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The pH-induced Metabolic Shift from Acidogenesis to Solventogenesis in *Clostridium acetobutylicum* – From Experiments to Models

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Abstract

The strictly anaerobic Gram-positive *Clostridium acetobutylicum* is able to ferment starchy material to acetone, butanol, and ethanol. Due to rising costs, dwindling resources, and environmental concerns regarding extraction and use of petroleum and natural gas, the academic and industrial interest in *C. acetobutylicum* has been renewed in recent years. However, an improved understanding of the clostridial metabolism and its regulations is a prerequisite for future industrial applications.

The COSMIC consortium, as part of the transnational SysMo initiative, is focusing on the pH-induced metabolic shift of *C. acetobutylicum* from acidogenesis to solventogenesis. During acidogenesis (high pH) the bacterium predominantly produces the acids acetate and butyrate, whereas the solvents acetone and butanol are fermented during solven-

togenesis (low pH). This metabolic phase transition is accompanied by changes in transcriptome, proteome, and metabolome which have been measured using a standardized experimental setup.
The information gathered is used to model this dynamic shift. Towards this end, we established a system of coupled differential equations, describing the biochemical reactions involved in AB fermentation and their dynamic changes found in recent experiments. Since *C. acetobutylicum* is not capable of maintaining a homoeostatic intracellular pH, the influence of a changing intracellular pH on enzyme activity and stability is of special interest for an improved understanding of AB fermentation. Such a model is able to predict product spectrum and metabolome during the pH-induced shift as well as for several mutants at solventogenesis.

Introduction

The obligatory anaerobic Gram-positive bacterium *Clostridium acetobutylicum* ferments starchy material to acetone, butanol, and ethanol which are important chemicals used in a plenty of industrial applications. The early industrial application if the acetone-butanol-ethanol (AB or ABE) fermentation is closely associated to Chaim Weizmann who helped to developed industrial facilities and held several patents related to this clostridial fermentation process. It became the second largest commercial biotechnological process ever performed until the early 1960 s when the production of these chemicals from petrochemicals became economically more favourable. Due to rising costs, dwindling resources, and environmental concerns regarding extraction and use of petroleum and natural gas, the academic and industrial interest in *C. acetobutylicum* has been renewed in recent years [1]. However, economic and ecological demands on the production of energy and (bio)chemicals require an optimization of the microbial fermentation. These demands include a large scale production of high-density, transportable energy that matches fossil fuels in convenience and utility. Here, *C. acetobutylicum* could be an adequate candidate for future CO_2-neutral energy production using (bio)butanol. First, it is non-pathogenic to humans, animals, and plants and produces no toxic by-products that may harm the environment. Second, butanol is a more attractive biofuel than ethanol which is currently used for that purpose [2, 3]. Its energy content is similar to that of pure gasoline and, thus, about 30 % higher than that of ethanol. Furthermore, butanol is less corrosive, sparingly soluble in water, and does not absorb water. Hence, contrary to ethanol, butanol can be added at the refinery, be transported and delivered through existing infrastructure, and does not require modifications to engines. Despite the usage as biofuel, butanol is also an important bulk chemical needed for a broad range of applications including surface coatings, adhesives/scalings, elastomers, textiles, superabsorbents, flocculants, fibres, and plastics [4]. In summary, butanol is an important resource and clostridial AB fermentation could be an alternative to the use of dwindling petrol chemicals for its production.

However, the present state of technology is insufficient for a large-scale application of this bacterial process. Future usage requires an increased efficiency of the fermentation, for example the enhancement of metabolic flow through the pathway and butanol produced per glucose equivalent. Furthermore, the control of AB fermentation by external means is poorly understood. Moreover, the obligatory anaerobic feature of *C. acetobutylicum* and its preference to sugars as energy and carbon source hinder future applications. Hence, an improved understanding of clostridial metabolism and its regulations is a prerequisite for future large-scale industrial applications.

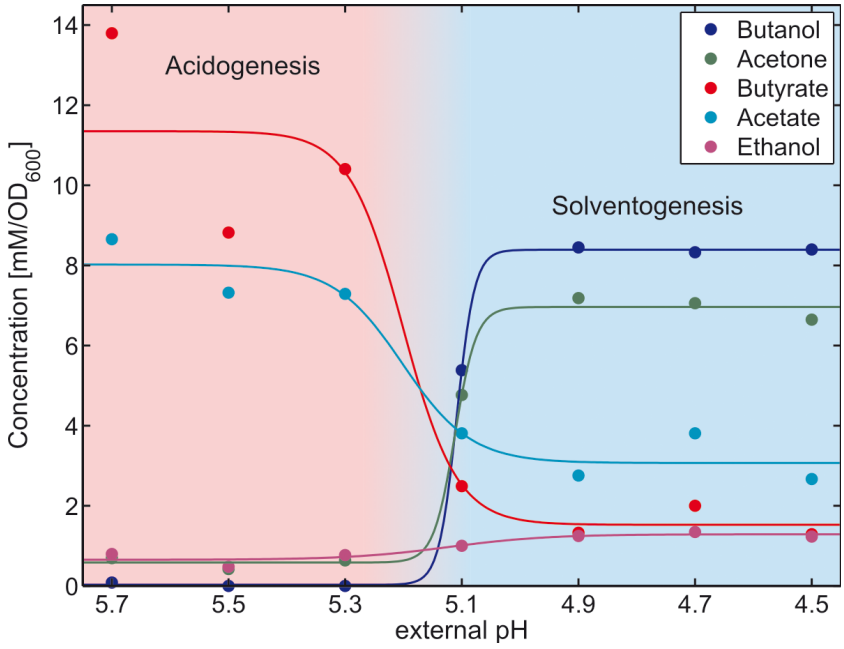

Figure 1. The steady-state product spectrum of AB fermentation in *C. acetobutylicum* as a function of the external pH. In a continuous culture under phosphate limitation, the bacterium changes its metabolic profile in response to variations of the external pH level. Experimental data (circles) are given in mM per optical density (OD_{600}). The subsequent normalization, using the optical density, was performed to account for fluctuations in the population size. Hyperbolic tangents are fitted to the experimental data (solid lines). Using their inflections points, we conclude that *C. acetobutylicum* is in acidogenic phase at pH < 5.1 and in solventogenic phase at pH < 5.1. A transition phase occurs in the range 5.2 < pH < 5.1.

Experimental evidence indicates that the extracellular pH level is crucial for the product spectrum and may, thus, enable an external control of the metabolic flow through the pathway. Interestingly, AB fermentation in *C. acetobutylicum* comprises two pH-dependent characteristic phases: acidogenesis and solventogenesis. During acidogenesis (high pH) the bacterium predominantly produces the acids acetate and butyrate, whereas the solvents acetone and butanol are fermented during solventogenesis (low pH). A systemic study of

the steady-state product spectrum as a function of the external pH level is shown in Figure 1. Furthermore, we fitted hyperbolic tangents on the experimental data to define quantitative measures for this pH-dependent phase transition. Using their inflection points, we found that the bacterium is in acidogenic phase at $pH > 5.2$ and in solventogenic phase at $pH < 5.1$. An intermediary transition phase emerges within the range $5.2 < pH < 5.1$ that differs from acidogenesis or solventogenesis. Furthermore, this metabolic phase transition is accompanied by changes in transcriptome, proteome, metabolome which have been measured using a standardized experimental setup.

The COSMIC consortium, as part of the European transnational SysMo initiative [5], is focusing on the pH-induced metabolic shift of *C. acetobutylicum* from acidogenesis to solventogenesis. A close collaboration between experimentalists and modellers realized an iterative cycle of data-driven models and model-driven design of experiments. This objective-driven approach will be discussed using the pH-induced metabolic shift in *C. acetobutylicum* as an example. Towards this end, we introduce recent and past experimental findings and the standardized experimental setup in the next section. Afterwards, we use this information to establish a dynamic mathematical model and discuss the assumptions applied in its formulation. Due to a lack of data, the pH-dependency of enzyme kinetic reactions involved in AB fermentation is neglected in current models. However, it is assumed that the pH-dependent specific activity is crucial for the kinetic regulation of the metabolic shift. Hence, we discuss its potential influence on the phase transition in the following section. Finally, we summarize our results and give an outlook to future research.

The pH-induced Metabolic Shift and the Metabolic Network of ABE Fermentation

In contrast to aerobic organisms that have established a prevalent tight regulation of intracellular pH [6 – 8], *C. acetobutylicum* is unable to maintain a constant intracellular pH, but rather the transmembrane pH gradient is kept constant [9, 10]. Experiments using batch and continuous cultures had been shown that the intracellular pH is higher with a difference $\Delta pH \approx 1$ [11, 12].

Under normal growth conditions *C. acetobutylicum* enters an exponential growth phase, which is characterized by the formation of acids, acetate and butyrate, as predominant liquid fermentation products. The increasing acid concentrations results in a decrease of the extracellular pH. Unable to maintain a homeostatic intracellular pH under this condition, the bacterium alters its metabolism to prevent a further reduction of the pH level. This phenomenon is referred to as the pH-induced metabolic shift and involves two processes: (i) the reutilization of previously secreted acids, and (ii) the production of pH-neutral solvents to prevent a recurrent decrease of the pH level. The data plotted in Figure 2 illustrate this phenomenon [11]. Furthermore, this batch experiment suggests that the internal pH follows the external one without significant delay.

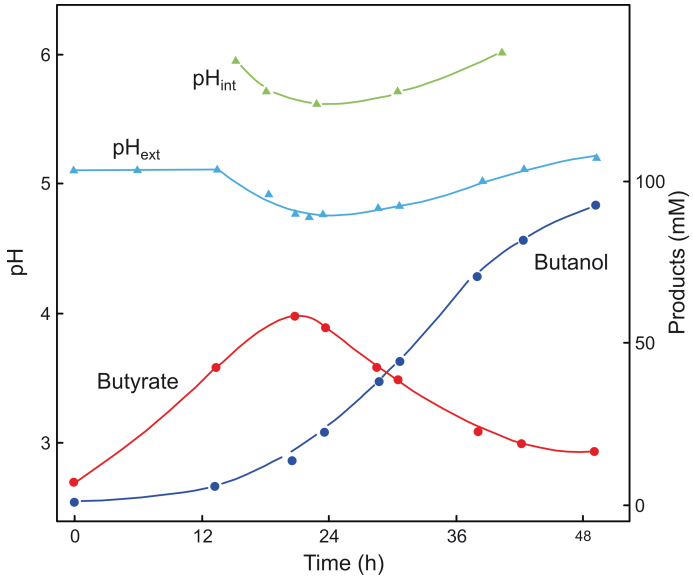

Figure 2. The extra- and intracellular pH and butyrate and butanol concentrations as a function of time in a growth experiment in batch culture. In contrast to aerobic organisms, the strict anaerobic *C. acetobutylicum* is unable to maintain a constant intracellular pH level, but rather keeps the transmembrane pH gradient constant. Thus, the intracellular pH follows the extracellular pH level without significant delay and an approximate difference $\Delta pH \approx 1$. Reproduced by courtesy of Springer from [11].

The preferred carbon and energy source of the bacterium is glucose, which is transported into the cell via a phosphoenolpyruvate-dependent phosphotransferase system [13, 14]. Then, the intracellular glucose is metabolized via glycolysis. The glycolytic steps form the backbone of acid and solvent formation and their activitiesare thus more or less pH-independent. Three key intermediates, acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA, are of particular interest for AB fermentation [15]. These intermediates are branching points which channel the metabolic flow either to acid or solvent formation (Figure 3).

The first branching point, acetyl-CoA, is the starting point for the formation of acetate and ethanol. Acetate is generated in two sequential reactions involving a phosphotransacetylase and an acetate kinase [9, 16]. The activity of the acetate kinase rapidly decreases to very levels at solventogenesis [17, 18]. Simultaneously, ethanol is formed via an acetaldehyde/alcohol dehydrogenase. Two antagonistically expressed NADH-dependent acetaldehyde/alcohol dehydrogenases, AdhE1 and AdhE2, play a crucial in the pH-induced metabolic shift [16, 19]. Whereas *adhe2* is transcribed during acidogenesis in a chemostat and its gene product only facilitates the formation of ethanol, AdhE1 is induced during solventogenesis and replaces AdhE2 [19]. Interestingly, the bifunctional AdhE1 promotes the production of both alcohols, ethanol and butanol [20].

The acetone formation starts from the second branching point, acetoacetyl-CoA, and is performed by the enzymes acetoacetyl-CoA transferase and acetoacetate decarboxylase, Adc, in two sequential reactions [9, 16]. The specific activity of Adc is very pH sensitive [21] and an increase by 38-fold from acidogenesis to solventogenesis was reported [17]. Furthermore, the uptake of acids during the shift requires active Adc [18, 22]. Interestingly, its concentration seems to more or less the same during both metabolic phases [23].

The last branching point, butyryl-CoA, is the starting point for the formation of either butyrate or butanol. Butyrate is produced in sequential reactions facilitated by two enzymes, phosphotransbutyrylase, Ptb, and butyrate kinase, which are most active during acidogenesis. Their specific activities decline during solventogenesis, 2-fold for Ptb and 6-fold for butyrate kinase [17]. The conversion of butyryl-CoA to butanol is promoted by sequential action of AdhE1. Importantly, experimental evidence indicates that AdhE1 is inactive in acid-producing cells, whereas its activity strongly increases during solventogenesis [19, 23, 24].

The involved CoA-transferase has fundamentally different role in clostridia compared to other bacteria [25]. Here, it is responsible for the uptake of formerly excreted acids, their conversion to the respective CoA-derivatives [17, 26], and acetone formation. In contrast to other enzymes involved in acid or solvent formation, it is insensitive to variations of the internal pH [27]. Because it operates in a sub-saturated regime, its activity is sensitive intracellular concentrations. Consequently, it is induced during solventogenesis.

The available information about ABE fermentation, summarized in the reduced network shown in Figure 3, suggests that the formation of acids and solvents is affected by pH-dependent kinetics and pH-dependent gene expression. Interestingly, the acid formation seems to be mostly modulated by changes of the kinetic properties, whereas the solvent formation requires both regulatory mechanisms.

Experimental evidence indicates that the pH-induced metabolic shift involves several levels of biological organization. However, the dynamic measurement of transcriptomic, proteomic, metabolic, and environmental changes is usually beyond the capacity of a single lab [28]. Thus, several labs with different specializations must closely collaborate to provide data fitting the needs of modelling. Towards this end, the COSMIC consortium established a standardized experimental setup and standard operation procedures.

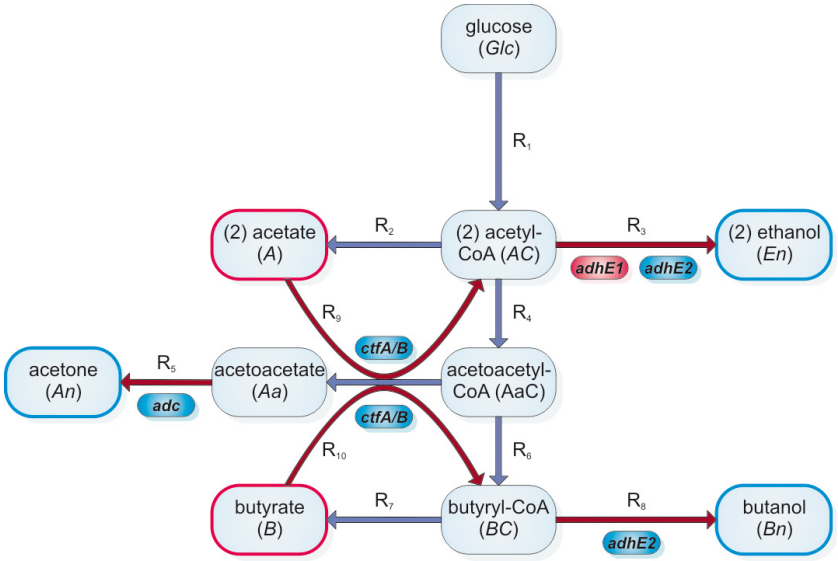

Figure 3. The reduced network of AB fermentation in *C. acetobutylicum*. The bacterium prefers glucose as carbon and energy source which is reduced in several glycolytic steps. During acidogenesis, the bacterium produces the acids acetate and butyrate (blue edges), whereas the solvents acetone and butanol (red edges) are the main liquid products during solventogenesis. This fermentative pathway comprises three key intermediate CoA-derivatives that are branching points of either acid or solvent production. Furthermore, recent experimental evidence indicates that several enzymes are induced in a pH-dependent manner. Those enzymes relevant for the AB fermentation are denoted on the reactions they facilitate. Furthermore, previously excreted acids are re-assimilated and converted to solvents during the metabolic shift. The abbreviations given in parentheses denote the variable names used in the mathematical model.

The continuous culture under phosphate limitation [29, 30] is superior to batch cultures, because it reduces the environmental and biological factors that could influence the bacterial population. Thus, it allows for a reasonable restriction of processes considered in the model and provides reproducible data which is a fundamental requirement for modelling. However, clostridial population may behave substantially different under these conditions than those in batch cultures [29]. The strain *C. acetobutylicum* ATCC 824 was grown under anaerobic conditions at 37 °C. The experiments were performed in a fermenter system, shown in Figure 4, with 0.5 mM KH_2PO_4 and 4% (wt/vol) glucose in the supplying medium and a dilution rate $D = 0.075\,h^{-1}$. The external pH in the culture medium was adjusted to and kept constant at pH 5.7 (acidogenic phase) and pH 4.5 (solventogenic phase) by automatic addition of KOH.

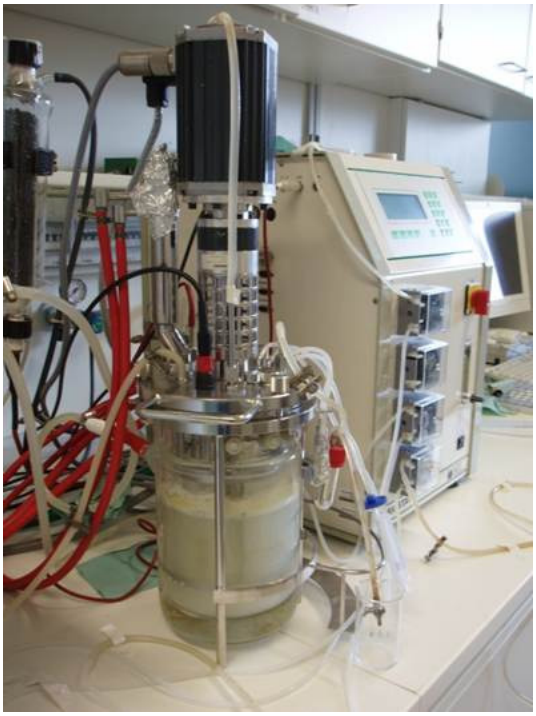

Figure 4. The standardized continuous culture setup used to investigate the influence of the external pH level on the metabolic state of *C. acetobutylicum.* The picture shows the well-stirred, pH-controlled fermenter and the control device which regulatesthe external pH. Photo by courtesy of Division of Microbiology, University of Rostock, Germany.

In three individual 'shift experiments', the pH level was changed from 5.7 to 4.5 to induce the metabolic shift. Data were taken for transcriptome, proteome, metabolome, product spectrum, and environome over the full length of the observation time. We used the gathered information to establish a mathematical model of the pH-induced metabolic phase transition in *C. acetobutylicum*. The model structure, underlying assumptions, and approximations are presented in the following section.

Modelling the Dynamic Shift from Acidogenesis to Solventogenesis

Here we present a kinetic model of the AB fermentation in *C. acetobutylicum* in continuous culture. Because there is a lack of published information on the kinetic parameters governing these reactions under the conditions used in experiments in the literature, we aggregate a number of reactions of the metabolic network [9], see also Figure 3. This leads to a reduced number of model parameters that need to be estimated from experimental data. Simulta-

neously, we focus upon those reactions which are most likely to be regulated by the changing intracellular pH level. The resulting metabolic model is given by a coupled system of nine differential equations:

$$\begin{aligned}
\frac{dAc}{dt} &= R_1 - R_2 - R_3 - 2R_4 + R_9 - D \cdot AC,\\
\frac{dA}{dt} &= R_2 - R_9 - D \cdot A,\\
\frac{dEn}{dt} &= R_3 - D \cdot En,\\
\frac{dAaC}{dt} &= R_4 - R_6 - R_9 - R_{10} - D \cdot AaC,\\
\frac{dAn}{dt} &= R_5 - D \cdot An,\\
\frac{dBC}{dt} &= R_6 - R_7 - R_8 + R_{10} - D \cdot BC,\\
\frac{dB}{dt} &= R_7 - R_{10} - D \cdot B,\\
\frac{dBn}{dt} &= R_8 - D \cdot Bn,\\
\frac{dAa}{dt} &= R_9 + R_{10} - R_5 - D \cdot Aa.
\end{aligned} \tag{1}$$

Note that we rearranged the reactions in comparison to the representation we published in [31].

The rate of change of considered intracellular metabolites and products is determined by the sum of the reactions R_i that either produce (positive sign) or consume (negative sign) the respective molecule. In addition, we introduced an out-flow term which is the product of the dilution rate D with the concentration of the corresponding metabolite, because we have a constant out-flow of both extra- and intracellular, intracellular products as a result of cell out-flow through the fermenter.

Due to the aggregation of reactions, five glycolytic steps were combined into one reaction (R_i), adopting the assumption that there is a constant flux from glucose to acetyl-CoA. Additionally, we reduce the number of steps in five other reactions. Here, we assume that the corresponding intermediates are in quasi-steady state. Thus, in the conversions of two acetyl-CoA into either two molecules of acetate (R_2) or two molecules of ethanol (R_3), of acetoacetyl-CoA into acetone (R_5), and of butyryl-CoA into either butyrate (R_7) or butanol (R_8), we reduce two steps into one. Furthermore, we represent the three steps in the conversion of acetoacetyl-CoA to butyryl-CoA by one (R_6).

The resulting reaction rates considered in the metabolic network (1) are

$$
\begin{aligned}
R_1 &= \frac{2V_1 \cdot Glc}{K_1 + Glc}, & R_6 &= \frac{V_6 \cdot AaC}{K_6 + AaC}, \\
R_2 &= \frac{V_2 \cdot AC}{K_2 + AC}, & R_7 &= \frac{V_7 \cdot BC}{K_7 + BC}, \\
R_3 &= a_3 \cdot AC \cdot adhe, & R_8 &= a_8 \cdot BC \cdot adhe, \\
R_4 &= \frac{V_4 \cdot AC}{2(K_4 + AC)}, & R_9 &= a_9 \cdot A \cdot AaC \cdot ctf, \\
R_5 &= a_5 \cdot Aa \cdot adc, & R_{10} &= a_{10} \cdot B \cdot AaC \cdot ctf,
\end{aligned}
\tag{2}
$$

here we include the stoichiometric constant of two in R_1 since two molecules of acetyl-CoA are formed from one of glucose. Similarly, the constant 0.5 in R_4 represent the formation of one acetoacetyl-CoA from two acetyl-CoA. In consequence, we introduced a factor of two in the kinetic equation for acetyl-CoA, see metabolic network (1), to keep the correct stoichiometry for this molecule.

In Equations (2), Michaelis-Menten-like equations describe reactions involving enzymes with pH-independent gene regulation and, thus, with constant intracellular concentrations. This type of reaction is determined by an apparent limiting rate V_i and an apparent Michaelis-Menten constant K_i. In contrast, solving-producing enzyme are induced during the metabolic shift, so that the assumption of constant total enzyme concentrations made in the derivation of the Michaelis-Menten equation does not hold for the reactions those enzymes facilitate. Hence, we only apply the quasi-steady-state approximation which leads us to apparently bimolecular and trimolecular reactions, where the apparent rate coefficient is defined as $\alpha = k_3/K_m$ [31, 32].

In addition to the metabolic network (1), we require thus the following pH-dependent equations to describe the induction of solventogenic enzymes:

$$
\begin{aligned}
\frac{dadhe}{dt} &= r_{\mathrm{ah}} + r_{\mathrm{ah}}^{+} \cdot F(pH) - D \cdot adhe, \\
\frac{dadc}{dt} &= r_{\mathrm{ad}} + r_{\mathrm{ad}}^{+} \cdot F(pH) - D \cdot adc, \\
\frac{dctf}{dt} &= r_{ct} + r_{ct}^{+} \cdot F(pH) - D \cdot ctf.
\end{aligned}
\tag{3}
$$

These rate equations comprise two different synthesis rates. During acidogenesis, the enzymes are expressed with a low basal rate r_i. Their synthesis increases by r_i^+ at solventogenesis. The pH-dependent function

$$F(pH) = -(pH(t) - 5.7)/1.2 \tag{4}$$

generates the shift between both modes of gene expression. It is coupled on the external pH level which is approximated using a hyperbolic tangent [31].

Finally, we estimated all model parameters from three 'shift' experiments. Towards this end, we normalized and scale the data across the dynamic shift interval to make comparisons between time points meaningful. Each data set was interpolated at identical time points, enabling the average of the three scaled sets to be calculated for parameter estimation.

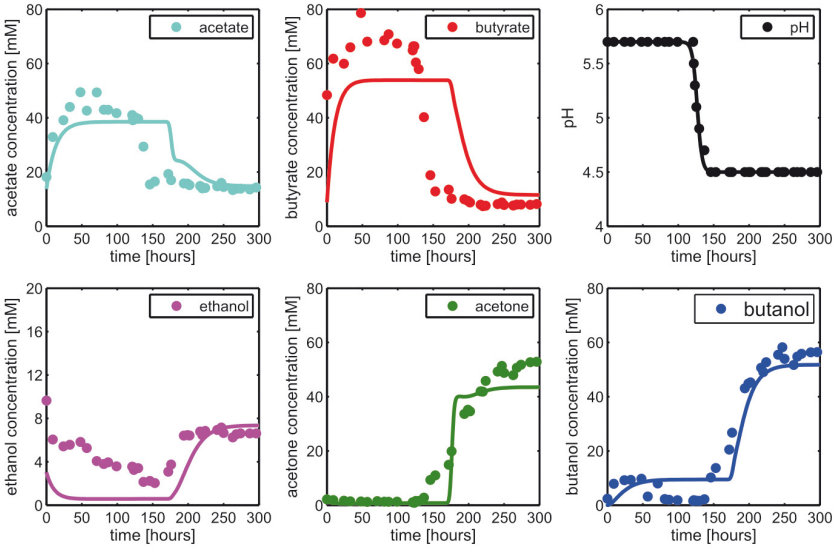

Figure 5. Comparison between experimental data and numerical simulation of a 'shift' experiment. The experiment (circles) started at acidogenic conditions (pH 5.7). During an initial phase (~100 hours), the culture established a steady state with the main products acetate and butyrate. At around 140 hours the pH control was switched off which results in the initiation of the metabolic switch. Finally, a solventogenic steady state is approached and the solvents butanol and acetone dominate the product spectrum. The results of the numerical simulation reproduce the measured steady states and describe the observed phase transition as a pH-induced rearrangement of the proteome involved in AB fermentation.

In Figure 5, we compare the numerical simulation results with data from a 'shift' experiment. The clostridial culture was maintained at acidogenesis (pH 5.7) for approximately 135 hours. During this period, an acidogenic steady state was established and the cells mainly produced the acids acetate and butyrate. Then, the pH control was stopped, allowing the natural pH-induced metabolic switch to solventogenesis to begin. During the shift, the product spectrum of the culture changed dramatically. The acid production dropped and

the cells re-assimilated previously excreted acids from the medium resulting in a rapid decrease of acid concentration. Simultaneously, the formation of solvents was initiated and the concentrations of acetone and butanol increased strongly. This shift lasted about a day until the culture reached a solventogenic pH of 4.5. However, the continuous culture had not yet approached a metabolic steady state at this point. It took further 100 hours to establish a solventogenic steady state.

The steady-state product spectrum predicted by our numerical simulation is in accordance with the experimental data. This agreement proofs that the pH-induced shift involves a rearrangement of the proteomic composition of *C. acetobutylicum*. During the phase transition the bacteria induces additional solvent-producing enzymes. Interestingly, the concentration of acid-producing enzymes changes insignificantly [23], whereas the transcription is increased for several genes encoding those enzymes [24]. These findings may reflect the behaviour of *C. acetobutylicum* in its natural habitat. Here, the pH-induced metabolic shift is initiated to countervail against a further decline of the environmental pH level. Eventually, the bacterium returns to an acidogenic state that requires the presence of acid-producing enzymes. The found increase in transcription might, thus, compensate for decreased mRNA and or protein stability at solventogenesis. Furthermore, the simulation deviates from the experimental data during the dynamic transition from acidogenesis to solventogenesis. The theoretical predicted transition time is much shorter than measured in experiments. The pH-dependent kinetic enzymatic properties neglected in the present model could be a plausible explanation for that observation. In particular, the dynamics of acetone formation is overestimated which coincides with the fact that the involved acetoacetate decarboxylase exhibits a remarkable pH-dependent behaviour [17, 21]. This evidence suggests that the influence of the changing pH level on kinetic properties of the components involved in AB fermentation is crucial for the metabolic conversion processes as well as for their intracellular regulation. Thus, we investigate in the next section, how some important network motifs respond on variations in the intracellular pH if pH-dependent kinetics is considered. This will shed a new light on cellular processes related to environmental factors, like temperature, pH, or salinity that directly affect the enzymatic properties.

pH-dependent Enzyme Kinetics and its Effect on Branch Points of the ABE Network

pH-dependence is not reflected in the common representation of the enzyme kinetic reaction shown in black in Figure 6, where the conversion of substrate S to product P is facilitated by enzyme E During this process an intermediary complex C is formed [33, 34]. The reaction becomes pH-dependent, if one considers that association and dissociation of hydrons, denoted in blue in Figure 6, change the structure of the enzyme and, thus, its specific activity.

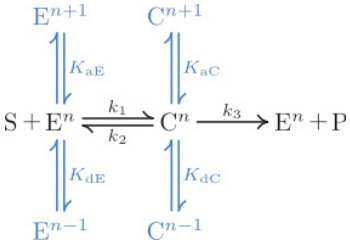

Figure 6. The effect of the hydrons on the enzyme kinetic reaction. Due to association and dissociation of hydron to and from the enzyme and the enzyme-substrate complex the enzymatic activity is changed. Here, we assume that only enzymes with n bound hydrons are active and that association and dissociation of hydrons is much faster than the enzymatic conversion.

The incorporation of the pH-dependent association/dissociation of hydrons into the enzyme kinetic reaction leads to a formal equivalent expression for the reaction rate [34, 35]

$$\frac{dP}{dt} = -\frac{dS}{dt} = \frac{V_{\max} \cdot S}{K_m + S}, \tag{5}$$

with a pH-dependent limiting rate $\mathcal{V}_{\max}$ and a pH-dependent Michaelis-Menten constant $\mathcal{K}_m$. Both parameters are determined by the equilibrium of dissociation and association of hydrons which is described by the dissociation constants $K_{(a,d)E}$ and $K_{(a,d)C}$, where the subscript 'a' denotes the association of a hydron and the subscript 'd' the dissociation, and the hydron concentration H^+. The pH-dependent limiting rate [35]

$$\mathcal{V}_{\max} = \frac{V_{\max}}{1 + H^+/K_{aC} + K_{dC}/H^+} \tag{6}$$

fulfils the relation $\mathcal{V}_{\max} \leq V_{\max}$, where $V_{\max} = k_3 \cdot E^T$ is the limiting rate of the standard Michaelis-Menten equation. It exhibits a typical bell-shaped form, Figure 7.

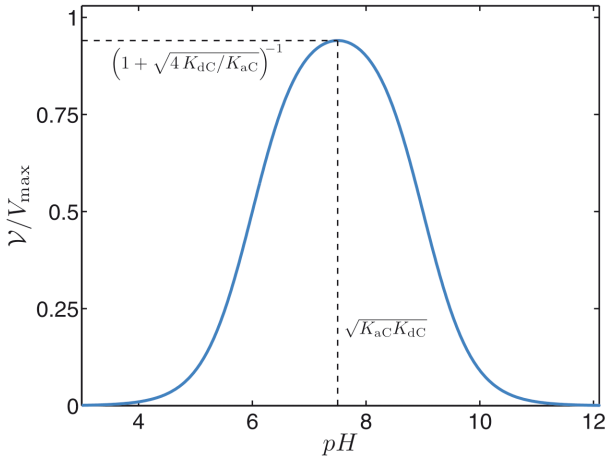

Figure 7. The apparent limiting rate ν as a function of the pH exhibits a typical bell-shaped form. Its maximum is determined by the dissociation constants K_{aC} and K_{dC} that describe the association and dissociation of a hydron to and from, respectively, the complex C.

The pH-dependent apparent Michaelis-Menten constant [35]

$$K_m = K_m \frac{1+H^+/K_{\mathrm{aE}}+K_{\mathrm{dE}}/H^+}{1+H^+/K_{\mathrm{aC}}+K_{\mathrm{dC}}/H^+}. \tag{7}$$

results from the multiplication of the standard Michaelis-Menten constant $K_m = (k_2 + k_3)/k_1$ with a rational pH-dependent expression. A further analysis of rate equation (5) reveals that the pH-dependency of those apparent Michaelis-Menten constant has to be considered for small substrate concentrations $S \ll K_m$. Thus, we neglect its effect on the reaction rate in our further investigations. Then, the reaction rate is directly proportional to the pH-dependent limiting rate and shares the same functionality.

We now apply this formalism developed for isolated enzymes to investigate the effects of a changing pH on chosen network motifs of the AB fermentation pathway. Towards this end, we consider the separate impact of the intracellular pH on the regulation of gene expression using a (de)activation cycle with two distinct pH-dependent states, Figure 8[a], and on metabolic reactions using a branching point, Figure 8[b], as an example.

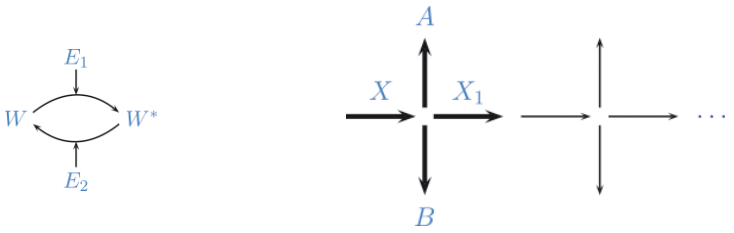

Figure 8. Schematic representation of two important motifs in the network of AB fermentation. Left: The (de)activation cycle of a protein W that comprises two distinct pH-dependent states. Here, we assume that the active form W^* may trigger the cellular adaption in response to changing pH levels, e. g. the induction of solvent-producing enzymes. The enzymes E_1 and E_2 activation and deactivate, respectively, the protein in pH-dependent reactions.Right: A metabolic branching point in the AB fermentation pathway. The intermediate X is converted either to product A or B in pH-dependent reactions promoted by the enzymes E_1 and E_2.

In the considered (de)activation cycle, a protein is activated by an enzyme E_1, e. g. by phosphorylation, and deactivated by enzyme E_2, e. g. by dephosphorylation, in a pH-dependent manner. Dependent on its activation state, the protein may trigger or inhibit specific cellular functions like transcription factor activity. Assuming that activation and deactivation are much faster than the cellular functions regulated by the protein, we focus on the steady state as a function of the external pH level and investigate how a shift of the position of the pH-dependent activity affects the steady-state concentration. For this purpose, we choose pH-dependent profiles with the same half width and height for activation and deactivation, respectively.

The steady-state concentrations as a function of the external pH are shown in Figure 9 for two different scenarios: [a] slightly differing pH-profiles, and [b] strongly differing pH-profiles. I accordance with experimental evidence, the latter setting assumes that the acid- and solvent-producing enzymes optimally operate either during acidogenesis or solventogenesis. Because activation and deactivation processes possess different pH-dependent activity, the steady state is a function of the external pH. Due to the slightly differing pH-dependent activities investigated in Figure 9[a], the system 'fine-tunes' the protein concentration to its current requirements. In our example, the maximum of the activation is located at a smaller pH value than the deactivation. Thus, the active form of the protein is decreasing with increasing pH, whereas the inactive form increases with pH. Very different maxima result in pH-sensitive steady states that provide a switch-like behaviour, Figure 9[b]. The protein may, thus, act as an intracellular pH sensor which triggers metabolic and physiological adaptations in *C. acetobutylicum*.

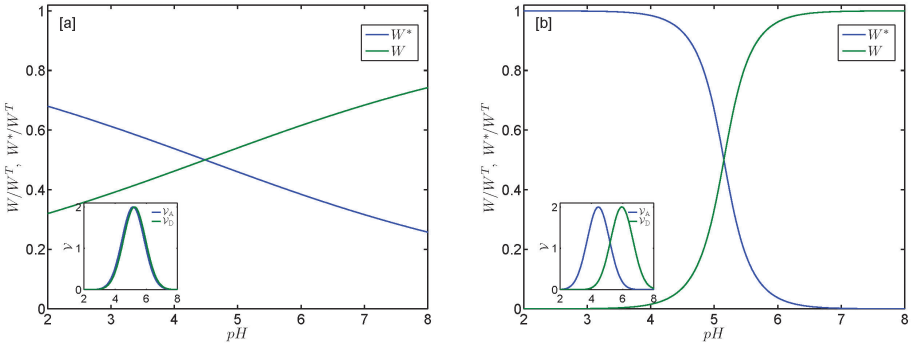

Figure 9. The fraction of the active and inactive form of protein W at steady state as function of the external pH. Two different situations are compared: **[a]** the maxima of the activities differ slightly ($M_A = 5.15$ and $M_D = 5.25$) and **[b]** the maxima differ-strongly from each other ($M_A = 5.45$ and $M_D = 5.95$). The corresponding specific activities, as a function of the pH, are shown as insets. With an increasing separation of the pH-profiles switch-like behavior emerges, which enables the (de)activation cycle to sense the intra- or extracellular pH level resulting an appropriate cellular response.

To investigate the influence of the intracellular pH on the product spectrum of a branching point, we consider a metabolite X, e. g. butyryl-CoA, which is converted to products A and B, e. g. butyrate and butanol. Additionally, metabolite X_1 shall be produced in both reactions that may be a reactant in further steps of a metabolic pathway, Figure 8[b]. The reactions are facilitated by enzymes E_1 and E_2. For the sake of simplicity, we assume that their concentrations are kept constant. Again, we consider two different scenarios, [a] slightly different specific activities and [b] strongly differing specific activities. Slightly differing specific result in a pH-dependent adaptation of the concentration of products A and B.

Interestingly, the concentration profile shows a switch-like behaviour for strongly differing activities. With an increasing pH level, the product which is produced in the enzyme kinetic reaction with the higher activity becomes dominant. In our example, this is the product *B*.

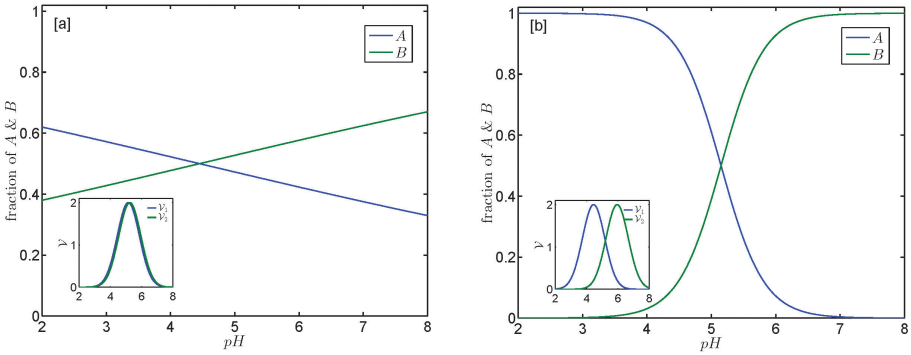

Figure 10. The fraction of products *A* and *B* with respect to the metabolite *X* as a function of the external pH value. Again, two situations are compared: **[a]** the maxima of the specific enzyme activities differ slightly and **[b]** the maxima differ strongly from each other. The corresponding specific activities as a function of the pH are shown in the insets. The diverging pH-dependent profiles lead to a pH-dependent product spectrum. Eventually, a changing pH shifts the product spectrum frommetabolite A to metabolite B and *vice versa*.

Interestingly, both scenarios representing genetic regulation and kinetic regulation exhibit a similar pH-dependent behaviour. However, they differ in their specific time scales. Kinetically regulated processes immediately respond to changes in the intracellular pH. In contrast, genetically regulated processes follow environmental variations with a delay caused by the time required for protein synthesis.

In summary, our investigation demonstrates that a changing pH level may lead to an altered product formation due to either changes in gene expression patterns or changes in specific activities of the reactions involved in the AB fermentation pathway of *C. acetobutylicum*.

Discussion

The bacterium *C. acetobutylicum* switches its metabolism between acidogenesis and solventogenesis in response to changes in the external pH. During acidogenesis (high pH), it dominantly produces acids, whereas the pH-neutral solvents acetone and butanol are the major fermentation products at solventogenesis. Several experimental studies, using a continuous culture under phosphate limitation, indicate that this metabolic adaptation involves changes on transcriptomic, proteomic, and metabolomic levels. However, the mechanisms underlying the regulation of the solventogenic shift remain unclear.

Using existent knowledge about the metabolic network and biochemical reactions, we established a kinetic model of AB fermentation in *C. acetobutylicum*. Incorporating gene regulation of solvent-producing enzymes furnishes a mechanistic representation of this pH-induced phase transition. Although, the model captures well the observed behaviour, including additional regulation may improve the fit. For instance, we have assumed that the induction of solventogenic enzymes is triggered at the same threshold pH value; assigning distinct threshold values to each encoding gene may improve the accuracy of the model.

Furthermore, considering that *C. acetobutylicum* is unable to maintain a constant intracellular pH level and experimental evidence indicates that the intracellular pH differs from the external pH by approximately $\Delta pH \approx 1$, we conclude that changes of the external pH affect the AB fermentation. Additionally, this idea is supported by observed strong pH-dependence of several enzymes involved in this metabolic process. Interestingly, the specific activity of the enzymes is optimal either during acidogenesis or solventogenesis. These findings suggest that pH-dependent kinetic properties of those enzymes may be important and could trigger the pH-induced metabolic switch.

Our investigations reveal that both regulatory mechanisms, genetic regulation, considered in the present model, and kinetic regulation may result in similar pH-dependent behaviour. Taken together experimental and theoretical knowledge, we conclude that kinetic and genetic regulation contribute to the cellular adaptation of *C. acetobutylicum* in response to changing pH levels.

Systems biology approaches involving alterations to gene expression levels are frequently adopted to investigate and exploit bacteria. For instance, we performed steady-state analyses to make predictions upon the effect of single mutations on the butanol yield during solventogenesis. By varying gene expression levels *in silico*, we infer that alterations in the expression of a single gene are insufficient to increase the butanol production significantly. Obviously, a more complex approach targeting two or more genes simultaneously is required.

The pH-dependency of enzymatic reactions has been neglected in previous models of clostridial AB fermentation potentially caused by missing knowledge about the pH-dependent kinetics of the enzymes and gene transcription. However, an isolated and independent consideration of kinetic and genetic regulation may be misleading. Further experimental and theoretical studies, therefore, require reliable information about pH-dependent enzymatic properties and pH-induced changes on transcriptomic, proteomic, and metabolomic levels. This information could provide the basis for a purposeful optimisation of the bacterium *C. acetobutylicum* for future industrial applications.

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