 1998). Around 1850, thousands of distilleries produced an estimated 24 million liters (90 million gallons) of “Camphene” (a camphor oil scented blend of turpentine and ethanol) per year (Kovarik, 1998).

Biofuels have also been used since the early days of the car industry. Even the invention of the first combustion engine, the “Otto cycle”, was performed with biofuels. German

engineer Nikolaus August Otto ran his early engines in the 1860s on ethanol, a fermentation product of yeasts. Interestingly, Otto's initial financing came from Eugen Langen, who owned a sugar refining company having links to the alcohol markets of Europe (Kovarik, 1998). Furthermore, Henry Ford's first prototype automobile, the "Quadricycle", in the 1880s could be operated with ethanol as fuel and his "Model T", the "Tin Lizzie", the most popular car produced between 1908 and 1927, was originally designed to run on pure ethanol. Ford was a big supporter of alcoholic fuels and told a New York Times reporter in 1925 (Ford, 1925): *"The fuel of the future is going to come from fruit like that sumach out by the road, or from apples, weeds, sawdust -- almost anything. There is fuel in every bit of vegetable matter that can be fermented. There's enough alcohol in one year's yield of an acre of potatoes to drive the machinery necessary to cultivate the fields for a hundred years."*

However, due to economic issues, pure ethanol was not able to prevail over gasoline. Before 1906, high taxes were levied on ethanol in the United States and later, gasoline became cheaply available due to the discovery of large oil reserves in Texas and Pennsylvania. Soon however, ethanol was recognized as an effective anti-knocking additive for combustion engines when mixed with gasoline. Blends such as "Agrol" (up to 17 % ethanol fermented from grain) in the United States, "Koolmotor" and "Cleveland Discol" (up to 30 % ethanol fermented from grain) in Britain, "Monopolin" (25 % ethanol fermented from potatoes) in Germany, "Benzalcool" (20 % ethanol) and "Robur" (30 % ethanol and 22 % methanol) in Italy, "Lattbentyl" (25 % ethanol fermented from paper mill wastes) in Sweden, "Moltaco" (20 % ethanol) in Hungary, "Benzolite" (55 % ethanol) in China, "Natalite" (up to 40 % ethanol fermented from sugar cane) in South Africa, "Gasonol" (20 % ethanol fermented from sugar cane) in the Philippines, "Shellkol" (up to 35 % ethanol fermented from molasses) in Australia, and "Espiritu" (20 % ethanol fermented from molasses) in Cuba were common between 1925-1945 (Kovarik, 1998; Giebelhaus, 1980; Finaly, 2004).

Ethanol was not the only biofuel used in the car industry at that time. Rudolph Diesel, inventor of the "Diesel oil-engine", tested his engine with peanut oil at the world's fair "Exposition Universelle Internationale" of 1900 in Paris (Nitske & Wilson, 1965; Knothe, 2001.) In 1912, Diesel published two articles (Diesel, 1912a, 1912b) in which he reflected: *"The fact that fat oils from vegetable sources can be used may seem insignificant to-day, but such oils may perhaps become in course of time of the same importance as some natural mineral oils and the tar products are now. (...) In any case, they make it certain that motor power can still be produced from the heat of the sun, which is always available for agricultural purposes, even when all our natural stores of solid and liquid fuels are exhausted."* During the 1920s however, diesel engine manufacturers altered their engines to petroleum-derived diesel fuel due to cheaper prices and lower viscosity (the viscosity of vegetable oil is about an order of magnitude higher), which led to better atomization of the fuel in the engine's combustion chamber (Knothe, 2001). This problem was solved when the Belgian patent 422,877 was granted on August 31<sup>st</sup> 1937 to George Chavanne of the University of Brussels (Chavanne, 1937). It describes the use of methyl and ethyl esters of vegetable oil, obtained by acid-catalyzed transesterification, as diesel fuel, being the first report on what is today known as biodiesel (Chavanne, 1943).

During World War II, vegetable oils and alcohols were used as supplementary or emergency fuels in most belligerent nations. For instance, it was reported that the Japanese battleship "Yamato" used refined soybean oil as bunker fuel (Knothe, 2001). Nevertheless, with the outbreak of WW II, virtually all resources were diverted from industrial alcohol production to synthetic rubber or ammunition (Finaly, 2004). After the war, gasoline dominated the market almost completely because of cheap Middle East oil. Only during

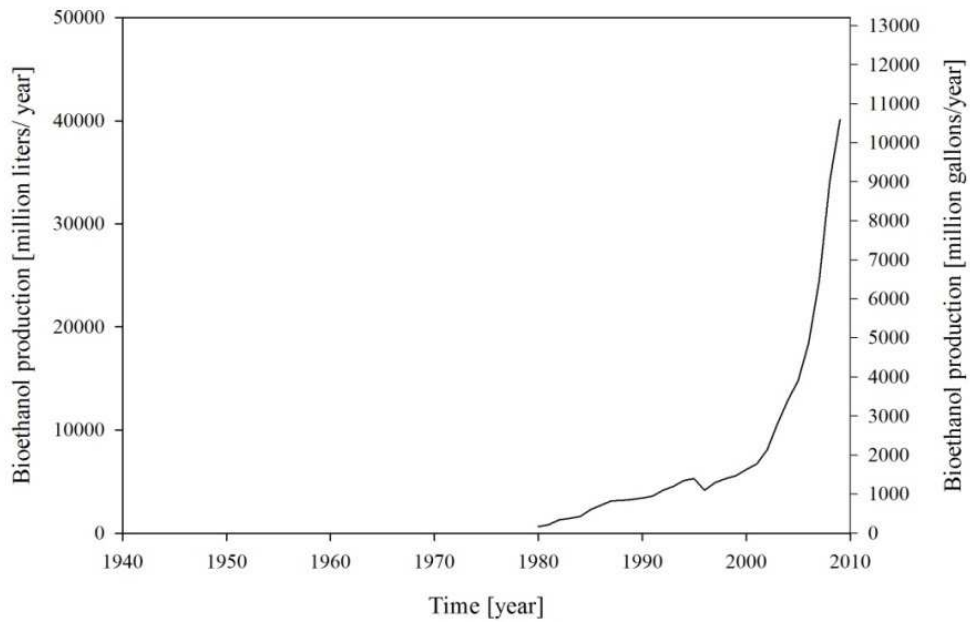
periods of scarcity such as the oil crises 1973-74 and 1979-80, many countries showed renewed interest in biofuels. The only nation that revived the bioethanol industry permanently was Brazil. Government, farmers, alcohol producers, and car manufactures cooperated in the 1970s to launch the alcohol program “Pró-álcool”. Brazil began to produce ethanol by fermentation of sugar cane. Initially, gasoline was blended with 20 % (“E20”) or 25 % (“E25”) ethanol, and after the second oil crisis pure ethanol (“AEHC” (“Álcool etílico hidratado combustível”, hydrated ethyl alcohol fuel) or “E96”) was also available as fuel, causing the car industry to implement the necessary engine modifications. The Brazilian bioethanol production increased from 600 million liters (160 million gallons) in 1975 to 13.7 billion liters (3.6 billion gallons) in 1997, by far the highest in the world (International Energy Agency [IEA], 2004).

Not until 2006, the United States surpassed the Brazilian bioethanol production with an annual capacity of 18.4 billion liters (4.9 billion gallons) compared to 17 billion liters (4.5 billion gallons). This was a dramatic increase taking into account that the US had produced only a comparatively low amount of 4.9 billion liters (1.3 billion gallons) bioethanol in 1997 (Figure 1a; Renewable Fuels Association [RFA], 2011). Today, the US doubled this number and produces around 40.1 billion liters (10.6 billion gallons) bioethanol (Figure 1a; RFA, 2011). The major comeback of biofuels in the United States and most other nations was driven by the enormous rise of the crude oil prices since the late 1990s (Figure 1b; EIA, 2011). Meanwhile, oil prices have reached sustained highs of over US\$ 80 per barrel and might well continue to increase due to political instability in the Middle East and concerns over the potential oil peak (highest production rate), demonstrated in July 2008 when the oil price surpassed US\$ 145 per barrel for a short time.

Other important drivers include the quest to gain energy independence (Schubert, 2006) and growing concerns on the effect of greenhouse gas emissions on the world’s climate, which could be counterbalanced by using renewable biomass for biofuel production. Also, MTBE (Methyl *tert*-butyl ether), an oxygenated anti-knocking additive for engines, was restricted in many countries and banned in some US states such as California and New York (which account for app. 45 % of the United States MTBE consumption) in response to environmental and health concerns (EIA, 2003; IEA, 2004). As a suspected carcinogenic agent, MTBE began turning up in significant amounts in ground water, since it is highly soluble in water, binds weakly to soil, and is not readily biodegradable in the environment (Squillance et al., 1997; EIA, 2003; IEA, 2004). This created an additional 10 billion liters (2.7 billion gallon) market for ethanol, which can be used as substitute directly or can be converted to the more environmental friendly ETBE (Ethyl *tert*-butyl ether) (EIA, 2003 & 2007). Moreover, many countries all over the world granted tax exemptions or paid subsidies for biofuels and set mandatory targets for the use of biofuels. The US congress for example established a renewable fuels mandate of 136 billion liter (36 billion gallons) by 2022 (Department of Energy [DOE], 2010), while the European Union agreed to satisfy 10 % of its transport fuel needs from renewable sources, including biofuels, hydrogen, and green electricity (EurActive Network, 2008). As a consequence, research and development of new, second generation biofuels got another push forward.

However, presently only first generation bioethanol and biodiesel are produced at a large industrial scale. While most countries produce mainly bioethanol, some European nations such as Germany, France, Spain and Italy focus on biodiesel, too. In 2009, Brazil and the United States produced app. 85 % of the world’s bioethanol, whereas Europe produced about 85 % of all biodiesel (Table 1; RFA, 2010; EIA, 2007; Biofuels platform, 2010; European Biodiesel Board, 2010).

A)



B)

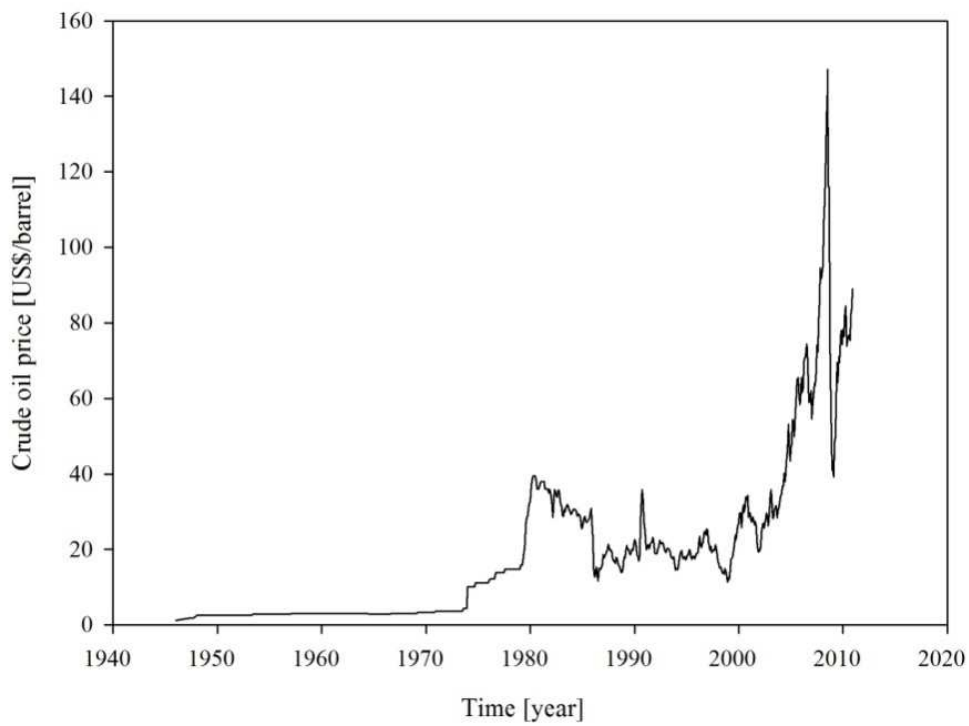


Fig. 1. Bioethanol production and crude oil price. A, development of the bioethanol production in the United States (RFA, 2010). B, development of the crude oil price (West Texas Intermediate) (EIA, 2011).

Biofuels are even used in motor sports nowadays. In 2007, the Scotsman Dario Franchetti won the iconic “Indianapolis 500” race with his 670-horsepower “Indy car” running on pure bioethanol. Moreover, in February 2008 a Virgin Atlantic Boeing 747-400 was the first commercial aircraft testing a 20 % blend of biofuel (a mixture of coconut and babassu nut oils) with 80 % fossil jet fuel (kerosene, C<sub>9</sub>-C<sub>18</sub>) in one of its four engines on a flight from London Heathrow to Amsterdam Schiphol airport (Virgin Atlantic, 2008). Airplanes in general have problems using ethanol because it freezes at an altitude of about 5 kilometers (16,400 feet). Long chain fatty acids, alkanes and also biobutanol have been identified as potential alternative by the Virgin Group, which announced to invest over US\$ 3 billion over the next 10 years in renewable energy initiatives (Ernsting, 2008). One of Virgin Fuels partners, Gevo, Inc. is working on production of this biofuel with metabolic engineered *Escherichia coli* and yeast (Atsumi et al., 2008a, 2008b, Liao et al., 2008; Gunawardena et al., 2008; Buelter et al., 2008; Hawkins et al., 2009).

Country	Bioethanol		Biodiesel		Total production	
	[MI]	[Mgal]	[MI]	[Mgal]	[MI]	[Mgal]
US	40,125	10,600	1,703	450	41,828	11,050
Brazil	24,900	6,578	n. d. a.		24,900	6,578
Germany	750	198	3,218	850	3,968	1,048
France	1,250	330	2,483	656	3,733	986
China	2,052	542	n. d. a.		2,052	542
Thailand	1,647	435	n. d. a.		1,647	435
Spain	465	123	1,089	288	1,554	410
Canada	1,102	291	n. d. a.		1,102	291
Italy	72	19	934	247	1,006	266
Belgium	143	38	527	139	670	177
Poland	166	44	421	111	587	155
Austria	180	48	393	104	573	151
Sweden	175	46	295	78	470	124
Netherlands	0	0	409	108	409	108
India	348	92	n. d. a.		348	92
Czech Rep.	113	30	208	55	321	85
Portugal	0	0	317	84	317	84
Hungary	150	40	169	45	319	84
Columbia	314	83	n. d. a.		314	83
Finland	4	1	279	74	283	75
UK	70	18	174	46	244	64
Australia	216	57	n. d. a.		216	57
Rest EU	163	43	549	146	712	189
Others	935	247	n. d. a.		935	247
<b>Total</b>	<b>75,340</b>	<b>19,903</b>	<b>13,168</b>	<b>3,481</b>	<b>88,509</b>	<b>23,381</b>

n. d. a. = no data available. Data were taken from RFA, 2010; EIA, 2007; Biofuels platform; 2010; European Biodiesel Board, 2010.

Table 1. Production of biofuels in 2009

The use of butanol as biofuel has already been reported in 2005, when David Ramey toured the United States in a 13-year old Buick fueled by pure butanol. Although consumption was 9 % higher, emissions of carbon monoxide (CO), hydrocarbons, and nitrogen oxides (NO<sub>x</sub>) were decreased enormously. He meanwhile started the company Butyl Fuel, LLC. While this is a fairly small enterprise, two major global players, BP and DuPont, also announced to start fermentative biobutanol production from sugar beet in June 2006 and formed the joint-venture Butamax™ Advanced Biofuels, LLC in 2009 with the aim to commercialize biobutanol by 2013. A first commercial plant with a capacity of 420 million liters (111 million gallons) will be built in Saltend, UK (Butamax, 2011a).

About 150 years ago, Louis Pasteur discovered that butanol can be formed by microbes (Pasteur, 1862). The culture he used for his experiments was probably a mixture of different clostridia (strictly or moderately anaerobic, spore-forming, Gram-positive bacteria, unable of dissimilatory sulfate reduction). More detailed studies on butanol producing bacteria were then conducted by Albert Fitz, who finally described the isolation of pure cultures of "*Bacillus butylicus*" from cow feces and hay (Fitz, 1876; Fitz, 1877; Fitz, 1878; Fitz, 1882). Other scientists, e. g. Martinus Beijerinck and Sergei Winogradsky, isolated further solvent-forming bacteria around 1900. These organisms received names such as "*Granulobacter saccharobutyricum*", "*Amylobacter butylicus*", and "*Bacillus orthobutylicus*", which are no longer taxonomically valid (Dürre & Bahl, 1996). Presumably all of them belong to the genus *Clostridium*, which was back then only used as a morphological description, meaning small spindle (Dürre, 2001).

Almost at the same time, considerable interest in synthetic rubber started as a result of the increase in the price of natural material due to its use in automobile tires. In 1910, the British company Strange and Graham, Ltd. launched a project to study butanol formation by microbial fermentation because of its use as precursor of butadiene, the starting material for the synthetic rubber production (as well as of isoamyl alcohol as a precursor of isoprene). The project was pursued by help of Auguste Fernbach and Moïse Schoen from the Institute Pasteur in Paris and William Perkins and Charles Weizmann from Manchester University. Fernbach isolated an acetone-butanol producer in 1911, but Weizmann separated in 1912, continuing his work at Manchester University. He succeeded in isolating an organism, later named *Clostridium acetobutylicum*, which produced significantly larger amounts of acetone and butanol than the strain isolated by Fernbach (McCoy et al., 1926). Patent applications were filed for the Fernbach process in 1911 and 1912 (Fernbach & Strange, 1911ab & 1912) and for the Weizmann process in 1915 (Weizmann, 1915). In 1913, Strange and Graham, Ltd. started production with the so-called ABE fermentation (for acetone-butanol-ethanol) based on the Fernbach process, first at Rainham, UK and later at King's Lynn, UK (Gabriel, 1928; Jones & Woods, 1986; Dürre & Bahl, 1996).

As an irony of fate, natural rubber became available at this time at much cheaper prices and in large quantities, because the new plantations in Asia started to be fully productive. However, the outbreak of World War I led to a sudden and large demand for acetone as a solvent for the production of cordite (smokeless gunpowder). The dominant source for acetone up to this time was calcium acetate imported from Austria, Germany, and the United States. As acetate imports from Austria and Germany were not available during that time and the production capacity in the US was almost negligible compared to the required quantities, Strange and Graham, Ltd. were contracted by the British War Office to supply acetone. However, their production was relatively inefficient, with an average capacity of about 440 kg (970 pounds) of acetone per week. Therefore, a switch to the Weizmann

process was requested. Thus, production could be increased to app. 900 kg (2,000 pounds) acetone per week. Hence, the disregarded by-product acetone helped the ABE fermentation process to an international breakthrough, becoming eventually the second largest biotechnological process ever performed (Jones & Woods, 1986; Dürre & Bahl, 1996). Due to the threat by German submarines, grain and corn could not be imported to the United Kingdom in the required quantities any longer. Therefore, the Weizmann process was transferred to Canada and the United States. Plants were built in Toronto in 1916 and Terre Haute, Indiana in 1917 (Gabriel, 1928; Ross, 1961; Jones & Woods, 1986; Dürre & Bahl, 1996). The constant supply of acetone was certainly a decisive factor in winning World War I. Weizmann declined any rewards or personal honors by the British government, but, being a member of the Zionist movement, clarified that his only wish was to see a home established for the Jews in Palestine. There is no doubt that this attitude affected the Balfour declaration of 1917, leading to the foundation of the State of Israel. In succession, Weizmann became its first president (Ross, 1961; Dürre & Bahl, 1996).

At the end of the war in 1918, there was no longer a high demand for acetone and consequently all production plants were closed. During the whole war, butanol (about twice the amount of the produced acetone) was considered a white elephant and simply stored in huge containers (Killeffer, 1927). However, the situation changed in 1920, when the United States implemented the prohibition. As a result no amyl alcohol, obtained as a by-product of the ethanol fermentation, was available for the production of amyl acetate, needed in large amounts by the rapidly growing automobile industry as solvent for lacquers. Butanol and its ester butyl acetate proved to be a well-suited alternative. The Commercial Solvents Corporation (CSC) was founded, obtained the patent rights to the Weizmann process, took over the plant at Terre Haute from the Allied War Board in 1919, and started butanol production in 1920. Despite the general recession of 1920, which forced a shutdown of several months, and a bacteriophage infection in 1923, which cut the yields dramatically, the plant was enlarged. Additionally, a new plant was opened in Peoria, Illinois in 1923, consisting of 32 189,000-liter (50,000-gallon) fermenters and enlarged in 1927 to 96 fermenters (Gabriel, 1928; Gabriel & Crawford, 1930; Ross, 1961; Jones & Woods, 1986; Dürre & Bahl, 1996).

After expiration of the Weizmann patent, many new strains were isolated (McCutchan & Hickey, 1954) and patented and new fermentation plants were built in the United States, Puerto Rico, South Africa, Egypt, the former Soviet Union, India, China, Japan, and Australia. Until 1945, two-thirds of the butanol in the United States was produced by fermentation. During World War II, the focus shifted to acetone production again (Jones & Woods, 1986; Dürre & Bahl, 1996). However, a few years after the end of the war, most of the plants in Western countries were closed because of rising substrate prices and competition by the growing petrochemical industry. The ABE fermentation was only continued in countries that were cut off from international supplies for political or monetary reasons. For instance, the South African apartheid regime ran a plant in Germiston with a capacity of 1,080 m<sup>3</sup> (11,625 cubic foot) until 1982 (Jones & Woods, 1986; Jones, 2001). The former USSR operated at least eight plants, some of them up to the late 1980s. Continuous fermentations with lignocellulose hydrolates as substrate and working volumes of more than 2,000 m<sup>3</sup> (21,530 cubic foot) were carried out. During the 1960s and 1970s more than 100,000 tons of butanol per year were produced (Zverlov, 2006). China also developed the continuous fermentation process and about 30 plants produced an annual amount of 170,000 tons of solvents at its peak in the 1980s. Afterwards the production decreased successively and the last plant was closed in 2004 (Chiao & Sun, 2007).



Meanwhile, China reopened some of its fermentation plants and constructed new ones, with an expected annual solvent production of up to 1,000,000 tons in the next five years (Chiao & Sun, 2007; Ni & Sun, 2009). New plants were also built or are planned in the United States (Gevo, 2009; Cobalt Technologies, 2010), the United Kingdom (Butamax, 2011a), Brazil (Afschar et al., 1990), France (Marchal et al., 1992; Nimcevic & Gapes, 2000), and Austria (Nimcevic & Gapes, 2000; Gapes, 2000). Thus, the biological butanol production faces bright prospects in the future.

### 3. Specifications for biofuels

Biofuels have to meet defined physical and chemical criteria for the use in modern combustion engines and for the use of the existing distribution infrastructure. First, the state of aggregation is of particular importance. Biofuels for transportation should be liquid at ambient temperature and atmospheric pressure which is true for alcohols, biodiesel, and renewable diesel. Gaseous biofuels such as hydrogen and methane (biogas) will require the development of a new infrastructure and modified engines. Second, biofuels should have the same properties as petrochemical-based fuels (Table 2).

Fuel	Gasoline	Ethanol	Butanol	Diesel	Biodiesel
Energy density [MJ/l]	32-35	21.2	29.2	35-42	32-42
Mileage [%]	100	61-66	83-91	100	90-100
Air-fuel ratio	14.6	9.0	11.2	15.0	13.8
Research octane number (RON)	91-99	129	96	-	-
Motor octane number (MON)	81-89	102	78	-	-
Cetane number (CN)	-	-	-	50-60	45-70
Vapor pressure [hPa]	35-90 (at 20 °C)	58 (at 20 °C)	6.7 (at 20 °C)	-	-
Flashpoint [°C]	< -20	12	35-37	55-60	100-190
Enthalpy of vaporization [MJ/kg]	0.36	0.92	0.43	-	-
Kinematic viscosity [mm <sup>2</sup> /s]	0.4-0.8 (at 20 °C)	1.5 (at 20 °C)	3.6 (at 20 °C)	1.2-3.5 (at 40 °C)	2-9.5 (at 40 °C)

Table 2. Physical and chemical properties of biofuels

Basically, biodiesel has similar characteristics as regular diesel. However, there are significant differences between biodiesel fuels produced from various vegetable sources (Fukuda et al., 2001), which can lead to damage of diesel engines. Critical points are dilution of motor oil, coking of piston rings, corrosion of hydraulic components, and depositions in

the injection system, resulting from the production process and fuel aging. Hence, this is perhaps the biggest problem of biodiesel fuels, causing a couple of automotive manufacturers to refuse the use of biodiesel in some of their models. Newly developed, highly efficient motor technologies require low sulfur and saturated hydrocarbons, without aromatic compounds. A way out of this problem will be the use of renewable diesel and diesel from BtL (biomass to liquid) biofuels.

Similarly, ethanol has a number of disadvantages that can be avoided when using butanol as a biofuel (see section 5.5). The reason why currently so many countries use biodiesel and ethanol despite a number of disadvantages is simply the fact that they are currently available in large quantities. In future, however, superior second generation biofuels such as renewable diesel, butanol, renewable gasoline, and BtL fuels will gain much more importance.

## 4. The present: First generation biofuels

### 4.1 Bioethanol

The bioethanol fermentation is by far the largest biotechnological process worldwide. Most common are batch fermentations with the yeast *Saccharomyces cerevisiae* from corn, sugar cane, cassava, wheat, or rye as substrate. Sugar beet has recently been introduced as well.

During the last few years, industrial production strains have been improved for higher ethanol yields, specific ethanol productivity, inhibitor insensitivity, and product tolerance (up to 20 % ethanol). Meanwhile, the bioethanol fermentation is a mature technology. Worldwide, a total of around 75 billion liters (20 billion gallons) bioethanol were used in 2009 as biofuel (Table 1). The world's largest bioethanol producer is POET, LLC ([www.poet.com/](http://www.poet.com/)) with over 27 plants producing more than 6 billion liters (1.5 billion gallons) (POET, 2011).

However, the increasing demands for sugar cane and especially corn are in serious competition with the food industry (food-vs-fuel discussion). Mexico has already seen huge demonstrations about the rising costs of the national dish tortillas, which are made from corn. A world bank report blamed the increasing biofuel use as one of the prime causes of raising food prices (Mitchell, 2008), and Oxfam claimed that current biofuel policies will push millions of people into poverty (Oxfam, 2008). The use of large land areas for growing monocultures is criticized as environmentally hazardous as well, and the sugar cane fields in Brazil are usually burned before harvest (to kill snakes and make the sugar cane easier to handle), releasing green house gases methane and nitrous oxide. Thus, corn and sugar cane ethanol can only be an interim solution, until second generation biofuels are commercialized, which are sustainable and independent of the use of food.

### 4.2 Biodiesel

Biodiesel is a chemically synthesized biofuel. Worldwide, more than 13.1 billion liters (3.5 billion gallons) have been used in 2009 as a pure fuel or fuel additive (Table 1). Biodiesel is defined as monoalkyl esters of fatty acids from vegetable oil or animal fats. It is produced by transesterification of the parent oil with an alcohol and this process is therefore also designated alcoholysis (Figure 2). The alcohol generally used for this process is chemically synthesized methanol because of its cheap price, but it is also possible to use higher alcohols from microbial fermentation such as ethanol or butanol. The resulting products are FAME (fatty acid methyl esters), FAEE (fatty acid ethyl esters), or FABE (fatty acid butyl esters),

respectively. The reaction is catalyzed by acids, alkalis, or lipase enzymes (Fukuda et al. 2001; Akoh et al., 2007).

Many different sources can be used as parent oil. Most common are vegetable oils. Primarily important is the oil yield per area, but climate and economics finally determine which vegetable oil is used. While the US rely on soybean oil (400-446 liter oil/ha (43-48 gallons per acre)) as major feedstock, European countries prefer rapeseed (canola) oil (1,100-1,190 liter oil/ha (118-127 gallons per acre)), and tropical countries palm oil (2,400-5,950 liter oil/ha (257-636 gallos per acre)). Sunflower oil (690 liter oil/ha (73 gallons per acre)), peanut/groundnut oil, cottonseed oil, castor oil, and safflower oil are also used commercially (Akoh et al., 2007; Chisti, 2007; Fairless, 2007).

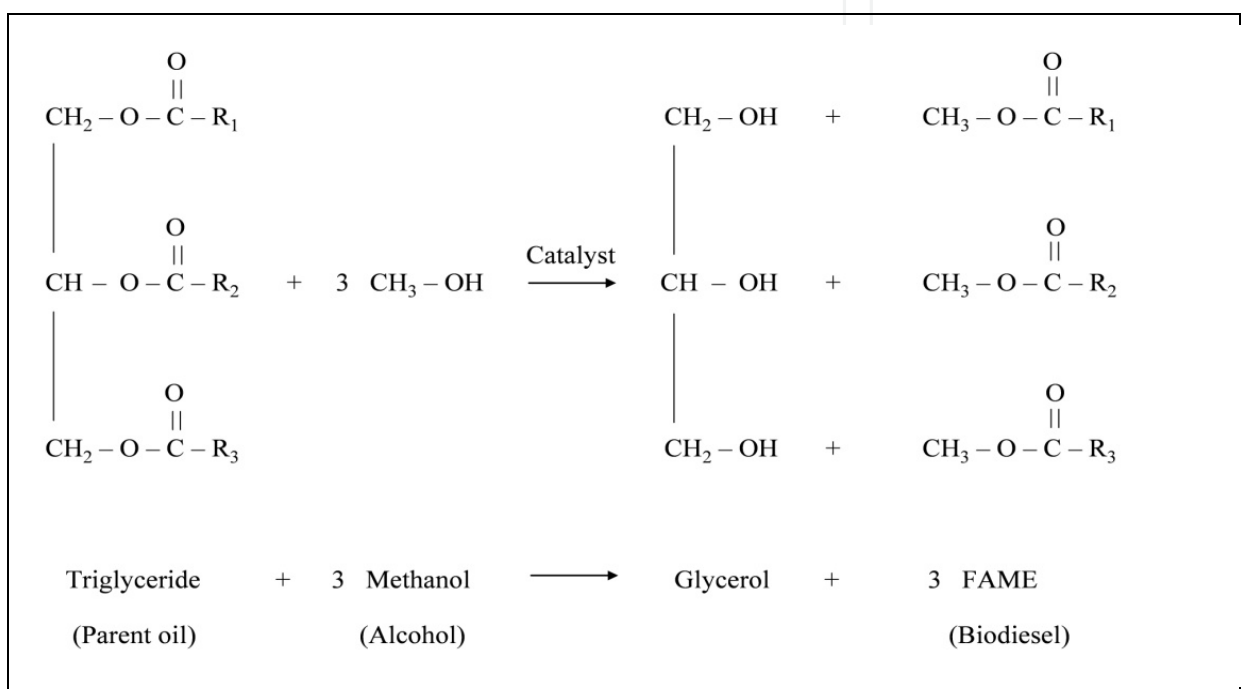


Fig. 2. Chemical reactions leading to biodiesel. R1-R3: saturated and/or unsaturated hydrocarbons of different chain length.

Moreover, the use of jatropha seed oil (1,300-1,892 liter oil/ha (139-202 gallons per acre)) seems to be very attractive because it does not compete with the food industry and the plant *Jatropha curcas* produces seeds containing up to 35 % oil and is resilient to pests and drought. Therefore, it can even grow in dry savanna. Hundreds of thousands hectares (respectively acres) are already in cultivation in South Asia, Africa, Middle and South America, and already in 2007, BP and D1 Oils launched a corresponding project (Fairless, 2007; BP, 2007). Jatropha-based biodiesel is also considered as aviation fuel (Air New Zealand, 2008). Other sources are animal fats (fish oils, blubber, lards, tallow, etc.) and even waste oils (frying oils, soapstocks, etc.).

However, microbial oils can also be used. Especially microalgal oils show great potential and are theoretically CO<sub>2</sub> neutral (like vegetable oils). Microalgae grow fast (biomass doubling time of 3.5 hours during exponential growth) and are rich in oil (up to 80 % weight of dry biomass). Photobioreactor experiments demonstrated an oil yield of 136,900 liter oil per hectare (14,635 gallons per acre) (with 70 % oil in biomass). Nevertheless, microalgal biomass production is generally considered more expensive than growing crops, despite

having a higher energy yield per area (Schubert, 2006; Chisti, 2007; Chisti, 2008) . Another big challenge is the high demand for water and especially fertilizers. As result, algae biofuels had a worse environmental footprint than corn ethanol in a recent life cycle analysis (Clarens et al., 2010). However, algae oils have big potential as jet fuel. Companies trying to commercialize algae biofuels include Algenol Biofuels ([www.algenolbiofuels.com/](http://www.algenolbiofuels.com/)), Aquaflow ([www.aquaflowgroup.com/](http://www.aquaflowgroup.com/)), Sapphire Energy, Inc. ([www.sapphireenergy.com/](http://www.sapphireenergy.com/)), or Solazyme, Inc. ([www.solazyme.com/](http://www.solazyme.com/)).

In addition, oil production with some yeasts, fungi, or bacteria has also been evaluated (Ratlidge, 1993). Metabolically engineered *E. coli* were not only shown to produce free fatty acids, but also biodiesel directly (up to 26 % FAEE of dry cellmass), referred to as microdiesel. The genes for the pyruvate decarboxylase (*pdh*) and alcohol dehydrogenase (*adhB*) of *Z. mobilis* have been introduced in *E. coli* to produce large amounts of ethanol. Additionally, the gene for the acyltransferase (*atfA*) of *Acinetobacter baylyi* strain ADP1 has been subcloned on the same plasmid in *E. coli*. The corresponding enzyme has an extraordinary low substrate specificity and is able to esterify ethanol with the acyl moieties of coenzyme A thioesters of fatty acids. However, supplementation of exogenic fatty acids (oleic acid) was necessary for a substantial FAEE yield, because the acyltransferase did not use the *de novo* synthesized fatty acids properly. Nevertheless, the feasibility of a new microbiological biodiesel production process was demonstrated and can now be further developed (Kalscheuer et al., 2006).

In general, biodiesel shows lower emissions of particulate matter and carbon monoxide (CO) than regular diesel fuel, but slightly enhanced nitrogen oxides (NO<sub>x</sub>) production. It is also only moderately more mutagenic. However, rapeseed oil used directly as a fuel for a diesel engine showed a strong increase in mutagenicity as measured by the Ames test (Bünger et al., 2006; Bünger et al., 2007). In general, vegetable oil represents currently only a niche application. Although pure fuel costs are lower than those for diesel, modification of engines is required and the higher viscosity leads to cold start problems.

#### 4.3 Renewable diesel

The expression "renewable diesel" became meanwhile standard in the US, while "hydrotreated vegetable oil" (HVO) is still more common in Europe. However, HVO is a term lacking precision, as except for vegetable oil also animal fat components such as lard and tallow are used for production. The triglycerides react with hydrogen at a catalyst, thereby forming propane (from the glycerol moiety), CO<sub>2</sub>, H<sub>2</sub>O, and hydrocarbons (from the fatty acids by splitting the ester bond and removal of the carboxy group) (Figure 3). Propane can be used as a fuel as well or as feedstock for the chemical industry. Gasoline (in fact: biogasoline; chain lengths from C<sub>4</sub> to C<sub>12</sub>) is produced as a side product. The major hydrocarbons of a chain length between C<sub>12</sub> and C<sub>20</sub> (well in the diesel range of C<sub>10</sub> to C<sub>25</sub>) are fully saturated, free of oxygen and aromatic compounds as well as low in sulfur content and producing less nitrous oxide upon burning, thus representing a superior bio-based diesel fuel, chemically equivalent to the crude oil-based product. Renewable diesel is about to enter the market in large quantities. ConocoPhillips ([www.conocophillips.com/](http://www.conocophillips.com/)) started in 2006 to produce renewable diesel commercially, now reaching 150,000 liters (39,600 gallons) per day in its Whitegate refinery in Cork, Ireland (Mason & Ghonasgi, 2008). Neste Oil Corporation ([www.nesteoil.com/](http://www.nesteoil.com/)) announced in May 2008 to produce an annual 170,000 tons of renewable synthetic diesel under the brand name NExBTL in its Provo refinery, Finland. Feedstocks include palm oil, rapeseed oil, and animal fats (Oja, 2008) .

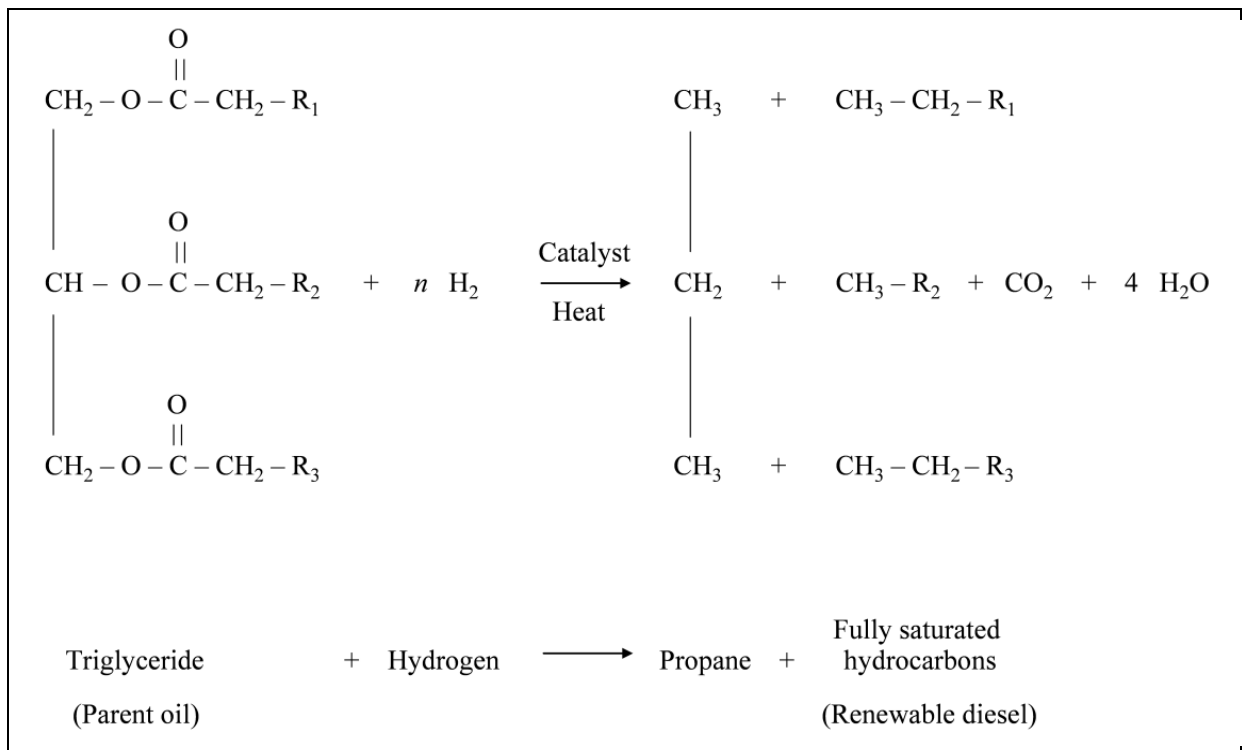


Fig. 3. Chemical reactions leading to renewable diesel. R1-R3: saturated and/or unsaturated hydrocarbons of different chain length.

## 5. The future: Second generation biofuels

### 5.1 Cellulosic biofuels

The use of biomass such as wood, dedicated energy crops, agricultural residues, and municipal solid waste would be a very attractive alternative. Production of up to 4,000 dry tons biomass per km<sup>2</sup> (1,545 tons per square mile) and year have been reported in field trials and an annual global biomass average of about 1,000 dry tons per km<sup>2</sup> (390 tons per square mile) is conservatively estimated (Ragauskas et al., 2006). The US can app. yield over 1 billion dry tons of biomass and continue to meet food, feed, and export demands (Perlack et al., 2005).

Whereas corn grain consist of starch and sugar cane of sucrose, biomass is composed of lignocellulose (typically 40-50 % cellulose, 25-35 % hemicellulose and 15-20 % lignin), which is the main component of the plant cell wall and therefore very resistant to degradation (Schubert, 2006; Gray et al., 2006). One approach towards degradation is based on thermochemical pretreatment and enzymatic hydrolysis of the lignocellulose into fermentable sugars and afterwards into so-called cellulose (or cellulosic) biofuels such as bioethanol.

However, the enzymatic hydrolysis reaction (most commonly with cellulases from the fungus *Trichoderma reesei*) is (still) too expensive (2.5-5 US cent per liter (10-20 US cents per gallon) of ethanol produced) and time-consuming (about 100-fold slower than the average fermentation rate with yeast) (Schubert, 2006; Gray et al., 2006). Many new enzymes from bacteria and fungi have been isolated and characterized in the last few years (Hildén & Johansson, 2004). Recently, the metagenome of the hindgut of the wood-feeding termite *Nasutitermes* has been analyzed with the aim to find novel cellulases (Warnecke et al., 2007). Moreover, well-known enzymes have either been engineered to improve their performance

or produced heterologously in an existing system such as *T. reesei* (Warnecke et al., 2007; Viikari, 2007). Companies such as Codexis, Inc. ([www.codexis.com/](http://www.codexis.com/)), Genencor® ([www.genencor.com/](http://www.genencor.com/)), or Novozymes ([www.novozymes.com/](http://www.novozymes.com/)) are working on efficient enzyme solutions, while others such as ZeaChem, Inc. ([www.zeachem.com/](http://www.zeachem.com/)) try to optimize the thermochemical breakdown route.

Cellulose consists exclusively of glucose, hemicellulose contains a complex mixture of carbohydrates with 15-50 % pentoses such as xylose and arabinose (Schubert, 2006; Gray et al., 2006). *S. cerevisiae* as well as the bacterial work horse *Zymomonas mobilis* (which has even a higher ethanol yield on starch than *S. cerevisiae*) are naturally not able to ferment pentose sugars. Therefore, new strains of both organisms have been developed in the last few years by metabolic engineering with additional pentose metabolic pathways (Hahn-Hägerdal, 2007; Jeffries & Yin, 2003; Dien et al., 2003). Furthermore, bacteria such as *E. coli* or *Klebsiella oxytoca* which can use a wide spectrum of sugars have been genetically modified towards ethanol production (Dien et al., 2003; Jarboe et al., 2007). Nevertheless, the industry is still relying on the yeast *S. cerevisiae* due to its robustness.

Already in 2004, Shell and Iogen ([www.iogen.ca/](http://www.iogen.ca/)) announced the successful production of cellulosic ethanol in one complete process for commercial use and are currently operating a demonstration plant with an output of 5,000 – 6,000 litres (1400-1600 gallons) of cellulosic ethanol per day (Iogen, 2011; Schubert, 2006). The world leading bioethanol producer POET, LLC is looking into production of cellulosic ethanol too.

Some microorganisms such as *Clostridium cellulolyticum*, *C. phytofermentans*, or *C. thermocellum* are also capable to ferment cellulose directly into ethanol, in so called consolidated bioprocessing process (CBP) (Lynd et al., 2005). Companies like Mascoma ([www.mascoma.com/](http://www.mascoma.com/)) and Qteros, Inc ([www.qteros.com/](http://www.qteros.com/)) are working to improve the conversion rates to commercial requirements. Heterologous cellulase expression in *Z. mobilis* and *S. cerevisiae* was successfully shown as well, but yields are nonsatisfying so far and there are still some limitations (Lynd et al., 2002; Demain et al., 2005; Schubert, 2006). However, biomass can also be gasified and then converted into a biofuels.

## 5.2 Biomass-to-Liquid (BtL) biofuels

One approach to convert lignocellulosic biomass into a biofuel borrows a technology from the coal industry, the “Fischer-Tropsch process”. This method was invented in the petroleum-poor but coal-rich Germany of the 1920s and describes the generation of synthesis gas (or syngas, consisting mainly of carbon monoxide and hydrogen) and its reaction into liquid alkanes, alkenes, and alcohols (Schubert, 2006). The desired product formation can be adjusted by process conditions and the use of appropriate catalysts (usually metals such as iron or cobalt).

While alcohols or diesel produced via such a “CtL” (coal-to-liquid) or “GtL” (natural gas-to-liquid) process is certainly not considered as biofuel, the syngas could also be generated from municipal solid waste or biomass in a so-called “BtL” (biomass-to-liquid) process. Renewable diesel produced this way is known under the brand names SunFuel® or SunDiesel® (cooperation of Choren Industries, Shell, Volkswagen, and Daimler; [www.sunfuel.de/](http://www.sunfuel.de/)). So far however, only pilot plants started to operate, by a series of companies such as Choren Industries ([www.choren.com/](http://www.choren.com/)), Enerkem ([www.enerkem.com/](http://www.enerkem.com/)), Fulcrum BioEnergy, Inc. ([www.fulcrum-bioenergy.com/](http://www.fulcrum-bioenergy.com/)), or Rentech ([www.rentechinc.com/](http://www.rentechinc.com/)). In 2008, Range Fuels ([www.rangefuels.com/](http://www.rangefuels.com/)) started to build a first commercial plant with a US\$ 76 million grant from the US Department of Energy. However,

instead a proposed goal of an annual 100 million gallon ethanol from wood chips, only 4 million gallons of methanol are produced (Energy Collective, 2011).

Besides huge problems with product specificity, sulfur gases and the accumulation of tar, which leads to consequential poisoning of the noble catalysts, are major issues for these processes. However, some acetogenic bacteria such as *Clostridium ljungdahlii* (Köpke et al., 2010) are also capable to ferment synthesis gas directly into bioethanol via the acetyl-CoA “Wood-Ljungdahl” pathway (Köpke et al., 2011; Tirado-Acevedo, 2010; Henstra, 2007). These bacteria are by far more specific and less affected by sulfur gases and tar (Ragauskas, 2006; Bredwell et al., 1999; Vega et al., 1990). Three companies, Coskata, Inc. ([www.coskata.com/](http://www.coskata.com/)), IneosBio ([www.ineosbio.com/](http://www.ineosbio.com/)), and LanzaTech NZ Ltd. ([www.lanzatech.co.nz/](http://www.lanzatech.co.nz/)), have already committed themselves to this technology and aiming to commercialize their process over the next few years. Coskata, Inc. is currently designing a 200 million liter (55 million gallon) per year facility in Greene County, Alabama with a US\$ 250 million loan guarantee by the USDA (Coscata, 2011), IneosBio has started to construct a 30 million liter (8 million gallon) per year plant near Vero Beach, Florida (IneosBio, 2011), and LanzaTech NZ Ltd. signed a commercial partnership deal towards the construction of a commercial plant at a steel mill in China (LanzaTech, 2010) with the aim to produce an annual 190 million liter (50 million gallons) bioethanol directly from steel mill off gases by 2013.

### 5.3 Methane from biogas

Methane or natural gas is already in use as a fuel for cars. 1 kg (2.2 pounds) of methane is equivalent to app. 1.4 liters (0.264 gallons) of gasoline. Thus, methane derived from biogas (mainly a mixture of CH<sub>4</sub> and CO<sub>2</sub>, plus minor amounts of water, H<sub>2</sub>S, and other gases) could be directly used without further modifications of the respective engines. However, purification of methane from biogas is required, and this process results in high costs. Also, due to the low percentage of cars currently running on methane, the existing infrastructure of distribution of gaseous fuels does not meet a large demand. Thus, methane from biogas is not expected to enter the market in relevant quantities within the next few years.

### 5.4 Hydrogen from biomass

Hydrogen can serve as a fuel in Otto-type combustion engines and in fuel cells. Thus, single solutions as well as so-called “hybrid cars” (combination of different engine types) will be suited to run on H<sub>2</sub>. Prototypes of cars with fuel cells already exist from several manufacturers, but mass production is not expected before app. 2015. Several processes have been developed to generate H<sub>2</sub> from biomass and respective pilot plants are in operation in e.g. Austria, Germany, and the US (Schindler, 2008). In principle, biomass is converted into synthesis gas (a mixture of mostly CO and H<sub>2</sub>), from which H<sub>2</sub> is further purified and compressed. Other ways of biohydrogen formation include photosynthetic production, fermentative production, and nitrogenase-mediated production (Levin et al., 2004; Nath & Das, 2004; Prince & Kheshgi, 2005). In general, the economics of the different systems are so far not sufficient to allow introduction of a large scale industrial process. A breakthrough at the laboratory level has been achieved recently: Highly efficient hydrogen production from any type of biodegradable organic matter became possible in electrohydrogenic reactors with mixed cultures of bacteria (Cheng & Logan, 2007).

## 5.5 Butanol (biobased-butanol, biobutanol)

While alcoholic fuels have almost the same properties as gasoline (Table 2), butanol provides a number of advantages over ethanol. Primarily, the energy density of butanol is significantly higher, resulting in an increased mileage. In addition, the air-fuel ratio of butanol is higher, which means that it can be run at richer mixtures and therefore produce more power. The octane rating of butanol is lower to that of ethanol, but similar to that of gasoline. Butanol has a lower vapor pressure and is thus safer to handle. Furthermore, the enthalpy of vaporization of ethanol is more than twice of that of butanol, which can lead to insufficient vaporization and cause problems during starts in cold weather. Additionally, ethanol is corrosive and hygroscopic. Especially, aluminum parts are attacked. Hence, ethanol cannot be distributed in pipelines and must be transported by tanker trucks, rail car, or river barge (IEA, 2004). Blending gasoline with ethanol at the production facility or refinery long before distribution is not feasible and must occur shortly before use (IEA, 2004), increasing the risk of a contamination of groundwater in case of spills. Butanol can be blended with gasoline well ahead of distribution and can be transported by the existing infrastructure. While ethanol can only be blended up to 85 % with gasoline, butanol can be blended in any concentration and used in existing car engines without any modification.

While butanol can also be produced by the Fischer-Tropsch process, the more economical synthesis route is by fermentation. This biotechnological procedure has a long-lasting history, as already described. The production organism usually used is *C. acetobutylicum*. During the last decades, intensive investigations led to a significant increase in our knowledge on physiology, metabolic regulation, and genetic manipulation of this organism, which in combination with improved fermentation and downstream processing will allow the revival of a large scale industrial bioprocess.

Next to the fermentative route, an alternative non-fermentative approach has been established by metabolic engineering of the amino acid biosynthesis pathways, which allows for production of 1-butanol and also iso-butanol. While most companies like Butalco GmbH ([www.butalco.com/](http://www.butalco.com/)), ButylFuel, LLC ([www.butanol.com/](http://www.butanol.com/)), Cobalt Technologies ([www.cobalttech.com/](http://www.cobalttech.com/)), Green Biologics, Ltd. ([www.greenbiologics.com/](http://www.greenbiologics.com/)), Metabolic Explorer ([www.metabolic-explorer.com/](http://www.metabolic-explorer.com/)), or Tetravita Bioscience, Inc. ([www.tetravita.com/](http://www.tetravita.com/)) are focusing on optimization of the classical ABE fermentation, Butamax™ Advanced Biofuels LLC ([www.butamax.com/](http://www.butamax.com/)) and Gevo ([www.gevo.com/](http://www.gevo.com/)) are pursuing the non-fermentative path, too.

### 5.5.1 Fermentative production of butanol (ABE fermentation)

#### 5.5.1.1 Metabolism and enzymes of *Clostridium acetobutylicum*

*C. acetobutylicum* is a Gram-positive anaerobe organism. Its metabolism is characterized by a biphasic fermentation (Figure 4), starting with the formation of the acids acetate and butyrate. As a consequence of the accumulation of free acids and the resulting pH drop, the essential proton gradient between inside and outside of the cell gets destroyed and *C. acetobutylicum* dies. The strategy of *C. acetobutylicum* to survive is to decrease acid production at the end of exponential growth and to switch the metabolism to solvent production. Acetate and butyrate are taken up from medium into the cell and metabolized into acetone and butanol. By conversion of acids into solvents, the pH increases again. Butanol, however, is also toxic to *C. acetobutylicum* and poses a serious threat to the cells, having damaging effects on membranes and some membrane proteins. Therefore, the



bacteria start with the formation of endospores at the same time. Byproducts of the fermentation are acetoin, ethanol, and lactate.  $\text{CO}_2$  and  $\text{H}_2$  are produced, too.

The main carbon sources for *C. acetobutylicum* are starch and sugars. Respective degradative enzymes have been found, purified, and characterized (e. g. an  $\alpha$ -amylase from strain ATCC 824) (Paquet et al., 1991; Annous & Blaschek, 1994). *C. acetobutylicum* is unable to feed on cellulose (Lee et al., 1985).

This is curious because the genome of *C. acetobutylicum* contains a number of genes, which code for cellulosome components (Nölling et al., 2001). By their overexpression in *E. coli* or *C. acetobutylicum* it could be shown that part of the respective proteins are functional (Lopez-Contreras et al., 2003; Sabathé & Soucaille, 2003). Phosphotransferase systems perform the uptake of many sugars. Glucose is degraded by glycolysis (Embden-Meyerhof-Parnas pathway) to pyruvate, which again is metabolized into acetyl-CoA by a pyruvate:ferredoxin-oxidoreductase (Nölling et al., 2001). This enzyme consists of an unknown number of 123 kDa subunits and is extremely oxygen-sensitive (Meinecke et al., 1989). Acetate is formed from acetyl-CoA. The latter one is also partly converted to butyrate.

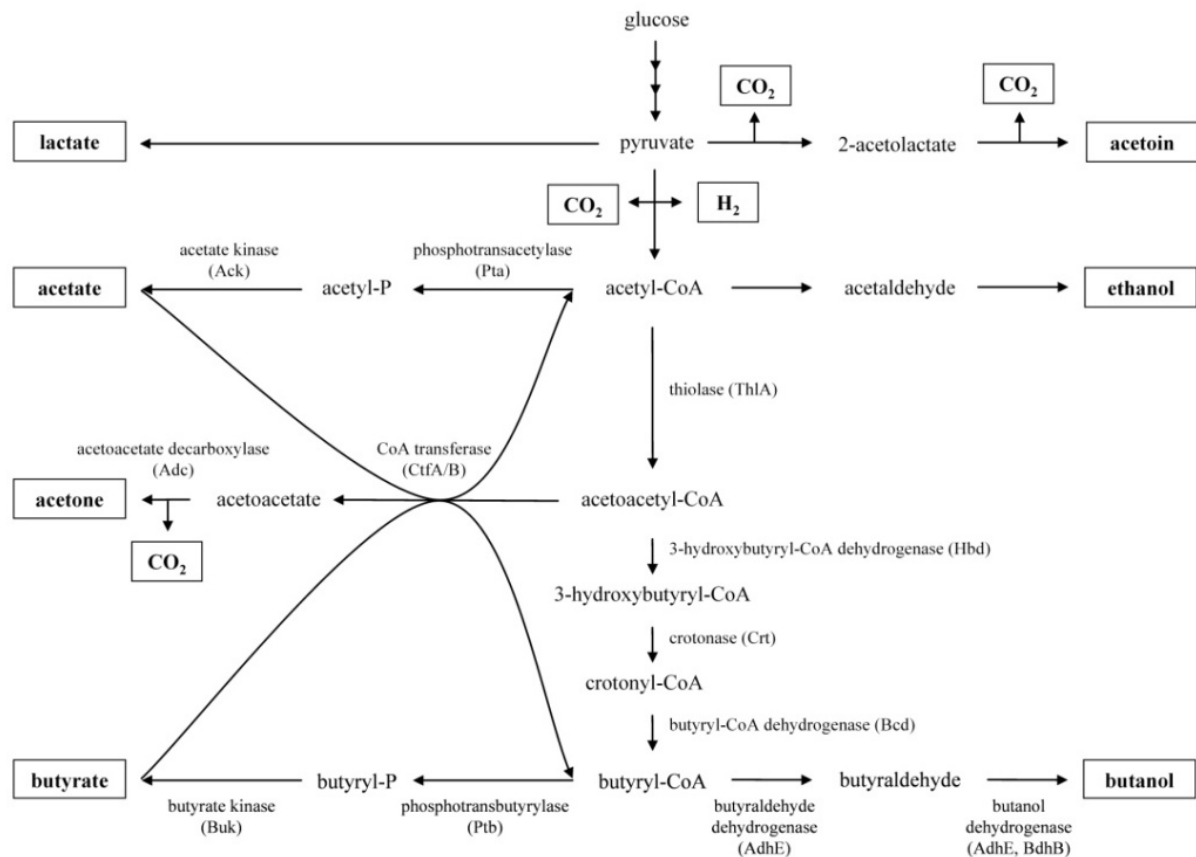


Fig. 4. Catabolic pathways of acid and solvent formation in *C. acetobutylicum*. The single reactions shown do not represent stoichiometric fermentation balances.

Phosphotransacetylase (Pta) and acetate kinase (Ack) (Gavard et al., 1957) are involved in acetate formation. Both are only strongly expressed during the acidogenic growth phase (Andersch et al., 1983). Pta was purified from *C. beijerinckii* and Ack from *C. acetobutylicum*. The former possesses a molecular mass of 56 to 57 kDa (Chen, 1993), the latter is a dimeric enzyme, consisting of identical subunits with a molecular mass of 43 kDa. The enzyme prefers the

substrates ATP and acetate (Winzer et al., 1997). Ack from *C. saccharobutylicum* P262 was also purified. The two subunits have each a molecular mass of 42 kDa (Diez-Gonzalez et al., 1997). Butyrate formation starts with the condensation of two molecules acetyl-CoA to one molecule acetoacetyl-CoA by a thiolase (ThlA). Acetoacetyl-CoA is reduced to 3-hydroxybutyryl-CoA with NADH by a 3-hydroxybutyryl-CoA-dehydrogenase (Hbd). By dehydration via a crotonase (Crt or Cch), crotonyl-CoA is formed. Crotonyl-CoA is reduced by a butyryl-CoA-dehydrogenase (Bcd) to butyryl-CoA. The formation of butyrate from butyryl-CoA is mediated by two enzymes, a phosphotransbutyrylase (Ptb) and a butyrate kinase (Buk). The thiolase of *C. acetobutylicum* is a tetramer formed of identical subunits, each with a molecular mass of 44 kDa (Wiesenborn et al., 1988). *C. acetobutylicum* and *C. beijerinckii* possess a different gene for a second thiolase (Winzer et al., 2000). The genes for two different thiolases have also been found in *Clostridium pasteuricum* (Berndt & Schlegel, 1975). 3-Hydroxybutyryl-CoA dehydrogenase (Hbd) from *C. beijerinckii* NRRL B593 is a protein consisting of several identical subunits, each with a molecular mass of 31 kDa. Together they compose an enzyme with a total mass of 231 kDa (Colby & Chen, 1992). A crotonase (crotonyl-CoA hydratase) was isolated from a *C. acetobutylicum* strain. This strain was not further specified (Waterson et al., 1972). The purified protein was composed of four identical parts. Each of these subunits possesses a molecular mass of 40 kDa. A remarkable feature of this enzyme is its limited substrate specificity and sensitivity towards high concentrations of crotonyl-CoA. It was found that the enzyme only acts on C4- and C6-enoyl-CoA (Waterson et al., 1972). A butyryl-CoA dehydrogenase has not been purified yet. Phosphotransbutyrylase (Ptb) from both *C. acetobutylicum* strain ATCC 824 and from *C. beijerinckii* has also been characterized. The molecular masses determined for the purified proteins were 264 kDa and 205 kDa, respectively. Ptb of *C. acetobutylicum* is composed of subunits with a molecular mass of 33 kDa. The enzyme of *C. beijerinckii* is formed from subunits of 31 kDa (Waterson, 1972; Thompson & Chen, 1990). Characterization of a butyrate kinase purified from *C. acetobutylicum* ATCC 824 showed a very low activity with acetate (only 6 % of that with butyrate). That enzyme possesses two subunits of high similarity, each with a molecular mass of 39 kDa (Hartmanis, 1987).

With the beginning of solventogenesis, *C. acetobutylicum* takes up butyrate and acetate. Butyrate, and to a lesser extent acetate, are converted into butyryl-CoA and acetyl-CoA by an acetoacetyl-CoA:acetate/butyrate-coenzyme A transferase (CoA transferase, CtfA/B), while acetoacetyl-CoA is simultaneously converted to acetoacetate. Acetone is formed by the decarboxylation of acetoacetate mediated by an acetoacetate decarboxylase (Laursen & Westheimer, 1966). In some *C. beijerinckii* strains, a further reduction of acetone to 2-propanol is catalyzed by a primary/secondary alcohol dehydrogenase. Sequencing analyses of the *C. acetobutylicum* strain ATCC 824 determined a molecular mass of 22.7 and 23.7 kDa for the two different subunits of the CoA transferase. A molecular mass of 23.6 kDa for both subunits of the protein in DSM 792 (Cary et al., 1990; Gerischer & Dürre, 1990; Petersen et al., 1993; Fischer et al., 1993) was found. Acetoacetate decarboxylase of *C. acetobutylicum* consists of 12 identical subunits, each of them with a molecular mass of 28 kDa, forming an holoenzyme of 330 kDa, while the enzyme from *C. beijerinckii* is only 200-230 kDa (Gerischer & Dürre, 1990; Petersen & Bennett, 1990; Chen, 1993).

In *C. acetobutylicum*, butanol formation is initiated by a bifunctional butyraldehyde/butanol dehydrogenase E (AdhE). Just prior to butanol synthesis the transcription of the respective gene is induced (Sauer & Dürre, 1995; Grimmier et al., 2011). After inactivation of *adhE* significantly less butanol was produced by *C. acetobutylicum*. A solvent-negative mutant

regained the ability to produce butanol after its transformation with *adhE* (Nair & Papoutsakis, 1994; Green & Bennett, 1996). Additionally, a butanol dehydrogenase (BdhB or BdhII) is involved in butanol production (Fischer et al., 1993; Petersen et al., 1991; Nair et al., 1994). This enzyme forms a dimer, consisting of two identical subunits. A molecular mass of 42 kDa was determined for each of them. Investigations of the enzyme activity showed that it is 46-fold higher with butyraldehyde than with acetaldehyde (Welch et al., 1989). The second butanol dehydrogenase is BdhA. Its enzyme activity is only twice as high with butyraldehyde than with acetaldehyde (Walter et al., 1992). Like BdhB it forms a dimer with a subunit size of 42 kDa. Both enzymes were purified from *C. acetobutylicum* ATCC 824.

*C. acetobutylicum* also possesses a second *adhE* gene (*adhE2*), which has a 66 % identity to *adhE* (Fontaine et al., 2002). It is transcribed in continuous culture only under acidogenic conditions (Grimmler et al., 2011) or in alcohologenic cultures (only butanol and ethanol formation) when grown at neutral pH on glycerol (Fontaine et al., 2002).

### 5.5.1.2 Genomic arrangement of elements required for sugar degradation, acid formation, and solventogenesis

The genome of *C. acetobutylicum* consists of a 3.94 Mbp chromosome and the megaplasmid pSOL1 (192 kbp) (Nölling et al., 2001). Phosphofructokinase (*pfk*) and pyruvate kinase (*pyk*) genes, whose products are involved in degradation of glucose, are arranged in one operon (Belouski et al., 1998). Genes for glyceraldehyde-3-phosphate dehydrogenase (*gap*), phosphoglycerate kinase (*pgk*), and triosephosphate isomerase (*tpi*) are grouped together in this order in a common operon. Although *gaf*, *pgk* and *tpi* are transcribed together, a separate transcript of *tpi* was also found (Schreiber & Dürre, 1999). The transcription start points are located in front of *gap* and *tpi*. Phosphotransacetylase (*pta*) and acetate kinase (*ack*) genes are clustered in one operon, with *ack* downstream of *pta* (Boynton, 1996). Thiolase (*thlA*) forms a monocistronic operon. Transcription of *thlA* is initiated at a typical  $\sigma^A$ -dependent promoter (Winzer et al., 2000; Stim-Herndon, 1995). An operon consisting of, at least, *thlR-thlB-thlC* includes the gene of a second thiolase (*thlB*), which shows a different transcription pattern in continuous culture than *thlA* (Grimmler et al., 2011). A vegetative sigma-factor dependent promoter seems to be responsible for transcription of *thlB* (Winzer et al., 2000). Crotonase (*crt*), butyryl-CoA dehydrogenase (*bcd*), and 3-hydroxybutyryl-CoA dehydrogenase (*hbd*) form a cluster together with *etfB* and *etfA* (gene products with homology to electron transfer flavoproteins). Except for a putative promoter upstream of *crt*, no further transcription start points were found upstream of the start codons of all these genes. This indicates that *crt-bcd-etfB-etfA-hbd* form one transcription unit, called the *bcs* operon (butyryl-CoA-synthesis) (Boynton et al., 1996). Phosphotransbutyrylase (*ptb*) and butyrate kinase (*buk*) are located in a common operon on the chromosome of *C. acetobutylicum* (Cary et al., 1988; Walter et al., 1993). The respective *ptb* promoter, located 57 bp upstream of *ptb*, initiates transcription of this operon during the acidogenic phase (Tummala et al., 1999; Feustel et al., 2004). Another butyrate kinase gene is also present in *C. acetobutylicum* (Huang et al., 2000).

The gene of the solventogenic enzyme acetoacetate decarboxylase (*adc*) is arranged in a monocistronic operon and is controlled by a  $\sigma^A$ -dependent promoter. Compared to the other promoters, which regulate transcription of solventogenic enzymes, the *adc* promoter allows highest expression (Feustel et al., 2004; Gerischer & Dürre 1992). The terminator of this operon is formed by a 28-bp stem-loop, 6 bp downstream of an UAA stop codon (Petersen et al., 1993; Gerischer & Dürre, 1992). This UAA is followed by another UAA stop codon. The

terminator of *adc* is located next to the *sol* operon which is transcribed by the RNA polymerase in the reverse direction of *adc*. It also terminates transcription of the *sol* operon and thus functions bidirectionally (Figure 5).

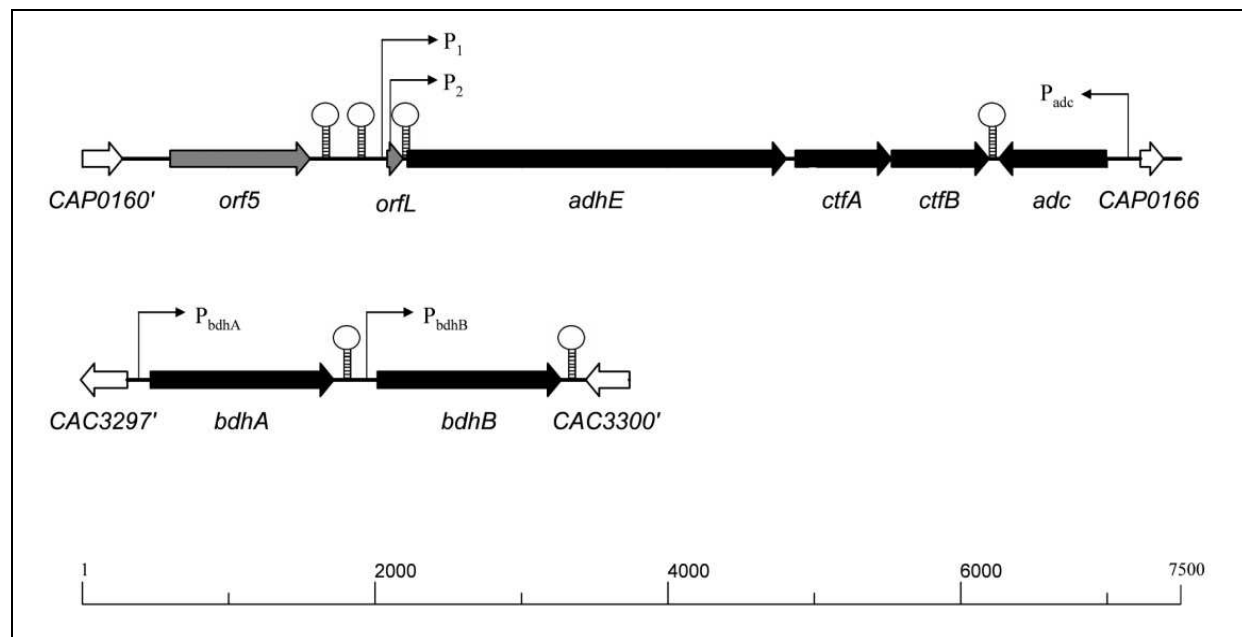


Fig. 5. Organization of megaplasmid and chromosomal gene regions encoding solventogenic enzymes in *C. acetobutylicum*. Promoter positions are indicated by  $P_{adc}$ ,  $P_1$ ,  $P_{bdhA}$ , and  $P_{bdhB}$ .  $P_2$  represents a mRNA-processing site. Possible stem-loop structures are indicated by hairpin symbols (Thormann et al., 2002).

A small peptide of unknown function (*orfL*), a butyraldehyde/butanol dehydrogenase (*adhE* or *aad*) and the two subunits of the CoA transferase (*ctfA* and *ctfB*) are encoded by this operon in the order *orfL-adhE-ctfA-ctfB* (Petersen et al., 1993; Fischer et al., 1993; Nair et al., 1994). The *sol* operon of other solventogenic clostridia (*C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*) includes the *adc* gene and contains an aldehyde dehydrogenase gene (*ald*) instead of *adhE* (Chen & Blaschek, 1999; Kosaka et al., 2007). The product of *adhE* is multifunctional. It has alcohol dehydrogenase activity (C-terminus) as well as aldehyde dehydrogenase activity (N-terminus), like the ethanol-forming *E. coli* enzyme. The latter one acts moreover as pyruvate:formate-lyase deactivase (Goodlove et al., 1989; Kessler et al., 1991).

*adhE2* forms a monocistronic operon. Two promoters  $S_1$  and  $S_2$  are deduced (Fontaine et al., 2002), but only the distal one ( $S_2$ ) shows a convincing homology to  $\sigma^A$ -dependent control regions (Dürre, 2004).

Some of the genes responsible for solventogenesis are carried on the megaplasmid pSOL1 (192 kbp) (*adc*, *sol*, and *adhE2*) (Nölling et al., 2001; Cornillot et al., 1997). The genes *bdhA* and *bdhB* for two butanol dehydrogenases have been identified on the chromosome. They form two consecutively located monocistronic operons. Their transcription is induced by  $\sigma^A$ -dependent promoters and stopped by rho-independent terminators (Petersen et al., 1991; Walter et al., 1992). As already mentioned, the genes for CoA transferase and acetoacetate decarboxylase are arranged in two different operons. The physiological conditions at the onset of solventogenesis might explain the separation of the genes for enzymes, which both

catalyze acetate formation. At that time, large amounts of acids are accumulated. To prevent a collapse of the proton gradient across the cytoplasmic membrane and cell death of the organism, the acids have to be disposed. After their uptake by *C. acetobutylicum*, a CoA transferase catalyzes formation of mainly butyryl-CoA and some acetyl-CoA (Dürre et al., 1995). The butyraldehyde/butanol dehydrogenase E subsequently mediates the conversion of butyryl-CoA into butanol. For this reason, the genetic information for *ctfA/B* and *adhE* is organized in a common transcription unit, the *sol* operon (Figure 5). Before butanol is formed, butyrate must be activated by its transformation into butyryl-CoA. Decarboxylation of acetoacetate is only needed to drive this initial reaction for thermodynamic reasons. Therefore, the *adc* gene is organized as a monocistronic operon.

### 5.5.1.3 Control mechanisms

At the beginning of solventogenesis in *C. acetobutylicum*, the enzymes required for solvent formation are induced or derepressed and the activity of some acidogenic enzymes is decreased (Andersch et al., 1983; Dürre et al., 1987; Hartmanis & Gatenbeck, 1984; Yan et al., 1988). Transcription of the respective genes for solventogenic enzymes starts several hours before solvents are being produced (Grimmler et al., 2011). *adc* is transcribed already with beginning of the exponential growth. Transcription is increased to its maximum in the stationary phase and slowed down afterwards (Sauer & Dürre, 1995; Gerischer & Dürre, 1992). The phosphorylated form of Spo0A initiates transcription of genes responsible for endospore formation and also solvent formation (Dürre & Hollergschwandner, 2004). There are Spo0A~P binding sites upstream of the *adc* and *sol* promoters of *C. acetobutylicum*. DNA-binding studies clearly revealed the participation of the phosphorylated transcription factor in regulation of solvent formation (Ravagnani et al., 2000). Spo0A inactivation reduced expression of genes responsible for solventogenesis (Harris et al., 2002). However, a complete removal of all binding motifs (0A boxes) for Spo0A upstream of the *adc* promoter caused reduction of the transcription of solventogenic enzymes, but did not abolish their expression completely. Consequently, an additional transcription factor must be involved in *adc* regulation (Böhringer, 2002). A surplus of carbon source, a pH below 4.3, limiting phosphate or sulphate concentrations, high concentrations of acetate and butyrate, and a higher temperature stimulate solvent production (Dürre & Bahl 1996; Dürre, 1998; Dürre et al., 2002; Bahl, 1983). Although signals triggering onset of solventogenesis are still unknown, all of the above mentioned factors change the topology of DNA (degree of DNA supercoiling), which influences the binding of regulatory proteins. DNA isolated from *C. acetobutylicum* during solventogenesis is more relaxed than the one extracted during acidogenesis (Wong & Bennett, 1996). Experiments with added novobiocin showed a dramatic increase of *adc* and *sol* transcription, thus supporting the essential influence of DNA topology (Ullmann, 1996; Ullmann & Dürre, 1998).

The induction of the *sol* operon takes place far before the bulk production of solvents (Sauer & Dürre, 1995; Feustel et al., 2004). The promoter P<sub>1</sub> is responsible for the transcription of *sol* (Thormann et al., 2002). *bdhB* is transcribed late during exponential growth when *sol* operon expression is already diminished. The gene product of *bdhB* thus is responsible for most of the butanol production (Sauer & Dürre, 1995). In accordance, upstream of the *bdhB* promoter a 0A box (5'-TGTAGAA) was found (Ravagnani et al., 2000). BdhA, an alcohol dehydrogenase, seems to be responsible for the removal of reducing equivalents (electron sink). This explains the constitutive expression of the respective gene under certain conditions (Sauer & Dürre, 1995).

The onset of solventogenesis is accompanied with the induction of other operons. The enzymes encoded by the *serCAXS* operon are needed for serine biosynthesis. Together with solvent formation, the induction of heat shock proteins such as DnaK, GroEL, and Hsp18 (Sauer & Dürre, 1995; Schaffer et al., 2002; Terracciano et al., 1988; Pich et al., 1990; Sauer & Dürre, 1993; Bahl et al., 1995) and a putative stress protein, PdxY, takes place (Schaffer et al., 2002). By microarray analysis, a lot of pioneering studies with *C. acetobutylicum* have been performed, leading to a better understanding of the regulation of solvent production and sporulation (Tomas et al., 2003a, 2003b; Alsaker et al., 2004; Tummala et al., 2003; Tomas et al., 2004; Borden & Papoutsakis, 2007; Alsaker & Papoutsakis, 2005; Jones et al., 2008; Grimmier et al., 2011).

#### 5.5.1.4 Improvement of the biological production of butanol

The historical fermentative production of butanol was economically inferior to the petrochemically synthesis. However, there is meanwhile a lot of potential to increase the competitiveness of the biotechnological process. Different strategies of metabolic engineering and process optimization offer a way to improve solvent productivity, solvent specificity, butanol tolerance, and substrate utilization:

- Butanol is fermented in a desired production organism such as *E. coli* or yeast. This requires the introduction of the genes for butanol synthesis into the respective organism. *E. coli* or yeast is a suitable host for the production of valuable metabolites as it is easy to manipulate and handle (Farmer & Liao, 2000; Martin et al., 2003; Causey et al., 2004; Kim et al., 2007). Engineering of a synthetic pathway for 1-butanol production was already successfully demonstrated by numerous groups in both organisms with genes from *C. acetobutylicum* or *C. beijerinckii* (Atsumi et al., 2008a; Inui et al., 2007; Donaldson et al., 2007b; Liao et al., 2008; Gunawardena et al., 2008; Buelter et al., 2008; Steen et al., 2008; Nielsen et al., 2009). While these first attempts resulted only in low butanol titers up to 16.2 mM, a recent study demonstrates butanol production of 30 g/l with a recombinant *E. coli* strain JCL166 ( $\Delta adhE$ ,  $\Delta ldhA$ ,  $\Delta frd$ ) (Shen et al., 2011), which is comparable to and even exceeds the native producer *C. acetobutylicum*. A very promising result was also the successful production of butanol directly from synthesis gas by a metabolically engineered *Clostridium ljungdahlii* strain (Köpke et al., 2010). Although the butanol yield was very low, it shows the potential of sustainable producing a superior biofuel such as butanol from an abundant non-food source.
- Another possibility is to modify *C. acetobutylicum* in a way to achieve higher butanol rates or eliminate undesired byproducts, creating a homobutanol producer (forming only butanol and some CO<sub>2</sub> and H<sub>2</sub>). With a growing number of genetic tools for *Clostridium* becoming available, some remarkable progress has been made with recombinant strains. The production of 238 mM butanol found with a *orf5*-negative strain overexpressing *adhE* represent the highest value ever reported (Harris et al., 2001). Inactivation of acetate and butyrate production of *C. acetobutylicum* can be achieved by mutations in the phosphotransacetylase and phosphotransbutyrylase genes or the corresponding kinase genes, respectively. Harris et al. inactivated the butyrate kinase gene (*buk*) (Harris et al., 2000). This led to solvent superproduction of 76 mM acetone and 225 mM butanol, when fermentation was carried out at pH 5 or below. It was the first time that the assumed barrier of biological solvent production of 200 mM was exceeded. In addition to *buk* inactivation, the *adhE* gene was overexpressed. This

mutant produced 66 mM acetone and 226 mM butanol. It could be shown that much more solvents are produced by *C. acetobutylicum* after a plasmid containing the *adc*, *ctfA* and *ctfB* genes were transformed into the bacterium. However, the control plasmid also caused a slight stimulation of the solvent production (Mermelstein et al., 1993). Another possibility of metabolic engineering is the improvement of the solvent tolerance of *C. acetobutylicum* (Tomas et al., 2003b; Tomas et al., 2004; Borden & Papoutsakis, 2007). Butanol tolerance and thus solvent production could be increased by overexpression of the chaperone-encoding *groESL* genes (Tomas et al., 2003b). By overexpression of the cyclopropane fatty acid synthase gene (*cfa*) the lipid composition of the membrane is altered, resulting in an increased butanol resistance. The disadvantage of this method is a significant lower butanol production (Zhao et al., 2003). Further improvements are possible by inactivation of genes leading to acetate, acetoin, acetone, ethanol, and lactate formation.

- Downstream processing is another way to improve economics of butanol fermentation. Distillation of butanol from the fermentation broth is very energy consuming. Alternative recovery methods might be better suited (Dürre, 1998; Santangelo & Dürre, 1996; Ezeji et al., 2004; Ezeji et al., 2007). With regard to the energy consumption, adsorption with molecular sieves (silicate) is much more efficient than gas stripping and pervaporation (Qureshi et al., 2005). During gas stripping, the products are eliminated from the fermentation media and then concentrated by condensation. The advantages of this method are that the microorganisms are not disturbed by the gases as well as the continuous working flow (Ezeji et al., 2004). During pervaporation, the product diffuses selectively across a membrane. Disadvantages of this method are the low selectivity and the incomplete removal of solvents from the fermentation broth. Moreover, the membranes are expensive. Another problem is fouling and clogging of the membranes. During liquid-liquid extraction the desired product is separated from the growth medium by mixing (and following dissolving) in a solvent. This method is only applicable with a solvent nontoxic to the bacteria. During perstraction, a membrane separates culture and extracting solvents, but it suffers from the same problems as pervaporation. Thus, a lot of recovery methods are available that make a more economic butanol purification possible.
- Over the last decades, several alternative carbon sources were evaluated as well. Processes with feedstocks such as apple pomace (fructose, glucose, sucrose) (Voget et al., 1985), whey (lactose) (Maddox et al., 1994), and lignocellulose (xylan and cellulose) (Maddox & Murray, 1983; Yu & Saddler, 1983; Yu et al., 1985; Fond et al., 1983) were developed. The butanol/acetone ratio after fermentation of whey (e.g. 100:1) is superior to that found with starch or molasses (2:1) (Bahl et al., 1986). An increased butanol content simplifies the product recovery process. The disadvantage of whey is its poor nutrient content. Consequently, a much lower productivity is found in comparison to molasses as substrate (Maddox, 1980; Welsh & Veliky, 1984; Ennis & Maddox, 1985; Linden et al., 1986). A fluidized bed reactor of bonechar-immobilized cells was used to improve fermentation with whey. The solvents were removed and concentrated by pervaporation (Maddox et al., 1994). Friedl et al. suggested using immobilized cells of *C. acetobutylicum* to optimize acetone-butanol fermentation (Friedl et al., 1991). The product was removed by pervaporation. With a lactose concentration of 380 mM in the feed solution, a stable high solvent productivity of 47 mM h<sup>-1</sup> was obtained. Lignocellulose as such cannot be used for fermentation, but pretreatment will release

hexoses and pentoses. Suitable pretreatments of lignocellulose are the Iogen process and methods that have been developed by Green Biologics (Abingdon, Oxfordshire, UK) (Green Biologics, 2007), and Green Sugar GmbH (Dresden, Germany) (Green Sugar GmbH, 2007).

### 5.5.2 Non-fermentative butanol production

Alternative routes to butanol have also been inspected. By expression of a 2-ketoacid decarboxylase Kivd and an alcohol dehydrogenase Adh from *Saccharomyces cerevisiae* in *E. coli*, 1-butanol and also iso-butanol (along with some other alcohols) could be produced non-fermentatively from intermediates of the amino acid biosynthesis (Atsumi et al., 2008b; Hawkins et al., 2009; Donaldson et al. 2006 & 2007a). Both, Butamax™ Advanced Biofuels LLC and Gevo, Inc. developed a respective technology, now having a lawsuit for patent infringement after Butamax's patent was granted (Butamax, 2011b).

### 5.6 Other biofuels

With the fast progress of synthetic biology (Peralta-Yahya & Keasling, 2010), companies such as Amyris, Inc. ([www.amyrisbiotech.com/](http://www.amyrisbiotech.com/)), Codexis, Inc. ([www.codexis.com/](http://www.codexis.com/)), LS9, Inc. ([www.ls9.com/](http://www.ls9.com/)), or OPX Biotechnologies, Inc. ([www.opxbiotechnologies.com/](http://www.opxbiotechnologies.com/)) are trying to develop a range of new biofuels with even superior properties, identical to those of gasoline or suitable as jet fuel.

## 6. Conclusion

Since fossil sources are limited and burning of these fuels leads to massive increase of the greenhouse gas CO<sub>2</sub> in the atmosphere, microbial production of biofuels became important again. First generation biofuels, however, have major drawbacks, as they compete with food industry or have unfavourable properties. Several second generation biofuels have been developed over the last few years and are on the way of commercialization, but need to be proven at scale. Biobutanol is one of the most promising second generation biofuels, providing a lot of advantages over bioethanol and has already been successfully used at large scale over decades. The acetone-butanol-ethanol fermentation looks back to a nearly 100 year old history and has already been used industrially at the beginning of the 19<sup>th</sup> century. Although a lot of fermentation plants were closed after the World War II, research regarding physiology, biochemistry, and genetics of *C. acetobutylicum* was continued. On the basis of these findings, the biological efficiency of solvent production is constantly been improved by metabolic engineering, downstream processing, and alternative substrates as surrogate for sugar. Several plants in China and Brazil are already operating again, and global players such as BP and DuPont dedicated themselves to production of biobutanol. Recently, metabolic engineering efforts demonstrated butanol production in high yields with *E. coli*, or from an alternative carbon sources such as syngas with *C. ljungdahlii*.

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