

Assessment of the Kinetics of Butanol Production by *Clostridium acetobutylicum*

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Preliminary results of a research activity aiming at investigating the feasibility of the acetone-butanol-ethanol (ABE) production by *Clostridium acetobutylicum* ATCC824 are reported. The contribution regards the characterization of the kinetics related to the ABE production process by free *C. acetobutylicum* ATCC824. Lactose solutions were adopted as medium with the aim of emulating cheese whey. The conversion process is characterized in terms of cells, acids, solvents, pH and total organic compounds as a function of time. Tests carried out under batch conditions show that: i) cells growth is constant for lactose concentration (C_L) smaller than 100 g/L; ii) the butanol specific production rate as a function of C_L may be represented by a Monod-like kinetics; iii) the lactose conversion - measured at the end of the solventogenesis phase - decreases with C_L ; iv) the selectivity of butanol with respect to total solvents increases with C_L and stabilizes at about 72%_w for C_L larger than 30 g/L.

1. Introduction

The economic scenario has revived the interest in Acetone-Butanol-Ethanol (ABE) fermentations. Recent developments in molecular techniques applied to solventogenic microorganisms in combination with recent advances in fermentation systems and downstream processing have contributed to improve ABE fermentation processes feasibility and competitiveness. The challenges raised over the last years as regards ABE production may be synthesized in: i) use of renewable resources as substrate; ii) selection of strains characterized by high ABE productivity; iii) development of new fermentation systems; iv) development of new downstream strategies for enhanced solvent recovery. The selection of unconventional substrates is favoured by the ability of clostridia strains to metabolize a wide range of carbohydrates like glucose, lactose, etc..., typically present in wastewater streams e.g. from the food industry. In particular, studies available in literature point out the potential of the ABE production process by fermentation of lactose (Welsh and Veliky, 1984) or cheese whey (Welsh and Veliky, 1986; Maddox, 1980).

Even though clostridia have been proven successful to produce ABE, information available in literature to support industrial scale-up is still lacking. The missing information regards either the kinetics of substrate conversion, cell growth and butanol production (Jones and Woods, 1986; Shinto et al., 2007) or the reactor systems.

Typically, the ABE fermentation process has been studied by means of reactor systems belonging to the batch and fed-batch typologies. Only a few attempts are reported in literature regarding continuous fermentation by means of clostridia strains confined in the reactor by immobilization (Huang et al., 2004; Qureshi et al., 2005; Ezeji et al., 2007) or cell-recycling (Tashiro et al., 2005). In a review on the state of art of the “white biotechnology”, Villadsen (2007) has specifically mentioned the ABE process as an example of how and how far a more fundamental based approach makes process development more effective and successful.

The present study reports the preliminary results of a research activity aiming at investigating the feasibility of the ABE production by *Clostridium acetobutylicum* ATCC824 in a continuous biofilm reactor adopting cheese whey as feedstock. The contribution regards the characterization of the kinetics related to the ABE production process by free *C. acetobutylicum* ATCC824. Lactose solutions were adopted as medium with the aim of emulating cheese whey. The conversion process is characterized in terms of cells, acids, solvents, pH and total organic compounds as a function of time. Results are worked out to assess the kinetics of the cells growth and of the ABE production. The yields of the carbon source in cells, acids and solvents are also assessed.

2 Materials and Method

2.1 Microorganism and culture media

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were reactivated according to the DSMZ procedure and stored at 4°C.

Synthetic medium adopted consisted of Yeast Extract (YE) at 5 g/L and of D-Lactose, typically at 50 g/L. CaCO₃ was added in the stock medium at concentration of 18 g/L. The medium was sterilized in autoclave.

2.2 Apparatus

Pre-cultures were carried out in 15 mL Hungate tubes. D-lactose bioconversion by *C. acetobutylicum* was investigated batchwise in screw-cap bottles (250 mL) housed in a thermostated room at 35°C.

2.3 Operating conditions and procedures

All tests were carried out at 35°C under anaerobic conditions and no pH control was adopted.

Pre-culture was prepared inoculating 2 mL of the stock culture into 5 mL of synthetic medium and incubated for two days. The pre-cultures were inoculated into the reactors containing synthetic medium consisting of YE and lactose at pre-fixed concentration (up to 110 g/L). Typically, the initial cells concentration was fixed at 4 mg_{DM}/L. The culture was periodically sampled to measure cell and metabolites concentrations, until lactose concentration approached a stationary state. Each measurement was carried out in triplicate.

2.4 Analytical methods

pH was measured off-line on 3 mL samples by a pHmeter (Hanna Instruments). Analysis of culture samples was carried out after centrifugation at 11,000 rpm for 10 min. The solid phase was characterized for biomass concentration. Liquid phase was characterized for lactose and metabolites concentration, and total organic carbon (TOC). 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. Lactose concentration was measured by means of an enzymatic kit (Biopharm). A GC apparatus was used, equipped with a FID, and outfitted with a capillary column poraplot Q (25 m x 0.32mm). External standards were adopted to assess acids and alcohols and their concentrations. The TOC was measured with a Shimadzu TOC 5000A analyzer.

3. Results

Figure 1 reports the time resolved profiles of the concentration of *C. acetobutylicum* cells (X), of lactose (C_L) and of metabolites (acetic acid, butyric acid, ethanol, acetone and butanol) as well as of the pH, measured during a batch culture carried out at 50 g/L initial lactose concentration (C_L^0). It is possible to identify two phases: the acidogenesis and the solventogenesis phases. The onset of solvents production marks the beginning of the solventogenesis phase ($t_A \cong 22$ h). Provided an 8 hour time-lag, the acidogenesis phase is characterized by: i) the steady increase of concentration of cells and of acids; ii) a cell concentration vs time profile that mirrors the lactose consumption; iii) the decrease of pH; iv) a molar ratio between acids (butyric/acetic) constant and equal to 1.5. The solventogenesis phase is triggered by pH=4, in agreement with previous results (Jones and Woods, 1986). The solventogenesis phase is characterized by: i) the gradual decrease of the lactose concentration approaching a stationary value; ii) the acid molar ratio constant and equal to 1.5.

The culture was further characterized in terms of: i) cells specific growth rate (μ) estimated according to usual procedures for kinetic analysis from batch culture data under the assumption of first-order growth kinetics with respect to biomass concentration in the exponential phase; ii) fractional yield of lactose-to-biomass estimated with reference to the acidogenesis phase ($(Y_{X/L})_{ac}$); iii) fractional yield of lactose-to-butanol ($(Y_{B/L})_{sov}$) with reference to the solventogenesis phase; iv) fractional yield of lactose-to-solvents ($(Y_{Sol/L})_{ov}$) and fractional yield of lactose-to-butanol ($(Y_{B/L})_{ov}$) with reference to the overall conversion test; v) overall lactose conversion (ξ_L). The maximum butanol specific rate (r_B) was estimated at the threshold of the solventogenesis phase as the slope of the butanol concentration (C_B) vs time curve (see Fig. 1). With reference to the run reported in figure 1 it results: $\mu=0.29$ h⁻¹; $(Y_{X/L})_{ac}=0.18$; $(Y_{Sol/L})_{ov}=0.20$; $(Y_{B/L})_{ov}=0.17$; $\xi_L=0.34$; $r_B=80$ mg_B/g_{DM}h.

The mass balance on carbon takes into account the carbon content of the liquid phase and *C. acetobutylicum* and that converted into CO₂, according to the Embden-Meyerhof

pathway (Jones and Woods, 1986). A compact form of the global balance, referred to the acidogenesis phase $[0, t_A]$, reads:

$$(\text{TOC}_0 + X_0 \cdot \alpha_C) - (\text{TOC}_A + X_A \cdot \alpha_C) - 4 * \frac{\text{MW}_C}{\text{MW}_L} (C_L^0 - C_{L,A}) = 0 \quad (1)$$

where α_C is the carbon mass fraction of *C. acetobutylicum* (0.4), MW_C and MW_L the molecular masses of carbon and lactose, X_A and $C_{L,A}$ the concentration of the cells and of lactose, respectively, at $t=t_A$, TOC_0 and TOC_A the total organic carbon at the beginning of the run and at $t=t_A$. The accuracy of the carbon balance is expressed as:

$$\frac{(\text{TOC}_A + X_A \cdot \alpha_C) + 4 * \frac{\text{MW}_C}{\text{MW}_L} (C_L^0 - C_{L,A})}{(\text{TOC}_0 + X_0 \cdot \alpha_C)} \approx \delta_{ac} \quad (2)$$

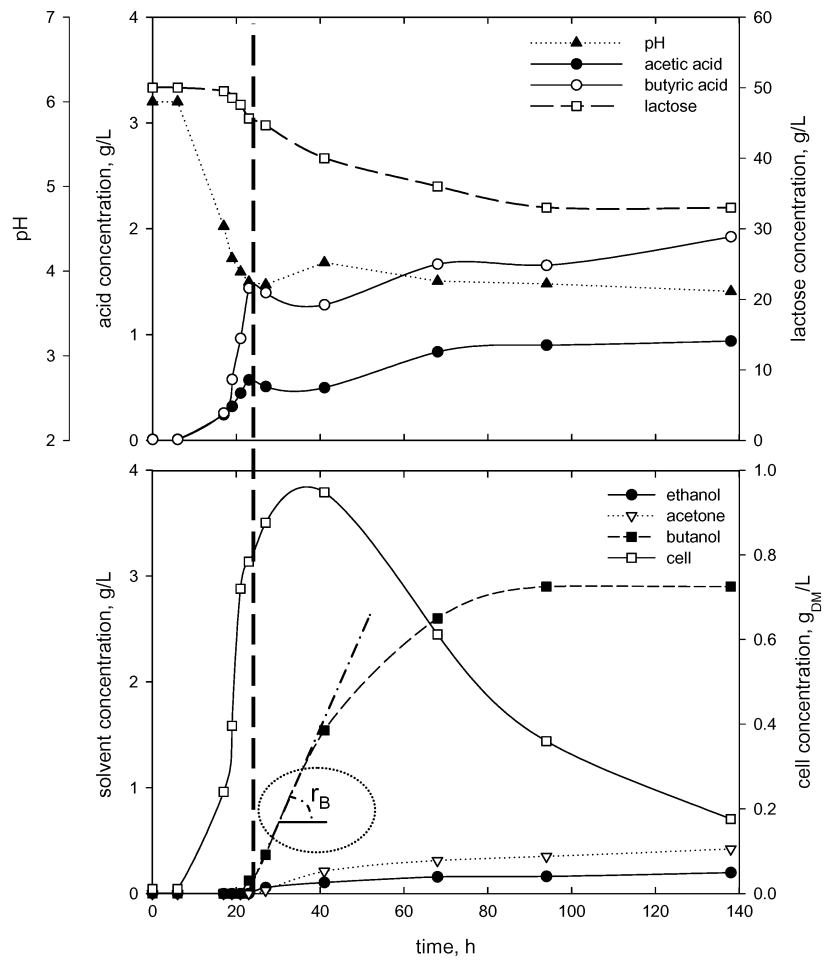


Figure 1 Data measured during a lactose conversion test: $C_L^0 = 50 \text{ g/L}$. The vertical dashed line marks the beginning of the solventogenesis phase.

The mass balance on carbon referred to the overall test takes into account the extra CO₂ released during acetone production (Jones and Woods, 1986). Assuming that the cell concentration decreases as a consequence of the sporulation without any cell leases, the global balance reads:

$$(\text{TOC}_0 + X_0 \cdot \alpha_C) - (\text{TOC}_A + X_{\max} \cdot \alpha_C) - 4 * \frac{\text{MW}_C}{\text{MW}_L} (C_L^0 - C_L) - \frac{\text{MW}_C}{\text{MW}_{Ac}} C_{Ac} = 0 \quad (3)$$

where X_{\max} is the maximum concentration and C_L and C_{Ac} the concentration of lactose and acetone and the end of the test. The accuracy of the test assessment has been expressed by δ_{ov} estimated as in eq. (2).

Data regarding the test reported in figure 1 are characterized by $\delta_{ac} = 0.96$ and $\delta_{ov} = 0.90$.

Figure 2 reports relevant data of lactose conversion as a function of the initial lactose concentration. The accuracy of the tests expressed in terms of δ_{ac} and δ_{ov} was always between 0.90 and 1. Main results are herein reported

Acidogenesis phase (figure 2A). Data of concentration of lactose, acetic and butyric acids measured at the end of the acidogenesis phase are reported. The process is characterized by cells specific growth rate, acid molar ratio and the $(Y_{X/L})_{ac}$ constant with C_L^0 , under operating conditions tested. Except for the run carried out at $C_L^0 = 2$ g/L, the pH at the threshold of the solventogenesis phase does not change with C_L^0 . During the run carried out at $C_L^0 = 2$ g/L, the pH decreases with the time and stabilizes at 4.4 when the conversion stops as a consequence of the lactose depletion. It is noteworthy that: i) the amount of lactose converted during the acidogenesis phase is about 4 g/L, if available; ii) the combination of $(Y_{X/S})_{ac}$ and of the amount of lactose converted until t_A results in a cell concentration at the threshold of the solventogenesis phase nearby constant and equal to 0.7 g_{DM}/L; cell growth is characterized by a zero-order kinetic with respect to the lactose with a specific growth rate equals to 0.29 h⁻¹ under the operating conditions tested.

Solventogenesis phase. Figure 2B reports data of butanol concentration and of lactose-to-butanol fraction yield estimated at the end of the solventogenesis phase. Analysis of the figure highlights that: i) the final butanol concentration increases with C_L^0 ; ii) the yield of lactose in butanol is characterized by a maximum at $C_L^0 = 50$ g/L.

Results regarding the overall conversion process carried out at C_L^0 ranging between 2 and 110 g/L (figure 2C) highlight some important features. The butanol fractional yield is characterized by a maximum of about 0.2 g_B/g_L at $C_L^0 = 50$ g/L, about the half of the maximum theoretical value (0.4 g_B/g_L). The lactose conversion degree and the overall selectivity with respect to the butanol $(Y_{B/S}/Y_{Sol/S})_{ov}$ decreases with C_L^0 approaching, respectively, 0.3 and 0.65 (molar basis).

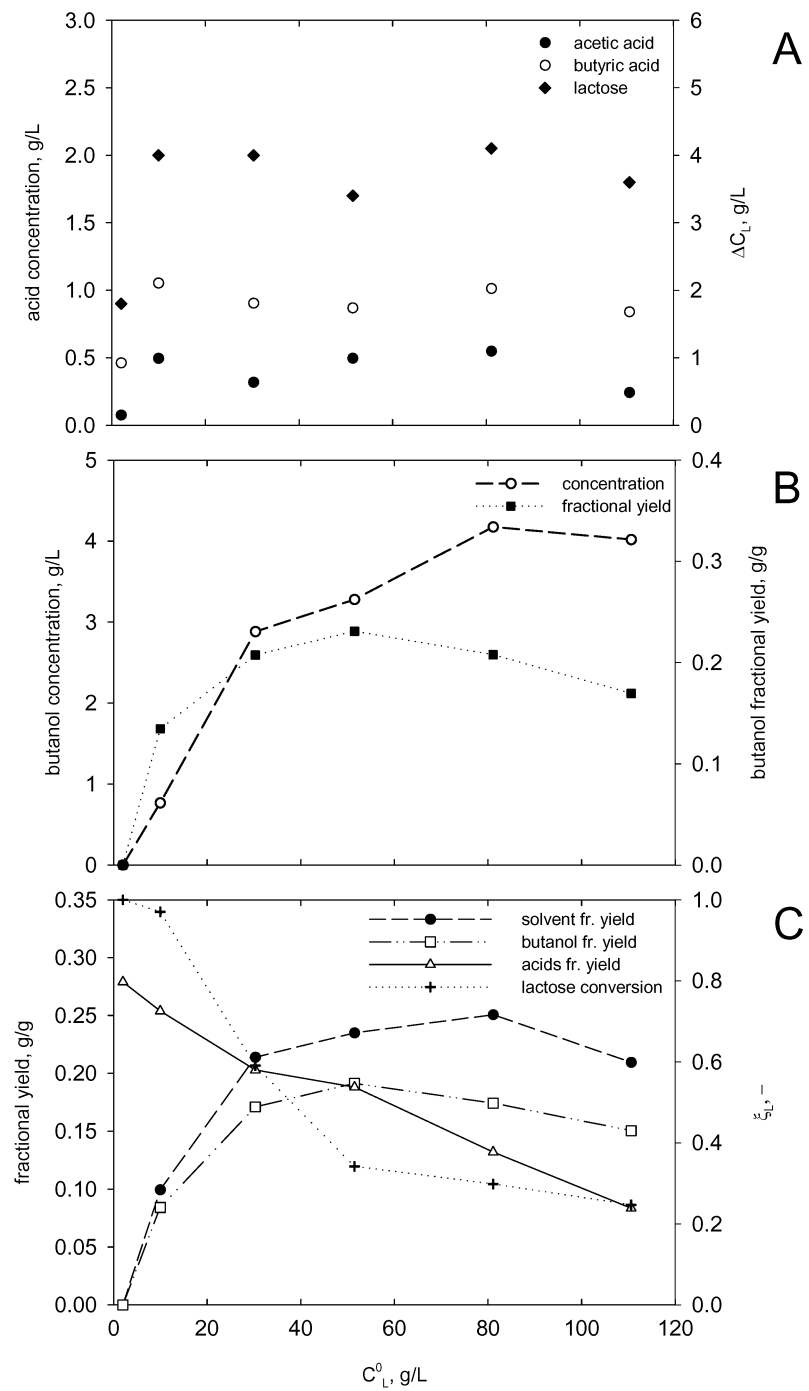


Figure 2 Relevant data assessed during lactose conversion as a function of the initial lactose concentration. A) Acidogenesis phase. B) Solventogenesis phase. C) Overall data.

The maximum butanol specific rate (r_B) as a function of the C_L measured on the verge of the solventogenesis phase is reported in Figure 3. It appears that the r_B may be described by a Monod-like model. The regression of data reported in figure 3 yields: $r_B = 128C_L / (28 + C_L)$ mg_B/g_{DM}h.

Some information about the optimal operating conditions to adopt in batch bioconversion may be drawn by working out data reported in the previous figures. The yield of butanol with respect to the initial lactose concentration (product of lactose conversion and of $Y_{B/S}$) is characterized by a maximum of about 0.10 at $C_L^0 = 30$ g/L.

Altogether, the butanol productivity by fermentation increases with C_L^0 even though the pureness becomes progressively lower. Therefore, the operating conditions to adopt for the butanol production should be optimized by taking into account both the fermentation and the recovery process. It should be remarked that for batch operations a fraction of lactose available is converted for stabilize the environment appropriate for the butanol production (pH lower than 4). In a continuous operation such fraction is just consumed during the reactor start-up.

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References

- Ezeji T.C., Qureshi N., Blaschek H.P., 2007, *Current Opinion in Biotechnology*, 18: 220-227
 Huang W.-C., Ramey D.E., Yang S.-T., 2004, *Appl. Biochem Biotechnol*, 113: 887-898

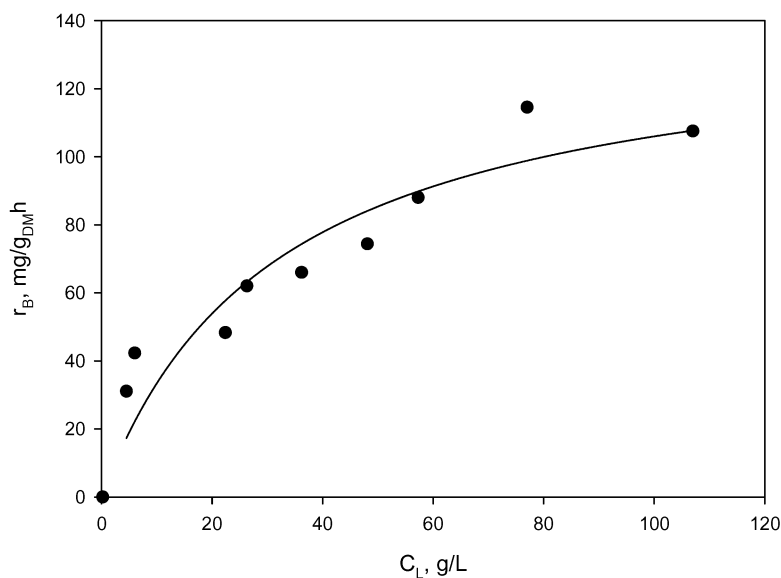


Figure 3 Specific production rate of butanol at the onset of the solventogenesis phase

- Jones D.T, Woods D.R., 1986, *Microb. Reviews*, Dec:484-524
- Maddox, I.S., 1980, *Biotechnology Letters*, Vol 2, 11, 61-64
- Qureshi N., Blaschek H.P., 2005, Eds Shetty K., Pometto A. and Paliyath G. Boca Raton, FL: Taylor & Francis Group: 525-551
- Shinto, H., Tashiro Y., Yamashita M., Kobayashi G., Sekiguchi T., Hanai T., Kuriya Y., Okamoto M., Sonomoto K. ,2007, *J. of Biotechnology*, 131: 45-56
- Tashiro Y., Takeda G., Kobayashi G., Sonomoto K., 2005, *J. of Biotechnology*, 120: 197-206
- Villadsen, J., 2007, *Chemical Engineering Science* 62, 6957-6968
- Welsh, F.W., Veliky, I., 1984, *Biotechnology Letters*, Vol 6, 1, 61-64
- Welsh, F.W., Veliky, I., 1986, *Biotechnology Letters*, Vol 8, 1, 43-46