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ORIGINAL PAPER

Butanol production by *Clostridium acetobutylicum* in a continuous packed bed reactor

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Abstract In this study, we report on a butanol production process by immobilized *Clostridium acetobutylicum* in a continuous packed bed reactor (PBR) using Tygon® rings as a carrier. The medium was a solution of lactose (15–30 g/L) and yeast extract (3 g/L) to emulate the cheese whey, an abundant lactose-rich wastewater. The reactor was operated under controlled conditions with respect to the pH and to the dilution rate. The pH and the dilution rate ranged between 4 and 5, the dilution rate between 0.54 and 2.4 h^{-1} (2.5 times the maximum specific growth rate assessed for suspended cells). The optimal performance of the reactor was recorded at a dilution rate of 0.97 h^{-1}: the butanol productivity was 4.4 g/Lh and the selectivity of solvent in butanol was 88%$_w$.

Keywords *Clostridium acetobutylicum* · Lactose · Butanol · Continuous fermentation · Biofilm · Packed bed reactor

List of symbols

AA, Ac, B, BA, Et, L	Concentration of acetic acid, acetone, butanol, butyric acid, ethanol, and lactose (g/L)
D	Dilution rate, 1/h
q	Productivity (g/L h)
R	Butanol acetone ratio (g/g)
X	Suspended biomass (g_{DM}/L)
$Y_{A/L}$	Lactose to acids fractional yield (g/g)
$Y_{S/L}$	Lactose to solvents fractional yield (g/g)

Subscripts

Et	Ethanol
Ac	Acetone
B	Butanol
IN	Feeding stream
OUT	Effluent stream

Greek letters

δ	Accuracy on carbon balance at steady state (g/g)
Φ	Butanol selectivity with respect to solvents (g/g)

Introduction

The economic scenario characteristic of the beginning of the third millennium revives the interest in strategy for bioconversion of industrial wastewaters in biofuels and bulk chemicals. However, the development of bio-processes suffers low specific production rates typical of fermentations, and huge reactors are necessary for processes operated at high throughput. Therefore, the development of industrial-scale, bio-based processes seeks innovative technologies aimed at intensified operations.

In this scenario, butanol is gaining much interest because of its many advantages with respect to other solvents [2]. In spite of the strong interest in butanol production by *Clostridia* fermentation, there are several factors that influence the economic competition with the petrochemical industry: the high cost of usual substrates

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(corn and molasses), the low butanol productivity, and the high cost of product recovery.

As reviewed by Jones and Woods [10], two pathways have been identified in the metabolism of *Clostridia* strains: acidogenesis, characterized by substrate conversion in acids (acetic and butyric acids), and high motile cells (acidogenic cells); solventogenesis, characterized by substrate and acids conversion in solvents (ABE), and by endospore cells. Noteworthy, the acid uptake is coupled with acetone production.

The proved ability of *Clostridia* strains to utilize a wide spectrum of carbohydrates [6] stimulated research in the use of renewable inexpensive feedstocks. The studies carried out on ABE fermentation adopting cheese whey—a potential inexpensive feedstock—or lactose as carbon source [3, 13, 25] have pointed out that the use of these substrates leads to low overall reactor productivities (0.1 g/Lh) when batch cultures are adopted. On the other hand, the butanol selectivity is larger than that typically assessed during the fermentation of conventional substrates, which reduces the costs of the butanol recovery.

Several attempts are reported in the literature regarding continuous fermentation by means of *Clostridia* strains confined in the reactor by immobilization [5, 9, 15, 20, 22, 24]. A variety of supports have been employed for biofilm development, such as sand [7, 8], granular activated carbon [11], anthracite coal [12], plastics [19], and various kinds of clays [22]. Nevertheless, the proved success to produce ABE, information available in the literature to support industrial demonstration and scale-up is still lacking [2].

The present study moves one further step toward the characterization of butanol production by *C. acetobutylicum*. In particular, the study is focused on the development of a continuous biofilm reactor. A packed bed reactor (PBR) has been investigated as a first step towards the development of a fluidized bed biofilm reactor. A complex media supplemented with lactose has been adopted to mime cheese whey wastewater. The butanol production process was characterized in terms of lactose and metabolites concentrations, solvent productivity, and butanol selectivity.

Materials and procedure

Microorganism and culture media

Clostridium acetobutylicum DSM 792 was supplied by DSMZ (Germany). Details on culture reactivation are reported in Napoli et al. [16].

The culture medium adopted consisted of yeast extract (Sigma-Aldrich) at 3 g/L and D-lactose at a concentration ranging between 15 and 30 g/L. The medium was sterilized in an autoclave (121°C, 20 min).

Apparatus

The apparatus adopted for the lactose fermentation consisted of a fixed bed reactor, liquid pumps, a heating apparatus, a device for the pH control, and on-line diagnostics. The glass-lined bioreactor (250-mL volume) was jacketed for the heat exchange. Nitrogen was sparged at the reactor bottom to support the anaerobic conditions. The device for the pH control consisted of a pH-meter, a peristaltic pump, a vessel with NaOH 1 M solution, and a controller.

The reactor, filled with medium and the carriers, was sterilized in an autoclave for 20 min at 121°C. The gas stream was sterilized by filtration while the medium contained in a stainless-steel tank was sterilized at 100°C for 1.5 h, flushing a saturated water steam at 3 atm in the internal coil.

Table 1 reports the characteristics of the carriers investigated.

Diagnostics

Details of the diagnostics adopted to measure suspended cells, lactose, and metabolites concentration are reported in Napoli et al. [16]. Distribution and morphology of the

Table 1 Features of the carriers tested and their performance regarding fermentation

Carrier	Diameter (μm)	Density (kg/m^3)		Suspended cell growth	Biofilm growth
Silica gel	600	720	Microporous	+	+
Pumice	500	1,050	Macroporous	+	±
Glass beads	400	2,540	Impervious	+	−
Silica sand	600	2,600	Impervious	−	+
Tygon®	(*)	1,180	Impervious	+	+
Teflon	(*)	2,200	Impervious	+	+
PVC (Kartell®)	(*)	1,300	Impervious	+	+

(*) The plastic rings had a length of 0.5 cm, an ID of 3.2 mm, and a thickness of 1.5 mm

biofilm was characterized by scanning electron microscopy (SEM). Particles sampled at the end of each run were repeatedly rinsed with 9 g/L NaCl solution pH 7 and were subsequently incubated for 1.5 h with 2.5%$_V$ glutaraldehyde at 4°C. After rinsing, the samples were dehydrated through extraction with ethanol aqueous solutions with the concentration of ethanol increasing progressively from 15%$_V$ up to 99.8%$_V$ [23]. After water extraction, the sample was air-dried at 60°C and covered with a gold layer as required by the SEM procedure. The samples were scanned and photographed with an electron microscope (Inspect, Fei).

Operating conditions and procedure

One milliliter of stock culture was transferred in a 15-mL screw-cap bottle containing 50 mL of culture media (15 g/L of lactose). The culture was incubated for 2 days under batchwise anaerobic sterile conditions, then 10 mL of active culture was inoculated in the reactor.

The selection of optimal biofilm carrier was carried out testing different materials in the PBR with the working volume at 150 mL. Typically, after 12–24 h of batch culture, the lactose-bearing (15 g/L) stream was fed to the reactor at the preset dilution rate (D). Samples of carriers harvested at the end of the tests were observed at SEM for biofilm characterization. Typically, the immobilization tests lasted 1 week in agreement with results reported by Qureshi et al. [19].

Tests to determine butanol production were carried out with the biofilm PBR operated at selected conditions. The mass of biofilm in the reactor was assessed at the end of the run in agreement with the following procedure: (i) the dry carrier was weighted before filling the reactor; (ii) the reactor was rinsed with sterile water to remove lactose and metabolites; (iii) the supports with the biomass were harvested and dried for 1 day, at a temperature of 40°C; (iv) the dried mass of the biomass and supports was weighted. The dried mass of the biofilm in the reactor was assessed as the difference between the weight of the supports-biofilm and the supports.

All tests were carried out at 35°C. The pH set-point was investigated in the range between 4.0 and 5.0.

Assuming the feeding aseptic and free of metabolites and that the gas stripping of metabolites is negligible, the metabolites concentration and lactose concentration measured during steady-state conditions were calculated out to assess the following data.

Accuracy

The reliability of the data measured during the tests was checked by means of the mass balance on carbon. The assumptions adopted to develop the carbon balance extended to the reactor were: (a) that the biofilm amount is constant; and (b) that the carbon conversion to CO_2 followed the Embden–Meyerhof pathway [10].

$$\mathrm{TOC}^{\mathrm{IN}} - \left(\mathrm{TOC}^{\mathrm{OUT}} + X \cdot \sigma_{\mathrm{C}}\right) - 4\frac{\mathrm{MW_C}}{\mathrm{MW_L}}\left(\mathrm{L}^{\mathrm{IN}} - \mathrm{L}^{\mathrm{OUT}}\right) + \frac{\mathrm{MW_C}}{\mathrm{MW_{Ac}}}\mathrm{Ac}^{\mathrm{OUT}} = 0 \quad (1)$$

where σ_c is the carbon mass fraction of *C. acetobutylicum* biomass, and $\mathrm{MW_C}$, $\mathrm{MW_L}$, and $\mathrm{MW_{Ac}}$ the molecular weight of carbon, lactose, and acetone, X, L, and TOC the concentration in the liquid phase of suspended biomass, lactose, and total organic carbon, respectively. Subscripts IN and OUT refer to the reactor feeding and effluent stream, respectively. The accuracy of the measurements is expressed by δ, defined as:

$$\frac{\mathrm{TOC}^{\mathrm{IN}} - \left(\mathrm{TOC}^{\mathrm{OUT}} + X \cdot \sigma_{\mathrm{C}}\right) - 4\frac{\mathrm{MW_C}}{\mathrm{MW_L}}\left(\mathrm{L}^{\mathrm{IN}} - \mathrm{L}^{\mathrm{OUT}}\right) + \frac{\mathrm{MW_C}}{\mathrm{MW_{Ac}}}\mathrm{Ac}^{\mathrm{OUT}}}{\mathrm{TOC}^{\mathrm{IN}}} = \delta \quad (2)$$

Yields

Lactose-to-acids ($Y_{A/L}$) and lactose-to-solvents ($Y_{S/L}$) fractional yields.

$$Y_{\mathrm{A/L}} = \frac{D\left(\mathrm{AA}^{\mathrm{OUT}} + \mathrm{BA}^{\mathrm{OUT}}\right)D}{D\left(\mathrm{L}^{\mathrm{IN}} - \mathrm{L}^{\mathrm{OUT}}\right)} \quad (3)$$

$$Y_{\mathrm{S/L}} = \frac{D\left(\mathrm{B}^{\mathrm{OUT}} + \mathrm{Ac}^{\mathrm{OUT}} + \mathrm{Et}^{\mathrm{OUT}}\right)}{D\left(\mathrm{L}^{\mathrm{IN}} - \mathrm{L}^{\mathrm{OUT}}\right)} \quad (4)$$

where D is the dilution rate, AA, BA, B, Ac, Et and L the acetic acid, butyric acid, butanol, acetone, ethanol, and lactose concentration, respectively.

Productivity of solvents

$$\text{butanol } q^{\mathrm{B}} = D\ \mathrm{B}^{\mathrm{OUT}} \quad (5)$$

$$\text{acetone } q^{\mathrm{Ac}} = D\ \mathrm{Ac}^{\mathrm{OUT}} \quad (6)$$

$$\text{ethanol } q^{\mathrm{Et}} = D\ \mathrm{Et}^{\mathrm{OUT}} \quad (7)$$

where q^{i} is the productivity of the solvent "i".

The butanol selectivity (Φ) was assessed as the ratio between the butanol expression rate and the sum of the production rate of all solvents:

$$\Phi = \frac{D\left(\mathrm{B}^{\mathrm{OUT}}\right)}{D\left(\mathrm{B}^{\mathrm{OUT}} + \mathrm{Ac}^{\mathrm{OUT}} + \mathrm{Et}^{\mathrm{OUT}}\right)} \quad (8)$$

Results

Selection of the carrier

Table 1 reports relevant results regarding the biofilm formation on the solids tested. The performances of the carriers were assessed in terms of suspended cell growth and of biofilm formation after 1 week of incubation. Except for silica sand, the investigated carriers did not suppress the growth of suspended cell cultures. Biofilm was observed on all solids except silica sand and on glass beads. The biofilm on the Tygon® rings (Tygon® LFL, Saint Gobain) appeared thicker and more widespread than that observed on the other solids. It should be noted that Tygon®, Teflon, and PVC (Isoflex® Kartell) are hydrophobic solids, a feature that promotes cell adhesion and biofilm formation [1].

Based on the results of the immobilization tests, Tygon® was chosen as the best-suited solid carrier for continuous lactose fermentation in the packed bed biofilm reactor. This choice was suggested by the consideration that Tygon® is stable, and by the favorable combination of carrier density and size, reflected by the good quality of the fluidization of these particles.

Biofilm reactor start-up

Figure 1 shows the start-up of the reactor loaded with 69 g of Tygon® rings. The concentration of metabolites and the pH are reported as a function of time. The reactor was inoculated at $t = 0$ and operated batchwise with respect to the liquid phase for 20 h. Thereafter, the reactor was switched to continuous operation by steadily feeding the lactose 15 g/L medium. The dilution rate was set at $D = 0.40\ h^{-1}$ and the pH was gradually increased from 5.0 to 5.5 to force fermentation under acidogenesis conditions. After about 2 days of incubation, the carriers were covered by a thin layer of biofilm visible to the direct observation and the dilution rate was increased to promote biofilm production with respect suspended cell growth. At $D = 0.80\ h^{-1}$, a value which is close to the maximum specific growth rate of suspended cells under the operating conditions adopted (data not published), the lactose concentration in the reactor decrease at a rate lager than the metabolites increase, probably as the consequence of biofilm growth.

The dilution rate was still increased at $t = 140$ h to compensate the gradual lactose depletion ($L < 2$ g/l). In particular, D was set at $2.4\ h^{-1}$, about 2.5 times the maximum specific growth rate. The biofilm reactor approached a steady-state regime since $t = 190$ h. Altogether, the biofilm reactor start-up took about 9 days and a remarkable amount of biofilm was formed.

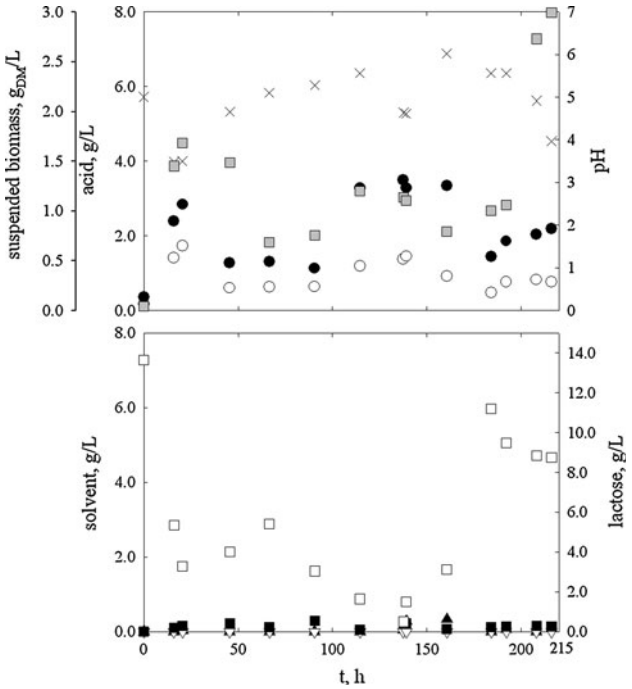

Fig. 1 Start-up of the PBR. Lactose concentration in the feeding 15 g/L. × pH, *filled circle* acetic acid, *open circle* butyric acid, *open square* lactose, *filled square* butanol, *inverted triangle* acetone, *filled triangle* ethanol, *gray filled square* suspended biomass

Butanol production

Provided there is a substantial amount of biofilm, at $t = 216$ h the bioreactor operating conditions were set to produce butanol: pH at 4.0 and the D at $0.54\ h^{-1}$. The value of pH was set in agreement with previous investigations carried out in batch reactor [16]: cells shift to the solvent production at pH = 4.0. As expected, the solvents were continuously produced, besides the acids, confirming the co-existence of *C. acetobutylicum* cells voted to produce ABE and of cells committed to produce acids and biomass. However, lactose conversion and solvents started gradually to decrease along the time, highlighting a progressive extinction of the fermentation process. Lactose conversion and solvent production were prompted recovered by increasing the pH up to 4.3 at $t = 287$ h. Steady-state conditions were approached in about 2 days and lasted for about 6 days (about 60 space–time).

Table 2 reports the main data regarding the steady-state characterized by $D = 0.54\ h^{-1}$ and pH = 4.3. The reactor performance was characterized in terms of lactose and metabolites concentration, lactose conversion degree, acid and solvent yield, solvent productivity, and butanol-to-solvents selectivity. The value of $\delta \approx 0$ supports the reliability of the steady-state assumption. Notwithstanding the operating conditions adopted promoted the solvents

production, the acid production (1.43 g/Lh) was still larger than the solvent production (0.77 g/Lh).

At $t = 472$ h, the operating conditions were changed in order to increase the solvent production. The lactose concentration in the feed was set at 30 g/L, close to the value characteristic of cheese whey. Mindful of the experience of pH increase from 4.0 to 4.3, the pH further increased at 5.0. After about 4 days of acclimatization to the new operating conditions, the D was set at 0.97 h^{-1}, in agreement with previous investigations that pointed out high solvent productivities at values of $D \approx 1$ h^{-1} [9, 21, 26]. The biofilm PBR approached a new steady-state condition ($t \approx 616$ h) and it was successfully operated for a further 134 h (about 140 space–time).

The biofilm PBR was stopped and the biomass concentration reached 74 g_{DM}/L, corresponding to a biomass-to-carrier ratio of 0.16 g_{DM}/g. The biomass concentration is larger than the value reported by Qureshi et al. [19] adopting bonechar as carriers (0.087g_{DM}/g). It is possible to infer that the Tygon® is a very promising support for *Clostridia* cell immobilization and butanol production.

Data related to the steady states are reported in Table 2. In general, the reactor performances improved with D and/or pH. The effects of the operating conditions on the reactor performances are now on order. In particular, attention is paid at both the butanol selectivity and the butanol productivity.

The average solvent concentration and the butanol/acetone mass ratio (R) increase with both the pH and D. In particular, R was 6 at pH = 4.3 and 88 at pH = 5.0.

Table 2 Steady-state cultures of *C. acetobutylicum* in PBR

Operating conditions		
D [h^{-1}]	0.54	0.97
pH	4.34	5.08
Lactose in the feeding [g/L]	15.0	30.0
Results		
Lactose [g/L]	6.3	11.8
Ethanol [g/L	0.05	0.55
Acetone [g/L]	0.18	0.05
Butanol [g/L]	1.20	4.59
Acetic acid [g/L]	0.53	0.67
Butyric acid [g/L]	2.12	1.48
δ, -	0.02	0.03
Ethanol productivity [g/Lh]	0.03	0.53
Acetone productivity [g/Lh]	0.10	0.05
Butanol productivity [g/Lh]	0.65	4.43
Butanol selectivity[g/g]	0.83	0.88
Solvents yield [g/g]	0.15	0.28
Acids yield [g/g]	0.28	0.12

The ratio R assessed at the highest pH is even larger than that estimated by Linden et al. [13] during the cheese whey fermentation that ranged between 12 and 20. In any case, R is larger than that typically estimated during glucose fermentation ($R = 3$) [10]. The lower concentration of acetone at higher pH and D is in agreement with the lower concentration of acids, taking into account that acetone production is promoted by high acid concentrations.

The butanol selectivity was larger than 80%$_w$ for the steady states investigated and reached 88% at the largest D, among the largest value reported in literature. The result is promising regarding the possible advantage in the recovery and concentration process of the butanol [4, 18]. The higher butanol concentration coupled with the high selectivity decrease the cost of the distillation train, typically adopted as downstream process [14, 17].

The analysis of the acid yield and of the solvent yield confirms the scenario highlighted. The acid yield is 2–3 times the solvent yield at low values of pH and D. At higher pH and D, the comparison of the yields is in favor of the solvents, supporting the marked shift of the lactose conversion towards the direct pathway.

Particular attention should be paid at the solvent productivity estimated at the steady states investigated. At $D = 0.97$ h^{-1} and pH = 5, the solvent productivity was about 5.0 g/Lh (butanol = 4.43 g/Lh), six times the value achieved at low pH and dilution rate. The maximum productivity achieved was still larger than that reported by Qureshi and Maddox [20], who assessed a solvent productivity of 4.5 g/Lh for a lactose fermentation carried out under similar operating conditions and adopting bonechar as the carrier.

Altogether, the results reported suggest that the performance of the biofilm reactor improves with pH, in agreement with results reported in the literature [9]. However, results appear to not agree with data reported by Jones and Woods [10] for batch and continuous culture of suspended cells. In particular, for suspended cells, the decrease of pH improves the solvent production. The agreement among the reported results is reconciled by taking into account the transport phenomena in the biofilm. Assuming a pH and metabolites gradient across the biofilm [19], the pH within the biofilm is lower than that in the bulk of the medium. As a consequence, setting pH = 4 in the bulk, the inner region of the biofilm experiences a pH lower than 4 with a consequent reduction of cell activity.

The results regarding the effects of pH on the reactor performance confirm the relevance of the transport phenomena in the biofilm. The decrease of pH moving deep in the biofilm requires that the pH be set in the bulk at a value higher than the optimal values assessed for suspended cell processes.

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