Biotechnology Advances 27 (2009) 764–781

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Metabolic pathways of clostridia for producing butanol

R. Gheshlaghi [a,b,*], J.M. Scharer [a], M. Moo-Young [a], C.P. Chou [a]

[a] Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1
[b] Department of Chemical Engineering, Ferdowsi University of Mashhad, Azadi Square, Pardis Campus, Mashhad, Postal Code 9177948944, Khorasan, Iran

ARTICLE INFO

Article history:
Received 18 November 2008
Received in revised form 4 June 2009
Accepted 5 June 2009
Available online 17 June 2009

Keywords:
Clostridium acetobutylicum
Clostridia
Metabolic pathway
Biobutanol
Enzyme kinetics
ABE fermentation

ABSTRACT

Worldwide demand for energy has been the impetus for research to produce alcohol biofuels from renewable resources. This review focuses on the biosynthesis of butanol, which is regarded to be superior to ethanol as a fuel. Although acetone/butanol fermentation is one of the oldest large-scale fermentation processes, butanol yield by anaerobic fermentation remains sub-optimal. Metabolic engineering provides a means for fermentation improvements. Consequently, a comprehensive assessment of the intermediary enzymes involved in butanol formation from carbohydrates by the saccharolytic bacterium, *Clostridium acetobutylicum* and other closely allied clostridia was performed to provide guidelines for potentially enhancing butanol productivity. The activity of the enzymes, their regulation and contribution to the metabolic pathways was reviewed. Published kinetic data for each important enzymatic reaction were assessed. For most enzymatic reactions, the systematic investigation of the kinetic data and the properties of the enzymes led to the development of rate equations that were able to describe activity as the function of the substrates, products, and allosteric effectors.

Contents

* Corresponding author. Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1. Tel.: +1 519 888 4567x36098; fax: +1 519 746 4979.
E-mail addresses: rgheshla@engmail.uwaterloo.ca, gheshlaghi@ferdowsi.um.ac.ir (R. Gheshlaghi), jscharer@uwaterloo.ca (J.M. Scharer), mooyoung@uwaterloo.ca (M. Moo-Young), cpchou@uwaterloo.ca (C.P. Chou).

0734-9750/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.biotechadv.2009.06.002

1. Introduction

Clostridia have a long history of being employed in several biotechnological processes, for instance, *C. acetobutylicum* in the conversion of renewable biomass for acetone/butanol production (Jones and Woods, 1986; Bahl et al., 1982b), *C. perfringens* for production of potent toxins such as enterotoxin and *C. botulinum* and *C. tetani* for neurotoxins (Rood et al., 1997), and *C. histolyticum* and *C. oncolyticum* to produce agents for cancer therapy (Schlechte and Elbe, 1988; Connell, 1935). The production of organic acids, alcohols, and other neutral solvents by the degradation of a wide range of polysaccharides by many species of clostridia has been reported (Mitchell, 1998; Mitchell et al., 1995; Cangenella and Weigel, 1993). Saccharolytic mesophilic species that are able to form butyrate, however, are the only species that are capable of producing butanol along with different amounts of acetone, isopropanol, and ethanol (Jones and Woods, 1989). In addition to *C. acetobutylicum*, other clostridia that are known to produce butanol as a major fermentation product are *C. aurantibutylicum*, *C. beijerinckii*, and *C. tetanomorphum* (Gottwald et al., 1984; George et al., 1983; George and Chen, 1983). Microbial butanol production was first reported by Louis Pasteur in 1861 and developed to an industrial production level by Chaim Weizmann using *C. acetobutylicum* in the early 20th century. A great number of studies (e.g., Robinson, 1922; Killeffer, 1927; Gabriel and Crawford, 1930; Davies and Stephenson, 1941; Langlykke et al., 1948) were performed in order to improve the process and fermentative process became competitive with chemical synthesis by the middle of the 20th century. Its application, however, declined during the 1950s and was overtaken by cheaper petrochemical-based processes by 1960 (Rose, 1961).

In the 1980s the reduced supply and escalating price of petroleum rekindled interest in fuel production by anaerobic bacteria (Rosenberg, 1980; Weigel, 1980; Zeikus, 1980) including acetone/butanol/ethanol (ABE) fermentation by various clostridial species (Calam, 1980; Haggstrom and Molin, 1980; Maddox, 1980). However, ABE fermentation using *C. acetobutylicum* strains has been the only large scale industrial fermentation that has been shown to have an economically viable potential (Jones and Woods, 1986). Despite these promising results, there are some drawbacks that affect its widespread use such as the high cost of substrates, low productivity, low concentrations (less than 20 g/l) due to solvent toxicity, and the high cost of product recovery (Gapes, 2000).

Since 1980s efforts have been made to improve ABE productivity by delineating the physiology of solventogenic clostridia, particularly *C. acetobutylicum*) (Girbal and Soucaille, 1994; Grupe and Gottschalk, 1992; Meyer et al., 1985; Bahl et al., 1982a) and genetics (Inui et al., 2008; Tomas et al., 2004; Harris et al., 2000; Cornillot et al., 1997; Blaschek and White, 1995). New possibilities for more sustainable solvent production via ABE fermentation with less expensive substrates have been proposed (Yu et al., 2007; Claassen et al., 1999). For instance, lignocellulosic materials such as domestic organic waste (Claassen et al., 2000) or fibrous corn wastes (Qureshi et al., 2006) can be used for ABE fermentation. Butanol is considered a promising product of biomass fermentations for potential industrial use as a solvent, chemical feedstock, and particularly liquid fuel (Durre, 2008). Therefore, it is very important to optimize the product yield and simultaneously reduce the level of other end products (i.e., H_2, CO_2, acetate, and butyrate). *I*t appears that the combination of trial-by-error experimentation and molecular biology are not sufficient enough to design an optimum strategy for the overproduction of the desired products (Zhao et al., 2003; Smits et al., 2000). Process optimization requires a strong theoretical and design framework (Alvarez-Vasquez et al., 2000).

The main objective of this review is to provide guidelines for improving the yield of butanol and other solvents produced by anaerobic fermentation by translating the metabolic observations, whenever possible, into appropriate kinetic expressions. To achieve this objective, it is essential to study the key enzymes involved and the regulation of their activity. The information regarding the key components and interactions of the biochemical system can be used for translating metabolism into a mathematical structure that is appropriate and convenient for optimization purposes. In this paper the enzymes involved in the metabolic pathways for acid and solvent formation in *C. acetobutylicum* are reviewed and based on the previously reported kinetic information the corresponding rate equations are presented. The equations are presented as reaction rate expressions (v_j), where j refers to the reaction number given in Appendix C. If there was no specific information for *C. acetobutylicum*, enzyme kinetic data from closely related clostridia were analyzed. We believe that a cohesive assessment of the somewhat scattered information is required to better understand the enzymatic regulation of the biochemical pathways.

2. Metabolism of *C. acetobutylicum*

C. acetobutylicum is a strictly anaerobic, heterofermentative, spore-forming bacterium (Girbal and Soucaille, 1994). Batch ABE fermentation by *C. acetobutylicum* can be divided into two distinctive phases, an acid production phase and a solvent production phase (Johnson et al., 1931). During the first phase, the cells grow rapidly and form carboxylic acids, mostly acetate and butyrate; the excretion of these acids lowers the external pH. These acids are suggested to act as inducers for the biosynthesis of the solventogenic enzymes during a second fermentative phase (Ballongue et al., 1985). The acids formed earlier re-enter the cells and act as co-substrates for the production of neutral solvents (Fond et al., 1985; Kell et al., 1973). At this point, the production of the acids ceases as well as cell growth, and the medium pH increases slightly due to the acid uptake (Terracciano and Kashket, 1986; Spivey, 1978). It has been suggested that the switch to solvent production is an adaptive response of the cells to the low medium pH resulting from acid production (Bahl et al., 1982a). The major end product of the fermentation is butanol, with acetone and ethanol being minor products. The bacterium, *C. acetobutylicum* is able to metabolize a great variety of carbon resources. Depending on the nature of the carbohydrate and the culture conditions, the extent of solvent conversions can vary (Compere and Griffith, 1979; Abou-Zeid et al., 1976; Taha et al., 1973). After the switch to solventogenesis, further carbon and electrons are directed to the formation of these solvents. At the end of the fermentation, the solvent concentrations may reach an inhibitory level and halt the metabolism (Bowles and Ellefson, 1985). The accumulation of solvents probably affects the membrane of *C. acetobutylicum* by altering both membrane function (Moreira et al., 1981) and membrane fluidity (Vollherbst-Schneck et al., 1984).

The biochemical pathways for both acidogenic and solventogenic metabolisms have been described in a general fashion and studies

have been performed to elucidate the factors affecting solvent production (Andreesen et al., 1989; Ljungdahl et al., 1989; Jones and Woods, 1986; Rogers, 1986). The metabolic pathways for acidogenesis and solventogenesis by *C. acetobutylicum* are depicted in Fig. 1. The complete list of the reactions is given in Appendix C. Hexose sugars are degraded to pyruvate by the Embden–Meyerhof–Parnas (EMP) pathway (Rogers, 1986). Kotze (1969) detected hexokinase (HK, EC 2.7.1.2), glucose-6-phosphate isomerase (G6PI, EC 5.3.1.9), and pyruvate kinase (PK, EC 2.7.1.40) activities in different groups of clostridia (i.e., saccharolytic, proteolytic, cellulolytic, or nitrogen-fixing), which were all able to degrade carbohydrates. The uptake of 1 mol of a hexose yields 2 mol of pyruvate with the net formation of 2 mol each of ATP and NADH (Gottschalk, 1986; Thauer et al., 1977; Doelle, 1975). The solvent producing clostridia also metabolize pentose sugars via the pentose phosphate pathway (Ounine et al., 1983; Cynkin and Delwiche, 1958). The resulting phosphorylated intermediates are converted by transaldolase and transketolase to fructose-6-phosphate and glyceraldehyde-3-phosphate, which enter the glycolytic pathway. The conversion of 3 mol pentose to pyruvate yields 5 mol ATP and 5 mol NADH (Rogers, 1986). *C. butyricum* is one of the best natural producers of 1,3 propanediol that exclusively uses glycerol dehydrogenase and dihydroxyacetone kinase for the conversion of glycerol to dihydroxyacetone phosphate which is then further metabolized via the latter steps of the EMP pathway

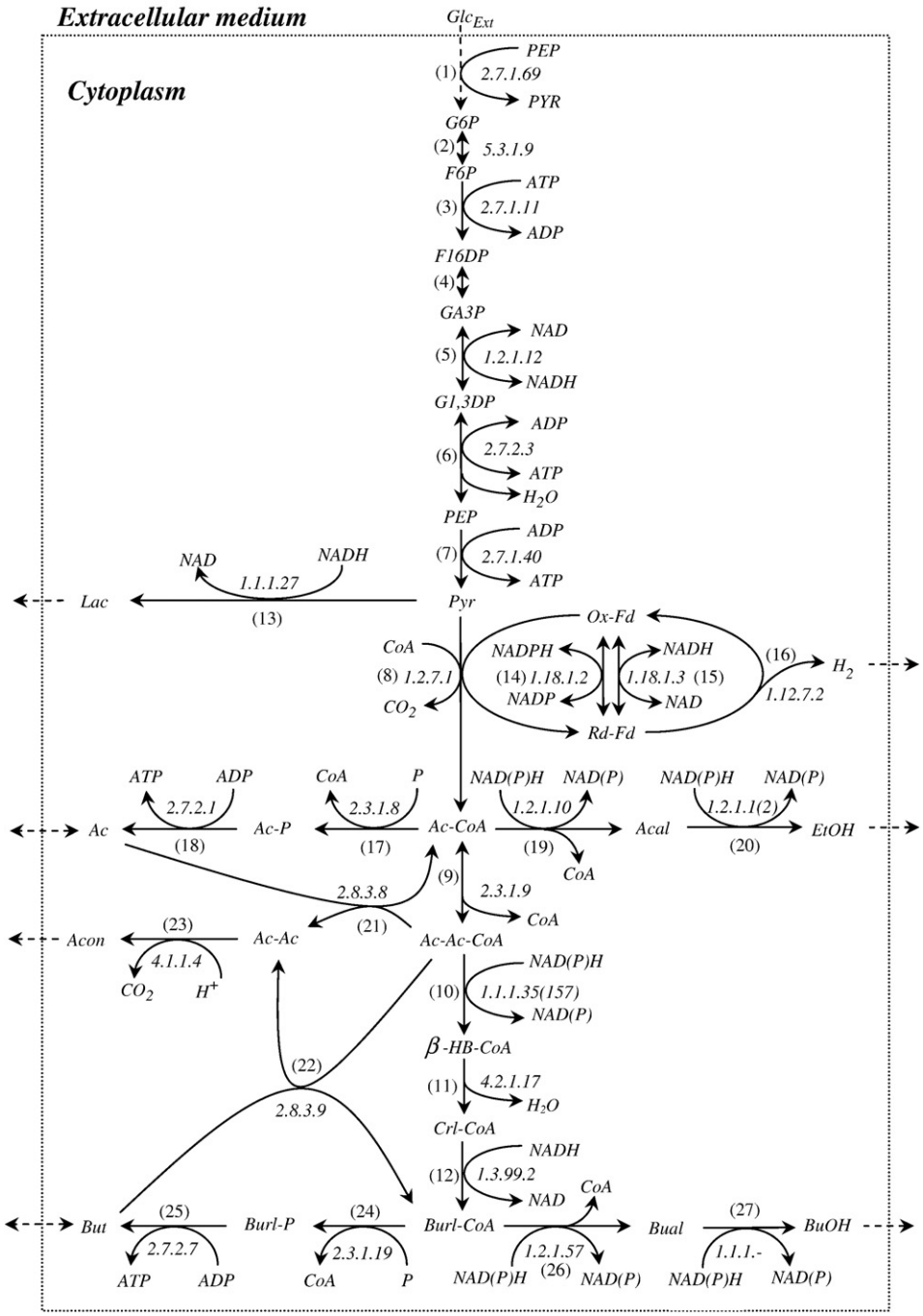

Fig. 1. Metabolic pathways of glucose degradation in *C. acetobutylicum*. The solid and dashed arrows represent intracellular reactions and transport processes, respectively. Reaction numbers are given in parenthesis. Enzymes are indicated by their EC numbers. Complete names of the enzymes, abbreviations, and reactions are given in the Appendices A, B, and C, respectively.

(Saint-Amans et al., 2001). Gonzalez-Pajuelo et al. (2006) introduced the genes for 1,3 propanediol production from *C. butyricum* VPI 3266 into *C. acetobutylicum* DG1, which was not able to grow on glycerol as the sole carbon source. In general, *C. acetobutylicum* cannot grow on glycerol only, but the recombinant strain grew on this carbon source and formed 1,3 propanediol as the main fermentation product. However, they observed a different pathway for glycerol oxidation. The recombinant strain of *C. acetobutylicum* used a glycerol kinase and a glycerol-3-phosphate dehydrogenase for oxidizing glycerol. The consumption of each mol of glycerol yields 1 mol ATP and 2 mol NADH. *C. acetobutylicum* is reported to possess several genes involved in cellulose degradation (Nolling et al., 2001), but it is not capable of using cellulose for growth (Mitchell, 1998). Although, xylose or lichenan as sole carbon sources can induce low but significant levels of extracellular cellulase activity in the medium, due to the lack of true cellulolytic properties this organism cannot degrade (hemi)cellulose (Lopez-Contreras et al., 2004).

Pyruvate is a key intermediate in clostridial metabolism. Under certain conditions, most saccharolytic clostridia are able to convert pyruvate to lactate by lactate dehydrogenase. However, pyruvate is mainly cleaved by pyruvate-ferredoxin oxidoreductase to form acetyl-CoA and CO_2 with concurrent reduction of ferredoxin (Uyeda and Rabinowitz, 1971a,b). Three oxidoreductases, namely, hydrogenase, NADH-ferredoxin reductase, and NADPH-ferredoxin reductase, are involved in electron transfer to various acceptors. The nature of the fermentation depends to a large extent on their relative enzyme activities. The reduced electron carrier ferredoxin plays an essential role: it can donate electrons to form either hydrogen via hydrogenase or the pyridine nucleotides via the ferredoxin-NAD(P) reductase (Kim et al., 1984). The acetyl-CoA generated by pyruvate-ferredoxin oxidoreductase can be further converted to either oxidized products (i.e., acetone, acetate, or CO_2) or reduced products (i.e., butanol, ethanol, or butyrate). Butyrate and butanol formation from acetyl-CoA occurs through acetyl-CoA, β-hydroxybutyryl-CoA, and Butyryl-CoA as intermediates (Hartmanis and Gatenbeck, 1984; Seedorf et al., 2008).

3. Enzymes characterization

3.1. EC 2.7.1.69: glucose phosphotransferase system

The major mechanism for carbohydrate uptake by clostridia is the phosphoenolpyruvate (PEP)-linked phosphotransferase system (PTS) in which the uptake and phosphorylation of substrates are simultaneous processes (Mitchell and Tangney, 2005). Similar to other well-studied Gram-positive bacteria such as *Staphylococcus aureus* (Hengstenberg et al., 1968), *Bacillus subtilis* (Marquet et al., 1971), and *Bacillus stearothermophilus* (Harris and Kornberg, 1972), the clostridial PTS consists of the general PTS phosphoryl-carrier proteins of EI and HPr as well as a membrane-associated and sugar-specific EII (Tangney and Mitchell, 2007). It was shown that both the soluble and the membrane fractions were required for PTS activity in *C. acetobutylicum*. Sugar phosphorylation and translocation occur according to a reaction series (Postma and Lengeler, 1985). First, phosphate from PEP is transferred to EI and then from phosphorylated EI (P-EI) to the carrier protein of HPr. In the second step, the phosphorylation and translocation of the sugar are carried out by the inducible membrane-associated EII complex with phosphorylated HPr (P-HPr) as a donor.

The specificity of the PTSs toward sugars differs amongst the clostridia. For example, the uptake of glucose, sucrose, maltose, lactose, and mannitol was mediated via PTS in *C. acetobutylicum* (Yu et al., 2007; Mitchell et al., 1995), whereas with *C. thermocellum* the PTS mechanism was operative for mannitol, but the uptake of glucose was mediated via a non-PTS system (Hernandez, 1982). The effect of butanol concentration on the activity of phosphotransferase (PT) of *C. acetobutylicum* has been studied (Hutkins and Kashket, 1986). A nonmetabolizable glucose analog, 2-deoxyglucose (DG), was used to study the activity of PT. The uptake of DG was only observed when an exogenous energy source such as fructose was also supplied. Similar to glucose uptake, higher uptake rates of DG were found during acidogenesis than during solventogenesis (Ounine et al., 1985). The addition of butanol decreased the level of 2-deoxyglucose-6-phosphate (DG6P) intracellularly. However, the addition of butanol did not decrease the total amount of DG6P produced since DG6P was extracellularly detected. The addition of PEP to butanol-treated cells significantly enhanced the phosphotransferase activity in the acidogenic-phase but not in the solventogenic-phase. The authors concluded that butanol acted primarily as a chaotropic agent to disrupt the cell membrane and caused the release of intracellular metabolites including PEP. Although, the cells had a decreased glucose phosphotransferase activity during solventogenesis, butanol was not responsible for the decrease.

The kinetic studies from the Enzyme II of *B. subtilis* with varying concentrations of P-HPr and methyl-α-D-glucopyranoside (αMG) suggested a ping-pong mechanism with the K_m values of 0.001 mM and 0.04 mM, respectively (Marquet et al., 1978). Likewise, the analysis of the kinetic data from the transphosphorylation reaction catalyzed by fructose PTS in *B. subtilis* showed a ping-pong reaction mechanism (Perret and Gay, 1979). The reported K_m values of some selected Gram-positive organisms are given in Table 1. The discrepancy between the reported K_m values of *C. acetobutylicum* for glucose could be due to strain differences, growth media, culture methods, and cell extract preparation. The K_m value for DG uptake was more than 7-fold higher than the average value (i.e., 0.023 mM) for glucose uptake. The lower affinity of DG uptake system could be due to energy depletion, because the cells easily exhausted their energy supplies (Hutkins and Kashket, 1986).

3.2. EC 2.7.1.11: phosphofructokinase (PFK)

Phosphofructokinase is an allosteric enzyme that catalyzes a unique step in glycolysis, namely the phosphorylation of fructose-6-phosphate (F6P) using ATP as phosphate donor to form fructose-1,6-diphosphate and ADP. This enzyme has been purified and analyzed from *C.*

Table 1
Michaelis constant for sugar metabolism from typical Gram-positive organisms.

Organism	Sugar	*Km* (mM)	Reference
C. acetobutylicum	D-deoxyglucose	0.170	Hutkins and Kashket (1986)
	Glucose	0.013	Ounine et al. (1985)
	Glucose	0.034	Mitchell et al. (1991)
B. stearothermophilus	Glucose	0.050	Harris and Kornberg (1972)
	Methyl α-glucoside	0.020	

pasteurianum (Uyeda and Kurooka, 1970). The enzyme, PFK reported to have a native molecular weight of 144 KDa with four identical subunits and its maximum activity was observed at pH values ranging from 7.0 to 8.2. The activity was approximately 60% of the optimum at pH 6 and 10 and no activity was detected at pH 5. This enzyme requires both Mg^{2+} and NH_4^+ or K^+. Magnesium cation forms a complex with ATP, which is perhaps the true substrate of the enzymatic reaction (Blangy et al., 1968). It has been suggested that NH_4^+ may stimulate glycolysis through the activation of PFK to increase the rate of ATP synthesis, which is necessary for nitrogen fixation (Uyeda and Kurooka, 1970). The K_m for NH_4^+ and K^+ were determined to be 0.18 mM and 20 mM, respectively.

The kinetics of PFK from *C. pasteurianum* was investigated with variable concentrations of ATP at fixed concentrations of F6P ranging from 0.12 to 0.6 mM (Uyeda and Kurooka, 1970). The double reciprocal plots were linear and provided a series of parallel lines. The PFK activity was not inhibited by ATP, which was in contrast with the activity of this enzyme from other sources such as yeast (Vinuela et al., 1963), *Escherichia coli* (Kemerer and Griffin, 1976), and mammalian tissues (Bloxham and Lardy, 1973). The K_m value for ATP was 0.055 mM, which was close to that from *E. coli* at 0.06 mM (Blangy et al., 1968). The catalytic activity of PFK responded sigmoidally with respect to F6P concentrations at an ATP range of 0.2–1 mM and pH levels of 6, 7, and 8.1. ADP was a positive effector and converted this sigmoidal response to a hyperbolic one. The Hill coefficients of F-6-P were lowered from 4.2 to 1.7 when the ADP concentration increased from zero to 10 mM in the presence of 1 mM ATP. The enzyme PFK was not significantly inhibited by PEP, F-1,6-DP, and phosphate. However, AMP at a concentration of 5 mM enhanced the enzymatic activity by 30%.

Belouski et al. (1998) cloned and sequenced the phosphofructokinase gene (*pfk*) from *C. acetobutylicum* ATCC 824. The allosteric PFK from *C. acetobutylicum* was composed of 319 amino acids with 76% similarity and 58% identity to its counterpart from *B. stearothermophilus*. An analysis of the kinetic data from *B. stearothermophilus* revealed that the mechanism of PFK from this organism followed a sequential random mechanism (Byrnes et al., 1994). They also identified the *pyk* gene encoding pyruvate kinase adjacent to *pfk* and showed that the two genes were organized within a single operon. Phosphofructokinase and pyruvate kinase catalyze two of the three irreversible reactions in the EMP pathway and have pivotal regulatory functions in this pathway (Boiteux and Hess, 1981).

3.3. EC 1.2.1.12: glyceraldehyde-3-phosphate dehydrogenase (GA3PDH)

Glyceraldehyde-3-phosphate dehydrogenase catalyzes the reversible phosphorylation of glyceraldehyde-3-phosphate to glycerate-1,3-diphosphate. This enzyme was determined to be specific for NAD rather than NADP with the NAD half saturation concentration of 0.39 mM for *C. pasteurianum* and 0.23 mM for *C. butyricum* (Jungermann et al., 1973). The NAD-linked GA3PDH from *C. acetobutylicum* DSM 792 has been purified (Schreiber and Durre, 1999). GA3PDH was inhibited by high intracellular NADH/NAD ratios and no activity was detected by NADH/NAD ratios greater than one (Girbal and Soucaille, 1994). Similar inhibition effects have been reported for GAPDH of *C. thermolacticum* (Collet et al., 2006). Schreiber and Durre (1999) measured the specific activity of the enzyme from crude extract of this organism to be 0.48 U/mg. These authors reported a native molecular weight of 160 kDa for GA3PDH and suggested a homotetrameric structure for this enzyme. Maximal pH-dependent activity of the enzyme was observed between pH = 8.5 and pH = 9.3. The specific activity of the enzyme from different stages of *C. acetobutylicum* fermentation revealed that this enzyme was not significantly regulated during the shift from acidogenic to solventogenic metabolism. A high level of similarity (96%) and identity (95%) were observed between NAD-linked GA3PDH of *C. acetobutylicum* and *C. pasteurianum*.

3.4. EC 1.2.7.1: pyruvate-ferredoxin oxidoreductase (PFOR)

The pyruvate resulting from glycolysis is cleaved by pyruvate-ferredoxin oxidoreductase (PFOR) in presence of coenzyme-A to yield carbon dioxide and acetyl-CoA with concomitant conversion of oxidized ferredoxin to its reduced form (Menon and Ragsdale, 1997). The ferredoxin of clostridia produces 1 mol of each acetyl-CoA and carbon dioxide per mole of ferredoxin reduced with concurrent transfer of two electrons (Uyeda and Rabinowitz, 1971a). Acetyl-CoA plays a central role in the branched fermentation pathways leading to both acid and solvent production. The formation of acetyl-CoA from pyruvate in aerobic bacteria is catalyzed by pyruvate dehydrogenase containing flavin and lipoic acid. In contrast, PFOR contains an iron–sulfur-chromophor in the redox center that shuttles the electron flow from pyruvate to ferredoxin. PFOR from *C. acetobutylicum* was purified and partially characterized (Meinecke et al., 1989). The enzyme contains thiamine pyrophosphate, iron, and sulfur. A thiamine pyrophosphate of 0.39 mol/mol of monomer was calculated based on apparent molecular weight of 123,000/monomer. Since, thiamine pyrophosphate was not covalently attached to the enzyme and was easily lost during purification, a thiamine pyrophosphate content of at least 1 mol/monomer could be assumed. The enzyme monomer contained 2.92 mol of sulfur and 4.13 mol of iron. The pyruvate-ferredoxin oxidoreductase of clostridia is a very unstable enzyme and very sensitive to the presence of oxygen. Upon the exposure to pure oxygen, the enzyme was 50% inactivated within an hour, while inactivation was not observed under a nitrogen atmosphere after 24 h.

Pyruvate and CoA are the substrates for PFOR with respective K_m values of 0.322 and 0.0037 mM for *C. acetobutylicum*(Meinecke et al., 1989). The enzyme can be inhibited by CoA at concentrations higher than 0.1 mM. The PFOR enzyme from *Halobacterium halobium* was also inhibited by high concentration of CoA; the mechanism was consistent with a competitive substrate inhibition of CoA versus pyruvate (Kerscher and Oesterhelt, 1981). The sequence of reactions catalyzed by PFOR in *C. thermoaceticum* (Menon and Ragsdale, 1997) and in *C. acidi-urici* (Uyeda and Rabinowitz, 1971b) revealed that the enzyme first reacts with pyruvate and releases carbon dioxide; then the acetyl group is cleaved by CoA to form acetyl-CoA. The above observations suggest a ping pong bi bi mechanism and the rate equation in the absence of CoA inhibition can be written as follows:

$$v_8 = \frac{V_{max,8}\,[\mathrm{Pyr}]\,[\mathrm{CoA}]}{[\mathrm{Pyr}]\,[\mathrm{CoA}] + 0.322\,[\mathrm{CoA}] + 0.0037\,[\mathrm{Pyr}]} \quad (1)$$

3.5. EC 2.3.1.9: thiolase (acetyl-CoA acetyltransferase)

Thiolase carries out the thermodynamically unfavorable condensation of two molecules of acetyl-CoA to one molecule of acetoacetyl-CoA, which is the precursor of the four-carbon products of *C. acetobutylicum* (Bennett and Rudolph, 1995). The change in the level of this enzyme in a batch of *C. acetobutylicum* has been studied (Hartmanis and Gatenbeck, 1984). They found high levels of thiolase activity throughout the fermentation, with the maximal activity occurring shortly after growth arrest. Thiolase competes with phosphate acetyltransferase (EC 2.3.1.8)

during acidogenesis and with acetaldehyde dehydrogenase (EC 1.2.1.10) during solventogenesis for the available pool of acetyl-CoA. The latter could influence the ratio of butanol plus acetone to ethanol. Formation of acetic acid yields twice as much ATP per mole of acetyl-CoA compared with butyric acid formation. Thus, the yield of ATP is highly dependent on the regulation of thiolase.

Thiolase from *C. acetobutylicum* ATCC 824 has been purified and characterized (Wiesenborn et al., 1988). This enzyme appears to be a tetramer with an estimated monomer size of 44 kDa. Thiolase activity from *C. acetobutylicum* has a very broad pH optimum ranging from pH 5.5 to 7.9. The internal pH of *C. acetobutylicum* remained within the range of 5.6 to 6.2 during the different stages of a typical batch fermentation (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985). Thus, the change in internal pH does not appear to affect the regulation of thiolase.

The Lineweaver–Burk plots for the thiolysis reaction suggested a ping-pong binding mechanism with the K_m values of 4.7 and 32 μmol respectively for CoA and acetoacetyl-CoA (Wiesenborn et al., 1988). The rate of condensation reaction was studied by varying the acetyl-CoA concentration from 0.099 to 1 mM. In order to study the effect of CoA on the condensation reaction, three concentrations of CoA (9.7, 19.3, and 38.6 μM) were employed. It was observed that with increasing concentration of CoA the acetyl-CoA saturation curve became increasingly sigmoidal. Consequently, the Lineweaver–Burk plot deviated from a straight line. This phenomenon has been reported with other thiolases (Oeding and Schlegel, 1979; Berndt and Schlegel, 1975). The kinetic data revealed that this thiolase was sensitive even to very low ratio of CoA to acetyl-CoA and in the presence of free CoA the reaction rate was less. We may conclude that this ratio is an important factor in modulation of the net rate of the condensation reaction. The intracellular concentrations of CoA and acetyl-CoA in extracts of *C. kluyveri* were estimated to be 0.24 and 0.9 mM, respectively (Decker et al., 1976). This level of CoA concentration in *C. acetobutylicum* would result in a strong product inhibition.

In the absence of CoA a Hill coefficient of 1 was observed, while this coefficient approached 1.45 in the presence of CoA. The above mentioned observations suggest a multi-substrate ping pong mechanism (Fromm, 1975). The following rate equation for two identical substrate molecules with product inhibition is proposed:

$$\frac{1}{v_9} = \frac{1}{V_{\max,9}} + \frac{K_{m,\mathrm{AcCoA}}}{V_{\max,9}\,[\mathrm{AcCoA}]}\left[1 + \frac{[\mathrm{CoA}]}{K_{I,\mathrm{CoA}}}\right] + \frac{K'_9}{V_{\max,9}}\frac{[\mathrm{CoA}]}{[\mathrm{AcCoA}]^2} \tag{2}$$

The kinetic constants of Eq. (2) were determined using the kinetic data reported by Wiesenborn et al. (1988) at 30 °C and pH 7.4 (Fig. 2 of their work). In the absence of CoA, the linear behavior of the condensation reaction on the double-reciprocal plot proved a Michaelis–Menten mechanism with $K_{m,\mathrm{AcCoA}}$ value of 0.275 mM. The maximum reaction rate, $V_{\max,9}$, was determined to be 0.0102 μmol/min. It can be seen from Eq. (2) that at fixed concentration of acetyl-CoA, when $1/v_9$ is graphed as a function of CoA, the slope term is:

$$\mathrm{Slope} = \frac{K_{m,\mathrm{AcCoA}}}{V_{\max,9}\,K_{I,P}}\frac{1}{[\mathrm{AcCoA}]} + \frac{K'_9}{V_{\max,9}}\frac{1}{[\mathrm{AcCoA}]^2} \tag{3}$$

A secondary plot of Slope [AcCoA] versus 1/[AcCoA] showed a linear behavior. The $K_{I,P}$ and K'_9 constants were determined to be 7.033 and 0.0086 mM, respectively. Consequently, the rate of condensation reaction can be written as:

$$v_9 = \frac{V_{\max,9}\,[\mathrm{AcCoA}]^2}{[\mathrm{AcCoA}]^2 + 0.275\,[\mathrm{AcCoA}]\left(1 + \frac{[\mathrm{CoA}]}{7.033}\right) + 0.0086\,[\mathrm{CoA}]} \tag{4}$$

Butyryl-CoA or ATP may act as feedback inhibitors if they accumulate to high internal concentrations. Relative to the control, the activity with butyryl-CoA was 58% at 1 mM and 85% at 0.2 mM, and activity with ATP was 59% at 10 mM and 90% at 2 mM (Wiesenborn et al., 1988).

3.6. EC 1.1.1.35(157): β-hydroxybutyryl-CoA dehydrogenase (BHBD)

The enzyme β-hydroxybutyryl-CoA dehydrogenase (oxidoreductase) in clostridia catalyzes the reduction of acetoacetyl-CoA by NAD(P)H. This reaction is a key step toward production of butyrate and butanol. The specific activity of BHBD in batch culture of *C. acetobutylicum* ATCC 824 increased during the exponential growth and reached a plateau after growth ceased (Hartmanis and Gatenbeck, 1984). The BHBD activity in phosphate limited chemostat cultures of *C. acetobutylicum* ATCC 824 showed eight-fold higher activity in solventogenic culture than acidogenic one (Vasconcelos et al., 1994). The BHBD from *C. kluyveri* utilizes NADPH as co-substrate; the K_m values were 0.05 and 0.07 mM for acetoacetyl-CoA and NADPH, respectively (Madan et al., 1973). The reaction rate with NADH as substrate was negligible (0.25%) in comparison to NADPH. The kinetic data suggested a ternary-complex mechanism. By close inspection, one could estimate the equal values of dissociation and the Michaelis constants for the substrates. The enzyme showed a maximum activity with a pH range from 5.5 to 7. In contrast, the analysis of this enzyme from *C. acetobutylicum* P262 (Youngleson et al., 1989) and from *C. acetobutylicum* ATCC 824 (Hartmanis and Gatenbeck, 1984) indicated it requires NADH as the reducing agent. The activity of both NADH- and NADPH-linked BHBD in the forward (physiological) direction was maximal at pH 5.0 and decreased steadily as the pH was increased to 8.0 (Colby and Chen, 1992). The specific activity of the NADH-linked enzyme was about 5-fold higher than that of NADPH-linked one. In the reverse (nonphysiological) direction, both NAD- and NADP-linked enzymes showed maximum activity at pH 8.0. Colby and Chen (1992) also reported the K_m values of 0.014 and 0.0086 mM for acetoacetyl-CoA (0.001 to 0.1 mM) and NADH (0.0095 to 0.07 mM), respectively, with evidence of substrate inhibition by acetoacetyl-CoA. We attempted to fit their data using typical mechanisms with substrate inhibition (e.g., Theorell–Chance, ordered, random, and ping-pong bi bi), but none of the models was suitable for describing the complex behavior of this enzyme. Finally, using the combination of the terms in these mechanisms, the following model was obtained by comprehensive regression analysis:

$$v_{10} = \frac{V_{\max,10}[\mathrm{AcAcCoA}]\,[\mathrm{NADH}]}{[\mathrm{AcAcCoA}]\,[\mathrm{NADH}] + 0.0143\,[\mathrm{NADH}]\left(1 + \frac{[\mathrm{NADH}]}{-0.990}\right) + 0.0072[\mathrm{AcAcCoA}]\left(1 + \frac{[\mathrm{AcAcCoA}]}{0.128}\right) - 6\times10^{-5}} \tag{5}$$

The maximum error associated with the model was less than 5%.

3.7. EC 4.2.1.17: enoyl-CoA hydratase (crotonase) (ECH)

In physiological (forward) direction, crotonase catalyzes the dehydration of β-hydroxybutyryl-CoA to crotonyl-CoA (Seedorf et al., 2008). This enzyme has been purified from *C. acetobutylicum* and analyzed in the non-physiological (reverse) direction (Waterson et al., 1972). This enzyme has a molecular weight of about 158 kDa and is composed of 4 subunits. The rate of hydration reaction was sensitive to high concentrations of crotonyl-CoA and showed a strong substrate inhibition mechanism for substrate concentrations higher than 0.071 mM. Waterson et al. (1972) also reported a maximum reaction rate of 4.1×10^4 μmol/min $mg_{protein}$ and a K_m value of 0.03 mM for crotonyl-CoA. An apparent equilibrium constant of 0.075 was determined for this enzymatic reaction.

3.8. EC 1.3.99.2: butyryl-CoA dehydrogenase (BCD)

Butyryl-CoA dehydrogenase is similar to acyl-CoA dehydrogenase (ACD) in eukaryotes (Youngleson et al., 1989). ACD contains flavin adenine dinucleotide (FAD) as a cofactor and catalyzes the oxidation of acyl-CoA to yield enoyl-CoA. Butyryl-CoA dehydrogenase in *C. acetobutylicum* acts in reverse direction and catalyzes the conversion of crotonyl-CoA to butyryl-CoA, which is a key branch point in butyrate and butanol formation. This enzyme has been shown to be present in many clostridia such as *C. butylicum*, *C. butyricum*, and *C. kluyveri* (v. Hugo et al., 1972). This enzyme from *C. acetobutylicum* has not been characterized. Hartmanis and Gatenbeck (1984) tried to purify the enzyme, but their attempts were not successful. They could not detect any activity with physiological electron donors of NADH and NADPH due to the problems with their assay methods. The gene *bcd*, which codes for BCD, along with an essential set of genes for butanol production were cloned and expressed in *E. coli* (Atsumi et al., 2008). Although the expression of the synthetic pathway produced 13.9 mg/l of butanol, the activity of BCD was not detected in crude extract of the cells. Plausible reasons have been proposed such as: 1) the enzyme is oxygen sensitive and inactivated during preparation (Hartmanis and Gatenbeck, 1984); 2) It is possible that BCD uses FAD as cofactor for electron transfer rather than NAD(P)H. The findings of Williamson and Engel (1984) and Whitfield and Mayhew (1974) support this possibility. They purified electron transfer flavoproteins from anaerobic bacteria together with BCD. Moreover, the properties of bacterial BCD seem to be similar to those associated with ACD from mammalian mitochondria that also require the flavoprotein cofactor (Boynton et al., 1996a).

3.9. EC 1.1.1.27: lactate dehydrogenase (LDH)

Lactate dehydrogenase catalyzes the reduction of pyruvate to lactic acid. Although, many clostridia contain this enzyme, lactate is often not the major fermentation product (Andreesen et al., 1989). However, under stressful conditions such as iron limitation (Bahl et al., 1986), sulfate limitation (Bahl and Gottschalk, 1984), or CO gassing (Simon, 1947) at neutral pH, lactate became the major fermentation product. Hence, lactate is not produced unless the conversion of pyruvate to acetyl-CoA is partially blocked. The blockage of pyruvate increases the intracellular concentration of F1,6DP, which in turn activates LDH. The clostridial LDHs are different from those of *E. coli* (Garvie, 1980). For instance, *E. coli* LDH is reversible and is not activated by F1,6DP, whereas clostridial LDH is a nonreversible enzyme that reduces pyruvate to lactate and is activated by F1,6DP (Contag et al., 1990).

An NADH-linked LDH has been partially purified and characterized from *C. acetobutylicum* (Freier and Gottschalk, 1987). In addition to F1,6DP, calcium and magnesium ions were positive effectors of this enzyme. The enzyme had an optimal pH of 5.8; little activity was measured at pH below 5. The native enzyme had an apparent molecular weight of 159 kDa and was shown to be tetrameric.

3.10. EC 1.18.1.3(2): ferredoxin-NAD(P) reductase

NAD(P)H-ferredoxin oxidoreductases are key enzymes for electron transfer in clostridia. These enzymes are able to catalyze the oxidation of NAD(P)H or reduction of NAD(P). The function of these enzymes in saccharolytic clostridia has been investigated (Jungermann et al., 1973). They concluded that the catabolic action of NADH-ferredoxin oxidoreductase is necessary to accomplish the reduction of ferredoxin, because the reduction of ferredoxin ($E_o=-400$ mV) by NADH ($E_o=-320$ mV) is thermodynamically unfavorable. Both NADH-ferredoxin oxidoreductase (catalyzing the reduction of ferredoxin by NADH) and ferredoxin-NAD reductase (catalyzing the reverse reaction: the reduction of NAD) activities have been reported in cell-free lysates of *C. pasteurianum*, and *C. butyricum*. The activity of the NADH-ferredoxin oxidoreductase was 3.3 and 4.2 times higher than the ferredoxin-NAD reductase in *C. pasteurianum* and *C. butyricum*, respectively. The NADH-ferredoxin oxidoreductase required acetyl-CoA as an obligatory activator. Petitdemange et al. (1976) studied the distribution and regulation of these reductases in butyric group of clostridia, namely, *C. acetobutylicum*, *C. pasteurianum*, and *C. tyrobutyricum* under well defined conditions. They found that the specific activity of ferredoxin-NAD reductase in butylic type clostridia (*C. acetobutylicum*) was lower than in the butyric type *C. pasteurianum*, which appeared to be contrary to the metabolic function. Furthermore, the activity of NADH-ferredoxin oxidoreductase in *C. acetobutylicum* was as high in *C. tyrobutyricum*, which is of a butyric type clostridium. This is not expected as the metabolism of *C. acetobutylicum* does not need NADH-ferredoxin oxidoreductase activity (Wood, 1961). The type of culture of *C. acetobutylicum* (i.e., solvent- or acid-producing) did not affect the abovementioned trends.

Table 2
Specific activities of NADH- and NADPH-ferredoxin oxidoreductase in (I) acid producing and (II) solvent producing chemostat cultures of *C. acetobutylicum*.

Enzyme	Specific activity (μmol/min mg)	
	I	II
NADH-ferredoxin oxidoreductase	0.1680	0.024
Ferredoxin-NAD reductase	0.0326	0.293
NADPH-ferredoxin oxidoreductase	<0.0003	<0.0003
Ferredoxin-NADP reductase	0.0512	0.199

Most clostridia appear to lack the enzymes required for oxidation of glucose-6-phosphate to produce NADPH, so it has been assumed that the major function of NADPH-ferredoxin oxidoreductase is to generate NADPH (Jungermann et al., 1973). The specific activities of these reductases in phosphate limited chemostat cultures of *C. acetobutylicum* are shown in Table 2 (Vasconcelos et al., 1994).

As shown in Table 2, the specific activity of NADH-ferredoxin oxidoreductase from *C. acetobutylicum* was more than five-fold higher than ferredoxin-NAD reductase in acid-producing culture, whereas it was about twelve-fold lower in solvent producing culture. The high activity of NADH-ferredoxin oxidoreductase in acid-producing culture was probably due to the regeneration of NAD which was required for the oxidation of glyceraldehydes-3-phosphate. This also prevents the accumulation of NADH, which in turn slows down the metabolic flux via glycolytic pathways. The higher activity of NAD reducing enzyme could be attributed to the higher demand for NADH during the solventogenesis. This organism showed a low NADPH-ferredoxin oxidoreductase activity and high ferredoxin-NADP reductase activity suggesting the possibility of ferredoxin-NADP reductase involvement in the production of NADPH. Similarly, when grown on glucose or sucrose in batch fermentations the butyric group clostridia exhibited low NADPH-ferredoxin oxidoreductase and high ferredoxin-NADP activities (Petitdemange et al., 1976). The kinetic properties of saccharolytic clostridia are presented in Table 3.

The reduction of ferredoxin by NADH required Ac-CoA as an obligate activator. The reduction of NAD appeared to be strongly inhibited by NADH in all clostridia. This competitive inhibitory effect, however, did not prevent the enzyme from functioning to generate NADH in *C. acetobutylicum* (Table 2) and other clostridium (Petitdemange et al., 1976) and was merely a very effective mode of regulation of the enzyme activity, which correlated this enzyme with glyceraldehydes-3-phosphate dehydrogenase. Among the three clostridia studied by Petitdemange et al. (1976), *C. tyrobutyricum* was the only one that was able to grow either on glucose or pyruvate/acetate. Ferredoxin-NAD reductase was the only enzyme detected when this organism was grown on pyruvate, while both NADH-ferredoxin and ferredoxin-NAD enzyme activities were detected when glucose was the sole carbon source. No NADH regulatory effect on ferredoxin-NAD reductase could be detected when grown on pyruvate/acetate. The authors concluded that NADH-ferredoxin oxidoreductase activity in this organism was highly dependent on the carbon source. Glyceraldehydes-3-phosphate dehydrogenase was not active when the organism was grown on pyruvate, so there was no accumulation of NADH by this enzyme. It seems that there is no need for the action of NADH-ferredoxin oxidoreductase and the inhibition of ferredoxin-NAD reductase by NADH to limit its activity in order to control the NADH and NAD levels in the cells.

During ferredoxin reduction by NADPH-linked oxidoreductase the reaction required NAD as an activator with *C. butyricum*, while none of the common nucleotides had any effect with *C. pasteurianum* and *C. acetobutylicum*. Jungermann et al. (1973) observed an inhibitory effect of NADPH in the NADP reduction by reduced ferredoxin (employing ferredoxin-NADP reductase) with the enzyme from *C. pasteurianum*, whereas Petitdemange et al. (1976) did not detect any such as regulation. However, they also observed the inhibition of the enzyme activity by NADPH when too much DEAE-cellulose was used during the removal of ferredoxin from the cell free extracts.

3.11. EC 1.12.7.2: hydrogenase

In bacteria, the formation or consumption of hydrogen is catalyzed by hydrogenases. They are classified according to the transition metal cofactors (i.e., NiFe, Fe, and FeFe hydrogenases) associated with the protein (Vignais et al., 2001). The clostridial [FeFe]-hydrogenases lack other transition metals. The active site consists of four iron–sulfur clusters in addition to an H-cluster and it often catalyzes the reduction of protons to yield hydrogen at high turnover numbers (Adams, 1990). These kinds of hydrogenases are very sensitive to carbon monoxide and oxygen. Carbon monoxide is a competitive inhibitor of this enzyme and oxygen degrades the H-cluster of the enzyme (Baffert et al., 2008). The physiological electron donor of hydrogenase in *C. acetobutylicum* (Demuez et al., 2007) and *C. pasteurianum* (Tamiya et al., 1966; Chen and Mortenson, 1974) were reported to be reduced ferredoxin. Hydrogenase competes with ferredoxin NAD(P) reductase to oxidize the reduced ferredoxin. The redox potentials of ferredoxin and hydrogen electrode are very small and approximately equal (−410 and −420 mV, respectively), so even in the presence of high hydrogen concentration, reduced ferredoxin can transfer electrons to hydrogenase and release hydrogen (Gottschalk, 1986). Girbal et al. (1994) showed that at neutral pH the hydrogenase of *C. acetobutylicum* was an important factor in the alkalization of the cytoplasm. They observed a positive ΔpH(i.e. $pH_i - pH_e$) (interior alkaline) during acidogenic metabolism and negative (interior acidic) ΔpH in the cultures that produced mainly alcohols. The hydrogen evolution rate in acidogenic cultures was significantly higher. Protons accumulate inside cytoplasm under conditions of low hydrogenase activity, which in turn acidifies the cytoplasm. Different methods have been used to decrease hydrogenase activity in vivo in order to change product distribution from acid toward alcohol formation such as: 1) elevated levels of hydrogen concentration (Yerushalmi et al., 1985); 2) sparging the culture with carbon monoxide (Kim et al., 1984) that acts as a reversible hydrogenase inhibitor; 3)

Table 3
Kinetic properties of NADH- and NADPH-ferredoxin reductases in (I) *C. acetobutylicum*, (II) *C. pasteurianum*, and (III) *C. butyricum*.

Enzyme		Half saturation concentration (mM)			
		I[a]	II[a]	II[b]	III[b]
NADH—ferredoxin oxidoreductase	[S]:NADH	—	—	≪0.3	—
	[A]:Ac—CoA	0.08	0.08	0.22	0.09
Ferredoxin—NAD reductase	[S]:NAD	0.08	0.47	0.37	0.33
	[I]:NADH	0.01*	0.02*	<0.02*	<0.02*
NADPH—ferredoxin oxidoreductase	[S]:NADH	—	—	—	—
	[A]:NAD	—	—	—	<0.5
Ferredoxin—NADP reductase	[S]:NADP	0.02	0.07	0.03	0.17
	[A]:NAD	n.d.	5#	—	—
	[I]:NADH	n.d.	0.08*	—	—
	[I]:NADPH	n.d.	n.d.	0.25#	—

n.d.: not detected.
*Competitive.
#Noncompetitive.
[S]:substrate, [A]:activator, [I]:inhibitor.
[a] Petitdemange et al. (1976).
[b] Jungermann et al. (1973).

limiting iron in cultures to decrease active hydrogenase concentration (Junelles et al., 1988); 4) and by growth on mixtures of glucose and glycerol (Vasconcelos et al., 1994).

Demuez et al. (2007) proposed an improved method to purify hydrogenase from *C. acetobutylicum* with 16-fold higher specific activity than the previously reported value by Girbal et al. (2005). The authors reported a K_m value of 0.0033 mM and $V_{max,16}$ of 122 μmol/min mg for the electron carrier of ferredoxin. The following kinetic model is proposed for hydrogen evolution rate by hydrogenase:

$$v_{16} = \frac{V_{max,16}\,[\mathrm{RdFd}]}{0.0033 + [\mathrm{RdFd}]} \tag{6}$$

The activity of hydrogenase was about the same in acid- and solvent-producing cells: 8.47 and 7.83 U/mg, respectively (Andersch et al., 1983). There was no detectable activity at pH values below 6.0. The hydrogenase of solvent-producing cells are presumably present in an inactive form, because the pH during this phase of fermentation is around 4.5 that is much lower than 6.0.

3.12. EC 2.3.1.8: phosphotransacetylase (PTA) (phosphate acetyltransferase)

During acidogenic metabolism, the enzyme PTA converts acetyl-CoA to acetyl phosphate (acetyl-P), which in turn is converted to acetate by acetate kinase. In batch fermentation of *C. acetobutylicum*, the activity of PTA significantly decreased as the culture switched to solventogenic metabolism with a reduced acetate formation and cell growth (Hartmanis and Gatenbeck, 1984). Despite the importance of PTA in the carbon and energy flow in solvent producing clostridia, only a few studies on this enzyme have been reported. The enzyme has a molecular weight of 40 kDa (Hibbert and Kyrtopoulos, 1971). To date, no kinetic information has been reported for this enzyme from *C. acetobutylicum*; the only studies performed were for *C. kluyveri* (Hibbert and Kyrtopoulos, 1971; Kyrtopoulos and Satchell, 1972, 1973). Kyrtopoulos and Satchell (1972) proposed a random bimolecular mechanism for the acylation of phosphate by acetyl-CoA. They found that CoA and acetyl phosphate were more strongly bound to the enzyme than acetyl-CoA and phosphate, respectively. Based on these findings and the experimental data, they found that CoA acted as a competitive inhibitor towards acetyl-CoA and as a noncompetitive inhibitor towards phosphate. Their experimental results on the inhibition of acetyl phosphate, however, revealed a complex behavior. They showed that at constant concentration of acetyl-CoA and increasing level of acetyl-P, a region of inhibition was followed by one of partial recovery of activity, which in turn was finally followed by further inhibition. The enzyme activity recovery was observed at high concentrations of phosphate (i.e.,>400 mM). The location of this unusual behavior shifted to higher concentrations of acetyl-P as the concentration of phosphate decreased from 900 to 400 mM. In our model, at low concentrations of phosphate (i.e., <150 mM) and acetyl-P (i.e., <400 mM) the activity recovery of the enzyme was ignored. Using the data presented by the authors, the following rate equation for acylation of phosphate by acetyl-CoA is proposed:

$$v_{17} = \frac{V_{max,17}\,[\mathrm{AcCoA}]\,[P]}{\left\{[\mathrm{AcCoA}]\left(1 + \frac{[\mathrm{CoA}]}{0.217}\right) + 0.21\left(1 + \frac{[\mathrm{CoA}]}{0.062}\right)\right\}\left\{[P]\left(1 + \frac{[\mathrm{AcP}]}{3.1}\right) + 288\left(1 + \frac{[\mathrm{AcP}]}{0.714}\right)\right\}} \tag{7}$$

The maximum reaction rate was 615 μmol/min $mg_{protein}$. Kyrtopoulos and Satchell (1972) reported an acetyl-CoA Michaelis–Menten constant of 1.1 mM. However, a review of their data yielded a K_m value of 0.21 mM as the best estimate. In addition, they proposed a simple competitive inhibition behavior for products, but the abovementioned model was much more accurate describing their data. It is worth to mention that the enzyme from both *C. acetobutylicum* (Hartmanis and Gatenbeck, 1984) and *C. kluyveri* (Kyrtopoulos and Satchell, 1973) were activated by K^+ and NH_4^+ and inhibited by Na^+.

3.13. EC 2.7.2.1: acetate kinase (AK)

Acetate kinase phosphorylates ADP using acetyl-P to form ATP and acetate. This reaction is important for energy conservation during the acid-producing stage of the fermentation. The activity of acetate kinase in batch fermentation of *C. acetobutylicum* was maximal at the early stage of the exponential phase and dropped significantly before an associated decrease in phosphotransacetylase activity and before growth stoppage (Hartmanis and Gatenbeck, 1984). The decrease in acetate kinase activity was concomitant with the increase of acetate concentration in the medium (Ballongue et al., 1986). Acetate kinase was synthesized by this organism when acetate concentration was less than 3 g/l. The higher concentrations of acetate caused a rapid drop in acetate kinase activity. The acetate kinase from different strains of clostridia had a strict requirement for magnesium and seemed to have a requirement for manganese (Diez-Gonzalez et al., 1997; Winzer et al., 1997). No activity was detected when butyrate was used as substrate. Since only cation-nucleotide complexes function as substrate in phosphotransferase reactions, no clostridial acetate kinase activity could be observed in the absence of divalent cations (Morrison, 1979).

The acetate kinase of *C. acetobutylicum* P262 was isolated and characterized (Diez-Gonzalez et al., 1997). The enzyme had a pH optimum of 8.0 and native molecular mass of 78 kDa and was probably a homodimer. The K_m values for ADP, acetyl-P, ATP, and acetate were 6, <1, 2.5 and 160 mM, respectively. With acetate as the substrate with variable concentration, acetate kinase from *C. thermoaceticum* showed a normal Michaelis–Menten kinetics, while with ATP as the substrate a sigmoidal reaction rate curve was observed (Schaupp and Ljungdahl, 1974). The K_m and $S_{0.5}$ values for acetate and ATP were 135 and 1.64 mM. A Hill coefficient of 1.78 was determined using this data, which was an indicator of two interacting substrate sites.

An acetate kinase specific activity of 3.61 and 1.12 U/mg was reported in acid- and solvent-producing cells of *C. acetobutylicum* DSM 1732 (Andersch et al., 1983). This was in agreement with the specific activity of 3.7 μmol/min mg from *C. acetobutylicum* DSM 1731 (Winzer et al., 1997). The molecular mass of this enzyme was in the range of 87–94 kDa with subunits of 43 kDa each that was in good agreement with the calculated value of 44.3 kDa by Boynton et al. (1996b) suggesting a dimer form. Acetate substrate specificity leading to acetyl-P formation was observed with ATP as the preferred phosphate donor. Hyperbolic saturation curves for the substrates in both directions were observed. The apparent K_m values from double reciprocal plots were reported to be 0.71, 0.58, 0.37, and 73 mM for ADP, acetyl-P, ATP, and acetate, respectively. These kinetic parameters were not similar to that of *C. acetobutylicum* P262.

3.14. EC 1.2.1.10: acetaldehyde dehydrogenase (AYDH)

In solvent producing *C. acetobutylicum* and *C. beijerinckii*, AYDH catalyzes the first specific reaction toward ethanol (or butanol). Generally, AYDHs may be distinguished on the basis of their coenzyme requirements: 1) the CoA independent, NAD(P) linked AYDH; 2) the CoA and NAD(P) independent; and 3) CoA and NAD(P) dependent isoezymes (Yan and Chen, 1990). The latter was found in clostridia. The CoA acylating AYDH from *C. beijerinckii* NRRL B592 was purified and found to be a regulated enzyme; its activity level increased 25-fold during the switch from acid production to solvent production (Yan and Chen, 1990). The enzyme was sensitive to oxygen both in the crude extract and purified forms. In the forward (physiological) direction the enzyme showed a broad peak pH activity between 6.5 and 7.0, while the activity of the enzyme in the reverse reaction increased monotonically when the pH increased from 6.5 to 9.0. The purified AYDH contained no alcohol dehydrogenase activity. The same AYDH can catalyze the formation of both acetaldehyde and butyraldehyde for ethanol and butanol production. Yan and Chen (1990) reported the true K_m and V_{max} values for acetyl-CoA, NADH, and NADPH in the forward direction. For the NADH-linked reaction, the maximum specific reaction rate was 3.4×10^{-2} μmol/min $mg_{protein}$; the K_m values were 0.154 and 0.0082 mM for acetyl-CoA and NADH, respectively. The K_m value of 0.0196 mM for acetyl-CoA and 0.206 mM for NADPH and the maximum specific reaction rate of 1.74×10^{-2} μmol/min $mg_{protein}$ were determined for the NADPH-linked reaction. During the kinetic studies 0.021 mg of purified AYDH was used. The K_m for NADPH was about 25-fold higher than that for NADH, and the V_{max}/K_m ratio with respect to coenzyme was about 50-fold lower with NADPH than with NADH. Therefore, NADH is the more effective coenzyme than NADPH for AYDH. In double-reciprocal plots of initial velocities versus NAD(P)H as the varying substrate and acetyl-CoA as the changing fixed substrate parallel lines suggested a ping pong mechanism for the physiological direction reaction. Based on the provided information the following rate equation for this reaction in physiological direction can be written:

$$v_{19a} = \frac{V_{max,19a}[\mathrm{AcCoA}][\mathrm{NADH}]}{[\mathrm{AcCoA}][\mathrm{NADH}] + 0.154[\mathrm{NADH}] + 0.0082[\mathrm{AcCoA}]} \tag{8}$$

$$v_{19b} = \frac{V_{max,19b}[\mathrm{AcCoA}][\mathrm{NADPH}]}{[\mathrm{AcCoA}][\mathrm{NADPH}] + 0.0196[\mathrm{NADPH}] + 0.206[\mathrm{AcCoA}]} \tag{9}$$

3.15. EC 1.1.1.1(2): NAD(P)H ethanol dehydrogenase (EDH)

Ethanol dehydrogenase catalyzes the reduction of acetaldehyde to ethanol. From the point of view of alcohol production, the acetone- and butanol-producing clostridia can be classified into two groups. The members of the first group (e.g., *C. acetobutylicum*) are able to produce primary (1°) alcohols only such as butanol and ethanol, while the second group consists of organisms (e.g., *C. beijerinckii*) that produce a secondary (2°) alcohol such as isopropanol in addition to the 1° alcohols (Chen, 1995). Although DNA homology and other physiological characters of *C. acetobutylicum* and *C. beijerinckii* showed these two species are distinct, their solvent-producing properties were very similar (Cummins and Johnson, 1971; George et. al., 1983). Nevertheless, conflicting results had been reported regarding the cofactor requirement of the enzyme. The nicotinamide nucleotide-dependent enzymes that differ in their specificity for nicotinamide nucleotides may have different metabolic control mechanisms. For the same physiological function, nicotinamide nucleotide-dependent enzymes of one organism may be specific for NAD and for another organism for NADP (Smith et al., 1983), but NADH and NADPH have different physiological functions in electron transport systems (Matthews et al., 1997). Hiu et al. (1987) have studied the properties of ethanol dehydrogenase from both isopropanol producing (NRRL 562) and non- producing strains of *C. beijerinckii* that were harvested from the solvent-producing phase of batch cultures. The activities of EDH from both strains were higher with NADPH than with NADH (six- and three-fold higher with EDH from NRRL 562 and NRRL 563, respectively). On the other hand, the specific activity of EDH with NADH were four- and six-fold higher in solvent and acid producing phosphate limited chemostat cultures of *C. acetobutylicum* (Vasconcelos et al., 1994). It has been reported that the centrifugation speed and pH had significant effects on the enzymatic activity of butanol dehydrogenase (Durre et al., 1987). The NADH-linked enzyme from low-speed centrifugation extracts had higher activity than the high-speed extracts, whereas NADPH-linked enzyme was not significantly affected by the centrifugation speed. In contrast to the NADPH-dependent enzyme that showed higher activities at higher pH values, the activities of NADH-dependent enzyme at pH 7.8 and 6.0 were nearly the same. The supernatant for the analysis of EDH activity was prepared at 37000 ×*g* by Hiu et al. (1987), whereas Vasconcelos et al. (1994) used 9000 ×*g*. Thus, the discrepancy in EDH cofactor requirement could be mainly due to different centrifugation speeds as well as other factors such as the differences in species, growth medium, and cultivation methods. The same conflicting findings were reported for butanol dehydrogenase as addressed and discussed below. The kinetic properties and coenzyme specificity of the purified EDH will need to be investigated in more systematic way (i.e., at the physiological pH).

Vasconcelos et al. (1994) measured 5 to 8 fold higher EDH activities in solvent producing culture than the acid producing one. A five-fold increment in enzyme activity in vitro caused the same level of increase of ethanol formation in vivo. Consequently, higher expression of EDH at the genetic level appeared to control ethanol production. The same qualitative trend of the enzyme activities was observed in chemostat cultures of this organism (Husemann and Papoutsakis, 1989). Zero activity was observed for EDH of strain NRRL 562 in ethanol oxidation direction with both NAD and NADP; the enzyme from strain NRRL 563 also showed little activity with both nucleotides (Hiu et al., 1987). These findings may suggest that EDH has a function in ethanol production in this organism.

3.16. EC 2.8.3.8 & EC 2.8.3.9: acetoacetyl-CoA-acetate/butyrate-CoA-transferase

The formation of acetoacetate is catalyzed by a CoA-transferase which transfers CoA from aceoacetyl-CoA to either acetate (Doelle, 1975) or butyrate (Andersch et al., 1983). Carboxylate specificity studies from *C. acetobutylicum* revealed that it could utilize straight chain C1–C7 and branched chain carboxylic acids, but no activity was observed with dicarboxylic acids (Hartmanis et al., 1984). The highest rates relative to acetate were associated to propionate, formate, and butyrate. This enzyme was inhibited by sodium ions. These authors determined the equilibrium

constants of the reaction catalyzed by transferase to be between 16 and 46. In addition, they performed a survey of the batch fermentations of this organism and concluded that transferase–decarboxylase pathway was solely responsible for uptake of acetate and butyrate during solventogenesis. This mechanism was energetically more favorable than the ATP consuming reactions catalyzed by acetate and butyrate kinases. The uptake of acetate and butyrate by CoA-transferase of *C. acetobutylicum* serves as a detoxification mechanism by reducing the inhibitory effect of these acids on cell growth (Costa, 1981). The activity of CoA-transferase from *C. acetobutylicum* in phosphate limited chemostat was much higher in solvent-producing than in acid-producing cells (Andersch et al., 1983). The CoA transferase from *C. acetobutylicum* ATCC824 has a native molecular weight of 93 kDa and was likely a heterotetramer of two a and two β subunits (Wiesenborn et al., 1989b). This reference analyzed the kinetic data of the CoA-transferases (*R*20 and *R*21). On double-reciprocal plot for acetate conversion, they did not detect any evidence of substrate inhibition at the level of substrates studied (i.e., acetate ranging from 60 to 300 mM and acetoacetyl-CoA from 2.8 to 25 μM). They proposed K_m values of 1200 and 0.021 mM for acetate and acetoacetyl-CoA, respectively. The kinetics mechanism can be represented by the following rate equation:

$$v_{21} = \frac{V_{\max,21}[\mathrm{Ac}][\mathrm{AcAcCoA}]}{[\mathrm{Ac}][\mathrm{AcAcCoA}] + 1200[\mathrm{AcAcCoA}] + 0.021[\mathrm{Ac}]} \tag{10}$$

At the moderate concentrations of acetoacetyl-CoA as the changing fixed substrate approximately parallel lines were observed and became hyperbolic concave-up at high concentrations of the varied substrate of butyrate. Non-linearity was also observed in the double-reciprocal plots of reaction rate versus acetoacetyl-CoA concentration at different constant levels of butyrate. Butyrate varied ranging between 33 and 300 mM and acetoacetyl-CoA ranging between 3.9 and 100 μM. The fitted line for the highest concentration of the fixed substrate intersected with the other lines to the right of the reciprocal rate axis. This behavior is known as double competitive substrate inhibition that is consistent with a Ping-Pong bi bi kinetic binding mechanism (Fromm, 1975; Segel, 1975). It was observed that the inhibitory effect of one substrate at high concentration diminished by increasing the level of the other substrate. Wiesenborn et al. (1989b) determined the Michaelis and inhibition constants for butyrate to be 660 and 410 mM, respectively, and for acetoacetyl-CoA to be 0.056 and 0.21 mM, respectively. The maximum reaction rate was 2.6×10^{-4} mM/min. The following rate equation for this reaction is accordingly proposed:

$$v_{22} = \frac{V_{\max,22}[\mathrm{But}][\mathrm{AcAcCoA}]}{[\mathrm{But}][\mathrm{AcAcCoA}] + 660[\mathrm{AcAcCoA}]\left(1 + \frac{[\mathrm{AcAcCoA}]}{0.21}\right) + 0.056[\mathrm{But}]\left(1 + \frac{[\mathrm{But}]}{410}\right)} \tag{11}$$

3.17. EC 4.1.1.4: acetoacetate decarboxylase (AADC)

The acetoacetate formed via CoA-transferase is subsequently decarboxylated by acetoacetate decarboxylase to yield acetone and carbon dioxide. The reaction is nearly irreversible. The large equilibrium constant (4×10^4 at 25 °C) implied that this reaction provided the driving mechanism for the uptake of acetate and butyrate from the medium (Hartmanis et al., 1984). They concluded that the formation of acetone was coupled with the uptake of acetate and butyrate. Acetoacetate decarboxylase is the key enzyme in the pathway of acetone formation from acetoacetyl-CoA. The mechanism of decarboxylation was proposed by a series of elegant studies (Westheimer, 1995; Highbarger and Gerlt, 1996). The decarboxylase is induced rather late in the fermentation when growth ceases (Autor 1970; Andersch et al., 1983). Although the CoA-transferase activity occurs earlier during the fermentation, no uptake of acetate and butyrate from the medium occurs until the decarboxylase is induced. The activity of the enzyme was almost 40-fold higher in solvent-producing cells (4.93 U/mg) as compared to acid-producing cells (0.13 U/mg) (Andersch et al., 1983). The optimum pH of the enzyme was reported to be 5 by Davies (1943) and 5.95 by Coutts (1972) so it well suited for acetone production at acidic pH values. Pyruvate causes 64% inhibition (competitive) at a concentration of 0.05 M. This enzyme was inhibited by low concentrations of a variety of monovalent anions such as HSO_3^-, NO_3^-, and Cl^- by combining with the identical active site, while divalent anions such as CO_3^{2-}, SO_4^{2-}, and HPO_4^{2-} did not inhibit apparently at 0.1 M (Fridovich, 1963). A normal Michaelis–Menten kinetics was observed for acetoacetate decarboxylase from *C. acetobutylicum* with fairly low affinity of the enzyme for acetoacetate; the K_m value for acetoacetate was reported to be 7.3 mM by Fridovich (1968) and 8.2 ± 0.8 mM by Highbarger and Gerlt (1996), which were in good agreement with the measured value of 8 mM by Davies (1943). The enzyme rate equation can be expressed by the following simple rate equation:

$$v_{23} = \frac{V_{\max,23}[\mathrm{AcAc}]}{8.2 + [\mathrm{AcAc}]} \tag{12}$$

3.18. EC 2.3.1.19: phosphotransbutyrylase (PTB) (phosphate butyryltransferase)

This enzyme catalyzing the interconversion of butyryl-CoA and butyryl-P and was purified from *C. acetobutylicum* (Gavard et al., 1957). It seems that two separate enzymes (PTB and PTA) are responsible for the formation of bytyryl-P and acetyl-P, respectively. The activity of PTB with acetyl-CoA was negligible (1.6% relative rate) compared with its activity with butyryl-CoA (Wiesenborn et al., 1989a). In addition to *C. acetobutylicum*, PTB was detected in other butyric acid forming clostridia such as *C. acetobutyricum*, *C. saccharoacetobutylicum*, and *C. sporogenes*, while *C. kluyveri* did not possess this enzyme (Valentine and Wolfe, 1960). PTB and butyrate kinase together enable the conversion of butyryl-CoA to butyrate. The specific activity of PTB from *C. acetobutylicum* DSM 1732 was two-fold higher in acid-producing cells (5.38 U/mg) than in solvent-producing cells (2.64 U/mg) in phosphate limited chemostat (Andersch et al., 1983). The activity of this enzyme in batch fermentation of *C. acetobutylicum* ATC 824 was not growth related and peaked during the early exponential phase and dropped significantly afterwards (Hartmanis and Gatenbeck 1984; Hartmanis et al., 1984). Although, the change in activity of this enzyme from *C. acetobutylicum* DSM 1732 differed; the activity of the enzyme increased during the exponential and deceleration phases and then decreased about 30% relative to its peak at the end of the fermentation (Andersch et al., 1983). The discrepancy could be due to using different strains of *C. acetobutylicum*, growth medium, and enzyme activity assay methods.

The enzyme, PTB from *C. acetobutylicum* ATC 824 has been purified almost to homogeneity (Wiesenborn et al., 1989a). These authors determined a native molecular weight of 264 kDa for this enzyme and assumed that it consisted of eight subunits of equal molecular weight. The enzyme had a pH optimum of 8.1 in the physiological (butyryl-P forming) direction. The activity of the enzyme was very sensitive to pH within the physiological range of 5.8 to 7 (Huang et al., 1985) with no apparent activity below pH of 6. The enzyme was less sensitive in the reverse (butyryl-CoA forming) direction and showed a broad pH optimum ranging from 7.5 to 8.7. Inhibition studies of nucleotides of the butyryl-P forming reaction revealed 18–20% inhibition by ATP, ADP, and NADP at the concentration levels of 2, 5, and 10 mM, respectively. The concentration of ATP as the product of acidogenic metabolism and NAD(P) as the product of solventogenic metabolism, however, were generally less than the levels considered significant for inhibitory effects (Vasconcelos et al., 1994). In their kinetic studies in the physiological direction, they tested the inhibitory effect of butyryl-P only and due to the practical difficulties they were not able to determine the inhibitory effect of CoA. The inhibitory effect of butyryl-P was tested at concentrations of up to 0.52 mM (two times of its K_m). The concentration of each substrate was fixed at twice the value of its K_m while the concentration of other substrate was varied up to quadruple the value of its K_m. Based on their analysis they concluded that the enzymatic reaction was consistent with a random bi bi binding mechanism. In physiological direction, inhibition by butyryl-P was competitive with respect to butyryl-CoA but noncompetitive with respect to phosphate. The inhibition by butyryl-P was competitive with respect to the both substrates in the reverse reaction, whereas phosphate inhibited noncompetitively. The K_m values at 30 °C and pH 8.0 for butyryl-CoA, Phosphate, butyryl-P, and CoA were 0.11, 14, 0.26, and 0.077 mM, respectively, and the dissociation constants were 0.22 mM, 28 mM, 0.54 μM, and 0.16 μM, respectively. The inhibition constant of butyryl-P was 0.35 mM. The following rate equation was fitted to the reported data of Vasconcelos et al. (1994) for the physiological reaction:

$$v_{24} = \frac{V_{\max,24}[\text{BurlCoA}][P]}{[\text{BurlCoA}][P] + K_{m,\text{BurlCoA}}[P]\left(1 + \frac{[\text{BurlP}]}{K_{I,\text{BurlP}}}\right) + K_{m,P}[\text{BurlCoA}] + K_{i,\text{BurlCoA}}K_{m,P}\left(1 + \frac{[\text{BurlP}]}{K_{I,\text{BurlP}}}\right)} \tag{13}$$

Upon substitution of the kinetics parameters, Eq. (13) can be written as follows:

$$v_{24} = \frac{V_{\max,24}[\text{BurlCoA}][P]}{[\text{BurlCoA}][P] + 0.11[P]\left(1 + \frac{[\text{BurlP}]}{0.35}\right) + 14[\text{BurlCoA}] + 3.08\left(1 + \frac{[\text{BurlP}]}{0.35}\right)} \tag{14}$$

The proposed model gave a maximum reaction rate of 0.0228 mM/min. The double-reciprocal plot of reaction rate versus phosphate exhibited a slight nonlinearity even at zero butyryl-P concentration and the deviation from linearity becomes more pronounced as the concentration of butyryl-CoA product increases. In the proposed equation the nonlinearity has been ignored, however, it was still able to predict the experimental data with a maximum relative error of 11%. The minimum and maximum intracellular concentrations of butyryl-CoA were reported to be 512 and 1002 μmol associated with the acidogenic and solventogenic batch fermentation of *C. acetobutylicum* (Boynton et al., 1994). These levels of concentrations should be able to saturate PTB, so that the maximal flux through this enzyme is expected. Since, the activity of this enzyme was negligible at pH below 6, the level of butyryl-CoA did not seem to be a significant factor in regulation of PTB, as reported previously (Wiesenborn et al., 1989a).

3.19. EC 2.7.2.7: butyrate kinase (BK)

Butyrate kinase from *Clostridium acetobutylicum* catalyzes the reaction by which butyryl-P is converted to butyrate with concomitant ADP phosphorylation. This phosphorylation reaction is an important energy source in butyric clostridia and *C. acetobutylicum* during the acid formation stage (Valentine and Wolfe, 1960; Hartmanis, 1987). Both PTB and butyrate kinase may play an important role in butyrate uptake. Hartmanis et al. (1984) suggested that butyrate uptake in *C. acetobutylicum* occurs only through CoA-transferase (EC 2.8.3.9). In contrast, Meyer et al. (1986) found that when continuous butyrate producing culture of this organism was sparged with carbon monoxide, there was a high uptake rate of butyrate with concomitant formation of butanol, but no acetone. The absence of acetone implied that CoA-transferase was not involved in butanol formation. This pathway was shown to be reversible in vitro (Valentine and Wolfe, 1960). Huang et al. (2000) claimed that butyrate kinase also catalyzes the reverse reaction by which butyrate is converted to butyryl-P using ATP as the phosphate donor. These conflicting findings suggest that the regulation of acid re-uptake and solvent formation is very complex.

The influence of acetic acid concentration up to 3 g/l did not affect butyrate kinase activity (Ballongue et al., 1986). The specific activity of butyrate kinase was more than 6-fold higher in acidogenic fermentation (3.02 U/mg) than during solventogenic metabolism (0.47 U/mg) in phosphate limited chemostat fermentation of *C. acetobutylicum* DSM 1732 (Andersch et al., 1983). The butyrate kinase activity was detected during all stages of batch fermentation of this organism with maximum activity at the end of exponential phase. The activity of this enzyme decreased to 56% of its maximum toward the end, when cell growth creased. In contrast, two distinct maxima for butyrate kinase activity were observed in batch fermentation for *C. acetobutylicum* ATC 824 (Hartmanis and Gatenbeck, 1984). The first maximum activity occurred late in the exponential phase. The second peak was much higher and appeared in the middle of stationary phase. The uptake of previously produced butyrate occurred at the second boost in the enzyme activity, but as discussed above, there was no decisive evidence that this enzyme was also involved in the uptake of butyrate during the solventogenesis when the activity of this enzyme decreased to very low levels towards the end of fermentation.

Butyrate kinase was partially purified from extracts of *C. tetanomorphum* (Twarog and Wolfs, 1963) and *C. butyricum* (Twarog and Wolfs, 1962) and purified to homogeneity from *C. acetobutylicum* (Hartmanis, 1987). Butyrate kinase from *C. butyricum* phosphorylated butyrate and propionate at equal rates, whereas its affinity for acetate was very low. The butyrate kinase (BKI) activity from *C. acetobutylicum* ATC 824 markedly decreased by disruption of the *buk* gene, however, the formation of butyrate was not eliminated (Green et al., 1996). This finding motivated Huang et al. (2000) to look for another gene responsible for butyrate formation. These researchers succeeded to identify, purify, and characterize a second butyrate kinase isoenzyme (BKII) which was highly homologous with BKI. They found that the kinetic properties of BKII were not consistent with a role in butyrate formation, so they concluded that another butyrate kinase with less sequence homology with BKI and BKII was responsible for butyrate production in the BKI deleted strain. The native molecular weight of BKI (Hartmanis, 1987) and BKII (Huang et al., 2000) were measured to be 85 and 80 kDa, respectively, and both were presumed to be dimers. In the butyryl-P direction the maximum

activity of BKI was near pH 7.5, while that of BKII was 8.5. The maximum specific reaction rate of BKI in butyryl-P formation direction was 402 U/mg, while the apparent K_m values of BKI were determined to be 14 mM and 1.4 mM for butyrate and ATP, respectively. The higher Michaelis constant of 620 mM for butyrate and lower maximum reaction rate of 165 U/mg for BKII were probably due to the fused thioredoxin protein (Huang et al., 2000).

3.20. EC 1.2.1.57: butyraldehyde dehydrogenase (BYDH)

Butyraldehyde dehydrogenase catalyzes the conversion of butyryl-CoA to butyraldehyde with concomitant formation of CoA. Andersch et al. (1983) detected a low level of BYDH activity (0.04 U/mg) in solvent producing cells of *C. acetobutylicum* and were not able to detect any activity in acid producing cells. Durre et al. (1987) developed a reliable assay method for detection and quantification of BYDH in extracts of this organism. They found that the centrifugation speed during extract preparation had a profound effect on the enzyme activity. The best results for BYDH activity was observed in the low-speed extracts. Apparently, the enzyme was inactivated by oxygen. In batch fermentation of *C. acetobutylicum* DSM 1732, the enzyme was synthesized 3 h before butanol production and reached to its maximum activity soon after butanol formation after which its activity dropped sharply to undetectable levels. BYDH was not active in the purely acidogenic chemostat culture of *C. acetobutylicum* (Husemann and Papoutsakis, 1989). The NADH-linked butyraldehyde dehydrogenase activity was not detected in chemostat culture of *C. acetobutylicum* at neutral pH in non-solvent producing culture when glucose was the only carbon source in the feed, but the enzyme was present in solvent producing cultures in which glucose–glycerol mixtures were used (Vasconcelos et al., 1994). The NADPH-linked enzyme was not detected in either of the cultures. The specific activity of BYDH rose 200-fold upon transition from acidogenesis to solventogenesis in batch cultures of this organism (Palosaari and Rogers, 1988). These authors concluded that butanol formation in chemostat of *C. acetobutylicum* was regulated at the genome level via butyraldehyde dehydrogenase expression. However, a comparison between the activity of the enzyme in vitro and in vivo showed that additional controls on butanol formation should operate at the enzyme level (Husemann and Papoutsakis, 1989). In addition, they showed that this enzyme was induced most strongly in the presence of carbon monoxide, followed by butyrate and glucose. The CoA-linked butyraldehyde from *C. acetobutylicum* B643 was purified by Palosaari and Rogers (1988). The active form of the enzyme was a homodimer and had a native molecular weight of 115 kDa. The authors found that the activity of butyraldehyde dehydrogenase co-purified with acetaldehyde dehydrogenase, since the proportion of the two activities during the purification process remained nearly constant (11.5 U/mg). Anion-exchange chromatography showed a single, symmetric peak of butyraldehyde dehydrogenase that co-eluted with acetaldehyde dehydrogenase, suggesting that a single enzyme protein carried both activities. The maximum activity of this enzyme in the butyryl-CoA forming direction was observed at about pH 9.0. In the physiological direction, the K_m values for butyryl-CoA, acetyl-CoA, NADH, and NADPH were 0.045, 0.055, 0.003, and 0.04 mM, respectively. The K_m values in the reverse direction were 1.6, 7.2, 0.045, 0.16, and 2.0 mM for butyraldehyde, acetaldehyde, CoA, NAD, and NADP, respectively. The apparent affinity of NADH and NAD for BYDH was far greater than that of NADPH and NADP, suggesting that this enzyme was NADH-specific. This was in agreement with the data reported by others (Durre et al., 1987; Vasconcelos et al., 1994). The maximum reaction rate was determined to be 18.1×10^{-3} mM/min, which was 5.5-fold higher than that for acetyl-CoA. The reaction was inhibited by CoA concentrations higher than 0.3 mM. The inhibition of CoA-linked aldehyde dehydrogenase was also reported for *C. kluyveri* (Smith and Kaplan, 1980). The kinetic dada for acetaldehyde dehydrogenase suggested a ping pong mechanism for the reaction (Yan and Chen, 1990; Smith and Kaplan, 1980). In the absence of CoA inhibition, a ping-pong mechanism for the forward reaction rate can be written as follows:

$$v_{26} = \frac{V_{\max.26}[\text{BurlCoA}][\text{NADH}]}{[\text{BurlCoA}][\text{NADH}] + 0.045[\text{NADH}] + 0.003[\text{BurlCoA}]} \tag{15}$$

3.21. EC 1.1.1: butanol dehydrogenase (BDH)

Butanol dehydrogenase catalyzes the final step in butanol synthesis, the reduction of butyraldehyde to butanol at the expense of a reduced NAD(P). The NADP-specific butanol dehydrogenase activity was reported to be induced at the same time with BYDH (Palosaari and Rogers, 1988). This enzyme was not found in acid-producing cells of *C. acetobutylicum* DSM 1732 and its activity of 0.06 U/mg was very low even in solvent-producing cells (Andersch et al., 1983). The use of different assays during the early BDH enzymatic investigations led to confusion regarding the coenzyme requirements of this enzyme (Andersch et al., 1983; Husemann and Papoutsakis, 1989; George and Chen, 1983; Girbal et al., 1995). The assay systems performed for butanol dehydrogenase from *C. acetobutylicum* DSM 1732 (Durre et al., 1987) and *C. acetobutylicum* ATCC 824 (Welch et al., 1989) clearly revealed that the enzyme had two different coenzyme requirements: the NADH-dependent butanol dehydrogenase (NADH-BDH) with higher activity at lower pHs and the NADPH-dependence with higher activity at more basic pHs. The activity of NADH-linked enzyme was higher in extracts obtained by low-speed centrifugation. The cloning of an NADPH-linked alcohol dehydrogenase from *C. acetobutylicum* P262 was reported (Youngleson et al., 1988). Ethanol and butanol were equally utilized by this enzyme and thus was classified as an alcohol dehydrogenase.

Welch et al. (1989) purified BDH-NADH dependent enzyme to near homogeneity and characterized this enzyme from *C. acetobutylicum* ATCC 824. This butanol dehydrogenase was later designated as BDH II (Petersen et al., 1991). The enzyme from crude extract had specific activities of 0.042 and 0.040 U/mg for NADH- and NADPH-dependent enzymes, respectively. Both activities required Zn^{2+} in the buffer to obtain a higher recovery of activities during the purification. The rate of butyraldehyde reduction by purified BDH II was 50-fold higher than the rate of butanol oxidation under the assayed conditions. This enzyme showed nearly 46-fold greater activity with butyraldehyde than with acetaldehyde, while another NADH-dependent butanol dehydrogenase, which was designated as BDH I, was only about twofold more active with butyraldehyde than acetaldehyde. The NADH-BDHs activity was maximal at pH 5.5 in butanol-forming direction and its activity decreased sharply in either sides of this optimum pH. The activity of enzyme in the reverse direction was maximal at pH 7. The two BDHs exhibited very similar properties, including a similar native molecular weight of slightly larger than 80 kDa that was composed of two equal molecular weight subunits (Walter et al., 1992).

Kinetic studies were conducted at the substrate concentration ranges of 0.05 to 1.2 mM for NADH and 2.5 to 50 mM for butyraldehyde (Welch et al., 1989). Inhibition studies were performed at butanol concentrations of 5 and 40 mM. The double-reciprocal plots were consistent with ternary complex mechanisms as the lines intersect at one point on the left of the $1/v$ axis. The slope and intercept of the secondary plots gave a maximum reaction rate of 7.04 ($K_{cat}=4.86\ s^{-1}$), and K_m values of 16 mM and 0.18 mM for butyraldehyde and NADH, respectively (Welch et al., 1989). Lineweaver–Burk plots of reaction rate versus butyraldehyde or NADH at different butanol concentrations revealed that butanol was a noncompetitive

inhibitor with respect to both butyraldehyde and NADH. These authors then concluded that the results were consistent with an ordered bi bi mechanism with NADH binding first and NAD being released last. They estimated a dissociation constant, $K_{i,BuOH}$, of 100 mM for BDH-butanol complex. We found that the data presented for product inhibition studies were not consistent with the data presented for the substrates data. The authors probably used different concentrations from different batches, but no corresponding concentration corrections have been made. More specifically, the data reported in product inhibition studies for zero level of product were not the same as the data reported in the plots that were presented earlier in the paper. Corrections of the data were made and the erroneously reported parameters were accordingly recalculated. The ordered bi bi mechanism with a product that shows noncompetitive inhibition for both substrates (Fromm, 1975) can be rearranged for the model under study as follows:

$$v_{27} = \frac{V_{max,27}[\text{Bual}][\text{NADH}]}{[\text{Bual}][\text{NADH}]\left(1 + \frac{[\text{BuOH}]}{213}\right) + 16[\text{NADH}] + 0.18([\text{Bual}] + 21)(1 + 0.017[\text{BuOH}])} \quad (16)$$

The maximum reaction rate was determined to be 7.04 μmol/min. When the intercepts of the double reciprocal plots of reaction rate versus butyraldehyde concentration were graphed as a function of butanol concentration, a linear behavior was observed that was completely consistent with the proposed model. The same figures of $1/v_{27}$ versus 1/[NADH] showed a parabolic concave downwards behavior, which was not explained by the proposed model. However, the maximum error due to assuming linear approximation was ± 13%. The addition of CoA and CoA containing compound such as acetyl- and butyryl-CoA at concentration levels of 0.2 mM caused a large decrease in the enzyme activity. Inhibition by addition of ATP was also observed, although not as large as in case of CoA. The inhibition by CoA, CoA containing compounds, and ATP was probably due to the competition of these metabolites with NADH for the coenzyme binding site (Welch et al., 1989).

4. Concluding remarks

The complex metabolism of *C. acetobutylicum* has been elaborated from different prospectives. Studies regarding solvent, particularly butanol production in *C. acetobutylicum* focused on different aspects such as the development of continuous fermentation technology (Bahl et al., 1982b; Gottschal and Morris, 1981a), improvements in strain tolerance towards butanol (Borden and Papoutsakis, 2007; Tomas et al., 2003), understanding of the physiological relationships between organic acid metabolism and the initiation of solvent production (Zhao et al., 2005; Gottschal and Morris, 1981b), and genetic modifications (Sillers et al., 2008; Gonzalez-Pajuelo et al., 2005; Tummala et al., 2003; Harris et al., 2001).

One of the main goals in biotechnology is the development of strains with enhanced yield of a desired product. Although, the traditional methods of random or targeted mutagenesis have been successful in the past, these have been largely superseded by genetic manipulations based on metabolic engineering principles (Bailey, 1991). Metabolic engineering comprises the elucidation of the metabolic system structure and kinetics of the intracellular enzymatic reactions as the first step and the implementation of the findings by genetic or environmental modifications of the selected organism as the following step (Torres and Voit, 2002). To accomplish the latter, one needs to alter not only the protein content of a given organism, but also its enzymatic profile. As a first component of metabolic engineering, it is essential to have a thorough understanding of the mechanisms and the associated regulations involved in the uptake of the substrates and their further metabolism. Modeling and collection of the key enzymatic reactions for butanol production in *C. acetobutylicum* are therefore an important first step in gaining insights into its complex metabolism that could lead to the construction of metabolically engineered production strains. The reported set of key kinetic information involved in the metabolic pathways of *C. acetobutylicum* can be used for this purpose. These kinetic studies can make a significant contribution to ABE fermentation as an industrial process. However, modeling of such a complex system in the framework of Michaelis–Menten formalism becomes overwhelming. The authors are aiming to translate the kinetic information in the present report into more comprehensive and integrated mathematical models such as Generalized Mass Action Systems or S-Systems (Voit, 2000). Using this approach, the dynamics of the system can be formulated by a set of differential equations, each representing the change in one dependent variable. The steady state equations of the S-system are linear when formulated in a logarithmic coordinate, which allows optimization of biochemical systems under steady state conditions. The development of the mathematical models would help to investigate the response of the system to change of metabolite and enzyme levels and to improve the production of the targeted products in cultures of this organism. In addition, it would be possible to estimate the optimum profile of enzyme concentrations contributing in the map to achieve a given goal and to search for minimum subset of the enzymes which may need to be modified to produce near optimal conditions (Sevilla et al., 2005). In order to represent a more accurate model for the metabolism of *C. acetobutylicum,* however, specific kinetic data for some regions of the metabolic pathway of this organism needs to be studied.

Acknowledgements

The authors thank CellNet and NSERC for financial support and acknowledge the cooperation of the Ministry of Science, Research, and Technology of Iran.

Appendix A. Enzyme EC number and name

EC number	Name	Abbreviation
2.7.1.69	Phosphotransferase system	PTS
5.3.1.9	Glucose-6-phosphate isomerase	G6PI
2.7.1.11	6-Phosphofructokinase	PFK
1.2.1.12	Glyceraldehyde-3-phosphate dehydrogenase	GA3PDH
2.7.1.40	Pyruvate kinase	PK
1.2.7.1	Pyruvate—ferredoxin oxidoreductase	PFOR
2.3.1.9	Thiolase	
1.1.1.35	β-hydroxybutyryl-CoA dehydrogenase (NADH-linked)	BHBD
1.1.1.157	β-hydroxybutyryl-CoA dehydrogenase (NADPH-linked)	BHBD
4.2.1.17	Crotonase (enoyl-CoA hydratase)	ECH
1.3.99.2	Butyryl-CoA dehydrogenase	BCD
1.1.1.27	Lactate dehydrogenase	LDH
1.18.1.2	Ferredoxin-NADP reductase NADPH—ferredoxin oxidoreductase	
1.181.3	Ferredoxin—NAD reductase NADH—ferredoxin oxidoreductase	
1.12.7.2	Ferredoxin hydrogenase (hydrogenase)	
2.3.1.8	Phosphotransacetylase (phosphate acetyltransferase)	PTA
2.7.2.1	Acetate kinase	AK
1.2.1.10	Acetaldehyde dehydrogenase	AYDH
1.1.1.1	Ethanol dehydrogenase (NADH-linked)	EDH
1.1.1.2	Ethanol dehydrogenase (NADPH-linked)	EDH
2.8.3.8	Acetoacetyl-CoA:acetate:CoA-transferase	
2.8.3.9	Acetoacetyl-CoA:butyrate:CoA-transferase	
4.1.1.4	Acetoacetate decarboxylase	AADC
2.3.1.19	Phosphotransbutyrylase (phosphate butyryltransferase)	PTB
2.7.2.7	Butyrate kinase	BK
1.2.1.57	Butyraldehyde dehydrogenase	BYDH
1.1.1	Butanol dehydrogenase	BDH

Appendix B. Chemicals abbreviation

Ac	Acetate
AcAc	Acetoacetate
AcAcCoA	Acetoacetyl-CoA
Acal	Acetaldehyde
AcCoA	Acetyl-CoA
Acon	Acetone
AcP	Acetyl phosphate
ADP	Adenosine-5-diphosphate
AMP	Adenosine-5-monophosphate
ATP	Adenosine-5-triphosphate
Bual	Butyraldehyde
BurlCoA	Butyryl-CoA; Butanoyl-CoA
BurlP	Butyryl phosphate; Butanoyl phosphate
BuOH	Butanol
But	Butyrate
CO_2	Carbon dioxide
CoA	Coenzyme-A
CrlCoA	Crotonyl-CoA
DEAE	Diehylaminoethyl
DG	Deoxyglucose
DG6P	Deoxyglucose-6-phosphate
EtOH	Ethanol
F1,6DP	Fructose-1,6-diphosphate
F6P	Fructose-6-phosphate
G1,3DP	Glycerate-1,3-diphosphate
G6P	Glucose-6-phosphate
GA3P	Glyceraldehyde-3-phosphate
Glc	Glucose
H	Hydrogen ion; proton
H_2	Hydrogen molecule
H_2O	Water
βHBCoA	β-Hydroxybutyryl-CoA
Lac	Lactate
αMG	methyl-α-D-glucopyranoside
NAD	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
Ox–Fd	Ferredoxin (oxidized)
P	Inorganic Orthophosphate
Pyr	Pyruvate
Rd–Fd	Ferredoxin (reduced)

Appendix C. Metabolic reactions

R1) $Glc_{Ext} + PEP \Rightarrow G6P + Pyr$
R2) $G6P \Rightarrow F6P$
R3) $F6P + ATP \Rightarrow F1,6DP + ADP$
R4) $F1,6DP \Rightarrow 2GA3P$
R5) $GA3P + NAD + P \Rightarrow G1,3DP + NADH + H$
R6) $G1,3DP + ADP \Rightarrow PEP + ATP + H_2O$
R7) $PEP + ADP \Rightarrow Pyr + ATP$
R8) $Pyr + CoA + OxFd \Rightarrow AcCoA + CO_2 + RdFd + 2H$
R9) $2\ AcCoA \Rightarrow AcAcCoA + CoA$
R10) $AcAcCoA + NAD(P)H + H \Rightarrow \beta\text{-}HBCoA + NAD(P)$
R11) $\beta\text{-}HBCoA \Rightarrow CrlCoA + H_2O$
R12) $CrlCoA + NADH + H \Rightarrow BurlCoA + NAD$
R13) $Pyr + NADH + H \Rightarrow Lac + NAD$
R14) $RdFd + NADP + H \Rightarrow OxFd + NADPH$
R15) $RdFd + NAD + H \Rightarrow OxFd + NADH$
R16) $RdFd + 2H \Rightarrow OxFd + H_2$
R17) $AcCoA + P \Rightarrow AcP + CoA$
R18) $AcP + ADP \Rightarrow Ac + ATP$
R19) $AcCoA + NAD(P)H + H \Rightarrow Acal + CoA + NAD(P)$
R20) $Acal + NAD(P)H + H \Rightarrow EtOH + NAD(P)$
R21) $AcAcCoA + Ac \Rightarrow AcAc + AcCoA$
R22) $AcAcCoA + But \Rightarrow AcAc + BurlCoA$
R23) $AcAc + H \Rightarrow Acon + CO_2$
R24) $BurlCoA + P \Rightarrow BurlP + CoA$
R25) $BurlP + ADP \Rightarrow But + ATP$
R26) $BurlCoA + NAD(P)H + H \Rightarrow Bual + CoA + NAD(P)$
R27) $Bual + NAD(P)H + H \Rightarrow BuOH + NAD(P)$

References

Abou-Zeid AA, Fouad M, Yassein M. Fermentative production of acetone-butanol by *Clostridium acetobutylicum*. Indian J Technol 1976;14:349–52.

Adams MW. The structure and mechanism of iron-hydrogenase. Biochim Biophys Acta 1990;1020:115–45.

Alvarez-Vasquez F, Gonzalez-Alcon C, Torres NV. Metabolism of citric acid production by *Aspergillus niger*: model definition, steady state analysis and constrained optimization of the citric acid production rate. Biotechnol Bioeng 2000;70:82–108.

Andersch W, Bahl H, Gottschalk G. Level of enzymes involved in acetate, butyrate, acetone, and butanol formation by *Clostridium acetobutylicum*. Eur J Appl Microb Biotechnol 1983;18:327–32.

Andreesen JR, Bahl H, Gottschalk G. Introduction to the physiology and biochemistry of the genus *Clostridium*. In: Minton NP, Clarke DJ, editors. Clostridia. New York: Plenum Press; 1989. p. 27–62.

Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, et al. Metabolic engineering of *Escherichia coli* for 1-butabol production. Met. Eng. 2008;10:305–11.

Autor AP. An investigation of acetoacetate decarboxylase. USA: Dissertation, Duke University; 1970.

Baffert C, Demuez M, Cournac L, Burlat B, Guigliarelli B, Bertrand P, et al. Hydrogen-activating enzymes: activity does not correlate with oxygen sensitivity. Angew Chem Int Ed 2008;47:2052–4.

Bahl H, Andersch W, Braun K, Gottschalk G. Effect on pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* growing in a continuous culture. Eur J Appl Microbiol Biotechnol 1982a;14:17–20.

Bahl H, Andersch W, Gottschalk G. Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. Eur J Appl Microbiol Biotechnol 1982b;15:201–5.

Bahl H, Gottschalk G. Parameters effecting solvent production by *Clostridium acetobutylicum* in continuous culture. Biotechnol Bioeng Symp 1984;14:215–23.

Bahl H, Gottwald M, Kuhn A, Rale V, Andersch W. Gottschalk, nutritional factors affecting the ratio of solvents produced by *Clostridium acetobutylicum*. Appl Environ Microbiol 1986;52:169–72.

Bailey J. Toward a science of metabolic engineering. Science 1991;252:1668–74.

Ballongue J, Amine J, Masion E, Petitdemange H, Gay R. Induction of acetoacetate decarboxylase in *Clostridium acetobutylicum*. FEMS Microbiol Lett 1985;29:273–7.

Ballongue J, Amine J, Petitdemange H, Gay R. Regulation of acetate kinase and butyrate kinase by acids in *Clostridium acetobutylicum*. FEMS Microbiol Lett 1986;35:295–301.

Belouski ED, Watson D, Bennett GN. Cloning, sequencing, and expression of the phosphofructokinase gene of *Clostridium acetobutylicum* ATCC 824 in *Escherichia coli*. Curr Microbiol 1998;37:17–22.

Bennett GN, Rudolph FB. The central metabolic pathway from acetyl-CoA to butyryl-CoA in *Clostridium acetobutylicum*. FEMS Microbiol Rev 1995;17:241–9.

Berndt H, Schlegel HG. Kinetics and properties of β-ketothiolase from *Clostridium pasteurianum*. Arch Microbiol 1975;103:21–30.

Blangy D, Buc H, Monod J. Kinetics of the allosteric interactions of phosphofructokinase from *Escherichia coli*. J Mol Biol 1968;31:13–35.

Blaschek HP, White BA. Genetic systems development in the clostridia. FEMS microbiol Rev 1995;17:349–56.

Bloxham DP, Lardy HA. In: Boyer PD, editor. Phosphofructokinase. The enzymes, 3rd editionUSA, NY: Academic Press, Inc.; 1973. p. 239–78.

Boiteux A, Hess B. Design of glycolysis. Phil Trans R Soc Lond B 1981;293:5–22.

Borden JR, Papoutsakis ET. Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. Appl Environ Microbiol 2007;73:3061–8.

Bowles LK, Ellefson WL. Effects of butanol on *Clostridium acetobutylicum*. Appl Environ Microbiol 1985;50:1165–70.

Boynton ZL, Bennett GN, Rudolph FB. Intracellular concentrations of coenzyme A and its derivatives from *Clostridium acetobutylicum* ATCC 824 and their roles in enzyme regulation. Appl Environ Microbiol 1994;60:39–44.

Boynton ZL, Bennett GN, Rudolph FB. Cloning, sequencing, and expression of clustered genes encoding β-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase, and butyryl-CoA dehydrogenase from *Clostridium acetobutylicum* ATCC 824. J. Bacteriol. 1996a;178:3015–24.

Boynton ZL, Bennett GN, Rudolph FB. Cloning, sequencing, and expression of genes encoding phosphotransacetylase and acetate kinase from *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 1996b;62:2758–66.

Byrnes M, Zhu X, Younathan ES, Chang SH. Kinetic characteristics of phosphofructokinase from Bacillus stearothermophilus: MgATP nonallosterically inhibits the enzyme. Biochem 1994;33:3424–31.

Calam CT. Isolation of *Clostridium acetobutylicum* strains producing butanol and acetone. Biotechnol Lett 1980;2:111–6.

Cangenella F, Weigel J. The potential of thermophilic clostridia in biotechnology. In: Woods DR, editor. The clostridia and biotechnology. MA, USA: Butterworth-Heinenmann; 1993. p. 393–429.
Chen JS, Mortenson LE. Purification and properties of hydrogenase from *Clostridium Pasteurianum* W5. Biochim Biophys Acta 1974;371:283–98.
Chen JS. Alcohol dehydrogenase: multiplicity and relatedness in the solvent-producing clostridia. FEMS Microbiol Rev 1995;17:263–73.
Claassen PAM, van Lier JB, Lopez-Contreras AM, van Niel EWJ, Sijtsma L, Stams AJM, et al. Utilization of biomass for the supply of energy carriers. Appl Microbiol Biotechnol 1999;52:741–55.
Claassen PAM, Budde MAW, Lopez-Contreras AM. Acetone, butanol, and ethanol production from domestic organic waste by solventogenic clostridia. J Mol Microbiol Biotechnol 2000;2:39–44.
Colby GD, Chen JS. Purification and properties of 3-hydroxybutyryl-Coenzyme A dehydrogenase from *Clostridium Beijerinckii* ("*Clostridium butylicum*") NRRL B593. Appl Environ Microbiol 1992;58:3297–302.
Collet C, Girbal L, Peringer P. Metabolism of lactose by *Clostridium thermolacticum* growing in continuous culture. Arch Microbiol 2006;185:331–9.
Compere AL, Griffith WL. Evaluation of substrates for butanol production. dev Ind Microbiol 1979;20:509–17.
Connell HC. The study and treatment of cancer by proteolytic enzymes. A preliminary report. Can Med Ass J 1935;33:364–70.
Contag PR, Williams MG, Rogers P. Cloning of a lactate dehydrogenase gene from *Clostridium acetobutylicum* B643 and expression in *Escherichia coli*. Appl Environ Microbiol 1990;56:3760–5.
Cornillot E, Nair RV, Papoutsakis ET, Soucaille P. The genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824 reside on a large plasmid whose loss leads to degeneration of the strain. J Bacteriol 1997;179:5442–7.
Costa JM. Solvent toxicity in the acetone-butanol fermentation. Proc Ann Biochem Eng Symp 1981;11:83–90.
Coutts SM. Ph.D. dissertation. USA: Harvard University; 1972.
Cummins CS, Johnson JL. Taxonomy of the clostridia: wall composition and DNA homologies in *Clostridium butyricum* and other butyric acid-producing clostridia. J Gen Microbiol 1971;67:33–46.
Cynkin MA, Delwiche EA. Metabolism of pentoses by clostridia. I. Enzymes of ribose dissimilation in extracts of *Clostridium perfringens*. J Bacteriol 1958;75:331–4.
Davies R, Stephenson M. Studies on the acetone-butyl alcohol fermentation. 1. Nutritional and other factors involved in the preparation of active suspensions of *Cl. acetobutylicum* (Weizmann). Biochem J 1941;35:1320–31.
Davies R. Studies on the acetone-butanol fermentation. 4. Acetoacetic acid decarboxylase of *C. acetobutylicum* (BY). Biochem J 1943;37:230–8.
Decker K, Rossle M, Kreusch J. The role of nucleotides in the regulation of the energy metabolism of *Clostridium Kluyveri*. In: Schlegel HG, Gottschalk G, Pfennig N, editors. Proceedings of the symposium on microbial production and utilization of gases. Gottingen, Federal Republic of Germany: Goltze; 1976. p. 75–83.
Demuez M, Cournac L, Guerrini O, Soucaille P, Girbal L. Complete activity profile of *Clostridium acetobutylicum* [FeFe]-hydrogenase and kinetic parameters for endogenous redox partners. FEMS Microbiol Lett 2007;275:113–21.
Diez-Gonzalez F, Russell JB, Hunter JB. The acetate kinase of *Clostridium acetobutylicum* strain P262. Arch Microbiol 1997;166:418–20.
Doelle HW. Bacterial metabolism. 2nd edition. USA, NY: Academic Press; 1975.
Durre P. Fermentative butanol production bulk chemical and biofuel. Ann N Y Acad Sci 2008;1125:353–62.
Durre P, kuhn A, Gottwald M, Gottschalk G. Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 1987;26:268–72.
Fond O, Matta-Ammouri G, Petitdemange H, Engasser JM. The role of acids on the production of acetone and butanol by *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 1985;22:195–200.
Freier D, Gottschalk G. L(+)-lactate dehydrogenase of *Clostridium acetobutylicum* is activated by fructose-1,6-biphosphate. FEMS Microbiol Lett 1987;43:229–33.
Fridovich I. Inhibition of acetoacetic decarboxylase by anions. J Biol Chem 1963;238:592–8.
Fridovich I. A study of interaction of acetoacetic decarboxylase with several inhibitors. J Biol Chem 1968;243:1043–51.
Fromm HJ. Initial rate enzyme kinetics. Berlin: Springer-Verlag; 1975.
Gabriel CL, Crawford FM. Development of the butyl-acetonic fermentation industry. Ind Eng Chem 1930;22:1163–5.
Gapes JR. The economics of acetone-butanol fermentation: theoretical and market considerations. J Mol Microbiol Biotechnol 2000;2:27–32.
Garvie EI. Bacterial lactate dehydrogenase. Microbiol Rev 1980;44:106–39.
Gavard R, Hautecoer B, Descouetieux H. Phosphotransbutyrylase de *Clostridium acetobutylicum*. CR Acad Sci Sev D 1957;244:2323–6.
George HA, Chen JS. Acidic conditions are not obligatory for onset of butanol formation by *Clostridium beijerinckii* (synonym. *C. butylicum*). Appl Environ Microbiol 1983;46: 321–7.
George HA, Johnson JL, Moore WEC, Holdeman LV, Chen JS. Acetone, isopropanol, and butanol production by *Clostridium beijerinckii* (syn. *Clostridium butylicum*) and *Clostridium aurantibutylicum*. Appl Environ Microbiol 1983;45:1160–3.
Girbal L, Soucaille P. Regulation of *Clostridium acetobutylicum* metabolism as revealed by mixed-substrate continuous cultures: role of NADH/NAD ratio and ATP pool. J Bacteriol 1994;176:6433–8.
Girbal L, Vasconcelos I, Scoucaille P. Transmembrane pH of *Clostridium acetobutylicum* is inverted (more acidic inside) when the in vivo activity of hydrogenase is decreased. J Bacteriol 1994;176:6146–7.
Girbal L, Croux C, Vasconcelos I, Soucaille P. Regulation of metabolic shifts in *Clostridium acetobutylicum* ATCC 824. FEMS Microbiol Rev 1995;17:287–97.
Girbal L, von Abendroth G, Winkler M, et al. Homologous/heterologous over-expression in *Clostridium acetobutylicum* and characterization of purified clostridial and algal Fe-only hydrogenases with high specific activity. Appl Environ Microbiol 2005;71: 2777–81.
Gonzalez-Pajuelo M, Meynial-Salles I, Mendes F, Soucaille P, Vasconcelos I. Microbial conversion of glycerol to 1,3-propanediol: physiological comparison of a natural producer, *Clostridium butyricum* VPI 3266, and an engineered strain, *Clostridium acetobutylicum* DG1 (pSPD5). Appl Environ Microbiol 2006;72:96–101.
Gonzalez-Pajuelo M, Meynial-Salles I, Mendes F, Andrade JC, Vasconcelos I, Soucaille P. Metabolic engineering of *Clostridium acetobutylicum* for the industrial production of 1,3-propanediol from glycerol. Metab Eng 2005;7:329–36.
Gottschal JC, Morris JC. Non-production of acetone and butanol by *C. acetobutylicum* during glucose-and ammonium-limitation in continuous culture. Biotechnol Lett 1981a;3:525–30.
Gottschal JC, Morris JC. The induction of acetone and butanol production in cultures of *C. acetobutylicum* by elevated cultures of acetate and butyrate. FEMS Microbiol Lett 1981b;12:385–9.
Gottschalk G. Bacterial metabolism. 2nd edition. USA, NY: Springer Verlag; 1986.
Gottwald M, Gottschalk G. The internal pH of *Clostridium acetobutylicum* and its effect on the shift from acid to solvent formation. Arch Microbiol 1985;143:42–6.
Gottwald M, Hippe H, Gottschalk G. Formation of n-butanol from D-glucose by strains of "*Clostridium tetanomorphum*" group. Appl Environ Microbiol 1984;48:573–6.
Green EM, Boynton ZL, Harris LM, Rudolph FB, Papoutsakis ET, Bennett GN. Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. Microbiology 1996;142:2079–86.
Grupe H, Gottschalk G. Physiological events in *Clostridium acetobutylicum* during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. Appl Environ Microbiol 1992;58:3896–902.
Haggstrom L, Molin N. Calcium alginate immobilized cells of *Clostridium acetobutylicum* for solvent production. Biotechnol Lett 1980;2:241–6.
Harris LM, Desai RP, Welker NE, Papoutsakis ET. Characterization of recombinant strains of the *Clostridium acetobutylicum* butyrate kinase inactivation mutant: needs for new phenomenological models for solventogenesis and butanol inhibition? Biotechnol Bioeng 2000;67:1–11.
Harris LM, Blank L, Desai RP, Welker NE, Papoutsakis ET. Fermentation characterization and flux analysis of recombinant strains of *Clostridium acetobutylicum* with an inactivated solR gene. J Ind Microbiol Biotechnol 2001;27:322–8.
Harris P, Kornberg HL. The uptake of glucose by a thermophilic *Bacillus* species. Proc R Soc Lond B 1972;182:159–70.
Hartmanis MGN. Butyrate kinase from *Clostridium acetobutylicum*. J Bio Chem 1987;262: 617–21.
Hartmanis MGN, Gatenbeck S. Intermediary metabolism in *Clostridium acetobutylicum*: levels of enzymes involved in the formation of acetate and butyrate. Appl Environ Microbiol 1984;47:1277–83.
Hartmanis MGN, Klason T, Gatenbeck S. Uptake and activation of acetate and butyrate in *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 1984;20:66–71.
Hengstenberg W, Egan JB, Morse ML. Carbohydrate transport in *Staphylococcus aureus*. VI. The nature of the derivatives accumulated. J Biol Chem 1968;243:1881–5.
Hernandez PE. Transport of D-glucose in *Clostridium thermocellum* ATCC 27405. J Gen Appl Microbiol 1982;28:469–77.
Hibbert F, Kyrtopoulos SA. Satchell PN. Kinetic studies with phosphotransacetylase. Biochim Biophys Acta 1971;242:39–54.
Highbarger LA, Gerlt JA. Mechanism of the reaction catalyzed by acetoacetate decarboxylase. Importance of lysine 116 in determining the pK_a of active-site lysine 115. Biochemistry 1996;35:41–6.
Hiu SF, Zhu C, Yan RT, Chen JS. Butanol-ethanol dehydrogenase and butanol-ethanol-isopropanol dehydrogenase: different alcohol dehydrogenases in two strains of *Clostridium beijerinckii* (*Clostridium butylicum*). Appl Environ Microbiol 1987;53:697–703.
Huang KX, Huang S, Rudolph FB, Bennett GN. Identification and characterization of a second butyrate kinase from *Clostridium acetobutylicum* ATCC 824. J Mol Microbiol Biotechnol 2000;2:33–8.
Huang L, Gibbins LN, Forsberg CW. Transmembrane pH gradient and membrane potential in *Clostridium acetobutylicum* during growth under acidogenic and solventogenic conditions. Appl Environ Microbiol 1985;50:1042–7.
Husemann MHW, Papoutsakis ET. Enzymes limiting butanol and acetone formation in continuous and batch cultures of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 1989;31:435–44.
Hutkins RW, Kashket ER. Phosphotransferase activity in *Clostridium acetobutylicum* from acidogenic and solventogenic phases of growth. Appl. Environ. Microbiol. 1986;51: 1121–3.
Inui M, Suda M, Kimura S, Yasuda K, Suzuki H, Toda H, et al. Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Appl Microbiol Biotechnol 2008;77:1305–16.
Johnson MJ, Peterson WH, Fred EB. Oxidation and reduction relations between substrate and products in the acetone-butylalcohol fermentation. J Biol Chem 1931;91:569–91.
Jones DT, Woods DR. Acetone-butanol fermentation revisited. Microbiol Rev 1986;50:484–524.
Jones TJ, Woods DR. Solvent production. In: Minton NP, Clarke DJ, editors. Clostridia. USA, NY: Plenum Press; 1989. p. 105–44.
Junelles AM, Janati-Idrissi R, Petitdemange H, Gay R. Iron effect on acetate-butanol fermentation. Curr Microbiol 1988;17:299–303.
Jungermann K, Thauer RK, Leimenstoll G, Decker K. Function of reduced pyridine nucleotide-ferredoxin oxidoreductases in saccharolytic clostridia. Biochim Biophys Acta 1973;305:268–80.

Kell DB, Peck MW, Rodger G, Morris JGX. On the permeability to weak acids and bases of the cytoplasmic membrane of *Clostridium pasteurianum*. Biochem Biophys Res Commun 1973;99:81–8.
Kemerer VF, Griffin CC. Phosphofructokinase from *Escherichia coli*. Methods Enzymol 1976;42:91–8.
Kerscher L, Oesterhelt D. Purification and properties of two 2-oxoacid:ferredoxin oxidoreductases from *Halobacterium halobium*. Eur. J. Biochem. 1981;116:587–94.
Killeffer DH. Butanol and acetone from corn. A description of the fermentation process. Ind. Eng. Chem. 1927;19:46–50.
Kim BH, Bellows P, Datta R, Zeikus JG. Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. Appl Environ Microbiol 1984;48:764–70.
Kotze JP. Glycolytic and related enzymes in clostridial classification. Appl Microbiol 1969;18:744–7.
Kyrtopoulos SA, Satchell PN. Kinetic studies with phosphotransacetylase. IV. Inhibition by products. Biochim Biophys Acta 1972;276:383–91.
Kyrtopoulos SA, Satchell PN. Kinetic studies with phosphotransacetylase. V. The mechanism of activation by univalent cations. Biochim Biophys Acta 1973;321:126–42.
Langlykke AF, van Lanen JM, Fraser DR. Butyl alcohol from xylose saccharification liquors from corncobs. Ind Eng Chem 1948;40:1716–9.
Ljungdahl LG, Hugenholtz J, Wiegel J. Acetogenic and acid-producing clostridia. In: Minton NP, Clarke DJ, editors. Clostridia. USA, NY: Plenum Press; 1989. p. 145–91.
Lopez-Contreras AM, Gabor K, Martens AA, Renckens BAM, Claassen PAM, van der Oost J, et al. Substrate-induced production and secretion of cellulases by *Clostridium acetobutylicum*. Appl Environ Microbiol 2004;70:5238–43.
Madan VK, Hillmer P, Gottschalk G. Purification and properties of NADP-dependent L(+)-3-hydroxybutyryl-CoA dehydrogenase from *Clostridium kluyveri*. Eur J Biochem 1973;32:51–6.
Maddox IS. Production of n-butanol from whey filtrate using *Clostridium acetobutylicum* NCIB 2951. Biotechnol Lett 1980;2:493–8.
Marquet M, Wagner MC, Dedonder R. Separation of components of the phosphoenolpyruvate-glucose phosphotransferase system from *Bacillus subtilis* Marburg. Biochimie 1971;53:1131–4.
Marquet M, Creignou MC, Dedonder R. The phosphoenolpyruvate: methyl-α-D-glucoside phosphotransferase system in *Bacillus subtilis* Marburg: kinetic studies of enzyme II and evidence for a phosphoryl enzyme II intermediate. Biochimie 1978;60:1283–8.
Matthews HR, Freedland R, Miesfeld RL. Biochemistry a short course. USA, NY: Willey-Liss Inc.; 1997.
Meinecke B, Bertram J, Gottschalk G. Purification and characterization of the pyruvate-ferredoxin oxidoreductase from *Clostridium acetobutylicum*. Arch Microbiol 1989;152:244–50.
Menon S, Ragsdale SW. Mechanism of the *Clostridium thermoaceticum* pyruvate: ferredoxin oxidoreductase: evidence for the common catalytic intermediacy of the hydroxyethylthiamine pyropyrosphate radical. Biochemistry 1997;36:8484–94.
Meyer CL, McLaaughlin JK, Papoutsakis ET. The effect of CO on growth and product formation in batch cultures of *Clostridium acetobutylicum*. Biotechnol Lett 1985;7:37–42.
Meyer CL, Roose JW, Papoutsakis ET. Carbon monoxide gassing leads to alcohol production and butyrate uptake without acetone formation in continuous cultures of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 1986;24:159–67.
Mitchell WJ. Physiology of carbohydrates to solvent conversion by clostridia. Adv Microbiol Physiol 1998;39:31–130.
Mitchell WJ, Tangney M. Carbohydrate uptake by the phosphotransferase system and other mechanisms. In: Durre P, editor. Handbook on clostridia. CRC Press; 2005. p. 165–85.
Mitchell WJ, Shaw JE, Andrews L. Properties of the glucose phosphotransferase system of *Clostridium acetobutylicum* NCIB 8052. Appl Environ Microbiol 1991;57:2534–9.
Mitchell WJ, Albasheri KA, Yazdanian M. Factors affecting utilization of carbohydrates by clostridia. FEMS Microbiol 1995;17:317–29.
Moreira AR, Ulmer DC, Linden JC. Butanol toxicity in the butylic fermentation. Biotechnol Bioeng Symp 1981;11:567–79.
Morrison JF. Approaches to kinetic studies on metal activated enzymes. Methods Enzymol 1979;63:257–94.
Nolling J, Breton G, Omelchenko MV, Makarova K, Zeng Q, Gibson R, et al. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J Bacteriol 2001;183:4823–38.
Oeding V, Schlegel HGX. β - Ketothiolase from *Hydrogenomonas eutropha* H16 and its significance in the regulation of poly-β-hydroxybutyrate metabolism. Biochem J 1979;134:239–48.
Ounine K, Petitdemange H, Raval G, Gay R. Acetone-butanol production from pentoses by *Clostridium acetobutylicum*. Biotechnol Lett 1983;5:605–10.
Ounine K, Petitdemange H, Raval G, Gay R. Regulation and butanol inhibition of D-xylose and D-glucose uptake in *Clostridium acetobutylicum*. Appl Environ Microbiol 1985;49:874–8.
Palosaari NR, Rogers P. Purification and properties of the inducible coenzyme A-linked butyraldehyde dehydrogenase from *Clostridium acetobutylicum*. J Bacteriol 1988;170:2971–6.
Perret J, Gay P. Kinetic study of a phosphoryl exchange reaction between fructose and fructose 1-phosphate catalyzed by the membrane-bound Enzyme II of the phosphoenolpyruvate-fructose 1-phosphotransferase system of *Bacillus subtilis*. Eur J Biochem 1979;102:237–46.
Petersen DJ, Welch RW, Rudolph FB, Bennett GN. Molecular cloning of an alcohol (butanol) dehydrogenase gene cluster from *Clostridium acetobutylicum* ATCC 824. J Bacteriol 1991;173:1831–4.
Petitdemange H, Cherrier C, Raval G, Gay R. Regulation of the NADH and NADPH-ferredoxin oxidoreductases in clostridia of butyric group. Biochim et Biophys Acta 1976;421:334–47.
Postma PW, Lengeler JW. Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. Microbiol Rev 1985;49:232–69.
Qureshi N, Li XL, Hughes S, Saha BC, Cotta M. Butanol production from corn fiber xylan using *Clostridium acetobutylicum*. Biotechnol Prog 2006;22:673–80.
Robinson GC. A study of the acetone and butyl alcohol fermentation of various carbohydrates. J Biol Chem 1922;52:125–55.
Rogers P. Genetics and biochemistry of *Clostridium* relevant to development of fermentation processes. Adv Appl Microbiol 1986;31:1–60.
Rood JI, McClane BA, Songer JG, Titball RW. The clostridia: Molecular biology and pathogenesis. USA, CA: Academic Press; 1997.
Rose AH. Industrial microbiology. UK, London: Butterworths; 1961.
Rosenberg SL. Fermentation of pentose sugars to ethanol and other neutral products by microorganisms. Enzyme Microbiol Technol 1980;2:185–93.
Saint-Amans S, Girbal L, Andrade J, Ahrens K, Soucaille P. Regulation of carbon and electron flow in *Clostridium butyricum* VPI 3266 grown on glucose–glycerol mixtures. J Bacteriol 2001;183:1748–54.
Schaupp A, Ljungdahl LG. Purification and properties of acetate kinase from *Clostridium thermoaceticum*. Arch Microbiol 1974;100:121–9.
Schlechte H, Elbe B. Recombinant plasmid DNA variation of *Clostridium* oncolyticum model experiments of cancerostatic gene transfer. Zbl Bakt Hyg A 1988;268:347–56.
Schreiber W, Durre P. The glyceraldehydes-3-phosphate dehydrogenase of *Clostridium acetobutylicum*: isolation and purification of the enzyme, and sequencing and localization of the gap gene within a cluster of other glycolytic genes. Microbiology 1999;145:1839–47.
Seedorf H, Florian Fricke W, Veith B, Bruggemann H, Liesegang H, Strittmatter A, et al. The genome of *Clostridium Kluyveri*, a strict anaerobe with unique metabolic features. Proc Natl Acad Sci 2008;105:2128–33.
Segel IH. Enzyme kinetics. USA, NY: John Wiley & Sons, Inc.; 1975.
Sevilla A, Vera J, Diaz Z, Canovas M, Torres NV, Iborra JL. Design of metabolic engineering strategies for maximizing L-(−)-Carnitine production by *Escherichia coli*. Integration of the metabolic and bioreactor levels. Biotechnol Prog 2005;21:329–37.
Sillers R, Chow A, Tracy B, Papoutsakis ET. Metabolic engineering of the non-sporulating, non-solventogenic *Clostridium acetobutylicum* strain M5 to produce butanol without acetone demonstrates the robustness of the acid-formation pathways and the importance of the electron balance. Metab Eng 2008, doi:10.1016/j.ymben.2008.07.005.
Simon E. The formation of lactic acid by *Clostridium acetobutylicum* (Weizmann). Arch Biochem 1947;13:237–43.
Smith LT, Kaplan NO. Purification, properties, and kinetic mechanism of coenzyme A-linked aldehyde dehydrogenase from *Clostridium kluyveri*. Arch Biochem Biophys 1980;203:663–75.
Smith EL, Hill RL, Lehman IR, Lefkowitz RJ, Handler P, White A. Principles of biochemistry, general aspects. 7th edition. USA, NY: McGraw-Hill Book Co.; 1983.
Smits HP, Hauf J, Muller S, Hobley TJ, Zimmermann FK, Hahn-Hagerdal B, et al. Simultaneous overexpression of enzymes of the lower part of glycolysis can enhance the fermentative capacity of *Saccharomyces cerevisiae*. Yeast 2000;16:1325–34.
Spivey MJ. The acetone/butanol/ethanol fermentation. Process Biochem 1978;13:2–5.
Taha SM, Mahmoud SAZ, Ishac YZ, El-Sawy M, El-Demerdash ME. Acetone-butanol fermentation in Egypt. II. Use of various raw materials. . Egypt J Microbiol 1973;8:15–27.
Tamiya N, Yamaguchi Y, Honya M, Yagi T. The electron carrier specificities of the hydrogenases of different origins. Biochem Biophys Res Comm 1966;22:43–7.
Tangney M, Mitchell WJ. Characterization of glucose phosphotransferase system in *Clostridium acetobutylicum* ATCC 824. Appl Microbiol Biotechnol 2007;74:398–405.
Terracciano JS, Kashket ER. Intracellular conditions required for initiation of solvent production by *Clostridium acetobutylicum*. Appl Environ Microbiol 1986;52:86–91.
Thauer RK, Jungermann K, Dekker K. Energy conservation in chemotropic anaerobic bacteria. Bacteriol Rev 1977;41:100–80.
Tomas CA, Beamish J, Papoutsakis ET. Transcriptional analysis of butanol stress and tolerance in *Clostridium acetobutylicum*. J Bacteriol 2004;186:2006–18.
Tomas CA, Welker NE, Papoutsakis ET. Overexpression of *groESL* in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Appl Environ Microbiol 2003;69:4951–65.
Torres NV, Voit EO. Pathway analysis and optimization in metabolic engineering. UK, Cambridge: Cambridge University Press; 2002.
Tummala SB, Junne SG, Papoutsakis ET. Antisense RNA downregulation of coenzyme A transferase with alcohol-aldehyde dehydrogenase overexpression leads to predominantly alcohologenic *Clostridium acetobutylicum* fermentations. J Bacteriol 2003;185:3644–53.
Twarog R, Wolfs RS. Enzymatic phosphorylation of butyrate. J Biol Chem 1962;237:2474–7.
Twarog R, Wolfs RS. Role of butyryl phosphate in the energy metabolism of *Clostridium tetanomorphum*. J bacteriol 1963;86:112–7.
Uyeda K, Kurooka S. crystallization and properties of phosphofructokinase from *Clostridium pasteurianum*. J Biol Chem 1970;245:3315–24.
Uyeda K, Rabinowitz JC. Pyruvate-ferredoxin oxidoreductase. III. Purification and properties of the enzyme. J Bacteriol Chem 1971a;246:3111–9.
Uyeda K, Rabinowitz JC. Pyruvate-ferredoxin oxidoreductase. IV. Studies on the reaction mechanism. J Bacteriol Chem 1971b;246:3120–5.
v. Hugo H, Schoberth S, Madan VK, Gottschalk G. Coenzyme specificity of dehydrogenases and fermentation of pyruvate by clostridia. Arch Microbiol 1972;87:189–202.

Valentine RC, Wolfe RS. Purification and role of phosphotransbutyrylase. J Biol Chem 1960;235:1948–52.
Vasconcelos I, Girbal L, Soucaille P. Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. J Bacteriol 1994;176:1443–50.
Vignais PM, Billoud B, Meyer J. Classification and phylogeny of hydrogenases. FEMS Microbiol Rev 2001;25:455–501.
Vinuela E, Salas ML, Sols A. End-product inhibition of phosphofructokinase by ATP. Biochem Biophys Res Commun 1963;12:140–5.
Voit EO. Computational analysis of biochemical systems. A practice guide for biochemists and molecular biologists. UK, Cambridge: Cambridge University Press; 2000.
Vollherbst-Schneck KJA, Sands A, Montencourt BS. Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 1984;47:193–4.
Walter KA, Bennett GN, Papoutsakis ET. Molecular characterization of two *Clostridium acetobutylicum* ATCC 824 butanol dehydrogenase isoenzyme genes. J Bacteriol 1992;174:7149–58.
Waterson RM, Castellino FJ, Hass GM, Hill RL. Purification and characterization of crotonase from *Clostridium acetobutylicum*. J Biol Chem 1972;247:5266–71.
Weigel J. Fermentation of ethanol by bacteria. A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. Experientia 1980;36:1434–46.
Welch RW, Rudolph FB, Papoutsakis ET. Purification and characterization of the NADH-dependent butanol dehydrogenase from *Clostridium acetobutylicum* (ATCC 824). Arch Biochem Biophys 1989;273:309–18.
Westheimer FH. Coincidences, decarboxylation, and electrostatic effects. Tetrahedron 1995;51:3–20.
Whitfield CD, Mayhew SG. Purification and properties of electron-transferring flavoprotein from *Peptostreptococcus elsdenii*. J Biol Chem 1974;249:2801–10.
Williamson G, Engel PC. Butyryl-CoA dehydrogenase from *Megasphaera elsdenii*: specificity of the catalytic reaction. Biochem J 1984;218:521–9.
Winzer K, Lorenz K, Durre P. Acetate kinase from *Clostridium acetobutylicum*: a highly specific enzyme that is actively transcribed during acidogenesis and solventogenesis. Microbiology 1997;143:3279–86.
Wiesenborn DP, Rudolph FB, Papoutsakis ET. Thiolase from *Clostridium acetobutylicum* ATC824 and its role in the synthesis of acids and solvents. Appl Environ Microbiol 1988;54:2717–22.
Wiesenborn DP, Rudolph FB, Papoutsakis ET. Phosphotransbutyrylase from *Clostridium acetobutylicum* ATCC 824 and its role in acidogenesis. Appl Environ Microbiol 1989a;55:317–22.
Wiesenborn DP, Rudolph FB, Papoutsakis ET. Coenzyme A transferase from *Clostridium acetobutylicum* ATCC 824 and its role in the uptake of acids. Appl Environ Microbiol 1989b;55:323–9.
Wood WA. In: Gunsalus IC, Stanier RY, editors. Fermentation of carbohydrates and related compounds. The bacteriaUSA, NY: Academic Press; 1961. p. 59–149.
Yan RT, Chen JS. Coenzyme A-acylating aldehyde dehydrogenase from *Clostridium beijerinckii* NRRL B592. Appl Environ Microbiol 1990;56:2591–9.
Yerushalmi L, Volesky B, Scczesny T. Effect of increased hydrogen partial pressure on the acetone-butanol fermentation by *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 1985;22:103–7.
Youngleson JS, Santangelo JD, Jones DT, Woods DR. Cloning and expression of a *Clostridium acetobutylicum* alcohol dehydrogenase gene in *Escherichia coli*. Appl Environ Microbiol 1988;54:676–82.
Youngleson JS, Jones DT, Woods DR. Homology between hydroxybutyryl and hydroxyacyl coenzyme A dehydrogenase enzymes from *Clostridium acetobutylicum* fermentation and vertebrate fatty acid β-oxidation pathways. J Bacteriol 1989;171:6800–7.
Yu Y, Tangney M, Aass HC, Mitchell WJ. Analysis of the mechanism and regulation of lactose transport and metabolism in *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 2007;73:1842–50.
Zhao Y, Hindorff LA, Chuang A, Monroe-Augustus M, Lyristis M, Harrison ML, et al. Expression of a cloned cyclopropane fatty acid synthase gene reduces solvent formation in *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 2003;69:2831–41.
Zhao Y, Tomas CA, Rudolph FB. Intracellular butyryl phosphate and acetyl phosphate concentrations in *Clostridium acetobutylicum* and their implications for solvent formation. Appl Environ Microbiol 2005;71:530–7.
Zeikus JG. Chemical and fuel production by anaerobic bacteria. Ann Rev Microbiol 1980;34:423–64.