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MINI-REVIEW

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New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation

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Abstract Clostridial acetone/butanol fermentation used to rank second only to ethanol fermentation by yeast in its scale of production and thus is one of the largest biotechnological processes known. Its decline since about 1950 has been caused by increasing substrate costs and the availability of much cheaper feedstocks for chemical solvent synthesis by the petrochemical industry. The so-called oil crisis in 1973 led to renewed interest in novel fermentation and product recovery technologies as well as in the metabolism and genetics of the bacterial species involved. As a consequence, almost all of the enzymes leading to solvent formation are known, their genes have been sequenced (in fact, *Clostridium acetobutylicum* has been recently included in the microbial genome sequencing project), the regulatory mechanisms controlling solventogenesis have begun to emerge and recombinant DNA techniques have been developed for these clostridia to construct specific production strains. In parallel, cheap agricultural-waste-based feedstocks have been exploited for their potential as novel substrates, continuous culture methods have been successfully established and new on-line product recovery technologies are now available, such as gas stripping, liquid/liquid extraction, and membrane-based methods. In combination with these achievements, a reintroduction of acetone/butanol fermentation on an industrial scale seems to be economically feasible, a view that is supported by a new pilot plant in Austria recently coming into operation.

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History

The first account of biological butanol synthesis stems from Louis Pasteur. In 1861 he isolated a butyric-acid-forming bacterium and named it *Vibrion butyrique*. This organism was unable to grow in the presence of air; later it became evident that oxygen was the proper toxic compound. This led to the term "anaerobic" to describe this type of metabolism (Pasteur 1861a, b, 1863). Further studies revealed that, in addition to butyrate, the solvent butanol was formed (Pasteur 1862). Pasteur did not investigate the physiology of this anaerobe, but a few years later Albert Fitz from Straßburg published a series of papers on bacterial glycerol fermentation. Major products found were butyrate and butanol, and Fitz designated his culture (presumably in pure form) *Bacillus butylicus* (Fitz 1876, 1878, 1882). The well-known dutch microbiologist Martinus Beijerinck also isolated two butanol-forming bacteria, *Granulobacter butylicus* and *Granulobacter saccharobutyricum*, the latter being probably identical to Fitz's organism (Beijerinck 1893). The bacterial production of acetone and isopropanol was discovered more than 10 years later (Pringsheim 1906a, b; Schardinger 1904, 1905, 1907). A detailed account of the history of the description and isolation of solvent-forming bacteria has been published recently (Dürre and Bahl 1996). The designation *Clostridium* was reintroduced by Adam Prazmowski, who referred to a former publication by Trécul (Prazmowski 1880). However, it was only in 1926 that the name *Clostridium acetobutylicum* was validly published (McCoy et al. 1926).

At the beginning of the 20th century, prices for natural rubber increased dramatically because of the high demand, which led to intensive research efforts to produce a synthetic substitute. In England, the company Strange & Graham Ltd., together with Professor William Perkin from Manchester University and his assistant Chaim Weizmann, later the president of the state of Israel, wanted to solve the problem by isolating a

bacterium that produced 3-methylbutan-1-ol (isoamyl alcohol) (Weizmann 1951; Jones and Woods 1986). It was during the course of this work that Weizmann isolated strain BY, subsequently identified as *C. acetobutylicum* and similar to Fitz's organism, which produced large amounts of acetone and butanol (Gabriel 1928). He patented the process (Weizmann 1915) and it turned out to play an important role in World War I. Acetone was needed for the manufacture of the smokeless gunpowder cordite, but the war meant that England was unable to import large amounts of calcium acetate, the feedstock for acetone production. The bacterial fermentation opened a way of synthesizing large quantities of this solvent, although it was necessary to transfer the technology to Canada and the United States, in 1916 and 1917 respectively, because of a shortage of the substrate maize in England (Jones and Woods 1986). Weizmann refused special honors by the British Government, but made clear that he was in favour of the idea of a Jewish homeland in Palestine. There is no doubt that his merits contributed substantially to the Balfour Declaration in 1917. As a consequence, the State of Israel was founded in 1948, and Chaim Weizmann became its first President.

After the armistice in 1918 there was no further demand for acetone, and the fermentation process was about to be abandoned. All the butanol that been produced along with acetone was considered a by-product and kept in large storage tanks. However another historic event caused an even greater interest in the biological solvent synthesis. Henry Ford introduced new methods of automobile production in the United States. As a consequence, more and more cars were produced, which needed to be painted, and amyl acetate served as a solvent for the various lacquers. The feedstock for this substance was amyl alcohol, which had been obtained as a by-product of alcoholic fermentation by yeast. However, in 1920 prohibition became effective in the USA, and no more amyl alcohol was available. Fortunately, it turned out that butyl acetate, derived from butanol as the feedstock, was ideally suited as a solvent for lacquers and could thus replace amyl acetate. New plants were built, and the isolation of molasses-fermenting strains even increased the plant capacity by 60% (Hastings 1978). At its peak capacity in 1927, the Commercial Solvents Corp. plant in Peoria (USA) ran 96 production fermentors with a volume of 189 250 l each (Gabriel 1928), representing the largest fermentation facility for solvent formation. In 1945, 66% of the total butanol and 10% of the total acetone production were obtained by the biotechnological process (Rose 1961), ranking it second in importance to ethanol fermentation by yeast. During World War II, the pendulum swung back towards acetone synthesis, but soon after 1945 the decline of the fermentation began as a result of economic competition from the petrochemical industry. In South Africa, National Chemical Products operated a plant with a capacity of twelve 90 000-l fermentors (Spivey 1978), which was closed in 1982 (Jones and Woods 1986), whereas in China about 50% of the acetone requirements are still met by fermentation (Santangelo and Dürre 1996).

In order to reintroduce an economically competitive biological process, three major drawbacks must be overcome

i. The high costs of the substrate (e. g. molasses)
ii. The low product concentration (about 2% because of solvent toxicity)
iii. The high product recovery costs (distillation has been used in the past).

This article will try to summarize the recent results of research on organisms, their physiology and genetics, and new developments in fermentation technology that could potentially allow the establishment of a new industrial process.

Strains and species

Starting with Pasteur, a variety of names have been associated with butanol-producing clostridia. In addition to the designations mentioned, impressive lists can be found in other reviews (Beesch 1952; Dürre and Bahl 1996). Many new isolates were described when molasses replaced starch as the substrate. The problem seemed to be solved by a tacit agreement to refer to acetone/butanol-producing species as *C. acetobutylicum* and to acetone/butanol/isopropanol-forming organisms as *C. beijerinckii* (formerly *C. butylicum*). However, it later became obvious that at least some *C. beijerinckii* strains did not synthesize isopropanol (George et al. 1983). Starting in 1990, international workshops on the regulation of metabolism, genetics and the development of the solvent-forming clostridia (*Clostridium* I–V) were regularly held (at Salisbury in 1990, Blacksburg in 1992, Evanston in 1994, Ulm in 1996, Toulouse in 1998) and brought together all the people working in this field. Participants soon realized that data reported for strains presumed to be identical were very different. This also included the type strain of *C. acetobutylicum* preserved in different culture collections (ATCC 824, DSM 792, NCIMB 8052). As a consequence, a detailed taxonomic and phylogenetic study of all available butanol-forming clostridial strains was launched. The results of the two groups working on this project were identical and clearly showed that there are four different major taxonomic groups (Johnson and Chen 1995; Jones and Keis 1995; Keis et al. 1995; Johnson et al. 1997). Of the abovementioned strains, ATCC 824 and DSM 792 still represent *C. acetobutylicum*, whereas NCIMB 8052 is now considered to belong to *C. beijerinckii*. Thus, one should be cautious when comparing older data from "*C. acetobutylicum*". Only strains of taxonomic group I represent this species. As a consequence, this review will only include data of properly described strains that can be classified correctly.

Enzymatic reactions of substrate degradation and product formation

C. acetobutylicum is able to use polymeric substrates such as starch and xylan, but not cellulose, for growth (Mitchell 1998). The degradation of starch is mediated by an α-amylase that has been purified (Paquet et al. 1991), and genetic data indicate the presence of at least one additional α-amylase gene (Verhasselt and Vanderleyden 1992; Gerischer and Dürre 1990). Larch wood xylan is only partially hydrolysed by the action of endoxylanases and a β-D-xylosidase (Lee et al. 1985, 1987, Lemmel et al. 1986). *C. beijerinckii* also grows on starch and employs the catalytic activities of glucoamylase and α-amylase (Ensley et al. 1975).

Glucose uptake in both species is mediated by phosphotransferase systems (Hutkins and Kashket 1986; Mitchell et al. 1991; Mitchell 1996). The pathway of sugar degradation has not been determined unequivocally, but it is likely that *C. acetobutylicum* and *C. beijerinckii* employ glycolytic reactions of the Embden-Meyerhof-Parnas type, as do most clostridia (Rogers and Gottschalk 1993). An important characteristic of this fermentation is that different phases are observed. During exponential growth acids are formed almost exclusively, whereas solventogenesis is initiated at the transition to the stationary growth phase, when even the previously produced acids are partly taken up again and transformed into solvents. A scheme of the reactions leading from starch or sugars to solvents, acids and gases is shown in Fig. 1.

The respective enzymes have been described extensively in recent reviews (Chen 1993; Dürre and Bahl 1996) and are therefore not dealt with in this article. Table 1 provides an updated list of purified proteins and their main properties from strains that can be unequivocally assigned to either *C. acetobutylicum* or *C. beijerinckii*. A remarkable feature is that many enzymes that have specialized in certain functions seem to exist in similar forms. Acetic and butyric acid are synthesized from the respective acyl-CoA derivatives by the consecutive action of phosphotransacylases and kinases. Of these four enzymes, phosphotransbutyrylase, butyrate kinase and acetate kinase have been purified from *C. acetobutylicum* (Table 1). Although the kinases are similar in size and catalyse an analogous reaction, they show an extremely high substrate specificity (Winzer et al. 1997). Another example is represented by the thiolase reaction, which is catalysed by two different enzymes, the physiological function of the second one still waiting to be determined (Winzer 1995). Also, besides the aldehyde dehydrogenase activity of the multifunctional aldehyde/alcohol dehydrogenase E, a separate NADH-dependent butyraldehyde dehydrogenase can be found in *C. acetobutylicum*, which has been recently purified (Schreiber and Dürre 1996). Finally, both *C. acetobutylicum* and *C. beijerinckii* employ at least three different alcohol dehydrogenases each. In the former, alcohol dehydrogenase E (AdhE) is active only at the onset of solventogenesis, whereas butanol dehydrogenase II (Bdh II) is the proper production enzyme. Bdh I seems to be involved in a low-level butanol formation (Sauer and Dürre 1995) that could be a kind of emergency reaction to get rid of reducing equivalents. In addition, there is an NADPH-dependent enzyme that catalyses ethanol formation (Sauer et al. 1993). In *C. beijerinckii*, a primary/secondary alcohol dehydrogenase is responsible for isopropanol production, whereas the physiological role of three different primary alcohol dehydrogenases remains to be determined (Chen 1995).

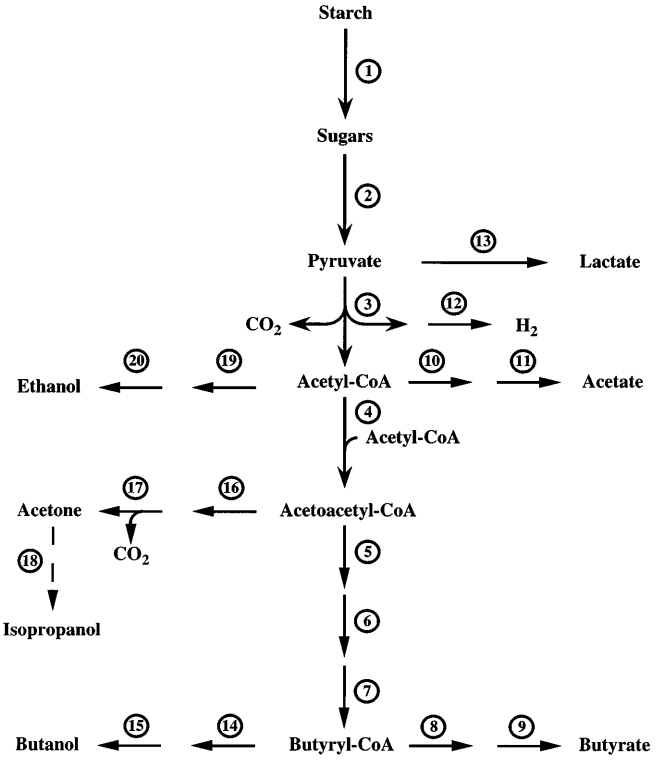

Fig. 1 Fermentation pathways in solventogenic clostridia. Circled numbers refer to the enzymes employed: *1* amylase; *2* phospho*enol*pyruvate phosphotransferase systems and glycolytic enzymes of the Embden-Meyerhof-Parnas pathway; *3* pyruvate:ferredoxin oxidoreductase; *4* thiolase; *5* 3-hydroxybutyryl-CoA dehydrogenase; *6* crotonase; *7* butyryl-CoA dehydrogenase; *8* phosphotransbutyrylase; *9* butyrate kinase; *10* phosphotransacetylase; *11* acetate kinase; *12* hydrogenase; *13* lactate dehydrogenase; *14* butyraldehyde dehydrogenase and aldehyde/alcohol dehydrogenase E; *15* butanol dehydrogenase and aldehyde/alcohol dehydrogenase E; *16* acetoacetyl-CoA:acetate/butyrate coenzyme-A transferase; *17* acetoacetate decarboxylase; *18* primary/secondary alcohol dehydrogenase; *19* acetaldehyde dehydrogenase; *20* ethanol dehydrogenase. The dashed line to isopropanol indicates that only some strains of *C. beijerinckii* are able to perform this reaction

Fermentation process and product recovery

In the past, acetone/butanol fermentation has been operated as a batch process followed by distillation to obtain the desired products. Typical yields were 15–18 g

Table 1 Purified enzymes of solventogenic clostridia involved in substrate degradation and product formation. *ND* not determined, – not available

Enzyme	Native molecular mass (kDa)/subunit size(s) (kDa)	Accession number of sequenced gene	Reference
C. acetobutylicum			
Endoxylanase A	65/65	–	Lee et al. 1987
Endoxylanase B	29/29	–	Lee et al. 1987
α-Amylase	83/84	M34078	Paquet et al. 1991
Xylosidase	224/85, 63	–	Lee and Forsberg 1987a
Arabinofuranosidase	94/94	–	Lee and Forsberg 1987b
Lactate dehydrogenase	159/36	–	Freier and Gottschalk 1987
Pyruvate:ferredoxin oxidoreductase	ND/123	–	Meinecke et al. 1989
Thiolase	ND/44	U08465	Wiesenborn et al. 1988
Crotonase	158/40	U17110	Waterson et al. 1972
Phosphotransbutyrylase	264/31	L14744	Wiesenborn et al. 1989a
Butyrate kinase	85/39	L14744	Hartmanis 1987
Acetate kinase	87/43	U38234	Winzer et al. 1997
Coenzyme-A transferase	93/24, 23	M34078, M93363	Wiesenborn et al. 1989b
Acetoacetate decarboxylase	330/28	M34078, M93363	Gerischer and Dürre 1990, Petersen and Bennett 1990
NADH:rubredoxin oxidoreductase	ND/41	–	Petitdemange et al. 1979
Butyraldehyde dehydrogenase	ND/40	–	Schreiber and Dürre 1996
Alcohol dehydrogenase (NADPH)	ND/44	–	Sauer et al. 1993
Butanol dehydrogenase I	ND/42	M96945	Petersen et al. 1991
Butanol dehydrogenase II	ND/42	M96946	Welch et al. 1989
C. beijerinckii			
β-Hydroxybutyryl-CoA dehydrogenase	213/31	–	Colby and Chen 1992
Phosphotransbutyrylase	205/33	–	Thompson and Chen 1990
Coenzyme-A transferase	85/28, 23	–	Chen 1993
Butyraldehyde dehydrogenase	115/56	–	Palosaari and Rogers 1988
	100/55	–	Yan and Chen 1990
Alcohol dehydrogenase 1	ND/42	–	Chen 1995
Alcohol dehydrogenase 2	ND/45, 42	–	Chen 1995
Alcohol dehydrogenase 3	ND/45	–	Chen 1995
Primary/secondary alcohol dehydrogenase	ND/39	M84723	Chen 1995

solvents/l within a period of 40–60 h (Woods 1995). It has been estimated that, under the same conditions, an increase to 22–28 g/l would be required to make the biological synthesis economically competitive again (Woods 1995). Market prices for fermentation products have been provided by Lenz and Moreira (1980), including butanol (U.S.$0.53/kg), acetone ($0.44/kg), ethanol ($0.40/kg), hydrogen ($0.29/kg) and carbon dioxide ($0.11/kg). Provision of substrates makes up about 63% of the total costs (Lenz and Moreira 1980), so that a variety of alternative compounds have been checked out for their ability to replace the now expensive molasses. As already mentioned, *C. acetobutylicum* is unable to degrade cellulose. Use of a coculture including a mesophilic cellulolytic *Clostridium* has been suggested (Fond et al. 1983) and genetic approach aimed at the transfer of cellulase genes from other organisms into the solventogenic clostridia in order to broaden the substrate spectrum (see next section for details). Traditional attempts to improve this part of the production process have involved the testing of a variety of alternative materials for use in the acetone/butanol fermentation. Peat and palm oil mill effluent proved to be partially consumed by *C. beijerinckii* and *C. aurantibutyricum*, respectively (Forsberg et al. 1986; Sombrutai et al. 1996), but probably could only play a role in very special cases. More emphasis should be given to lignocellulose and waste products of the agricultural and dairy industry (Table 2). Substrate costs could thus be drastically reduced, especially if the chosen strain allows versatility in its usage of different carbon sources.

The other possibility to improve the traditional process is to reduce costs of product recovery. Membrane-based systems, such as reverse osmosis, perstraction, pervaporation and membrane evaporation, as well as liquid/liquid extraction, adsorption and gas stripping, have been compared (Ennis et al. 1986; Groot et al. 1992). There is no easy answer to the question of which technology is most suitable (Table 3). Membrane-based systems show a high selectivity for solvents, but might suffer from clogging and fouling and seem to be more suited for use with immobilized cells. Liquid/liquid extraction also has a high selectivity, but emulsions might form that render the process less suitable. On the other hand, gas stripping does not lead to complete removal of solvents from the fermentation broth (Mollah and Stuckey 1993), but nor does it suffer from particulate substrates or from clogging or fouling by biomass. All these procedures can be designed to allow on-line product recovery, so that butanol toxicity, the third major problem of the traditional process, is reduced. So far, there exist no published reports on strains that are

Table 2 Economically interesting novel substrates for clostridial solvent fermentation

Substrate	Carbon components	Organism	Reference
Apple pomace	Fructose, glucose, sucrose	*C. beijerinckii*	Voget et al. 1985
Jerusalem artichokes	Polyfructans	*C. beijerinckii*	Marchal et al. 1985
Lignocellulose	Cellulose, xylan	*C. acetobutylicum*	Maddox and Murray 1983, Marchal et al. 1986
Whey	Lactose	*C. acetobutylicum* *C. beijerinckii*	Maddox et al. 1993
Low-grade potatoes	Starch	*C. beijerinckii*	R. Gapes, personal communication
Rye	Starch	*C. beijerinckii*	R. Gapes, personal communication
Peat	Mainly cellulose, xylan	*C. beijerinckii*	Forsberg et al. 1986
Palm oil mill effluent	Mainly oil, cellulose, starch, xylan	*C. aurantibutyricum*	Sombrutai et al. 1996

highly butanol-resistant and still produce large amounts of solvents.

Recently an attempt has been made to reconstitute an industrially viable process by use of the new developments described above. Richard Gapes from the Technical University of Vienna initiated the construction of a pilot plant for acetone/butanol fermentation within the facilities of a distillery in Starrein, Austria. The process is based on the group's experience with solvent formation by *C. beijerinckii* NRRL B-592 (Gapes et al. 1996). The pilot plant will be operated as a continuous two-stage culture, with reactor working volumes of approximately 50 l and 300 l and two feed tanks of 3500 l each (R Gapes, personal communication). Substrates will be low-cost agricultural starchy material (low-grade potatoes, potato cutting waste, potato pulp and juice from starch production, maize, rye and, later on, possibly hydrolysates, domestic waste etc.). Potatoes are steam-exploded, treated with α-amylase and sterilized, whereas maize and rye are milled before sterilization. No further growth additives are required (R Gapes, personal communication). Product separation is planned by gas stripping with heating of the effluent to approximately 70 °C and condensation of the solvent/water

Table 3 Novel product recovery techniques for clostridial solvent fermentation, Data are based on reports by Ennis et al. (1986), Gapes et al. (1996), Groot et al. (1992), and Maddox et al. (1993)

Method	Principle	Advantage	Disadvantage
Adsorption	Adherence of solvents to e.g. silicalite or ion-exchange resins, heat regeneration		High price of material, low capacity, low selectivity, possible fouling
Gas stripping	Heating of effluent, purging with gas, condensation of solvent/water vapours	Simple to perform, low chance of clogging or fouling	Low selectivity, no complete removal of solvents, more energy required compared to membrane-based processes
Liquid/liquid extraction	Contact of water-immiscible solvent with fermentation broth, recovery of dissolved acetone/butanol/isopropanol by distillation	High capacity, high selectivity, low chance of clogging or fouling	Expensive to perform, possible forming of emulsions
Membrane evaporation	Selective diffusion of solvents across a porous membrane, recovery of evaporated vapours by applying vacuum and condensation	Smaller membrane area required compared to pervaporation, simple to perform	Possible clogging or fouling
Perstraction	Similar to liquid/liquid extraction, with a membrane separating fermentation broth and extractant	High selectivity, simple to perform	Large membrane area required, possible clogging or fouling
Pervaporation	Selective diffusion of solvents across a non-porous membrane, recovery of evaporated vapours by applying vacuum or sweep gas	High selectivity compared to membrane evaporation, simple to perform	Lower membrane flux compared to membrane evaporation, possible clogging or fouling
Reverse osmosis	High-pressure separation of dilute aqueous solution into a concentrated one and pure water by use of a semipermeable membrane, distillation of concentrated solution	Lower costs than conventional distillation	Possible clogging or fouling

vapours. Testing of the plant is already under way, and the operational start is planned for spring 1998. The results are expected to provide conclusive evidence of the costs of a ''novel'' acetone/butanol fermentation and its economic competitiveness with petrochemical solvent synthesis.

Genetics and metabolic engineering

Cloning of genes from a clostridial solvent producer was first reported in 1986 from David Woods' laboratory in Cape Town (for a review see Young et al. 1989), although the organism used (strain P262) is no longer considered to belong to *C. acetobutylicum*. Similarly, another early report on cloning (Efstathiou and Truffaut 1986) referred to a bacterium that has not yet been properly classified. Thus, phosphotransbutyrylase and butyrate kinase were the first genes to cloned from *C. acetobutylicum* (Cary et al. 1988). During the last decade, sequences of almost all the genes of solvent- and acid-forming enzymes have become known (Papoutsakis and Bennett 1993; Bennett and Rudolph 1995; Dürre et al. 1995; Boynton et al. 1996a). Physical maps of the chromosome have been established for both *C. acetobutylicum* and *C. beijerinckii* (Cornillot et al. 1997a; Wilkinson and Young 1995). In addition, a variety of nucleotide sequence data referring to heat-shock response, potassium transport, primary metabolism, sigma factors, sporulation and other areas have been published that cannot be dealt with in the limited space of this article. Most important: *C. acetobutylicum* has been included in the list of microbes the genomes of which are going to be sequenced. Recently, Genome Therapeutics Corp. Genome Sequencing Center (USA), as a component of the Department of Energy (DOE) Microbial Genome Project (USA), has made publically available the raw data obtained so far through the internet (http://www.cric.com/htdocs/sequences/clostridium/clospage.html), and the interested reader is referred to this site for additional information. Given the speed of modern sequencing technology the complete genome of *C. acetobutylicum* will probably be published in 1998.

Genes of acidogenic enzyme pairs such phosphotransacetylase/acetate kinase and phosphotransbutyrylase/butyrate kinase are clustered in both *C. acetobutylicum* and *C. beijerinckii* (Boynton et al. 1996b; Walter et al. 1993; Oultram et al. 1993). Similarly, genes encoding solventogenic proteins are grouped together, as in the so-called *sol* operon, *adhE-ctfA-ctfB*, of *C. acetobutylicum* (encoding a multifunctional aldehyde/alcohol dehydrogenase and a coenzyme-A transferase). Adjacent to this locus, but with convergent direction of transcription, the monocistronically organized acetoacetate decarboxylase gene has been found (Dürre et al. 1995). Recently it was reported that this whole region is located on a 210×10^3-base-pair circular plasmid (Cornillot and Soucaille 1996; Cornillot et al. 1997b), the potential loss of which nicely explains the long known effect of strain degeneration (decreased ability or complete loss of solvent formation upon repeated subculturing) (Stim-Herndon et al. 1996; Cornillot et al. 1997b). Genes for the other two butanol dehydrogenases (*bdhA* encoding Bdh I, *bdhB* encoding Bdh II) are also clustered, but form different operons (Walter et al. 1992) residing on the chromosome (Cornillot et al. 1997a). RNA analyses verified that the *adc*, *bdhA*, *bdhB* and, in addition, *thlA*, *hsp18* and *lyc* genes (the latter three encoding a thiolase, a heat-shock protein and an autolysin) as well as the *sol* operon are induced shortly before solvents can be detected in the medium (Gerischer and Dürre 1992; Walter et al. 1992; Sauer and Dürre 1995; Winzer 1995). However, it is not yet known how this transcriptional regulation is mediated. A variety of different environmental conditions is required for solventogenesis to start, which include excess of substrate, pH below 4.5, threshold concentrations of acetate and/or butyrate and growth-limiting amounts of suitable salts (phosphate or sulphate) that all are known to affect DNA topology directly. Therefore, it has been proposed that DNA supercoiling might be the trigger for the onset of solvent formation (Dürre et al. 1995). Support for this hypothesis comes from recent reports stating that DNA from *C. acetobutylicum* becomes less negatively supercoiled when entering the solventogenic stage and that transcription of genes encoding solventogenic enzymes is specifically induced upon inhibition of DNA gyrase, an enzyme that introduces negative supercoils (Wong and Bennett 1996; Ullmann et al. 1996). The recent cloning of the clostridial gyrase genes (Ullmann and Dürre 1996) should allow a modulated expression in vivo, and thus unequivocal evidence for an essential role of the degree of DNA supercoiling in the induction of acetone and butanol synthesis. It should be kept in mind, however, that changes in DNA topology can only affect the first step of the regulatory cascade (transcription of the *adc* and *sol* operons; Sauer and Dürre 1995). Genes that are turned on later (such as *bdhB*) must be induced by help of regulator proteins. Several different effectors and models have been suggested that might be involved in this stage of control (Boynton et al. 1994; Girbal et al. 1995; Grupe and Gottschalk 1992; Meyer and Papoutsakis 1989; Rao and Mutharasan 1989).

Enormous progress has been made in the last few years with respect to genetic manipulation of the solventogenic clostridia (Dürre and Bahl 1996). Transposon mutagenesis has been established (Bertram and Dürre 1989; Bertram et al. 1990; Woolley et al. 1989). Plasmids based on pAMβ1, pIM13, or pCBU2 replicons are all maintained stably in *C. acetobutylicum* and represent the vectors of choice (Lee et al. 1992; Minton et al. 1993). In vivo methylation of plasmid DNA before transformation is important to prevent restriction by a clostridial endonuclease (Mermelstein and Papoutsakis 1993). Electroporation has proven to be an efficient and relatively easy method of DNA transfer into *C. acetobutylicum* and *C. beijerinckii* (Mermelstein et al. 1992;

Oultram et al. 1988; Birrer et al. 1994). Plasmid copy number in *C. acetobutylicum* varies between approximately 7 (pIM13 replicon) and 14 (pCBU2 replicon) (Lee et al. 1993), but it should be noted that there is a distinct effect of the presence of vectors on product formation. Such plasmid-carrying strains produce more solvents and fewer acids than the wild type (Walter et al. 1994a). The opposite effect (low levels of solvent synthesis of recombinant strains) has also been found when the tetracycline-resistance marker *tetM* is used instead of an erythromycin-resistance-encoding gene (Ullmann 1995). Using this repertoire of techniques several approaches to metabolic design have been recently studied. Inactivation of *adhE*/*aad* drastically reduced butanol production (Green and Bennett 1996) but, upon transformation of this gene into a solvent-negative mutant, the alcohol-forming ability could be restored (Nair and Papoutsakis 1994). Similarly, a decrease of acid synthesis caused by disruption of either phosphotransacetylase or butyrate kinase genes has been reported (Green et al. 1996), while increased production of butyrate could be achieved in a recombinant strain carrying a plasmid with additional phosphotransbutyrylase and butyrate kinase genes (Walter et al. 1994b). Enhanced acetone production could be observed upon transformation with artificially constructed acetone formation operons (consisting of the *adc* and *ctfA*/*B* genes under control of the *adc* promoter) (Mermelstein et al. 1993; Guillot 1997). Finally, the first steps have been taken to broaden the substrate range of solventogenic clostridia to include utilization of cellulose by transfer of endoglucanase genes from *C. cellulovorans* and *C. thermocellum* (Minton et al. 1993; Kim et al. 1994). However, so far no cellulolytic recombinant strains have been constructed.

Conclusion

This article has tried to summarize the recent discoveries and developments in both scientific and engineering aspects of the historically important clostridial solvent fermentation. Basic scientific research has elucidated most of the respective metabolic reactions and their regulation and provided the basis for strain and process improvement by genetic manipulation and substrate choice. Novel developments in product recovery opened the way for a competitive biological solvent synthesis. The establishment of a pilot plant might mark the first step to a successful reintroduction of this biological process on an industrial scale. However, it needs to be mentioned that there are additional uses of the solventogenic clostridia. Even if a generally enhancing role in oil recovery (Behlulgil et al. 1992) might be questioned, exciting recent results document the feasibility of using *C. beijerinckii* as a highly specific delivery system for cancer gene therapy (Minton et al. 1995; Fox et al. 1996; Lemmon et al. 1997). Thus, the biotechnological potential of solventogenic clostridia is gaining industrial importance again, not only in bulk chemical production but also for medical treatments.

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