

MFA of *Clostridium Acetobutylicum* Pathway: the Role of Glucose and Xylose on the Acid Formation/Uptake

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Acetone-Butanol-Etanol is typically produced during the second stage of batch fermentations of some *Clostridium* strains under selected operating conditions: acids are consumed along with the carbon source and pH increase. This contribution reports a MFA regarding *Clostridium acetobutylicum* DSM 792 fermentation adopting reference sugars (glucose and xylose) as carbon sources. The attention on these sugars is particularly relevant because they are the main components of hydrolyzed lignocellulosic biomass. The results have pointed out that the butyrate formation pathway plays a fundamental role both in the accumulation of butyrate and in butyrate uptake without acetone formation.

1. Introduction

The concerns regarding energy and environmental issues have revalued the interest in the use of biomass as a renewable energy source. According to this scenario, studies have bloomed in the scientific literature regarding the production of energy vectors from a wide spectrum of biomass. Solvent-producing clostridia could produce acetone, butanol, and ethanol (ABE) from several biomasses such as palm oil waste (Lee et al., 1995), agro-industrial waste(water)s (Raganati et al., 2013), and agricultural crops (Qureshi et al., 2001). The remarkable features of the butanol – e.g. hydrophobicity, high energy density, storage and transportation consistent with current structures – make this alcohol a potential substitute and/or supplement of gasoline (Table 1) (Cascone, 2008; Masiero et al., 2011).

Table 1: Main features of biofuels vs. gasoline

Characteristics	Butanol	Ethanol	Gasoline
Density (kg/m ³ at 20 °C)	810	789	743
Net Calorific value (MJ/kg)	32	27	43
Octane Number	86	100	87
Solubility in water (% at 25 °C)	9	100	<0.01

ABE is produced during the fermentations of some *Clostridium* strains - saccharolytic butyric acid-producing bacteria - under appropriate operating conditions (*C. saccharoperbutylacetonicum*, *C. acetobutylicum*, *C. beijerinckii*, *C. aurantibutyricum*). These strains are able to metabolize a great variety of substrates, pentoses, hexoses, mono-, di- and polysaccharides (Jones and Woods, 1986). Under batch conditions, the fermentation process of solvent-producing clostridium strains proceeds with the production of cells, hydrogen, carbon dioxide, acetic acid and butyric acid during the initial growth phase (acidogenesis) (Jones and Woods 1986). As the acid concentrations increase (pH decrease), the metabolism of cells shifts to solvent production (solventogenesis) and acidogenic cells – able to reproduce themselves - shift to the solventogenesis state with a morphological change (Jones and Woods, 1986). Two different physiological states must be taken into account for Clostridia: one for the acidogenic phase, and one for the solventogenic phase. During the acidogenesis the acids production ensures high ATP and

NADH yields. During the solventogenesis the active cells become endospores unable to reproduce themselves.

The spectrum of sugars present in the most promising feedstock for the ABE fermentation is quite large and studies available in the literature have pointed out that the fermentation performances depend on the sugar assortment (Ezeji and Blaschek, 2008; Raganati et al., 2012). Despite this large spectrum of feedstocks, models of the clostridia metabolism for the sugars are very few. Papoutsakis (1984) developed a stoichiometric model for the ABE production pathway from glucose. The model could be used to calculate or estimate the rates of reactions of the pathway in several ABE-producing clostridia. Desai et al. (1999) investigated the contribution of acid formation pathways in the metabolism of *C. acetobutylicum* ATCC824T according to the metabolic flux analysis (MFA) and using glucose as carbon source.

A research program is active in Napoli regarding production of butanol by ABE fermentation (Napoli et al., 2010; Napoli et al., 2012; Raganati et al., 2013). In this study the MFA has been adopted to investigate the role of the main reaction steps of the *Clostridium acetobutylicum* metabolic pathway to convert reference sugars of hydrolyzed lignocellulosic biomass into butanol. Results of batch fermentation tests carried out using glucose and xylose as carbon source have been adopted for the flux assessment. The MFA has been implemented with reference to glucose and xylose as carbon source. The comparison of fluxes has suggested the relative role of each reaction step as a function of the carbon source investigated.

2. Materials and methods

2.1 Microorganism and medium

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were reactivated according to the DSMZ procedure. Reactivated cultures were stored at -80°C. The thawed cells were inoculated into 12 mL synthetic medium containing glucose (30 g/L) and Yeast Extract (YE) (5 g/L) in 15 mL Hungate tubes (pre-cultures). Cells were grown under anaerobic conditions for 48 h at 37 °C, then they were transferred into fermentation bottles. The fermentation medium consisted of 5 g/L YE and 5 g/L of CaCO₃ supplemented to P2 stock solution: buffer) 0.25 g/L KH₂PO₄, 0.25 g/L K₂HPO₄, 2 g/L ammonium chloride; mineral) 0.2 g/L MgSO₄·7H₂O, 0.01 g/L MnSO₄·H₂O, 0.01 g/L FeSO₄·7H₂O (Napoli et al., 2011). The medium was sterilized in autoclave prior to the carbon addition. The carbon source (single sugar) was supplemented to the medium and sterilized by filtration. Two sugars – glucose and xylose - were investigated. The overall initial concentration of each sugar was set at 60 g/L.

2.2 Batch fermentation

Pyrex screw capped bottles (100 mL) containing 75 mL medium were used as fermenters. All experiments were carried out in fermenters at rest, at 37 °C, without pH control. The medium was inoculated with 6.25 %_v suspension of active growing pre-cultures. 3 mL of cultures were sampled periodically for cell/metabolites characterization.

2.3 Analytical procedures

pH was measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments). Analysis of culture samples was carried out after centrifugation at 10,000 rpm for 10 min. The liquid phase was characterized in terms of sugar and metabolite concentrations. Cell density was determined by measuring the absorbance at 600 nm (Cary-Varian mod. 50 scan UV-VIS spectrophotometer). Calibration tests indicated that the optical density is proportional to *C. acetobutylicum* dry mass under the operating conditions tested, in particular 1 OD₆₀₀ corresponded to 0.4 g_{DM}/L. Sugar concentration was determined by high performance liquid chromatography (HPLC) using an Agilent 1100 system (Palo Alto, CA). The sugars were separated on a 8 μm Hi-Plex H, 30 cm 7.7 mm at room temperature and detected with a refractive index detector. Deionized water was used as mobile phase at a flow rate of 0.6 mL/min. A GC apparatus equipped with a FID, and outfitted with a capillary column poraplot Q (25 m x 0.32 mm) was used.

3. Metabolic flux analysis

According to the metabolic flux analysis (Papoutsakis, 1984), the assessment of in vivo fluxes has been carried out by processing the time-resolved concentration of substrates and products. The analysis has been based on the known metabolic pathway (Fig. 1) translated into a set of reactions, e.g. a set of linear equations. The reaction set regarding the glucose conversion is reported in Table 2 as Eq.s (T.1.1) through (T.1.13): reversible reactions are indicated with '+/-', irreversible reactions are indicated with '+' (Desai et al., 1999). The r_{PTAAK} and r_{PTBBK} refer to the reactions catalysed by the enzyme couples PTA-AK and PTB-BK, respectively.

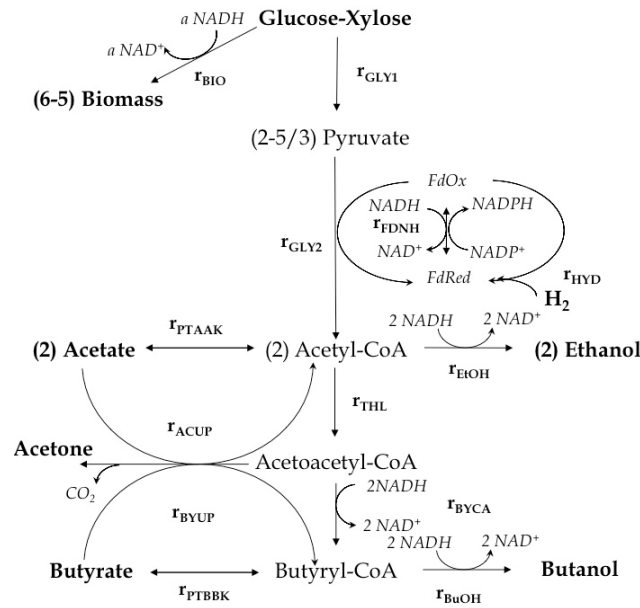


Figure 1: *C. acetobutylicum* metabolic pathway and relevant fluxes. The conversion between major carbon containing species is depicted (cofactors are not reported).

Table 2: Reaction step of *Clostridium acetobutylicum* metabolic pathway

Reaction Step	Flux
$\text{GLU} \xrightarrow{\frac{6 \cdot 12}{\sigma_X \cdot Y_{X/ATP}} \text{ATP} - a \text{NADH}} 6\text{BIO}$	$a = \frac{6}{2}(\gamma_X - \gamma_{\text{GLU}})$ $+/-r_{\text{BIO}}$ (T.1.1)
$\text{GLU} \longrightarrow 2\text{PYR} + 2\text{ATP} + 2\text{NADH}$	$+r_{\text{GLY1}}$ (T.1.2)
$\text{PYR} \longrightarrow \text{ACoA} + \text{CO}_2 + \text{FdRed}$	$+r_{\text{GLY2}}$ (T.1.3)
$\text{ACoA} + 2\text{NADH} \longrightarrow \text{E}$	$+r_{\text{EtOH}}$ (T.1.4)
$\text{ACoA} \longrightarrow \text{AA} + \text{ATP}$	$+/-r_{\text{PTAAK}}$ (T.1.5)
$2\text{ACoA} \longrightarrow \text{AACoA}$	$+/-r_{\text{THL}}$ (T.1.6)
$\text{AACoA} + \text{AA} \longrightarrow \text{A} + \text{ACoA} + \text{CO}_2$	$+r_{\text{ACUP}}$ (T.1.7)
$\text{AACoA} + \text{AB} \longrightarrow \text{BCoA} + \text{A} + \text{CO}_2$	$+r_{\text{BYUP}}$ (T.1.8)
$\text{AACoA} + 2\text{NADH} \longrightarrow \text{BCoA}$	$+/-r_{\text{BYCA}}$ (T.1.9)
$\text{BCoA} \longrightarrow \text{AB} + \text{ATP}$	$+/-r_{\text{PTBBK}}$ (T.1.10)
$\text{BCoA} + 2\text{NADH} \longrightarrow \text{B}$	$+r_{\text{BuOH}}$ (T.1.11)
$\text{FdRed} \rightarrow \text{H}_2$	$+r_{\text{HYD}}$ (T.1.12)
$\text{FdRed} \rightarrow \text{NADH}$	$+/-r_{\text{FDNH}}$ (T.1.13)
$\text{XYL} \xrightarrow{\frac{5 \cdot 12}{\sigma_X \cdot Y_{X/ATP}} \text{ATP} - \frac{5}{2}(\gamma_X - \gamma_{\text{XYL}}) \text{NADH}} 5\text{BIO}$	$+/-r_{\text{BIO}}$ (T.1.14)
$\text{XYL} \longrightarrow \frac{5}{3}\text{PYR} + \frac{5}{3}\text{ATP} + \frac{5}{3}\text{NADH}$	$+r_{\text{GLY1}}$ (T.1.15)

The MFA of xylose fermentation has been proposed by substituting the reaction steps (T.1.1) and (T.1.2) with (T.1.14) and (T.1.15). The reactions regarding ATP have not been considered in the present model. It has been assumed that the produced ATP is consumed by cell growth process and cell non-growth maintenance. The flux r_{BIO} has been adopted as a measure of both growth and lysis of cells and it is based on a dynamical shifting of the stoichiometric coefficients of glucose/xylose and NADH: i) for positive values

of r_{BIO} , the coefficients reported in table 2 were used; for negative values of r_{BIO} , the coefficient of glucose/xylose and NADH were set at 0.

The stoichiometric matrix of the model was characterized by a singularity that prevented the assessment of a unique set of fluxes of the metabolic activity. The non linear constraint proposed by Desai et al. (1999) was adopted to solve the model equation set. The constraint relates the uptake fluxes of acetate (r_{ACUP}) and butyrate (r_{BYUP}):

$$\frac{r_{\text{BYUP}}}{r_{\text{ACUP}}} = 0.315 \frac{\text{[butyrate]}}{\text{[acetate]}} \quad (1)$$

The stoichiometric model of solventogenic clostridia has been restructured as a constrained minimization problem of the objective function Eq. (2):

$$\|A \cdot r - x\|^2 + \left(r_{\text{BYUP}} \text{[acetate]} - 0.315 \text{[butyrate]} \right)^2 \quad (2)$$

where the first term of Eq. (2) is the sum of weighted squared residuals, and the second term is from the Eq. (1). A is the stoichiometric matrix, x the species accumulation vector, and r the pathway flux vector.

4. Results and discussion

Figure 2 shows metabolite concentration measured during batch fermentations of *C. acetobutylicum* on both glucose and xylose as carbon source. As expected, acetate and butyrate were produced/accumulated at the beginning of the fermentation, and acetone, butanol and ethanol were produced/accumulated at a second stage. The A:B:E molar ratio was about 3:6:2 and 1:6:1 for glucose and xylose respectively.

The concentration time series were processed to assess the specific net rates (production/consumption) of acetate, butyrate, and acetone (Fig. 3 A-C) for both glucose and xylose fermentation. The specific net rates referred to the unit biomass concentration expressed as OD. The vertical dotted lines mark the transition to the solventogenesis phase. The analysis of the specific net rates referred to glucose (Fig. 3A) pointed out that: i) acetate and butyrate rates were maximum at the beginning of the fermentation and gradually decreased during the transition to the solventogenesis phase; ii) acetate and butyrate rates were lower than zero during the solventogenesis phase to point out the uptake of both acids; iii) the rate of acetone increased at the solventogenesis threshold and it reached its maximum value late during the solventogenesis phase.

The rates reported in Fig. 3C were calculated for the fermentation tests carried out adopting xylose as carbon source (Fig. 2B). The main dynamics observed for the test carried out adopting glucose as carbon source are still observed. The main differences are: i) the butyrate specific net rate was definitively negative during the solventogenesis phase and the acetate uptake rate is nearby zero, that is only butyrate is definitively converted during solventogenesis; ii) the acetone production rate is quite low throughout the test.

It is worth to note that the specific net rate of acetone departs from zero before the acidogenesis phase ended. This observation confirms that measured uptake of acetate and butyrate are net values and acid formation rates are higher than the measured specific net values. The specific net rates do not accurately describe metabolic activity.

Metabolic flux analysis has been adopted to assess the un-observable patterns of the metabolic activity. The specific in vivo fluxes as estimated according to the MFA are reported in Figs 3 B and 3D. The rate r_{PTAAK} assessed for both glucose and xylose metabolisms decreased gradually throughout the fermentation and approached zero. The observed r_{PTAAK} vs. time profile is consistent with the acetate specific net rate vs. time profile. However, a fundamental difference may be noted between glucose and xylose fermentation as reported hereinafter.

Glucose – i) it has been assessed that the acetate is continuously produced (not consumed) according to the reaction (T.1.5) throughout the fermentation, indeed r_{PTAAK} is always positive during the fermentation (Fig. 3B); ii) it has been measured that the acetate is converted during the late solventogenesis phase (Fig. 3A). The analysis of results suggests that acetate uptake is via the acetone formation pathway, r_{ACUP} .

Xylose – i) it has been assessed that the acetate is continuously produced (not consumed) according to the reaction (T.1.5) throughout the fermentation, indeed r_{PTAAK} is always positive during the fermentation (Fig. 3D); ii) the acetate conversion (r_{PTAAK}) vs. time (Fig. 3C-D) looks like the acetate specific net production rate. The analysis of results suggests that the acetone formation pathway, r_{ACUP} , is negligible.

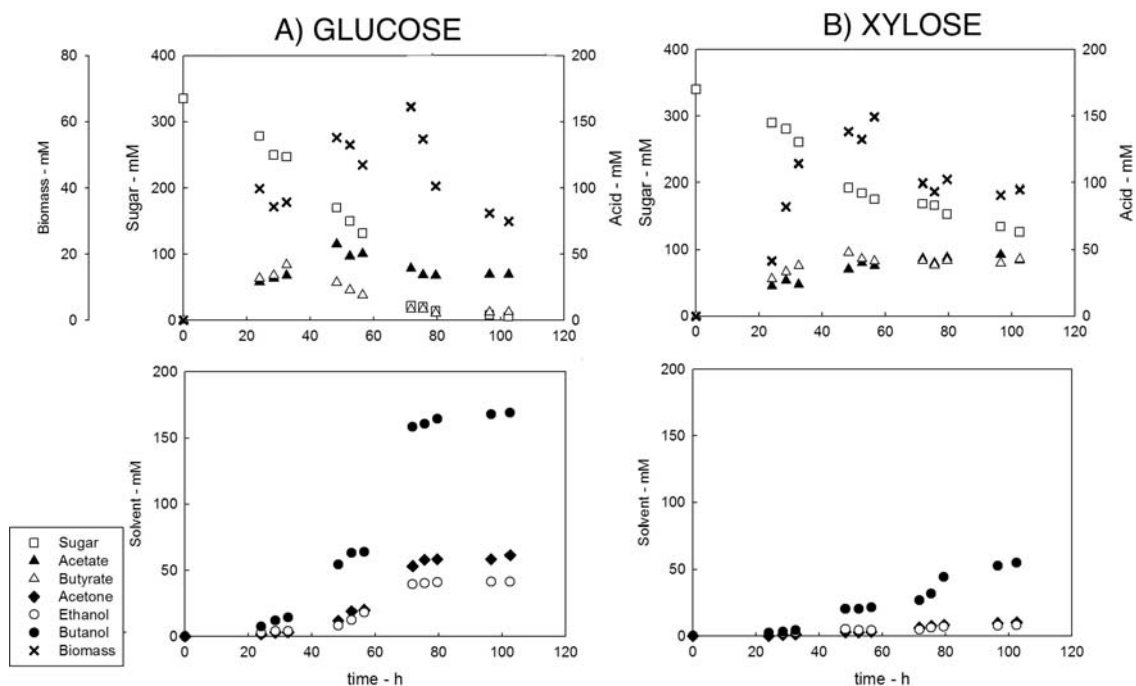


Figure 2: Time resolved concentration of metabolites measured during *C. acetobutylicum* batch fermentation.

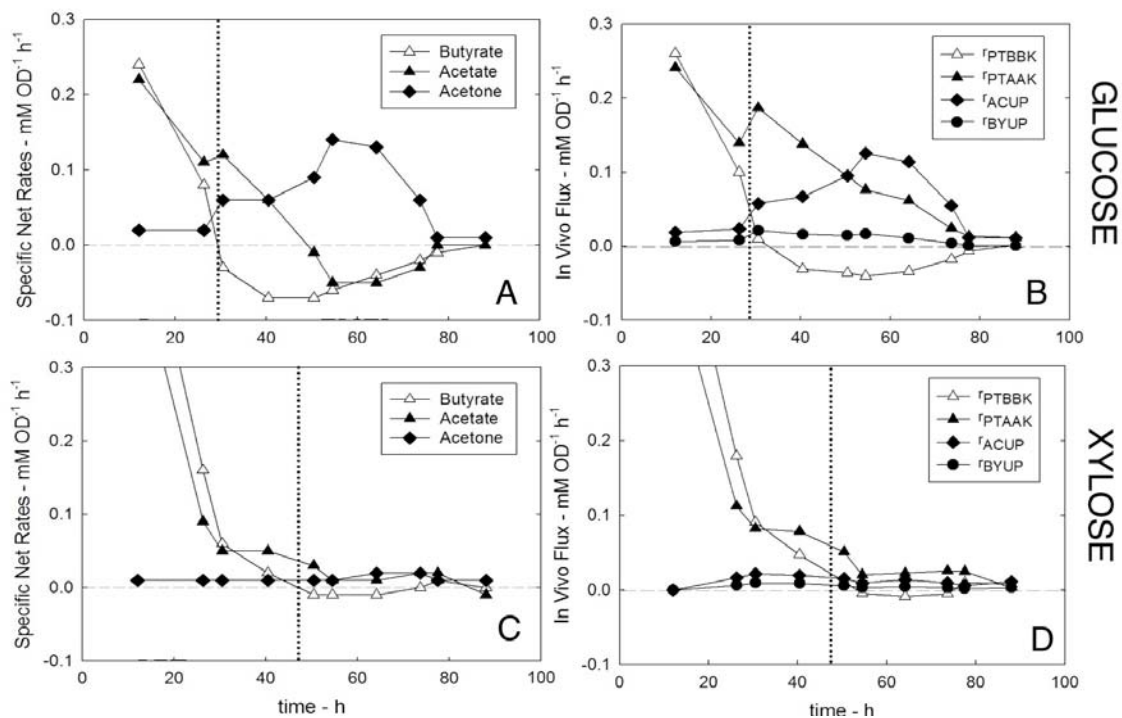


Figure 3: A-C) Specific net rates of production (>0) or consumption (<0) of acetate, butyrate and acetone calculated by processing data reported in Fig. 2. B-D) Specific in vivo fluxes assessed by MFA. Dashed horizontal lines mark zero net rate or in vivo flux. Vertical dotted lines mark the transition to the solventogenesis.

The butyrate formation pathway appears to change during the different phases of the cultures. The r_{PTBBK} assessed for both carbon sources decreases gradually since the beginning of the fermentation tests and it is a proof of that the butyrate is produced according to the butyrate formation pathway. As the solventogenesis starts, the butyrate formation pathway acts to uptake the butyrate (negative values of r_{PTBBK} , Fig.s 3B and 3D). Although the reconversion of butyrate has been assessed for both carbon sources, this process is more marked in glucose fermentation than in xylose fermentation (Fig. 3B vs. Fig. 3D). As regards the role of the acetone formation pathway in the fate of the butyrate, the butyrate formation pathway appears to dominate the uptake with respect to the acetone formation pathway. The analysis of reported results regarding the butyrate fate points out that PTB and BK are still active during solventogenesis and they are responsible of the butanol production at a low acetone/butanol ratio. This scenario is still more marked for xylose fermentation. The higher (absolute) values of r_{PTBBK} calculated for glucose fermentation under solventogenic phase with respect to those calculated for xylose fermentation would suggest that a high butyryl-CoA production is expected for glucose fermentation. The higher values of butyryl-CoA concentration are in agreement with the higher butanol concentration measured at the end of the glucose fermentation (Fig. 2).

5. Final remarks

Clostridium acetobutylicum fermentation of both glucose and xylose has been successfully carried out to produce butanol. Results have been interpreted according the metabolic flux analysis (MFA). The analysis of the MFA has pointed out the roles played by acid formation enzymes in the complex primary metabolism of solventogenic clostridia. In particular, the effects of the carbon source on the relevance of each step of the acid formation/uptake pathway have been highlighted. The butyrate formation pathway plays a fundamental role both in the production of butyrate and in butyrate uptake without acetone formation.

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