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Butanol Production by *Clostridium acetobutylicum* in a Continuous Packed Bed Reactor Fed with Cheese Whey

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This research work reports on the feasibility of bio-butanol production by fermentation of cheese whey in a continuous packed bed reactor (PBR). The anaerobic solventogenic bacterium *Clostridium acetobutylicum* DSM 792 was adopted for the fermentation processes and commercial cheese whey powder was adopted as substrate.
The fermentation plant consisted of a PBR, liquid pumps, a thermostatic unit, and a pH control unit. The PBR was a 4 cm ID, 16 cm high glass tube with a 8 cm bed of 3 mm Tygon rings, as carriers. The pH ranged between 4.5 and 5.5, the dilution rate (D) between 0.4 h^{-1} and 0.64 h^{-1}. The PBR feedstock was a solution of deproteinized cheese whey powder.
Results show that the best performance (butanol productivity 2.66 g/Lh, butanol concentration 4.93 g/L, butanol yield 0.26 g/g, butanol selectivity of the overall solvents production 82%$_w$) was at D=0.54 h^{-1}.

1. Introduction

The Acetone-Butanol-Ethanol (ABE) fermentation is receiving renewed interest as a way to upgrade renewable resources into valuable base chemicals and liquid fuels (Ezeji et al, 2007a; Dürre, 2008). Butanol offers several advantages over ethanol for gasoline-alcohol blending because of its high energy content, low miscibility with water, and low volatility (Bohlmann, 2007; Cascone, 2008). It can replace gasoline without any modification of the current vehicle and engine technologies (Dürre, 2008).
ABE is typically produced during the later stage of batch fermentations of some Clostridium strains - saccharolytic butyric acid-producing bacteria - under appropriate operating conditions (*Clostridium saccharoperbutylacetonicum, C. acetobutylicum, C. beijerinckii, C. aurantibutyricum*). Under batch conditions, the fermentation process of solvent-producing clostridia proceeds with the production of cells, hydrogen, carbon dioxide, acetic acid and butyric acid during the initial growth phase (acidogenesis) (Jones and Woods, 1986). The increase of the acid concentration (pH decreases) causes the metabolism of cells to shift to solvent production (solventogenesis) and the acidogenic cells – able to reproduce themselves – to shift to the solventogenesis state with a morphological change (Jones and Woods, 1986). Under solventogenesis, the active cells become endospores unable to reproduce themselves. Accordingly, two different physiological states must be taken into account for clostridia: one for the acidonegenic phase, and one for the solventogenic phase.
Despite the aforesaid remarkable advantages of butanol as a bioproduct, its industrial production via fermentation is not developed for several issues: feedstock cost and availability, low value of the yield and productivity, low concentration of butanol in the broth, degeneration of butanol-producing strains (Kumar and Gayen, 2011). Moreover, the low butanol concentration in fermentation broth makes its recovery and concentration quite complex (Liu and Fan, 2004; Ezeji et al., 2007b; Napoli et al., 2012a). From the process point of view, performance enhancements were proved by continuous ABE production in reactors with immobilised clostridium strains (Qureshi et al., 2000; Lee et al., 2008; Napoli et al., 2010). As regards feedstocks, economic progress may be made if waste(water) streams are adopted.

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The potential of the cheese-whey as feedstock for butanol production has been pointed out by several authors (Napoli et al., 2011; Qureshi and Maddox, 1987). However, results reported in the literature regarded cheese whey supplemented with extra nutrients (Qureshi and Maddox, 1987) and batch fermentation (Foda et al., 2010). No investigation has been carried out with cheese whey without extra nutrients in continuous reactor.
This research work reports about the set-up and operation of a biofilm reactor packed bed to produce bio-butanol by continuous conversion of cheese whey. *Clostridium acetobutylicum* DSM 792 was adopted for the fermentation processes. Process performances were reported in terms of butanol production rate, butanol selectivity and butanol yield.

2. Materials and Methods

2.1 Microorganism and medium

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Reactivation procedure and pre-culture for inoculum were reported in Napoli et al. (2010).
The synthetic medium adopted consisted of 23 g/L lactose and 5 g/L YE supplemented with P2 stock solution: buffer) 0.5 g/L KH2PO4, 0.5 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 (Qureshi and Blaschek, 2000). The lactose-YE solution was sterilized in autoclave (20 min at 121 °C).
The cheese whey powder (CWP) was from the Sierolat S.p.A., an Italian enterprise. The composition of the CWP is reported in table 1. The CWP was solubilized in deionized water (powder concentration 40 g/L, lactose 28 g/L) and deproteinized by heat treatment at 90 °C for 30 min (Raganati et al., 2013). The precipitate was removed by centrifugation at 5'000 rpm and 10 °C for 30 min and the supernatant was sterilized in autoclave (121 °C, 20 min) to be used as fermentation medium.

Table 1: Composition of the cheese whey powder (determined by the supplier)

lactose	proteins	ashes	lactic acid	moisture
69 $\%_W$	12.5 $\%_W$	7.5 $\%_W$	4 $\%_W$	2.5 $\%_W$

2.2 Apparatus

Pyrex screw capped bottles (100 mL) containing 75 mL medium were used for batch fermenters. Tests were carried out at rest without pH control. The medium was inoculated with 6.25 % (v/v) suspension of active growing pre-cultures. 3 mL of cultures were sampled periodically for the concentration measurement of lactose and metabolites.
The apparatus adopted for the cheese whey fermentation consisted of a fixed bed reactor, liquid pumps, a heating apparatus, a device for the pH control, and on-line diagnostics (Figure 1). The fixed bed was at the bottom of a 200 mL glass lined pipe (4 cm ID, 16 cm high) jacketed for the heat exchange. Nitrogen was sparged at the reactor bottom to support anaerobic conditions. The device for the pH control consisted of a pH-meter, a peristaltic pump, a vessel with NaOH 0.3 M solution, and a controller. The reactor with the carriers (8 cm bed) was sterilized in autoclave at 121 °C for 20 min. The gas stream was sterilized by filtration. The sterile medium was fed at the bottom of reactor by means of a peristaltic pump.
Tygon rings (3 mm external diameter, 1 mm internal diameter) were selected as biofilm carriers according to previous investigation (Napoli et al., 2010).

2.3 Analytical Procedures

The pH of batch cultures 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 lactose and metabolite concentrations, total organic carbon (TOC), and total nitrogen (TN). Lactose concentration was determined by high performance liquid chromatography (Agilent 1100 system - Palo Alto, CA) equipped with a refractive index detector. A GC apparatus (Agilent 7890A GC) equipped with a FID and with a capillary column poraplot was used for the metabolite analysis. Hexanoic acid was adopted as internal standard to assess acids and alcohols and their concentrations. A Shimadzu TOC 5000A analyzer was adopted to measure TOC/TN concentration.

2.4 Procedures and Operating Conditions

Tests were carried out at 37 °C. A volume of 300 microliter of stock culture was transferred in 4 Hungate tubes (15 mL) containing the culture media (23 g/L of lactose). The cultures were incubated for 1 day under batchwise anaerobic sterile conditions, then 40 mL of active culture were inoculated in the reactor.

The biofilm in the fixed bed reactor was grown adopting lactose-based medium at the beginning of the test and setting the pH compatibly with acidogenic phase. The feeding was switched to CWP-based medium and after a pre-set time the pH was definitively decreased for solvent production.
Tests aimed at the butanol production were carried out with the biofilm PBR operated at selected conditions. The dilution rate (D) – ratio between the feeding volumetric flow rate and the volume of the fixed bed – ranged between 0.54 and 0.64 1/h. Each steady state was characterized in terms of metabolites and lactose concentration.
Lactose and metabolite concentrations were worked out to assess the data reported hereinafter. It was assumed that the feeding was aseptic and free of metabolites and that the gas stripping of metabolites was negligible. Lactose conversion (ξ_L), lactose-to-"i-species" fractional yield coefficient ($Y_{i/L}$), butanol productivity (W_B), ABE productivity (W_{ABE}), butanol selectivity (Φ) were assessed according to the equations:

$$\xi_L = \frac{(L^{IN} - L^{OUT})}{L^{OUT}} \quad (1)$$

$$Y_{i/L} = \frac{i^{OUT}}{(L^{IN} - L^{OUT})} \quad (2)$$

$$W_B = D \cdot B^{OUT} \quad (3)$$

$$W_{ABE} = D \cdot ABE^{OUT} \quad (4)$$

$$\Phi = \frac{D \cdot B^{OUT}}{D \cdot (A^{OUT} + B^{OUT} + E^{OUT})} \quad (5)$$

where L, A, B and E are the concentration of lactose, acetone, butanol and ethanol, respectively, measured in the feeding (suffix IN) and in the effluent (suffix OUT). The performances were assessed working out the concentration of lactose and metabolites, provided that the steady-state was stabilized for at least 10 times the reactors mean residence time (1/D).

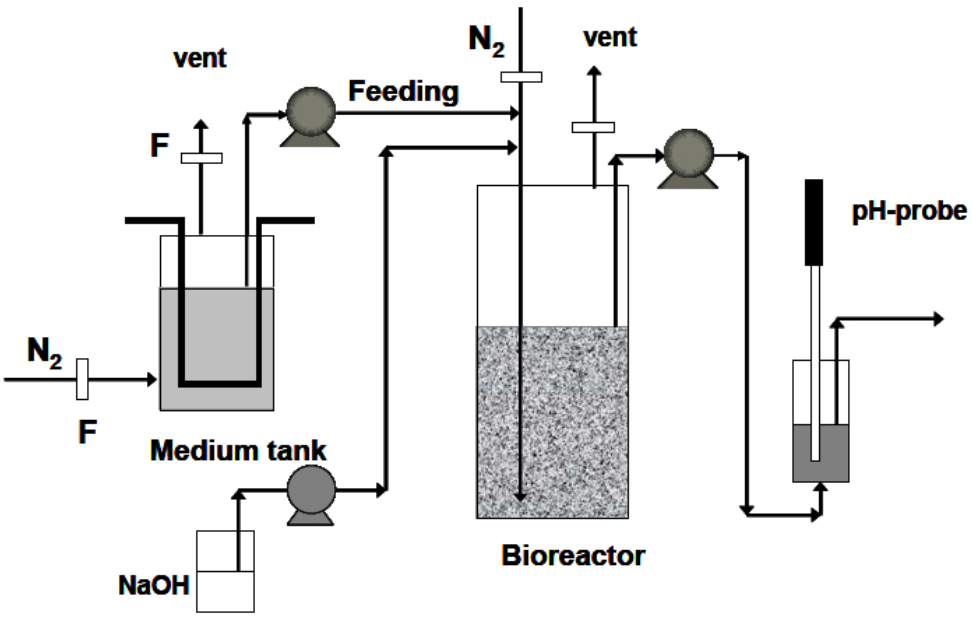

Figure 1: Outline of the apparatus adopted for continuous test equipped with a packed bed biofilm reactor. F) gas sterilization filter.

3. Results and Discussion

Biofilm reactor start-up

Tygon rings - 39.2 g – were adopted to prepare a packed bed reactor 8 cm high.. The volume of the reactor was set at 100 mL by means of the overflow duct. The reactor was inoculated with actively growing culture. No chemical was adopted to assist cell immobilization on the selected support. The start-up process was carried out employing the synthetic medium with 23 g/L lactose.

Figure 2 reports time series of metabolite concentration and pH. The reactor was inoculated at t = 0, operated under batch conditions with respect to the liquid phase for 24 h (data non reported) and then under continuous conditions. The dilution rate was set at 0.40 h^{-1} and the pH was gradually increased from 5.0 to 5.5 to operate the fermentation under acidogenesis conditions (Napoli et al., 2011). A visible biofilm layer on carriers was formed in about one week and at t= 7 day the dilution rate was increased

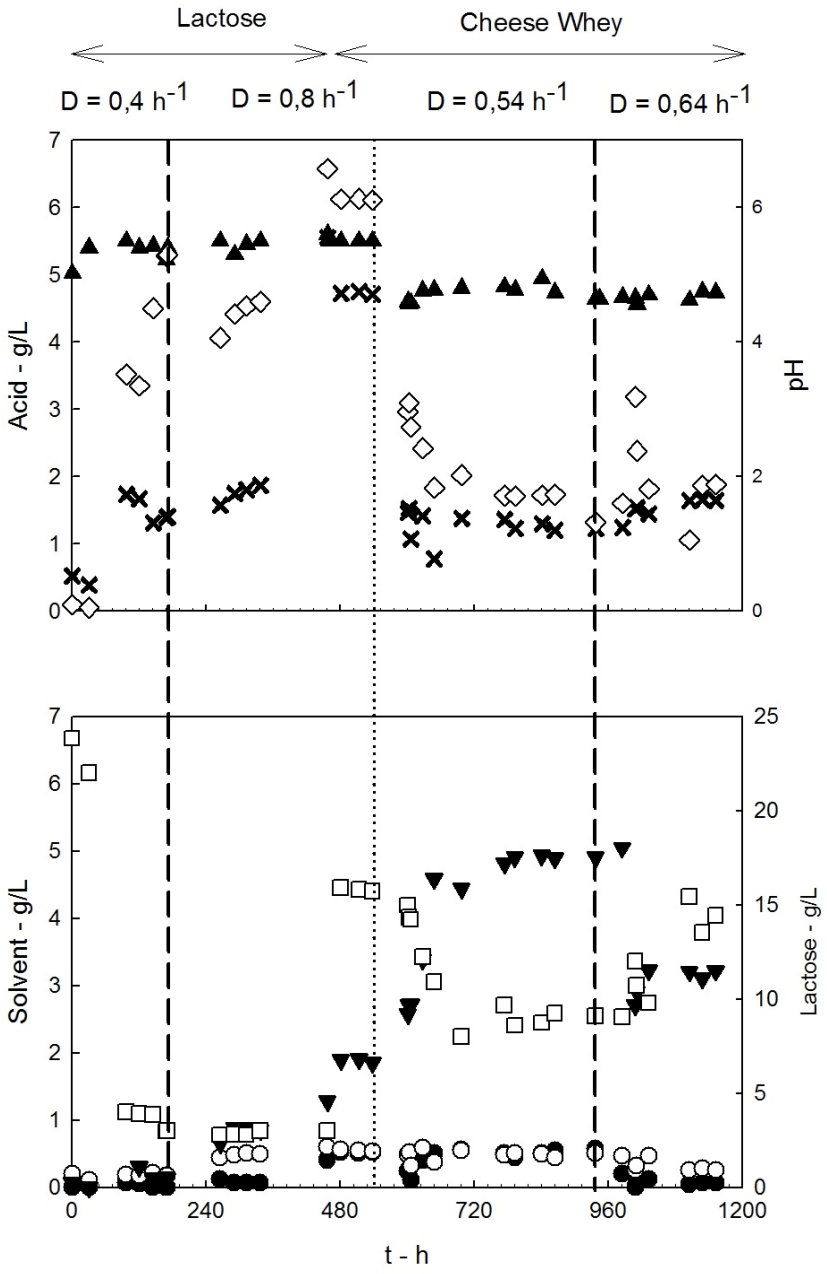

Figure 2: Main concentration data measured during PBR continuous operation. The vertical dotted line marks the instant at which the pH set-up of the controller was changed from 5.5 to 4.5. pH ▼, acetic acid ✖, butyric acid ◊, acetone ●, ethanol ○, butanol ▼, lactose □.

(D = 0.8 1/h) to promote biofilm production with respect to suspended cell growth. It is interesting to note that solvent production started even though the bulk pH was still 5.5. It can be assumed that the pH in the biofilm decreased moving from the liquid-biofilm towards the inner region (Qureshi et al., 2005; Napoli et al., 2010; Olivieri et al., 2011; Rai et al., 2012), promoting the solventogic shift of cells.
At t = 18.5 d the carriers appeared covered by abundant biofilm. The total acid (acetic + butyric) yield resulted about 0.35 $g_{acid}/g_{lactose}$, in good agreement with data assessed by Napoli et al. (2012b). The feeding stream was switched from lactose-synthetic medium to CWP solution (28 g/L lactose) to accustom cells to the complex medium. A new steady-state regime was approached in about three days even though it differed with respect to that assessed during the previous stage (equal D, different medium). On one hand, the lactose conversion measured operating with CWP-based medium was lower with respect to the previous medium. On the other hand, the metabolites - acids and solvents - production rate resulted higher when the reactor was operated with CWP-based medium.
Altogether, the biofilm reactor start-up took about 21 days and a remarkable amount of active biofilm was formed.

Butanol production
The bioreactor operating conditions were changed at t = 21.7 d to produce butanol: pH=4.7, D=0.54 h^{-1}. The value of the pH was set according to previous investigations (Napoli et al., 2010). It was higher than the value at which cells shift to solvent production occurs, pH = 4 (Napoli et al., 2009), because pH and metabolites gradient across the biofilm is expected (Qureshi et al., 2005; Napoli et al., 2010).
The reactor approached a steady-state regime within 7 d. As expected, solvents and acids were continuously produced, confirming the co-existence of both *C. acetobutylicum* cells voted to produce ABE and cells committed to produce acids and biomass (Napoli et al., 2009). PBR performances were characterized in terms of metabolite concentration, lactose conversion degree, yield of acids, butanol and ABE, productivity of butanol and of ABE, and butanol to solvents selectivity (Table 2). The performances were assessed working out the concentration of lactose and metabolites according to the equations reported in the “Procedures” section, provided that the steady-state was stabilized for at least 10 times the

Table 2: Steady-state cultures of C. acetobutylicum

Operating Conditions		
D [h^{-1}]	0.54	0.64
pH	4.7	
Lactose in the feeding [g/L]	28	
Results		
Lactose [g/L]	9.1	14.4
ξ_L [%]	68	49
Acetone [g/L]	0.58	0.07
Butanol [g/L]	4.93	3.23
Ethanol [g/L]	0.50	0.25
Acetic acid [g/L]	1.23	1.65
Butyric acid [g/L]	1.32	1.89
Butanol productivity, W_B [g/Lh]	2.66	2.06
ABE productivity, W_{ABE} [g/Lh]	3.24	2.27
Butanol yield, $Y_{B/L}$ [g/g]	0.26	0.24
ABE yield, $Y_{ABE/L}$ [g/g]	0.32	0.26
Acids yield, $Y_{acids/L}$ [g/g]	0.14	0.26
Butanol selectivity, Φ [g/g]	0.82	0.91

reactors mean residence time (1/D=1.9 h). It is noteworthy that the lactose concentration was 9 g/L, about half the affinity constant (28 g/L) assessed by Napoli et al. (2009).
At t= 38 d the dilution rate was increased at 0.64 h^{-1}. After about 7 d the PBR approached a new steady state. Performances of the new regime are reported in Table 2.
The analysis of results reported in Table 2 points out that butanol productivity reduced with D even though a high butanol selectivity was recorded at high D. The productivity was comparable with that measured by Qureshi and Maddox (1987) operating a continuous biofilm reactor and adopting whey permeate supplemented with yeast extract.

4. Final Remarks

The butanol production in a continuous biofilm PBR was investigated. *Clostridium acetobutylicum* was employed to convert cheese whey in acetone-butanol-ethanol without any extra nutrients. The reactor was successful operated for about two months. The butanol productivity reduced with D. Butanol selectivity increased with D.

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