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CONTINUOUS GLUCOSE FERMENTATION BY *CLOSTRIDIUM ACETOBUTYLICUM* – KINETICS UNDER ACIDOGENESIS AND SOLVENTOGENESIS CONDITIONS*

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Abstract. *The paper reports the characterization of the cell growth kinetics and of the specific butanol production rate of Clostridium acetobutylicum DSM 792 adopting glucose as carbon source. As regards the acidogenesis phase, the attention was focused on the cell growth kinetics as well as on energetics and yield of the fermentation. Tests were carried out in a CSTR operated under controlled pH. As regards the solventogenesis phase, the attention was focused on the specific butanol production rate. Tests were carried out in a CSTR equipped with a microfiltration unit. The biomass in the broth was classified as an heterogeneous cell population consisting of acidogenic cells, solventogenic cells, and spores.*

Keywords: growth rate, butanol production rate, *clostridium acetobutylicum*, glucose

1. Introduction

The apprehension over greenhouse gas emission from fossil fuels leading to global warming has caused the biofuels to have gained significant attention by the scientific and the industrial communities. Among the various biofuels, butanol is a promising one because it is characterized by high energy content, low miscibility

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with water and low volatility [1]. Butanol can be produced by *Clostridia* strains from a great variety of sugars [2, 3].

Clostridia batch fermentations are characterized by two successive phases: during the first phase (acidogenesis), cell grow and produce butyric acid (BA), acetic acid (AA), CO_2 and H_2. The production of acids causes a decrease in the pH of the medium, an environmental condition not favorable to bacterial growth. *Clostridia* cells respond to the harsh conditions by a metabolic and morphological shift (solventogenesis): i) the exponential growth phase ends; ii) the active cells become endospores, unable to grow; iii) the acids and the substrate are converted to solvents, Acetone-Butanol-Ethanol (ABE). The fermentation process ends as the concentration of ABE approach the inhibition threshold [4].

The design and optimization of a fermenter for butanol production ask for kinetic of both the cell growth and the solvent production. In particular, the assessment under continuous fermentation conditions is characterized by higher realiability with respect to that carried out under batch conditions [5]. The effects of alcohols and acids on the kinetics of cell growth and metabolite production are a key issue for the industrial development of the ABE fermentation [6]. Previous investigations proved that the kinetics depend on the sugar. Indeed, kinetic characterization was assessed for xylose [7,8] and lactose [9,10]. To the authors knowledge, investigations reported in the literature regarding *Clostridia* kinetics on glucose did not include cell population balance during the different fermentation phases.

This paper reports the assessment of the growth kinetics and of the specific butanol production rate of *Clostridium acetobutylicum* DSM 792 using glucose as carbon source. In particular: i) the acidogenesis characterization was carried out operating a CSTR under controlled pH. The effects of acids and solvents on the growth rate of *C. acetobutylicum* cells under acidogenesis phase conditions was investigated. The dilution rate (D, the ratio between the fed volumetric flow rate and the reactor volume) ranged over a wide interval; ii) the solventogenesis characterization was carried out by means of a CSTR equipped with a microfiltration (MF) unit to confine the solventogenic (no-growing) cells in the reactor. The D and the recycle ratio (R, the ratio between the volumetric flow rate through the microfiltration unit and the feed flow rate) ranged over wide intervals. The reactor performance was assessed to measure the heterogeneous cell population (acidogenic, solventogenic, spores) in the reactor and the butanol production rate referred to the mass unit of solvetogenic cells.

2. Materials and methods

2.1. Microorganism, media and analytical methods

Stock cultures reactivation, inoculation procedures and synthetic medium composition and analytic procedures are reported in Napoli et al. [10].

2.2. Apparatus

Apparatus for acidogenesis and solventogenesis characterization are reported in Napoli et al. [10] and Procentese et al. [7-9], respectively. The volume of the reactor was 0.4 L for both the characterization tests.

2.3. Operating conditions and procedures

Acidogenesis phase

The D was tuned between 0.07 and 1.7 h^{-1}. The glucose concentration in the reactor feeding was set at 40g/L. The set-point for the pH controller was set at 5.5.

Solventogenesis phase

Two streams were withdrawn from the reactor: Q_P) the (sterile) stream filtered by the MF unit; Q_{OUT}) the broth stream spilled from the fermenter. The volumetric flow rate of the former was controlled by means of a peristaltic pump; the volumetric flow rate of the latter was measured at the overflow duct exit. The recycle stream between the reactor and the MF unit was characterized in terms of the recycle ratio (R) defined as Q_P/Q_0 [7,9].

The glucose concentration in the feeding to the reactor was set at 50 g/L. The set-point for the pH controller was set at 4.7. The investigation interval of D and R was 0.02–0.22 h^{-1} and 10–70%, respectively, according to previous studies [7,9]. A set of runs was carried out by setting R at 15% and changing D between 0.02 and 0.22 h^{-1}. A test campaign was carried out by setting D at a pre-fixed value (0.07 and 0.1 h^{-1}) and changing R between 10% and 40%. More details on operating conditions and procedures are reported in Procentese et al. [6].

3. Theoretical framework

3.1. Acidogenesis phase

Growth rate model, energetics and product yields

The specific cell growth rate μ was assessed according to the balanced growth condition (D=μ), being the reactor feeding a sterile stream. In particular, an unstructured unsegregated model for the cell growth rate was assumed. The μ of *C. acetobutylicum* is expected to be characterized by products inhibition as reported by Procentese et al. [8]. In particular, the μ is expected to be fully

inhibited as the concentration of inhibitor metabolites approaches a critical value (P_{max}, where P is the generic metabolite). The interactive multiproduct-inhibited model reported by Procentese et al. [8] was adopted to describe the μ *C. acetobutylicum* with glucose as carbon source:

$$\mu = \mu_{max}.\frac{Glu}{Glu+K_{Glu}}.\left(1-\frac{AA}{AA_{max}}\right)^{n_{AA}}.\left(1-\frac{BA}{BA_{max}}\right)^{n_{BA}}.\left(1-\frac{A}{A_{max}}\right)^{n_{A}}.\left(1-\frac{E}{E_{max}}\right)^{n_{E}}.\left(1-\frac{B}{B_{max}}\right)^{n_{B}} \quad (1)$$

where μ_{max} is the maximum specific growth rate, K_{Glu} the Monod coefficient for glucose, and *Glu, AA, BA, A, E, B* the concentration of glucose, acetic acid, butyric acid, acetone, ethanol and butanol, respectively. Due to the high number of parameters to be determined, a multistep parameter inference procedure was used to infer the kinetic parameters as reported in Procentese et al. [8]. As regards the effect of the pH on the process, the model reported by Napoli et al. [10] was used:

$$\mu_{max} = \frac{\overline{\mu_{max}}}{1+\frac{H^+}{K_H}+\frac{K_{OH}}{H^+}}.\left(1-\frac{10^{(pH_{OPT}-pH)}}{10^{\Delta pH}}\right) \quad (2)$$

where H^+ is the concentration of hydrogen ions in the medium and μ_{max}, K_H and K_{OH} are model parameters. pH_{OPT} is the pH at which the cell growth rate is maximum. ΔpH is the transmembrane pH (difference of pH between the medium and the intracellular value) and it was set at 1.5. The effects of pH on the maximum specific growth rate and on the critical concentrations of metabolites were assessed by processing the data measured during tests carried out at pH ranging between 4 and 7.

$Y_{X/Glu}$, $Y_{BA/Glu}$ and $Y_{AA/Glu}$ may be expressed as a function of μ being Y_{ATP} (the amount of dry weight of microorganism produced per g mol of ATP) a function of the specific growth rate, as reported by Procentese et al. [8]:

$$Y_{ATP} = Y_{ATP}^{MAX}\frac{\mu}{\mu+Y_{ATP}^{MAX}\times m_1\left(1-\left(\mu/\mu_{\max}\right)\right)} \quad (3)$$

$$Y_{X/Glu} = \frac{1.5}{1+\alpha}\frac{MW_X}{MW_{Glu}}g_{DM}/g \quad (4)$$

$$Y_{BA/Glu} = \frac{0.75\alpha}{1+\alpha}\frac{MW_{BA}}{MW_{Xyl}}g/g \quad (5)$$

$$Y_{AA/Glu} = \frac{0.5\alpha}{1+\alpha}\frac{MW_{AA}}{MW_{Glu}}g/g \quad (6)$$

where $\alpha = 3\left(MW_X / Y_{ATP}\right) / 6.5 = 46.62 / Y_{ATP}$ (7)

3.2. Solventogenesis phase

The data measured in the fermenter under steady state conditions were processed to assess the kinetics of solventogenic cells and the specific butanol production rate (r_B) within the theoretical framework reported by Procentese et al. [7]. The basic biomass pathway reported in Procentese et al. [7], adapted to glucose, was addressed to assess the microbial population: the concentration of acidogenic cells (X_A), solventogenic cells (X_S), and spore (X_D).

The values of X_A, X_S, X_D, μ_s (specific rate of solventogenic cell formation) and r_B (production rate of butanol referred to the mass unit of X_S) were assessed by processing the measured concentration of sugar, cells and metabolites as reported by Procentese et al. [7]. The data of r_B (D^*B/X_S) were interpreted taking into account that glucose, acetic acid and butyric acid are the substrates and butanol is the inhibition product. In particular, the Monod–Boulton model proposed for processes inhibited by products [7,9] was used:

$$r_B = r_{B,MAX}\left(\frac{Glu}{K_{Glu,B}+Glu}\right)\left(\frac{AA}{K_{AA}+AA}\right)\left(\frac{BA}{K_{BA}+BA}\right)\left(\frac{K_B}{K_B+B}\right) \quad (8)$$

The value of $r_{B,MAX}$, $K_{Glu,B}$, K_{AA}, K_{BA} and K_B were assessed by a parametric inference procedure.

4. Results and discussion

4.1. Acidogenesis phase

Fig. 1A reports the concentration of glucose, biomass and acids measured during continuous fermentation tests carried out in a CSTR under controlled pH. The concentration of reported species was measured provided that steady state conditions established for more than four space-time. The diagram of substrate/product concentrations vs. D typical of processes characterized by product inhibition was produced [8].

Some tests were carried out supplementing acids (3 g/L *BA*, 1 g/L *AA*) and solvents (3 g/L *B*, 10 g/L *E*, 20 g/L *A*) to the glucose bearing medium to assess the effects of these metabolites on the cell growth (data not reported). All the data were processed according the multi-step procedure reported by Procentese et al. [8] to assess the critical acid and solvent concentrations and exponents (reported in Table 1). The error between the expected value (μ) and the experimental value

(D) is less than 10%. This observation suggests that the kinetics assessed according to Eq. (1) and the multistep parameter inference procedure adopted is reliable.

Results of tests carried out at different pH values (data not reported) were processed according to Eq. (1) to assess the μ_{max}. Computations corresponding to Eq. (2) are reported in Fig. 1B as solid line. The agreement with experimental data is satisfactory over the entire range of pH tested.

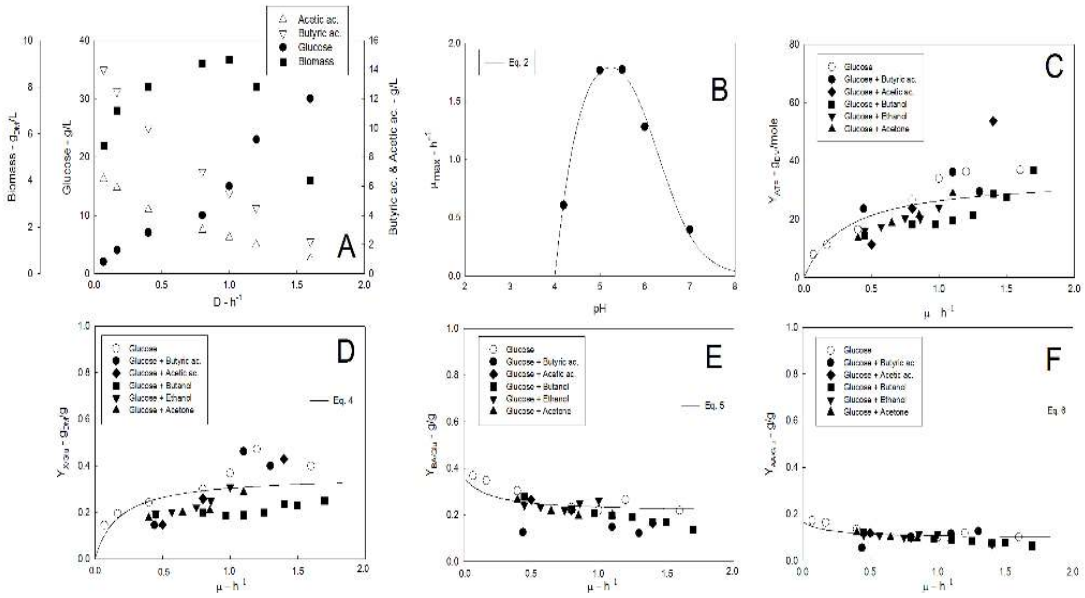

Fig. 1. A) Biomass, glucose and acid concentration vs. D measured during continuous acidogenesis fermentation under steady state conditions. Feeding: 40 g/L glucose. B) μ_{max} vs. *pH*. C) Y_{ATP} vs. μ. D-E-F) $Y_{X/Glu}$, $Y_{BA/GLu}$, $Y_{AA/Glu}$ vs μ. Lines are plots of the theoretical values.

Data measured during the runs reported in Fig. 1A and those measured when supplementing acids and solvents to the glucose bearing stream were processed to assess $Y_{X/Glu}$, $Y_{BA/GLu}$, $Y_{AA/Glu}$, Y_{ATP}, Y_{ATP}^{MAX} and m_1. The Y_{ATP} was calculated according to Procentese et al. [8] (Y_{ATP}=29.5*$Y_{X/Acids}$) for all operating conditions investigated and reported vs. μ (Fig. 1C). The regression of data (μ, Y_{ATP}) was carried out according to Eq. (3) and it yielded: Y_{ATP}^{MAX} =30 g_{DM}/mol_{ATP} and m_1= 0.009 $mol_{ATP}/g_{DM}h$.

Figures 1D, 1E, and 1F report $Y_{X/Glu}$, $Y_{BA/Glu}$, and $Y_{AA/Glu}$ calculated for all the tests as a function of the μ. These figures also show the plots of the expected value of each yield assessed according the Eqs. (4)–(6) and Eq. (3) assuming the value of

Y_{ATP}^{MAX} and m_1 reported above. The agreement between the experimental data and the theoretical values confirms the soundness of theoretical framework.

4.2. Solventogenesis phase

Figs. 2A and 2B report the concentration of metabolites, glucose and total cells, measured during the steady states of the fermentation as a function of D at R=15%. Glucose conversion and the concentration of products decreased with D.

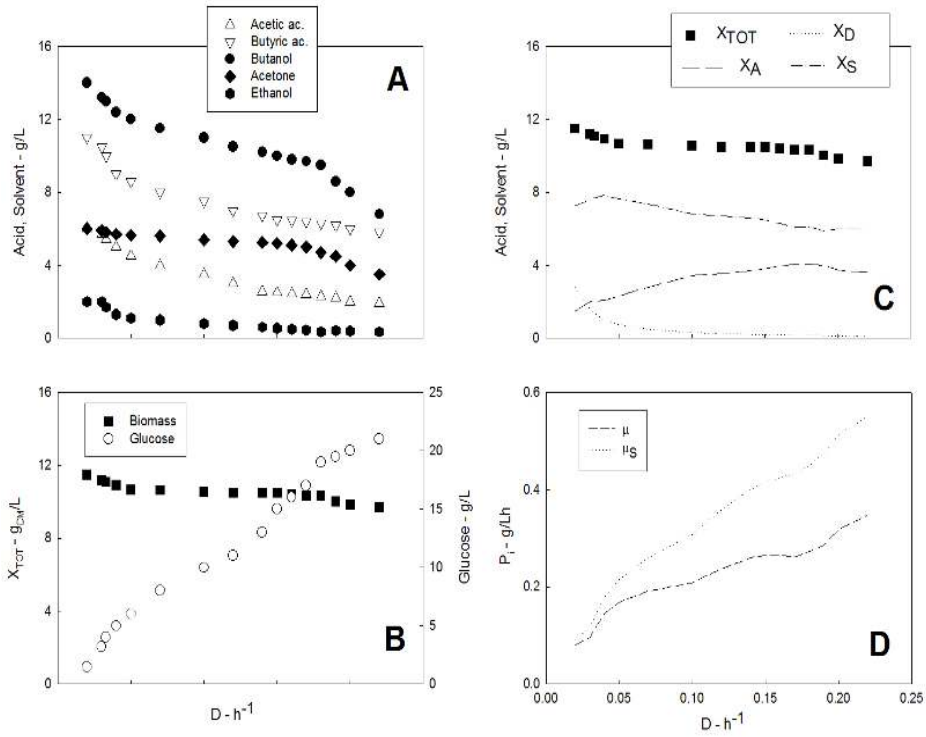

Fig. 2. A-B) Biomass, glucose and acid concentration vs. D measured during continuous solventogenesis fermentation under steady state conditions; feeding: 50 g/L glucose, R=15%. C) X_{TOT} measured (points) and X_A, X_S, X_D calculated (lines) for steady states of continuous fermentations vs D; R = 15%. D) μ (Eq. 1) and μ_S ($D_{OUT}= \mu - \mu_S$).

The concentration of X_A, X_S and X_D were assessed according to the mechanistic framework described in Procentese et al. [7]. Fig. 2C reports X_{TOT} measured during the continuous fermentation tests as well as the calculated X_A, X_S and X_D at R = 15% and D ranging between 0.02 and 0.22 h^{-1}. The concentration of X_A increases linearly with D while the X_D decreases exponentially with D. The concentration of X_S is characterized by a maximum at D=0.04 h^{-1}. Fig. 2D reports

the specific growth rate of X_A (μ) and the solventogenic cell production rate (μ_S). They both increased with D. The deviation of model predictions from experimental data points is fairly small, typically within 20%.

r_B was calculated for all the tests (D**B*/X_S). The kinetic constants were assessed by data regression according to Eq. (8). The results of the best-fit procedure are reported in Table 1. $K_{Glu,B}$ =2 g/L suggests that the butanol production rate does not significantly depend on carbon source. The error between the model prediction and the experimental data is typically within 20%. Therefore, the soundness of the proposed theoretical framework may be claimed.

Table 1. Kinetic parameters assessed according to Eq. (2)

Growth rate																
Eq. 1-2	μ_{max} h^{-1}	K_G g/L	AA_{max} g/L	BA_{max} g/L	A_{max} g/L	B_{max} g/L	E_{max} g/L	n_{AA} -	n_{BA} -	n_A -	n_B -	n_E -	K_H	K_{OH}	pH_{OPT}	$\overline{\mu_{max}}$ h^{-1}
	2.07	0.05	9	18.5	67	19	40	0.9	1.1	0.91	0.37	0.91	$3.33*10^{-4}$	$5*10^{-7}$	5.5	2.1
Butanol production rate																
Eq. 7	$r_{B,MAX}$ $g_B/g_{DM}h$		$K_{G,B}$ g/L		K_{AA} g/L			K_{BA} g/L					K_B g/L			
	6		2		0.2			0.1					0.4			

Conclusions

The μ and the $r_{B,}$of *C. acetobutylicum* on glucose was successfully investigated. In particular: i) as regards the μ, the experimental results were analyzed in terms of a multiple product-inhibited and interacted growth model; a tool to assess the yields of biomass and acids was also described; ii) as regards the r_B, the biomass present in the broth was identified as: acidogenic cells, solventogenic cells and spores. The r_B was referred only to the mass unit of solventogenic cells.

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