Continuous cultures of *Clostridium acetobutylicum*: culture stability and low-grade glycerol utilisation

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Abstract

Continuous cultures of two strains of *Clostridium acetobutylicum* were stable for over 70 d when grown on glucose/glycerol mixtures. Butanol was the major fermentation end-product, accounting for 43 to 62% (w/w) of total products. Low-grade glycerol [65% (w/v) purity] could replace commercial glycerol [87% (w/v) purity], leading to a similar fermentation pattern: a butanol yield of 0.34 (mol/mol), a butanol productivity of 0.42 g l^{-1} h^{-1} and a 84% (w/w) glycerol consumption were attained when cultures were grown at pH 6 and D = 0.05 h^{-1}; butanol accounted for 94% (w/w) of total solvents. These values are among the highest reported in literature for *C. acetobutylicum* simple chemostats.

Introduction

The acetone/butanol/ethanol fermentation, although economically viable in the past, is no longer competitive with products derived from the petrochemical industry. However, in recent years there has been a renewed interest in this fermentation, which has led to the development of work on the metabolism and genetics of solventogenic clostridia and to the improvement of fermentation and product recovery technologies (Woods 1995, Dürre 1998).

The economic balance of this fermentation could be improved by using cheaper raw materials. For example, molasses, if used as a substrate, may account for about 50% of the final cost of the product (Vandecasteele & Marchal 1993). The capacity and versatility of the solventogenic bacteria in degrading different alternative fermentation substrates, alone or in mixtures, can be an important feature for the implementation of an industrial process and, therefore, research in this area is needed.

A potential alternative substrate is glycerol. This compound is a secondary product of several processes, namely the conversion of fatty oils (sunflower, rapeseed) to bio-fuels, which yields 10% (w/v) glycerol (Eggersdorfer *et al.* 1992, Chowdhury & Fouhy 1993). Moreover, glycerol utilisation by *Clostridium acetobutylicum* ATCC 824 directs the electron and carbon flows to butanol formation (Vasconcelos *et al.* 1994). However, glycerol can only be used by these bacteria in continuous cultures, in mixtures with a more oxidised substrate like glucose.

A drawback of continuous cultures by solventogenic clostridia is culture instability and strain degeneration. Obviously, for an industrially attractive process, the problem of strain degeneration has to be solved. Culture degeneration, characterised by a progressive loss of solvent production capability, has been known for a long time and has been observed in both continuous and batch cultures (Kutzenok & Aschner 1952, Finn & Nowrey 1959, Jöbses & Roels 1983, Stephens *et al.* 1985). A condition that seems to contribute to the stability of continuous cultures of *C. acetobutylicum* is to use phosphate limitation. Long-term solvent production, for over one year, has been reported with *C. acetobutylicum* DSM 1731 in a phosphate-limited chemostat culture at pH 4.3 (Bahl *et al.* 1982). However, phosphates are naturally

present in complex substrates that could be used for solvent production on an industrial scale and, for its removal, an extra step would be required in medium preparation.

The first objective of this work was to access the stability of continuous cultures of *C. acetobutylicum* on glucose/glycerol mixtures without phosphate limitation. A second objective was to evaluate the possibility of using low-grade glycerol as carbon source without any prior purification. The *C. acetobutylicum* strains chosen to perform this work were: the type strain ATCC 824 and strain ATCC 4259, which was reported as a non-lactic acid producer (Kim *et al.* 1984).

Materials and methods

Micro-organisms and culture conditions

Clostridium acetobutylicum ATCC 824 and ATCC 4259 were grown on a synthetic medium with the following composition per litre of deionised water: glucose, 50 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_20$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; acetic acid, 2.2 g; biotin, 0.04 mg; *p*-aminobenzoic acid, 8 mg. The medium pH was adjusted to 6.3 with NH_4OH 14 N. The strains were stored in spores form at $-20\,^{\circ}C$.

Medium used in the continuous feed contained the following components per litre of deionised water: glucose, 15 or 30 g; glycerol, 15 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.028 g; NH_4Cl, 1.5 g; $COCl_2 \cdot 6H_2O$, 0.01 g; biotin, 0.04 mg; *p*-aminobenzoic acid, 8 mg; H_2SO_4 9.7 M, 0.1 ml. Low-grade glycerol, obtained from the transesterification process using rapeseed oil (Novance, Compiègne, France), contained the following components (as provided by the supplier): glycerol, 65% (w/v); sodium salts, less than 5% (w/v); non-glycerol organic matter, less than 1% (w/v); metals, less than 1 g l^{-1}; heavy metals, less than 5 mg l^{-1}.

A two litre fermenter Biostat MD (Braun, Melsungen, Germany) was used; the operating conditions were as follows: working volume, 1.4 l; dilution rate, 0.05 h^{-1}; temperature, 35 °C; stirring, 200 rpm; pH 6.5 (maintained constant by automatic addition of 6 M NH_4OH).

After sterilisation, medium in the fermenter was flushed with sterile O_2-free N_2 until room temperature was reached. The fermentation was first carried out batchwise. A growing culture, taken at the early exponential growth phase, was used as inoculum (10% v/v). The inoculum medium and the initial medium in the reactor were identical to the one used to store spores. After sterilisation, the feed medium was also flushed with sterile O_2-free N_2 until room temperature was reached. During the experiment the feed tank was maintained under N_2 at 30 mbar, to avoid O_2 entry. All tubing were made of butyl rubber and the bioreactor gas outlet was protected with a pyrogallol arrangement (Vasconcelos *et al.* 1994). Continuous feeding was started when the culture in the reactor reached the exponential growth phase.

Analytical procedures

Cell concentration was measured turbidometrically, at 620 nm and correlated with cell dry weight determined directly.

Concentrations of glucose, glycerol, butanol, ethanol, acetic, butyric and lactic acids were determined by HPLC using an Aminex HPX-87H column (Biorad, Richmond, CA) and detection was assessed by refractive index. The operating conditions were as follows: mobile phase, H_2SO_4 0.5 mM; flow rate, 0.5 ml min^{-1}; temperature, 30 °C.

Samples were taken daily with daily external control of pH and cell morphology (by optical microscopy).

Results and discussion

Clostridium acetobutylicum ATCC 824 was grown in continuous culture, with a mixture of 15 g glucose l^{-1} and 15 g glycerol l^{-1} in the feed medium and without phosphate limitation (phosphate higher than 0.74 mM, according to Bahl *et al.* 1982). Butanol, ethanol and organic acids (acetic, butyric and lactic) were produced at pH 6.5 and dilution rate 0.05 h^{-1}. Butanol was the major fermentation end-product (4.2 g l^{-1}) and its production was continuous. The culture was glucose-limited, but only 42% (w/w) of the feed glycerol was consumed (Table 1). After 52 d, glucose was increased to 30 g l^{-1} in the feed medium, while keeping the glycerol concentration constant. Butanol and organic acids production increased up to 6.6 g l^{-1} and 6.7 g l^{-1} respectively, and remained constant until the fermentation was stopped. Again, no residual glucose was detected and only 53% (w/w) of the feed glycerol was metabolised. The fermentation was stopped 72 d

Table 1. Fermentation results of *Clostridium acetobutylicum* ATCC 824 and ATCC 4259 grown in chemostat cultures on glucose and commercial glycerol [87% (w/v) purity] mixtures, at dilution rate 0.05 h^{-1} and pH 6.5.

Strain (ATCC No.)	Substrate feed ($g\ l^{-1}$)	Consumed glycerol ($g\ l^{-1}$)	Butanol ($g\ l^{-1}$)	Total solvents ($g\ l^{-1}$)	Total acids ($g\ l^{-1}$)	$Y_{butanol}$	$Q_{butanol}$ ($g\ l^{-1}\ h^{-1}$)
824	Glucose (14.8) Glycerol (14.6)	6.2	4.2	6.5	3.2	0.32	0.21
	Glucose (29) Glycerol (14.9)	7.9	6.6	7.9	6.7	0.29	0.33
4259	Glucose (15.1) Glycerol (16)	6.2	5.4	6.5	2.2	0.41	0.27
	Glucose (33) Glycerol (14)	8.5	7.6	8.6	7.6	0.31	0.38

$Y_{butanol}$ – butanol yield (carbon mole of butanol produced/carbon mole of glucose and glycerol consumed); $Q_{butanol}$ – butanol productivity.

after initiating the continuous feeding without any sign of acid drift or morphological changes, characteristics of strain degeneration.

C. acetobutylicum ATCC 4259 was also grown under the conditions described for strain ATCC 824 cultures. A similar fermentation profile was observed, except that no lactate production was detected. After 16 d, glucose was increased to 30 g l^{-1} in the feed medium, while glycerol remained at 15 g l^{-1}. Butanol production increased and stabilised at 7.6 g l^{-1}, continuing to be the major fermentation end-product 40 d after the continuous feed was started. No residual glucose was detected, but only 38 and 61% (w/w) of the feed glycerol were consumed, respectively in cultures with 15 and 30 g glycerol l^{-1} (Table 1). No degeneration signals were observed while the culture was maintained.

Butanol yields obtained for both strains varied between 0.29 and 0.41 mol butanol produced/mol substrates consumed.

It was thus possible to maintain stable continuous cultures of *C. acetobutylicum* ATCC 824 and ATCC 4259 on glucose/glycerol mixtures for long periods of time (for over 70 and 40 d, respectively), in spite of the absence of phosphate limitation and of the high working pH (6.5). However, cultures were maintained at a low dilution rate (0.05 h^{-1}) and were not submitted to high concentrations of butanol (above 9 g l^{-1}), which might otherwise promote culture instability (Fick *et al.* 1985, Barbeau *et al.* 1988).

Cornillot *et al.* (1997) showed that solventogenic genes encoding the enzymes required for acetone and butanol formation are carried on a plasmid (pSOL1) whose loss leads to degeneration. *C. acetobutylicum* DG1, a degenerated strain which does not carry the pSOL1 plasmid (Cornillot *et al.* 1997), did not produce butanol when grown on the conditions reported here (data not shown). Moreover, a gene encoding a NADH-dependent aldehyde/alcohol dehydrogenase, responsible for butanol production in alcohologenic cultures of *C. acetobutylicum* ATCC 824, was characterised and found to be carried by the pSOL1 plasmid (Fontaine *et al.* 2002). Therefore, it seems that the maintenance of butanol production in continuous cultures of *C. acetobutylicum* ATCC 824 and 4259 is due to the presence of the plasmid in the cells. The excess of reducing equivalents generated by glycerol metabolism may constitute the selection pressure for plasmid maintenance and culture stability.

In order to be economically attractive as a fermentation substrate, low-grade glycerol should be used without prior purification steps. Petitdemange *et al.* (1995) reported that low-grade glycerol, obtained from the transesterification of rapeseed oil, could be toxic as several strains of *C. butyricum* failed to grow on this type of glycerol when used as the sole carbon source. To evaluate the possibility of using low-grade glycerol, obtained from the biodiesel producing process, continuous cultures ($D = 0.05\ h^{-1}$) of *C. acetobutylicum* ATCC 4259 were performed with 30 g glucose l^{-1} and 13 g low-grade glycerol l^{-1} (without any prior purification) in the feed medium, at pH 6 and 6.5. This strain was chosen as it presented better butanol yield and productivity than *C. acetobutylicum* ATCC 824. Butanol was the major fermentation end-product, attaining a yield of 0.34 (mol/mol) and a productivity of 0.42 g l^{-1} h^{-1}, when the culture was grown at pH 6 (Table 2). Cultures were glucose-

Table 2. Fermentation results of *Clostridium acetobutylicum* ATCC 4259 grown on glucose/low-grade glycerol mixtures and comparison with other continuous butanol fermentations reported in literature.

Microorganism	Butanol ($g\,l^{-1}$)	Total solvents ($g\,l^{-1}$)	$Y_{butanol}$	$Q_{butanol}$ ($g\,l^{-1}\,h^{-1}$)	Carbon source	Culture medium	Reference
C. acetobutylicum ATCC 4259	7.4	8.1	0.3	0.37	Mixt.	Synt. (pH 6.5)	This work
C. acetobutylicum ATCC 4259	8.6	9.2	0.34	0.42	Mixt.	Synt. (pH 6)	This work
C. acetobutylicum DSM 1731	9.6	14.5	0.33	0.24	Glu.	Synt.	1
C. acetobutylicum ATCC 824	9	13	0.36	0.54	Glu.	Semidef.	2
C. beijerinckii BA 101	8.8	15.6	n.d.	0.44	Glu.	Semidef.	3
C. acetobutylicum ATCC 824	7.6	12	0.28	0.23	Glu.	Synt.	4
C. acetobutylicum ATCC 824	9.2	10	0.45	0.92	Glu.	Semidef. + BV	5

$Y_{butanol}$ – butanol yield (carbon mol of butanol produced/carbon mol of substrates consumed); $Q_{butanol}$ – butanol productivity; Mixt. – mixtures of 30 g glucose l^{-1} and 13 g low-grade glycerol l^{-1}; Synt. – synthetic; Glu. – glucose; Semidef. – semidefined; BV – Benzyl viologen; n.d. – not determined, insufficient data available; 1 – Bahl *et al*. (1982); 2 – Fick *et al.* (1985); 3 – Formanek *et al.* (1997); 4 – Monot & Engasser (1983); 5 – Rao & Mutharasan (1988).

limited and 84 and 73% (w/w) of low-grade glycerol were consumed, respectively at pH 6 and 6.5. The results achieved with low-grade glycerol (Table 2) were similar to those obtained with commercial glycerol (Table 1).

Examining existing data on simple chemostats reported in literature (Table 2), it can be concluded that butanol concentrations achieved in this work, with commercial and low-grade glycerol, are in the range of the highest values reported for this type of cultures. The best results reported were obtained using semidefined media, usually supplemented with yeast extract (Fick *et al.* 1985), or in the presence of toxic compounds such as benzyl viologen (Rao & Mutharasan 1988). Another important feature is the effect of glycerol on product selectivity, with the final solvents composition consisting almost exclusively of butanol [94% (w/w) of total solvents]. This effect is only comparable to the butanol selectivity related to total solvents obtained with benzyl viologen (Rao & Mutharasan 1988).

Conclusions

It is possible to maintain continuous cultures of Clostridium acetobutylicum for long periods of time on glucose/glycerol mixtures, in the absence of phosphate limitation. Butanol was the major fermentation end-product, accounting for 43 to 62% (w/w) of total products, and its production was continuous over the test period.

Low-grade glycerol from the biodiesel production process could be used in continuous cultures of *C. acetobutylicum* with results similar to those obtained with commercial glycerol. The butanol yield (0.34 mol/mol) and productivity (0.42 g l^{-1} h^{-1}) obtained in this work are among the highest values reported in literature for *C. acetobutylicum* simple chemostats.

The possibility of glycerol utilisation on the acetone/butanol/ethanol fermentation is dependent on the availability of low-grade glycerol and on its price. As a carbon feedstock for fermentations, glycerol can be competitive if its price is not higher than 0.3 US$ kg^{-1} (Claude *et al.* 2000). Another issue to be considered is that, although the results presented here were obtained with pure glucose, it is unlikely that this substrate may have an industrial application. Therefore, to further evaluate the possibility of a practical application of low-grade glycerol, the use of cheaper sources of glucose like starches or molasses, in mixtures with low-grade glycerol, should be investigated. To our knowledge there are no reports in literature on the use of this kind of substrates in continuous acetone/butanol/ethanol fermentations. Technical problems, such as the lack of homogeneity or increasing viscosity of media and the possible interference (catabolic repression) of such complex substrates with glycerol metabolism, are likely to occur and should be investigated before envisaging an industrial butanol production.

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