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Production of 1,3-propanediol by *Clostridium butyricum* in continuous culture with cell recycling

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Abstract The continuous fermentation of 1,3-propanediol from glycerol by *Clostridium butyricum* was subjected to cell recycling by filtration using hollow-fibre modules made from polysulphone. The performance of the culture system was checked at a retention ratio (dilution rate/bleed rate) of 5, dilution rates between 0.2 h^{-1} and 1.0 h^{-1} and glycerol input concentrations of 32 g l^{-1} and 56 g l^{-1}. The near-to-optimum propanediol concentration of 26.5 g l^{-1} (for 56 g l^{-1} glycerol) was maintained up to a dilution rate of 0.5 h^{-1} and then decreased while the propanediol productivity was highest at 0.7 h^{-1}. The productivity could be increased by a factor of four in comparison to the continuous culture without cell recycling. By application of the model of Zeng and Deckwer [(1995) Biotechnol Prog 11: 71–79] for cultures under substrate excess, it was shown that the limitations resulted exclusively from product inhibition and detrimental influences from the cell recycling system, such as shear stress, were not involved.

Introduction

The method of cell retention by crossflow filtration and recycling has been under investigation for more than 15 years (Belfort 1989): The culture passes a permeable membrane through which cell-free liquid can be drawn off while the more concentrated cell suspension returns to the reactor. The advantages are well-known: the fermentation runs at a considerably higher productivity than a conventional culture, and a cell-free culture liquid is available for product processing.

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The method has been investigated mainly for the alcoholic fermentation (e.g. Lee and Chang 1987), but also for the acetone/butanol fermentation (Afschar et al. 1985; Schlote and Gottschalk 1986; Pierrot et al. 1986; Ferras et al. 1986; Mulchandani and Volesky 1986), for the 2,3-butanediol fermentation (Ramachandran and Goma 1988; Zeng et al. 1991) and for lactic and acetic acids production.

In recent years the 1,3-propanediol fermentation that uses glycerol as substrate has been successfully revived by several research groups since the product is of interest as a feedstock for polyester production. Improvement in the performance of the fermentation has been achieved by immobilization on polyurethane carriers (Pflugmacher and Gottschalk 1994), but the potential of cell recycling is still unknown for this fermentation. Its optimization is the subject of this investigation using a constant concentration factor and varied dilution rates.

Materials and methods

Strain and medium

Clostridium butyricum DSM 5431, obtained from the German Collection of Microorganisms and Cell Cultures, was used throughout. It was cultivated in 100-ml anaerobic flasks with rubber septa for syringe operation containing 50 ml medium under nitrogen. The medium was described by Homann et al. (1990) and was also used for precultures.

For the fermentations in continuous culture, the chemically defined medium of Reimann et al. (1996) was used, which contained glycerol as carbon source in the concentrations indicated, NH_4Cl as nitrogen source in 5% of the amount of the glycerol concentration, as well as biotin and pantothenic acid as growth factors.

Setup of the continuous culture with cell recycling

Continuous cultivation was carried out in a 1-l glass fermenter with a working volume of 0.5 l (Fig. 1) under continuous sparging with highly purified nitrogen. The temperature was maintained at 35 °C and the pH at 7.0, using 2.5 M KOH for correction. For cell retention two hollow-fibre modules made from polysulphone

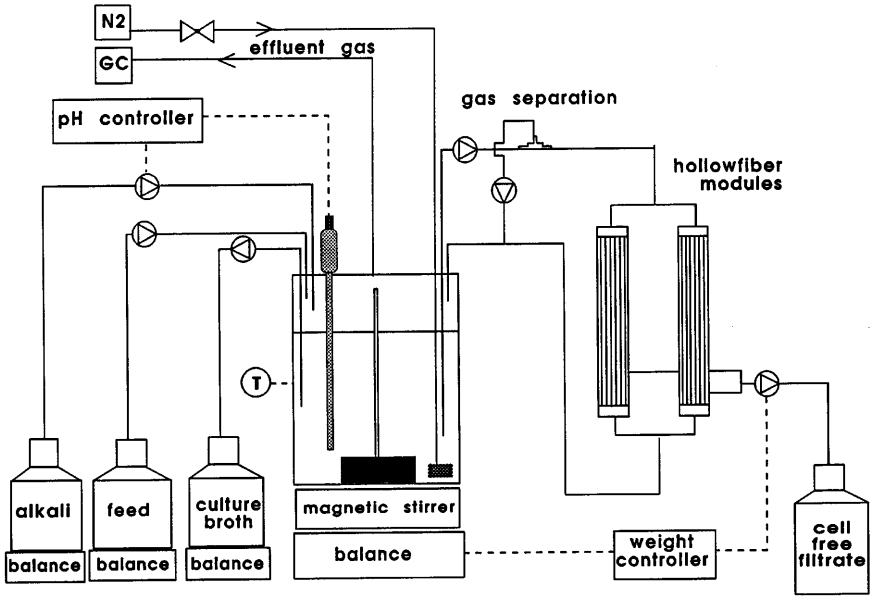

Fig. 1 Schematic view of the continuous culture assembly with the cell-recycling equipment

(Fresenius St. Wendel GmbH, Germany, catalogue no. SPS 7005-8, fibre inner diameter 0.5 mm, effective filtration area 0.7 m^2, pore size 0.01 μm inside to 2 μm outside) were used in parallel, one for the current fermentation, the other in case the first one was plugged. The culture was circulated through the membrane tubes at a rate of 0.5 l/min. A constant filtration rate independent of the state of the membranes was achieved by controlling the fermenter weight by the filtrate pump and by gas removal prior to the filtration modules. For the weight control, scales and a control unit from Sartorius, Göttingen, Germany were used. Cell-containing culture was pumped off at a constant rate ("bleed rate"). For medium supply, filtrate and bleed, as well as for medium circulation, peristaltic pumps were used.

Analytical procedures

The cell density was measured as dry weight; the carbon source, glycerol, was determined by an enzymatic method according to the instructions of the test kit manufacturer (Boehringer, Mannheim, Germany). The dissolved fermentation products were determined gas-chromatographically (Biebl 1991) as well as the fermentation gases CO_2 and H_2 (Reimann et al. 1996). The concentration of viable cells was estimated according to the most probable number method (Gerhardt et al. 1981) in 15-ml Hungate tubes.

Results

The fermentations were first run as conventional continuous cultures at a dilution rate that was about half the maximum growth rate (0.3 h^{-1}). When the conditions were stable the cell retention system was set into operation, the culture dilution rate (bleed rate) being adjusted to one-fifth of the medium dilution rate (dilution rate). Along with this retention ratio, the dilution rate and the bleed rate were increased stepwise until the performance of the culture declined. The experiment was carried out with two glycerol concentrations, 32 g l^{-1} (350 mmol l^{-1}) and 56 g l^{-1} (610 mmol l^{-1}). A concentration of 92 g l^{-1} (1000 mmol l^{-1}), which is tolerated in batch culture, was attempted but did not support sufficient growth. At a glycerol concentration of 56 g l^{-1} and a dilution rate of 1.0 h^{-1} a steady state was achieved at a retention ratio of 10, but, because of membrane plugging, the culture could not be continued at a higher dilution rate.

Growth and propanediol formation

The complete results are shown in Table 1. At both substrate concentrations the cell density increased almost in line with the retention ratio, i.e. 4- to 5-fold for 32 g l^{-1} and 3.4- to 4-fold for 56 g l^{-1}, in comparison to the culture without cell recycling. At a retention ratio of 10, the cell mass did not increase further. The propanediol productivity displayed an optimum curve that peaked at a dilution rate of 0.7 h^{-1} for both substrate concentrations (Fig. 2). At higher dilution rates the culture was obviously increasingly inhibited by the fermentation products.

Also shown in Fig. 2 is the propanediol content of the culture, which began to decrease at the same dilution rate as the productivity. The concentration of 350 mmol (= 26.6 g) l^{-1} obtained with 55 g l^{-1} glycerol in the medium seems to be close to the achievable maximum, as the glycerol in the culture was not completely used up and trials with higher substrate concentration failed.

Cell activity

It is generally a problem in cell-recycle cultures that the activity of the retained cells tends to decrease as a result of accumulation of dead and inactive cells. Attempts to estimate the viable cells by a most probable number method, using Hungate tubes, did not provide sufficiently precise results. Therefore, we used the specific consumption rate of the substrate and the specific

Table 1 Fermentation of glycerol by by *Clostridium butyricum* in continuous culture with and without cell recycling. D_M medium dilution rate, D_B bleed rate, q specific 1,3-propanediol production rate, Q 1,3-propanediol production rate

Glycerol, S_0 (mmol l^{-1})	Glycerol, S (mmol l^{-1})	D_M (h^{-1})	D_B (h^{-1})	D_M/D_B	Cell mass (g l^{-1})	Acetate (mmol l^{-1})	Butyrate (mmol l^{-1})	1,3-Propanediol (mmol l^{-1})	q (mmol g^{-1} h^{-1})	Q (g l^{-1} h^{-1})
330	129	0.31	0.31	1.0	1.35	36	12	131	30.8	3.1
328	10	0.31	0.07	4.7	5.85	17	50	180	8.9	4.2
334	19	0.49	0.10	4.8	6.85	32	38	189	13.6	7.0
370	67	0.70	0.14	4.9	5.47	46	19	178	22.8	9.5
385	230	0.97	0.20	4.9	3.06	27	7	97	30.6	7.2
620	165	0.20	0.20	1.0	1.70	32	47	273	32.3	4.2
626	42	0.30	0.06	5.3	5.72	22	73	341	18.1	7.8
600	47	0.50	0.09	5.3	6.20	22	63	350	28.1	13.3
604	131	0.72	0.13	5.5	6.98	32	43	300	31.1	16.4
621	381	0.99	0.19	5.2	4.15	17	25	147	35.1	11.1
610	156	1.00	0.09	11.1	5.14	38	39	275	53.8	20.9

formation rate of the products. As shown in Table 1, the specific propanediol formation rate decreased drastically when the culture was switched over to cell recycling conditions and then increased with the dilution rate and the bleed rate, indicating that the cell activity is determined by the cell dilution rate and not by the medium dilution rate. The relation of the specific glycerol consumption rate and the product formation rates to the cell dilution rate, which is identical with the growth rate, is shown in Fig. 3 for the lower glycerol concentration. As expected, glycerol consumption and propanediol and acetate formation first increased linearly with the growth rate but then levelled off. The linear phase was up to 0.17 h^{-1} for the lower and 0.09 h^{-1} for the higher glycerol concentration (not shown).

Obviously the conversion capacity of the cells not only depends on the growth rate but also on the accumulation of substrate, indicating inhibition by the products. In Fig. 4 the specific conversion rates are plotted against the residual glycerol concentrations for the steady states listed in Table 1, including those without cell recycling. The specific activity can be seen to be relatively low at low residual glycerol concentrations and reaches its highest level when more than 110 mmol/l or 10 g/l glycerol has accumulated in the culture. On the basis of a concept of Tsai and Lee (1990), Zeng and Deckwer (1995) have recently modelled anaerobic fermentations with substrate excess. They showed that the conversion rate q under these conditions consists of a minimum rate q^* occurring under substrate limitation and an excess rate q^E:

$$q = q^* + q^E$$

The term q^* was formulated according to the Pirt equation, while for formulation of q^E the maximum additional rate under substrate excess and a substrate-sufficient saturation constant K_s^* had to be introduced. For substrate consumption this is

$$q_s = m_s + \frac{\mu}{Y_s^{max}} + \Delta q_s^{max} \cdot \frac{c_s - c_s^*}{c_s - c_s^* + K_s^*}$$

where q_s is the specific substrate consumption rate, Δq_s^{max} the maximum additional substrate consumption rate under substrate excess condition, m_s the substrate consumption for maintenance, μ the growth rate, Y_s^{max}

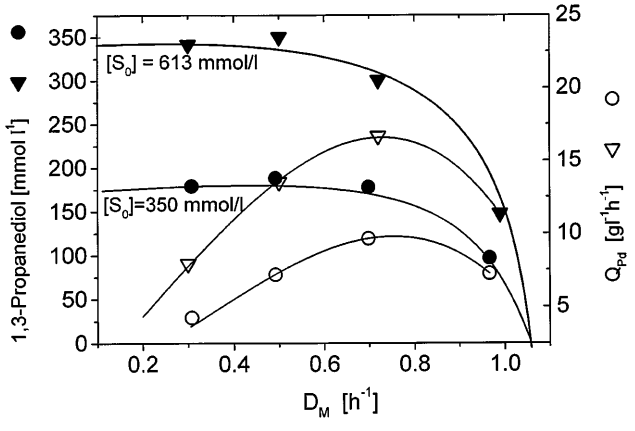

Fig. 2 Product concentration and volumetric productivity Q in continuous culture with cell recycling at increasing dilution rate, with two glycerol input concentrations. The bleed rate was set to 1/5 the dilution rate

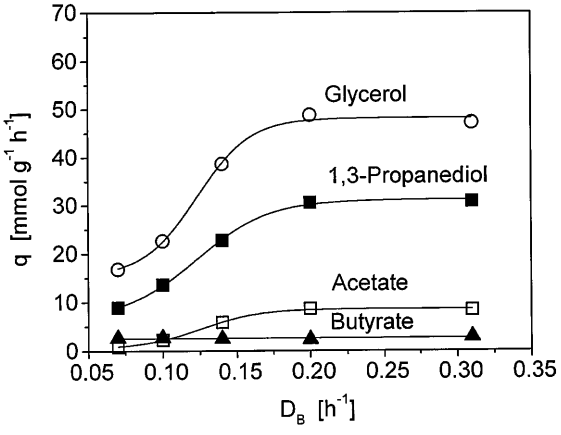

Fig. 3 Influence of of the bleed rate D_B on the specific conversion rate q at a glycerol input concentration of 350 mmol l^{-1}

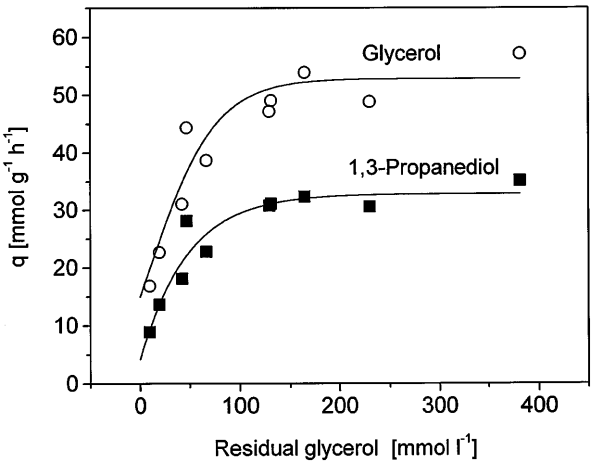

Fig. 4 Influence of the residual glycerol in the reactor on the specific conversion rates. The two steady states without cell retention (Table 1) are included

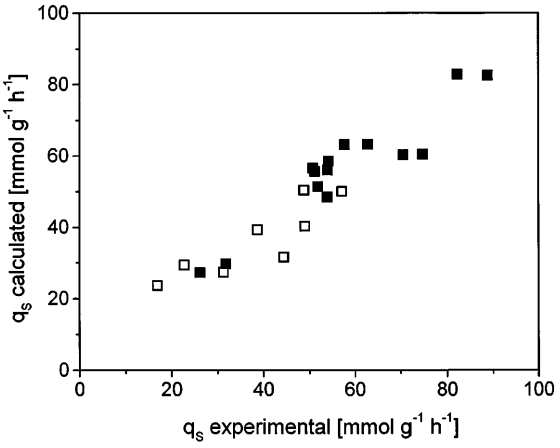

Fig. 5 Comparison of the specific glycerol consumption rates calculated with the substrate-excess model of Zeng and Deckwer with the experimental values showing steady states from continuous culture with (□) and without cell recycling (■)

the maximum biomass yield, c_s the actual substrate concentration, c_s* the substrate concentration under substrate limitation and K_s* the saturation constant for substrate excess conditions.

To confirm that the observed phenomena resulted from product inhibition and subsequent substrate accumulation, the steady-state substrate consumption rates were compared with those expected from the model of Zeng and Deckwer (1995). The necessary constants were obtained from a number of steady states of continuous cultures without cell recycling (Reimann 1997). From the parameter estimation included in the Excel software, m_s was determined as 11.5 mmol g^{-1} h^{-1}, Y_s^{max} as 0.0079 g $mmol^{-1}$ Δq_s as 15.8 mmol g^{-1} h^{-1} and K_s* as 39.0 mmol l^{-1}. The calculated specific rates were corrected for the respective specific growth rates. Plotting the calculated specific glycerol consumption rates of the chemostat steady states with and without cell recycling against the experimental values shows that the rates of both culture types satisfactorily fit into the applied model, indicating that cell-recycle cultures depend on the same limiting factors as the conventional continuous culture (Fig. 5). A similar parity plot was obtained for the propanediol formation rate (not shown).

Discussion

The method of cell recycling by continuous filtration has been applied to almost all current anaerobic fermentations of economic interest, most extensively to acetone/butanol fermentation (see Introduction for references). Increases in productivity by a factor of 3–5 were usually achieved. However, the limits of the method soon became obvious. Undoutedly the main cause of malfunction is clogging of the membrane, which could be overcome to some degree by regular backflushing as well as by the appropriate choice of the filter material, which should be compatible with the cell wall properties of the respective organisms (Schlote and Gottschalk 1986). Shear stress, to which the cells are exposed when the culture is quickly pumped through the narrow tubes or plate interfaces of the filtration modules, is another unfavourable factor. In any case a loss in specific cell activity was observed as the cell density increased and the fraction of viable cells often fell drastically. Although product formation is always faster than in corresponding cultures without cell recycling, the increase in productivity is often not proportional to the increase in cell mass.

Application of cell recycling to the clostridial 1,3-propanediol fermentation from glycerol exhibited results that are in line with those of other anaerobic fermentations. The 4-fold productivity increase, in comparison to the uninfluenced continuous culture, and the final concentration of 26.5 g l^{-1} appear to be satisfactory. There is only one comparable report dealing with glycerol fermentation, in which cell retention was achieved by immobilisation on a polyurethane carrier using a strain of *Citrobacter freundii* (Pflugmacher and Gottschalk 1994). Here a 2-fold increase in productivity at a 1,3-propanediol concentration of 16.3 g l^{-1} was obtained. Probably the high fraction of non-growing cells occurring in the carriers may be the reason for the lower performance achieved by this technique.

In spite of the productivity increase, the cell-recycle culture of the glycerol fermentation with *C. butyricum* could be performed only in a narrow section of the theoretical range. With a glycerol concentration of 56 g l^{-1}, maximum conversion of the substrate occurred only up to a (medium) dilution rate (D_M) of 0.5 h^{-1}, although up to (D_B/D_M) $\mu_{max} = 5 \times 0.6 = 3$ h^{-1} (where D_B is the bleed rate) should be possible, which would mean a productivity of 80 g l^{-1} h^{-1} instead of 13.3 g l^{-1} h^{-1} that was actually achieved. It is shown in this investigation that, in the cell-recycle culture of glycerol-fermenting clostridia, no other factors were operative than those affecting conventional continuous culture, in which the determining factor is the inhibition by the

products. This means that the capacity of a cell-recycling culture can be predicted from a comparable continuous culture without cell recycling when the bleed rate is used instead of the dilution rate. Now it can also be easily explained why the specific rates decrease so drastically when the culture is switched to cell recycling, although the dilution rate is maintained. Product formation by the cells is determined by the bleed rate, which is only a small fraction of the dilution rate.

It is often anticipated that cell-recycle culture increases not only productivity but also product concentration. However, in view of the above conclusions, substantial improvement cannot be expected if a continuous culture that is carefully optimized for product concentration is used for comparison, and not the start-up culture. The published data mostly indicate somewhat higher values for the cell-recycle culture. For the acetone/butanol fermentation, Bahl et al. (1982) achieved 18.3 g l^{-1} solvents in a two-stage fermentation at an effective dilution rate of 0.03 h^{-1}, while the same group (Schlote and Gottschalk 1986) obtained 22 g l^{-1} at a dilution rate of 0.1 h^{-1} with total cell recycling. For the butanediol fermentation Zeng et al. (1991) found a maximum concentration of 43 g l^{-1} butanediol + acetoin for the culture without and 54 g l^{-1} with cell recycling. For the clostridial 1,3-propanediol fermentation the final product concentration could not be increased by cell recycling (26.6 compared to 28.4 g l^{-1} in unrecycled cultures).

The steady-state concentrations for 1,3-propanediol, found for both types of continuous culture, are relatively low if compared to the final concentrations of batch cultures, which amount to 56 g l^{-1}. Possibly they cannot be increased further since, with the higher glycerol concentrations applied, the substrate was not completely used up, and higher input concentrations did not support growth. In the glycerol fermentation by *Klebsiella pneumoniae,* 1,3-propanediol concentrations of up to 48 g l^{-1} and glycerol consumptions of up to 101 g l^{-1} have been achieved in a conventional continuous culture (Menzel et al. 1997), although the highest batch values correspond to those of the *Clostridium* culture. This high performance is partly paid for by high residual glycerol concentrations or excessive formation of by-products; nevertheless, the relatively low values for *C. butyricum* may indicate unknown limitation, as pointed out by Zeng (1996).

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