Cultivation of an L-Lactate Dehydrogenase Mutant of *Bacillus stearothermophilus* in Continuous Culture with Cell Recycle

R. San Martin,* D. Bushell, D.J. Leak and B.S. Hartley
Centre for Biotechnology, Imperial College of Science, Technology and Medicine, London, United Kingdom

Received December 1, 1993/Accepted December 23, 1993

Continuous fermentation with cell recycle proved very effective in increasing the ethanol volumetric productivity of the thermophilic facultative anaerobe, *Bacillus stearothermophilus* strain LLD-15, on sucrose at 70°C. When complete cell recycle was used, cell viability decreased after a few residence times and sucrose consumption was reduced. Operation using a constant bleed rate resulted in greater stability and higher ethanol volumetric productivities. A mathematical model based on maintenance energy requirements provided an adequate description of the system. © 1994 John Wiley & Sons, Inc.
Key words: cell recycle • thermophilic ethanol fermentation • *Bacillus* • fermentation

INTRODUCTION

Despite recent research, the production of ethanol by fermentation still remains more expensive than gasoline or ethanol produced by ethylene hydration. Major improvements will plausibly come from the use of novel microorganisms, with the ability to ferment pentoses and hexoses simultaneously, allowing for the integral use of relatively cheap lignocellulosic substrates. Unfortunately, this has not been achieved with newly isolated pentose-fermenting yeasts such as *Pichia, Pachysolen*, and *Candida* species, primarily due to their long fermentation times.[20] An interesting possibility is to use ethanologenic thermophilic bacteria with an ability to ferment hexoses and pentoses at 60° to 70°C, where ethanol separation costs should be reduced. In recent years, several new strains have been isolated from natural environments and their fermentation potential evaluated.[21] Although some fermentation yields are comparable with those obtained using yeasts, most experiments have been performed in small fermentation volumes and in media containing large amounts of complex substances and low substrate concentrations. Moreover, most attempts to establish continuous cultures, where operation at high temperature offers important potential advantages (e.g., continuous ethanol removal), have resulted in poor growth and low ethanol yields.[17]

Our own approach has been to examine the potential for ethanol production of the best known thermophile, *Bacillus stearothermophilus*. This thermophilic facultative anaerobe has been extensively studied due to its importance as a contaminant in the beverage, food, and canning industries, and it has frequently been used to study the nature of thermophily.[21] Under anaerobic conditions, the wild-type strains of *B. stearothermophilus* produce significant amounts of lactate via an NAD-linked L-lactate dehydrogenase and minor amounts of formate, acetate, and ethanol via the pyruvate formate lyase pathway (PFL). However, a mutant strain LLD-15, lacking L-lactate dehydrogenase activity, compares favorably with other ethanologenic thermophilic strains and can be studied in continuous cultures both aerobically and anaerobically.[1,20]

Strain LLD-15 was selected on the basis of resistance to fluoropyruvate, from a parental strain that exhibited high growth rates, both aerobically and anaerobically, at 70°C on a wide range of sugars, including sucrose, pentoses, and the principal hexose sugars present in lignocellulosic hydrolyzates.[8,15] Anaerobically, it metabolizes pyruvate primarily through the PFL pathway, to yield (in moles per mole sucrose): 4, formate; 2, acetate; and 2, ethanol. However, under certain cultivation conditions (e.g., acid pH), formate and acetate production drop, whereas ethanol yields increase above the values predicted by the PFL balance. Significant CO_2 production and a high pyruvate dehydrogenase activity (PDH) in anaerobic cell extracts, have suggested that strain LLD-15 metabolizes pyruvate both via the PFL and PDH pathways. Furthermore, batch experiments have shown that the anaerobic PDH pathway is more active at high sucrose concentrations and low pH, probably due to problems in the cellular excretion of acetate and formate.[8] The resulting decrease of carbon flux through the PFL pathway induces a higher activity of the PDH pathway, resulting in ethanol yields comparable with those obtained with yeasts (e.g., 3.5 mole ETOH/mol sucrose).

We have recently developed a minimal medium for the anaerobic cultivation of strain LLD-15 on sucrose.[18] Continuous culture experiments have shown that this strain produces ethanol in good yields only up to moderate dilution rates (i.e., $D \leq 0.2\ h^{-1}$) and low sucrose concentrations ($C_{si} < 2.0\%$ w/v). Outside this range, lactate is the major anaerobic product, due to takeover by rever-

*To whom all correspondence should be addressed at present address: Catholic University, Department of Chemical Engineering, Vicuña Mackenna 4860, P.O. Box 6177, Santiago, Chile

CCC 0006-3592/94/01021-08

tant strains, which are always present in the bioreactor due to the low, but significant, reversion frequency of the mutation in the L-lactate dehydrogenase gene. Analysis of the specific sucrose consumption rate q_s vs. growth rate, has revealed that lactate production only occurs at q_s values above 4 to 4.2 g sucrose/g cell · h.[19] A plausible explanation is that, at high q_s values, the main anaerobic pathways saturate (i.e., PDH and PFL). Increasing q_s further by imposing a higher dilution rate in continuous culture, or by poorly regulated sucrose import, results in the accumulation of pyruvate in LLD-15, but offers a significant competitive advantage to the revertant, which can direct the excess carbon flux to lactate production.

To increase the stability of strain LLD-15 in continuous culture, we have examined the use of a system with cell recycle, using sucrose as the carbon source. The principle is that, by operating at higher cell densities, the specific sugar consumption rate can be maintained below the reversion value. Moreover, cell recycle allows for operation at high dilution rates, without washout of the culture, increasing the ethanol volumetric productivity, provided substrate consumption remains high. The results obtained have been used to evaluate a model to predict the cell density based on the concept of maintenance energy.

THEORETICAL ASPECTS

Continuous Culture with Partial Cell Recycle

Figure 1 depicts a continuous culture system with cell recycle. The broth is circulated through a "cell-separator" (e.g., centrifuge, settler, or ultrafiltration membrane), to remove filtrate at a similar rate to the incoming feed. On a laboratory scale, cell recycle can be attained by using tangential flow ultrafiltration. This type of equipment has already been used successfully for mesophilic ethanologenic microorganisms, such as *Saccharomyces cerevisiae*[5,12] and *Zymomonas mobilis*.[10,13] Also, it has been used with other thermophiles, such as *Clostridium thermosaccharolyticum*.[14] In principle this system could be operated with complete cell retention, but cell viability decreases with extended operation times.[10,13] This can be remedied through the use of partial

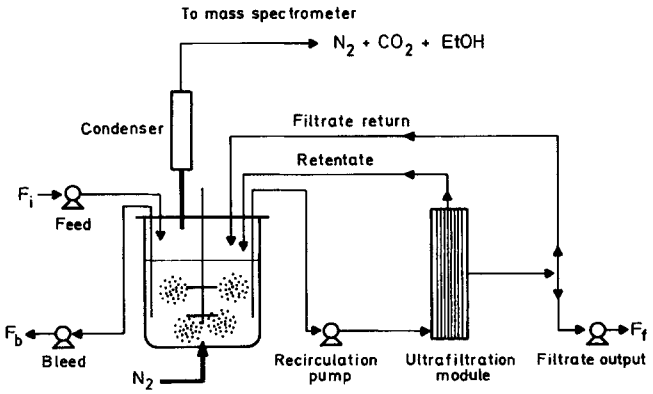

Figure 1. Experimental configuration for cell recycle studies.

cell recycle. In such a system, minimal cell growth is maintained by a continuous bleed stream with flow rate F_b. This removes a small proportion of the total biomass, thereby allowing an equivalent proportion of the feed to be used for cell growth.

Based on the maintenance model postulated by Pirt[16] the balance equations for biomass and substrate in a system with cell recycle can be expressed as follows:

$$dC_x/dt = \mu C_x - D_b C_x \quad (1)$$

$$dC_s/dt = D_b(C_{si} - C_s) - (1/Y_{x/s})\mu C_x - m_s C_x \quad (2)$$

where D_b is the bleed dilution rate. At steady state, dC_x/dt and dC_s/dt equal zero, and the equations yield:

$$\mu = D_b = F_b/V \quad (3)$$

$$q_s = (1/Y_{x/s})\mu + m_s \quad (4)$$

Eq. (3) indicates that, in a system with partial cell recycle, μ is determined by the bleed rate F_b, rather than by the rate of incoming feed F_i, as in conventional continuous cultures ($\mu = D = F_i/V$). This allows for operation at dilution rates above μ_{max}, without washout of the culture. In Eq. (4), q_s refers to the specific rate of sucrose consumption as defined by:

$$q_s = D(C_{si} - C_s)/C_x \quad (5)$$

This model has been extended to predict the kinetics of the increase in cell concentration when the system is switched from open continuous culture to operation with cell recycle.[9] Assuming that, under substrate limited conditions, a pseudo-steady state with respect to substrate concentration is valid, the following expression for $C_x(t)$ can be derived:

$$C_x(t) = \left(C_{x\text{in}} - \frac{Y_{x/s}D(C_{si} - C_s)}{m_s Y_{x/s} + D_b}\right)e^{-(m_s Y_{x/s} + D_b)t} + \frac{Y_{x/s}D(C_{si} - C_s)}{m_s Y_{x/s} + D_b} \quad (6)$$

where $C_{x\text{in}}$ is the steady state cell concentration before operation with complete cell recycle. This expression can also be used to estimate the cell concentration in the bioreactor under steady state conditions ($t \rightarrow \infty$):

$$C_x = \frac{Y_{x/x}D(C_{si} - C_s)}{m_s Y_{x/s} + D_b} \quad (7)$$

Continuous Culture with Complete Cell Recycle

After switching to a continuous system with complete cell recycle, the cell concentration rises until it reaches a plateau, where the rate of substrate input (g sucrose/h) matches the biomass maintenance requirements (g sucrose/g cell · h). This results in a zero growth rate and eventual loss of viability at prolonged retention times. However, it also provides an alternative method to estimate the maintenance energy coefficient m_s,[2] although it should be recognized that an apparent zero growth rate may represent a dynamic balance between cell death and

growth. Hence, based on Eqs. (4) and (5), the maintenance coefficient at zero growth rate ($\mu = D_b = 0$), can be estimated as:

$$q_{so} = m_s = D(C_{si} - C_s)/C_{xo} \quad (8)$$

where C_{xo} is the cell concentration when the culture reaches zero growth rate. Monitoring the level of residual limiting substrate (e.g., sucrose) allows an indirect estimate of the culture viability with operation time. This determines the maximum retention time or minimum bleed rate, required to maintain adequate substrate conversion in the bioreactor.

MATERIALS AND METHODS

Strain and Inoculum Development

All experiments were started with cultures of *Bacillus stearothermophilus* strain LLD-15 (NCIMB 12428), maintained at –18°C in glycerol (1 mL culture, 0.8 mL 80% glycerol).[3] The inoculation and start-up procedure have been described elsewhere.[18,19]

Media

Experiments were carried out at 1.0%, 1.5%, and 2.0% (w/v) input sucrose, using a minimal medium.[18] All salts were analytical grade (BDH, Dagenham, U.K.) and distilled water was used in all preparations. To avoid caramelization, media and sucrose were autoclaved separately, at 121°C for 1 h. The final volume of liquid in the medium reservoir was 20 L. Amino acid and vitamins (Sigma, Poole, Dorset, U.K.) were filter sterilized using 0.2-μm filters (Gelman Sciences Ltd., Northampton, U.K.).

Bioreactor Configuration

The bioreactor was operated following as described previously.[18,19] The bioreactor configuration is shown in Figure 1. All experiments were carried out at 70°C in a LSL Biolafitte 2-L bioreactor (Life Sciences Laboratories, Luton, U.K.) with 1.5-L working volume. Cell recycle was achieved by recirculating the broth with a peristaltic pump (170 rpm, Model 501UR, Watson-Marlow, Falmouth, U.K.) through a hollow fiber ultrafiltration system (Model DC-2, Amicon, Stonehouse, U.K.). High-shear-resistant tubing was used to prevent breakage at 70°C (Santoprene, Monsanto).

The flow of cell-free filtrate F_f was controlled by a peristaltic pump (Model 101-UR, Watson-Marlow, Falmouth, U.K.); the excess filtrate was recirculated to the bioreactor. This arrangement allowed filter operation up to its maximum capacity. The bleed rate F_b was maintained constant using an identical peristaltic pump. Steady state was attained after three to five retention times.

Table I shows the characteristics of the hollow fiber cartridges (Diaflo, Amicon). The cartridges were sterilized in situ by recirculation with 20% (v/v) ethanol for 1 h, followed by sterile distilled water rinsing under aseptic conditions. After each run, the cartridges were cleaned by recirculation and backflushing with 0.1N sodium hydroxide solution. Antifoam was not used to avoid membrane fouling.[10]

Analyses

Sample preparation and periodic analyses of biomass, sucrose, ethanol, lactic, formic, acetic, and succinic acids were performed using the procedures described previously.[18] Experimental ethanol values to aqueous ethanol (i.e., ethanol in the broth), and do not consider ethanol losses in the vapor phase. The latter were estimated based on the theoretical ethanol molar yields, calculated as described below.

Theoretical Ethanol and CO_2 Molar Yields

Significant ethanol losses occurred due to excessive foaming in the headspace of the bioreactor and incomplete condensation of the exiting gases. However, the use of low nitrogen sparging rates (e.g., 40 mL/min) to reduce foaming prevented adequate measurement of dissolved and gaseous CO_2. For this reason, the ethanol and CO_2 molar yields were calculated theoretically based on the carbon and generalized degree of reduction balances and the anaerobic pathways of strain LLD-15.[19]

RESULTS

Continuous Culture with Complete Cell Recycle

The system with complete cell recycle was studied at different dilution rates and sucrose concentrations. The data was used to determine the maximum retention time in the bioreactor before loss of cell viability, and to evaluate the parameters of the kinetic model.

Figure 2 shows the effect of varying the dilution rate, at a fixed input sucrose concentration of 1% (w/v). At 0.1 h^{-1}, the residual sucrose increased 10 to 12 h after the cells had attained zero growth rate. Increasing the dilution rate

Table I. Hollow fiber cartridge specifications.

Cartridge type[a]	Nominal cutoff (MW)	Surface area (m^2)	Recirculation rate (L/min)	Filtrate rate[b] (mL/min)
H1P 10-43	10,000	0.03	1.2	7.5
H1P 100-20	100,000	0.06	0.6	15

[a]Material: polysulfone.

[b]Average filtrate rate after repeated operation cleaning; mean transmembrane pressure, 10 psig.

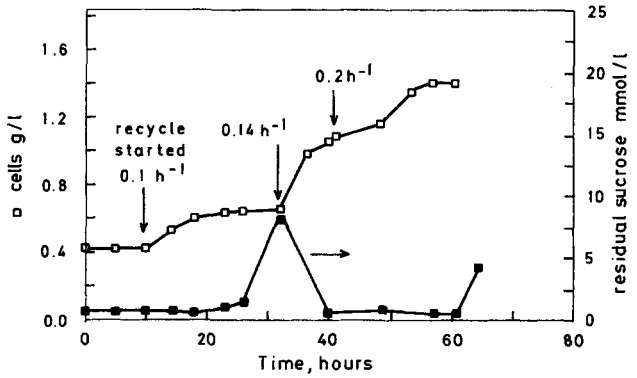

Figure 2. Continuous cultivation of strain LLD-15 with complete cell recycle: cell density and residual sucrose at different dilution rates. Input sucrose 1% (w/v), pH 7.0, 70°C, 400 rpm.

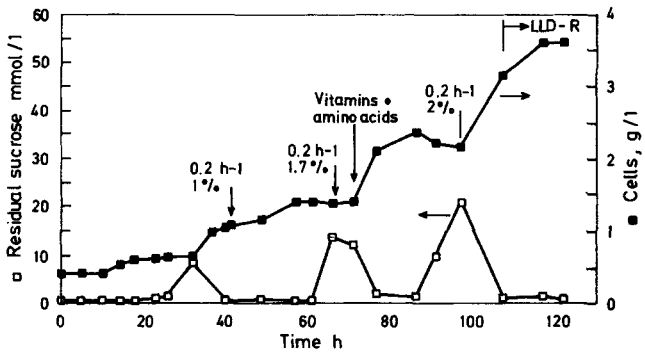

Figure 3. Continuous cultivation of strain LLD-15 with complete cell recycle: effect of increasing the incoming sucrose concentration, at a constant dilution rate of 0.2 h^{-1}, pH 7.0, 70°C, 400 rpm.

resulted in additional cell growth, as previously observed; at 0.2 h^{-1}, the residual sucrose increased approximately 10 h after the cells had reached zero growth rate.

Figure 3 shows the effect of increasing the incoming sucrose concentration, at a constant dilution rate of 0.2 h^{-1}. At 1.7% (w/v) input sucrose, it was necessary to increase the concentration of amino acids and vitamins in the reservoir to obtain adequate growth. Nevertheless, a drop in cell viability was again observed approximately 10 h after zero growth rate was attained. These results indicated that average retention times of 10 h or less should be used to maintain adequate cell viability in the bioreactor.

Increasing the input sucrose concentration to 2% (w/v) caused takeover by a lactate-producing revertant.[18,19] Microscopic observation revealed considerable cell lysis during the takeover period. The possibility that the dissolved ethanol concentration contributed to this was investigated in a system with partial cell recycle and removal of ethanol with nitrogen sparging, as described below.

Continuous Culture with Partial Cell Recycle and Stability of LLD-15

Due to the low stability of the system with complete cell recycle, the effect of a continuous bleed F_b was examined. The latter was determined based on the maximum time that the cells remained viable in the system with complete cell recycle. Thus, a mean residence time of 10 h, corresponding to D_b = 0.1 h^{-1} was chosen. Figure 4 shows the results of an experiment with partial cell recycle using 1% (w/v) input sucrose and a constant bleed rate of D_b = 0.1 h^{-1}. The overall dilution rate was gradually increased from 0.14 to 0.62 h^{-1}, allowing for steady state to be achieved at each level. At 0.62 h^{-1}, the system was operated for approximately 30 h and the total operation period was 8 days. The maximum dilution rate was determined by the capacity of the filter.

Table II shows product concentrations and molar yields for the same experiment. The acid yields were similar to those obtained in open continuous culture experiments,[19] indicating a similar activity of the PFL, PDH, and succinate pathways. However, the experimental ethanol yields were significantly lower, primarily due to incomplete condensation of the exiting vapors in the presence of foam.

Nevertheless, the most important result was that this system could be operated at high dilution rates without takeover by strain LLD-R, as observed in conventional continuous cultures. This resulted in ethanol volumetric productivities significantly higher than those obtained in conventional continuous fermentations, even without considering important ethanol losses in the vapor (i.e., 1.4 g ethanol/L · h, vs. 0.6 g ethanol/L · h, based on aqueous ethanol). Based on the theoretical yields, the ethanol volumetric productivity was as high as 2.6 g ethanol/L · h (see Table II).

The stability of strain LLD-15 in the system with partial cell recycle can be explained by examining the independence of the specific rate of sucrose consumption, q_s, from the overall dilution rate, D. According to the maintenance model, in conventional continuous cultures, $\mu = D$, and

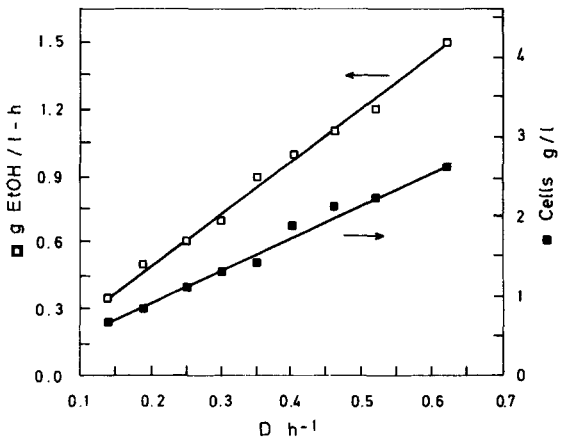

Figure 4. Continuous cultivation of strain LLD-15 with partial cell recycle: cell density and ethanol volumetric productivity at different dilution rates; 1% (w/v) input sucrose, pH 7.0, 70°C, 400 rpm. Bleed rate D_b = 0.1 h^{-1}. Ethanol values are based on aqueous ethanol (ethanol detected in the broth), and are not corrected for ethanol losses in the vapor.

Table II. Continuous cultivation of strain LLD-15 with partial cell recycle: product concentrations and molar yields at 1% (w/v) input sucrose (pH 7.0, 70°C, 400 rpm).

Input sucrose 1% (w/v), 33 mmol/L; concentrations in mmol/L							
D h^{-1}	Sucrose consumed	EtOH	Form	Ac.	Succ.	Pyr.	Lact.
0.14	30.2	54.4	47.8	14.4	4.0	0.0	0.33
0.19	32.4	58.3	46.5	19.0	2.6	0.5	0.32
0.30	32.4	51.8	45.2	18.3	2.6	0.0	0.33
0.40	31.6	50.6	44.7	17.6	2.4	0.0	0.35
0.52	31.7	50.7	55.4	22.2	3.8	0.7	0.33
0.62	31.8	50.9	46.9	19.3	3.0	0.0	0.33

Molar yields (moles ethanol/mol fermented sucrose)										
	Ethanol[a]									
D h^{-1}	Aqueous	Theor.	Form.	Ac.	Succ.	Pyr.	Lact.	Cells	CO_2[b]	C-bal[c]
0.14	1.8	3.0	1.6	0.5	0.13	0.00	0.011	0.67	2.07	99.20
0.19	1.8	3.1	1.4	0.6	0.08	0.02	0.011	0.53	2.30	99.95
0.30	1.6	3.1	1.4	0.6	0.08	0.00	0.010	0.54	2.29	99.44
0.40	1.6	3.1	1.4	0.6	0.08	0.00	0.011	0.61	2.27	99.41
0.52	1.6	2.9	1.8	0.7	0.12	0.02	0.011	0.54	1.87	99.80
0.62	1.6	3.1	1.5	0.6	0.09	0.00	0.010	0.54	2.21	99.40

	Aqueous EtOH	Vol. productivity[d]	Cells g/L		Spec. productivity[d]	Spec. consumption[d]
D h^{-1}	(g EtOH/L)	(g EtOH/L · h)	Exp.	Model	(g EtOH/g · h)	(g sucrose/g cell · h)
0.14	2.5	0.35	0.7	0.6	0.50	2.1
0.19	2.7	0.51	0.8	0.8	0.64	2.6
0.30	2.4	0.72	1.3	1.3	0.55	2.6
0.40	2.3	0.92	1.9	1.7	0.48	2.3
0.52	2.3	1.20	2.2	2.1	0.54	2.6
0.62	2.3	1.40	2.6	2.7	0.55	2.6

[a]Aqueous ethanol refers to ethanol detected in the broth. Theoretical ethanol calculated as described in ref. 21.
[b]Theoretical CO_2 yields, calculated as described in ref. 21.
[c]Carbon balance based on theoretical ethanol and CO_2 yields.
[d]Values calculated based on aqueous ethanol and experimental cell densities.

q_s is directly proportional to the overall dilution rate, D [see eq. (4)]:

$$q_s = (1/Y_{x/s})D + m_s \quad (9)$$

whereas, in a system with partial cell recycle, q_s is independent of D, but determined by the cell growth rate, D_b, as demonstrated from Eqs. (3) and (4):

$$q_s = (1/Y_{x/s})D_b + m_s \quad (10)$$

Thus, in a system with partial cell recycle, q_s can be maintained at a value where takeover by strain LLD-R is not a problem, by adequately controlling D_b.

Figure 5 compares the specific sucrose consumption rates for the system operated in conventional continuous culture and with partial cell recycle. It can be seen that, by operating at $D_b = 0.1$ h^{-1}, the average q_s was maintained at 2.5 g sucrose/g cell · h, which was significantly lower than the q_s value at which reversion was observed in conventional continuous culture (i.e., $q_s \leq 4.0$ to 4.2 g sucrose/g cell · h).

Continuous Culture with Partial Cell Recycle and Ethanol Removal

Ethanol can be a severe growth and metabolic inhibitor of thermophilic bacteria and, in most cases, the ethanol tolerance does not exceed 0.5% to 1% (v/v).[6] Thus, at the aqueous-phase ethanol concentrations observed in the previous experiments, ethanol inhibition may have been significant. To investigate this, an experiment at 2% (w/v) input sucrose ($D_b = 0.1$ h^{-1}) was done using continuous ethanol removal by nitrogen sparging, as described previously.[17]

Table III shows that, when the system was operated at a dilution rate of 0.31 h^{-1}, no significant lysis or takeover by revertants was observed suggesting that, at this sucrose concentration in the complete cell recycle experiment, ethanol could have reached inhibitory levels. However, the fact that the lactate-producing revertant took over under conditions of high ethanol concentration suggests that the major effect was probably end-product inhibition of LLD-15 reducing the metabolic flux below the rate required for maintenance (and thus accelerating cell death and lysis). If the ethanol toxicity resulted from general membrane disruption this

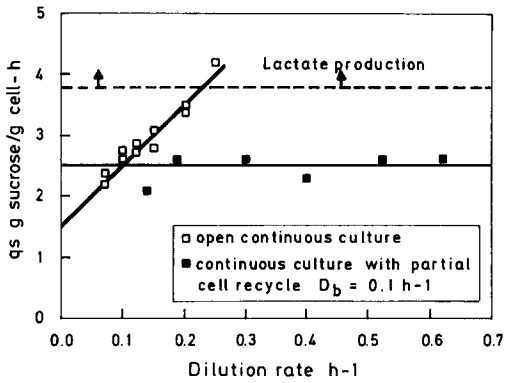

Figure 5. Comparison of q_s for conventional continuous culture and continuous culture with partial cell recycle of strain LLD-15. Input sucrose 1% (w/v), 70°C, pH 7.0, 400 rpm.

should have been equally true for the revertant strain, i.e., it would not have possessed a growth advantage.

Although ethanol production was not determined accurately, due to carryover of foam from the head of the bioreactor into the external ethanol-recovery vessel, the molar yields of formate and acetate were lower than when using 1% (w/v) input sucrose, indicating a higher activity of the PDH pathway. This is consistent with results in batch cultures, where at higher sugar concentrations the activity of the PDH pathway increases, while the carbon flow through the PFL pathway decreases.[8] The latter is probably due to a lower rate of formate and acetate export at higher extracellular acid concentrations.

Continuous Culture with Partial Cell Recycle at Acid pH

Even though the system with partial cell recycle significantly improved the ethanol volumetric productivity of strain LLD-15, ethanol yields were still below the maximum theoretical value (i.e., 4 mol EtOH/mol fermented sucrose). Based on the observation that acid pH increased ethanol yields,[8] one experiment was performed at pH 6.5 and 1% (w/v) sucrose input concentration (D_b = 0.1 h^{-1}).

This experiment showed that, after approximately 15 h of operation, lactate was detected in the broth as observed in conventional continuous cultures under similar conditions. It has been proposed that the revertants have a selective growth advantage under conditions where flux from pyruvate is becoming rate limiting. The observation that ethanol flux through the PDH pathway increases at low pH is believed to reflect the increased difficulty in acid excretion which effectively puts a lower maximum flux on the PFL pathway. One consequence of this would be that the total flux from pyruvate in LLD-15 would become rate limiting at lower q_s, i.e., the q_s at which revertants appear is pH-dependent.

Estimation of Maintenance Energy Coefficient and Performance of the Maintenance Model

The maintenance energy coefficient m_s was estimated from experiments with complete cell recycle at 0.1 h^{-1} (Figure 6) and 0.15 h^{-1}, using Eqs. (5) and (6). The values shown in Table IV were comparable with those estimated from continuous culture experiments (i.e., 1.6 g sucrose/g cell · h), indicating that the assumption that m_s is independent of the growth rate was essentially correct under the conditions studied. The values estimated for conventional continuous culture experiments were calculated by linear regression analysis of the data presented in Figure 5.

The performance of the maintenance model was tested for both complete and partial cell recycle bioreactor configurations using the parameter values m_s = 1.6 g sucrose/g cell · h and $Y_{x/s}$ = 0.105. Figure 6 shows the prediction for cell concentration based on Eq. (6) when the system was switched from open continuous culture to operation with complete recycle. Table II compares experimental and predicted values for steady cell concentrations when the system was operated with partial

Table III. Continuous cultivation of strain LLD-15 with partial cell recycle: product concentrations and molar yields at 2% (w/v) input sucrose (pH 7.0, 70°C, 400 rpm).

Input sucrose: 61 mmol/L. Concentrations in mmol/L								
D h^{-1}	S_0	S_1	Ethanol[a]	Formate	Ac.	Succ.	Pyr.	$R_{form/ac}$[b]
0.31	61	5	44	67.8	28.1	0.0	3.1	2.41

[a]Values do not consider ethanol losses due to nitrogen sparging.
[b]$R_{form/ac}$ = molar ratio formate to acetate.

Molar yields (mol/mol fermented sucrose) and cell concentration										
	Ethanol[c]								Cells (g/L)	
D h^{-1}	Aqueous	Theor.	Formate	Ac.	Succ.	Pyr.	Lact.	CO_2^d	Exp.	Model
0.31	0.80	2.96	1.2	0.5	0.0	0.06	0.014	2.4	2.1	2.3

[c]Aqueous ethanol refers to ethanol detected in the broth. Theoretical ethanol calculated as described in ref. 21.
[d]Theoretical CO_2 yields, calculated as described in ref. 21.

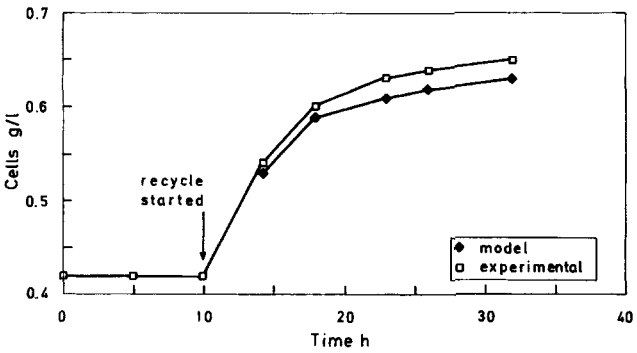

Figure 6. Continuous cultivation of strain LLD-15 with complete cell recycle: kinetics of cell concentration; 1% (w/v) input sucrose, pH 7.0, 70°C, 400 rpm. $D = 0.1\ h^{-1}$.

cell recycle. In both cases, model predictions compared favorably with the experimental values.

Performance of Hollow Fiber Cartridges

The hollow fiber cartridges used in this work were designed to operate at 50°C (see Table I). However, during the course of the experiments it was possible to operate at 65° to 70°C for 5 to 6 days before membrane fouling occurred. Replacement with a new cartridge allowed for continuous operation for prolonged periods of time.

After adequate cleaning, the cartridges were reused with good filtration capacity, although occasional fiber breakage occurred when H1P 100-20 cartridges were used more than three times. The H1P 10-43 cartridges proved to be more robust, but their filtration capacity was lower.

In preliminary experiments, silicone antifoam (1510, Dow Corning) was used in minimal amounts, but the filtration rate decreased dramatically, in accordance with previous results.[10] The use of high-shear-resistant tubing for broth recirculation improved the reliability of the system, because preliminary use of commercial silicone tubing resulted in breakage after 10 to 12 h of operation at 70°C.

DISCUSSION

The study of strain LLD-15 in a system with complete cell retention indicated that it was not possible to maintain the viability of the biomass for periods above 10 to 15 h, at 70°C. This eventually resulted in low sugar consumption, making the system inoperable. This problem was solved by the use of partial cell recycle, where a small bleed provided sufficient cell growth to maintain adequate viability in the bioreactor.

The use of partial cell recycle allowed for stable operation with strain LLD-15 at high overall dilution rates. This stability was explained by the independence between the overall dilution rate D and the specific sucrose consumption rate, q_s. The latter was controlled with the bleed rate, D_b, and was fixed at a value much lower than that where takeover by strain revertants was normally a problem. This significantly increased the ethanol volumetric productivity, particularly in relation to conventional continuous cultivation. The results were also significant in relation to other ethanologenic thermophilic bacteria, such as *Thermoanaerobium brockii*[22] and *Thermoanaerobacter ethanolicus*[11], where the establishment of continuous culture has been difficult. The values also compare well with the highest volumetric productivity reported for thermophiles in cell recycle systems, i.e., 2.2 g/L · h for *Clostridium thermosaccharolyticum*.[14] However, the volumetric productivities are still much lower than those obtained with cell recycle of *Saccharomyces cerevisiae*[5,12] (approximately 100 g EtOH/L · h from 10% w/v glucose), or *Zymomonas mobilis*[10,13] (50 g EtOH/L · h from 10% w/v glucose). Thus, future experiments should consider the use of more concentrated sugar feeds and operation under conditions that maximize ethanol production (e.g., acid pH). To avoid ethanol toxicity these experiments will also require continuous ethanol removal by gas sparging or operation under mild vacuum.

Partial cell recycle could also prove useful in improving ethanol production with other thermophilic bacteria that favor acid production at high rates of sugar consumption. For example, at high growth rates on glucose. *Clostridium thermohydrosulfuricum* shifts its metabolism from ethanol production to lactate production, probably due to the activation of the enzyme lactate dehydrogenase by high intracellular levels of 1,6 fructose diphosphate.[6] This could be remedied by using cell recycle and a low bleed rate to maintain the specific sugar consumption below lactate production levels.

The maintenance energy requirements, estimated from experiments with complete cell recycle, were in good agreement with the values obtained in continuous cultures. This suggested the validity of a kinetic model with a maintenance energy coefficient independent of the growth rate, μ. This was also confirmed by the good prediction capacity of the model for transient and steady state conditions, in systems operated with complete and partial cell recycle. This allowed for the prediction of biomass concentrations in the bioreactor based on kinetic (i.e., maintenance energy coefficient, m_s) and operational parameters (i.e., dilution rate, sugar concentration, bleed rate).

The main experimental problems related to the accurate measurement of ethanol and CO_2 and the production of lactate under conditions that maximize ethanol production (e.g., acid pH). The inability to use antifoam with the

Table IV. Estimation of maintenance model kinetic parameters m_s and $Y_{x/s}$ for strain LLD-15 at 1% (w/v) input sucrose (70°C, pH 7.0).

Bioreactor configuration	m_s (g sucrose/g cell · h)	$Y_{x/s}$ (g cell/g sucrose)
Complete cell recycle		
$D = 0.1\ h^{-1}$	1.60	—
$D = 0.15\ h^{-1}$	1.50	—
Continuous culture		
$D = 0.1 - 0.25\ h^{-1}$	1.64	0.105

ultrafiltration membranes caused excessive foaming, even at the low nitrogen sparging rates used. This in turn prevented good removal of dissolved CO_2 and analysis of the exiting gases, because the equipment normally used for this purpose (i.e., mass spectrometer or infrared gas analyzer) require minimum gas flow rates of 70 to 100 mL/min. We believe this could be solved by operating a larger volume bioreactor, that would allow the use of adequate foam breaking equipment, better condensation of the exiting gases, and the use of higher nitrogen sparging rates.

Lactate production at acid pH indicated that the q_s for selection of revertants was pH-dependent and that the imposed D_b necessary to maintain viability resulted in an excessive q_s at pH 6.5. To resolve this we are developing more stable mutants by gene disruption techniques and using deletion mutagens such as di-epoxybutane. However, even with the use of a nonrevertant strain, the continuous production of ethanol at acid pH will probably require a two-stage process in which fresh cells are constantly fed to the bioreactor to account for loss of cell viability and poor growth. This system has already been proposed by Hartley[7] and it has already been tested on a laboratory scale.[17]

R.S.M. is grateful to the British Council for financial support

NOMENCLATURE

C_s	concentration of limiting carbon source (g/L)
C_{si}	incoming concentration of limiting carbon source (g/L)
C_x	concentration of cells (g/L)
$C_{x\text{in}}$	concentration of cells in conventional continuous culture before use of cell recycle (g/L)
X_{xo}	concentration of cells at zero growth rate (g/L)
D	overall dilution rate (h^{-1})
D_b	bleed dilution rate (h^{-1})
F_b	volumetric bleed rate (L/h)
m_s	maintenance factor based on substrate requirements (g substrate consumed/g cell · h)
q_s	specific sucrose consumption rate (g substrate consumed/g cell · h)
q_{so}	specific sucrose consumption rate at zero growth rate (g substrate consumed/g cell · h)
μ	specific growth rate (h^{-1})
$Y_{x/s}$	yield factor of biomass on substrate (g cell/g substrate consumed)

References

1. Amartey, S., Leak, D., Hartley, B.S. 1991. Development and optimization of a defined medium for aerobic growth of *Bacillus stearothermophilus* LLD-15. Biothechnol. Lett. **13**: 621–626.
2. Beyeler, W., Rogers, P.L., Fiechter, A. 1984. A simple technique for the direct determination of maintenance energy coefficient: an example with *Zymomonas mobilis*. Appl. Microbiol. Biotechnol. **19**: 277–280.
3. Brock, T.D. 1978. Thermophilic microorganisms and life at high temperatures. Springer-Verlag, New York.
4. Charley, R.C., Fein, J.E., Lavers, B.H., Lawford, H.G., Ross, G. 1983. Optimization of a process design for continuous ethanol production by *Zymomonas mobilis*. Biothechnol. Lett. **5**: 169–174.
5. Cheryan, M., Mehaia, M.A. 1984. Ethanol production in a membrane recycle bioreactor, conversion of glucose using *Saccharomyces cereviseae*. Proc. Biochem. **15**:204–208.
6. Germain, P., Toukourou, F., Donaduzzi, L. 1986. Ethanol production by anaerobic thermophilic bacteria: regulation of lactate dehydrogenase activity in *Clostridium thermohydrosulfuricum*. Appl. Microbiol. Biotechnol. **24**: 300–305.
7. Hartley, B.S. 1988. Thermophilic ethanol production, International Patent Application UK 8712410.
8. Hartley, B.S., Shama, G. 1987. Novel ethanol fermentations from sugar cane and straw. Phil. Trans. R. Soc. Lond. **A321**: 555–568.
9. Jobses, I.M., Hiemstra, H.C., Roels, J.A. 1987. Fermentation kinetics of *Zymomonas mobilis* near zero growth rate. Biotechnol. Bioeng. **29**: 502–512.
10. Khorakiwala, K.H., Cheryan, M., Mehaia, M.A. 1985. Ethanol production in a membrane recycle bioreactor: comparison of performance of *Saccharomyces cereviseae* and *Zymomonas mobilis*. Biotechnol. Bioeng. Symp. **15**: 250–255.
11. Lacis, L.S., Lawford, H.G. 1988. Effect of growth rate on ethanol production by *Thermoanaerobacter ethanolicus* in glucose or xylose limited continuous culture. Biotechnol. Lett. **10**: 603–608.
12. Lafforge, C., Malowinski, J., Goma, G. 1987. High yeast concentration in continuous fermentation with cell recycle obtained by tangential microfiltration. Biotechnol. Lett. **9**: 347–352.
13. Lee, K.J., Lefebvre, M., Tribe, D.E., Rogers, P.L. 1980. High productivity ethanol fermentations with *Zymomonas mobilis* using continuous cell recycle. Biotechnol. Lett. **2**: 487–492.
14. Mistry, F.R., Cooney, C.L. 1989. Production of ethanol by *Clostridium thermosaccharolyticum*: I. Effect of cell recycle and environmental parameters. Biotechnol. Bioeng. **34**: 1295–1304.
15. Payton, M.A., Hartley, B.S. 1985. Mutants of *Bacillus stearothermophilus* lacking NAD-linked L-lactate dehydrogenase. FEMS Microbiol. Lett. **26**: 333–336.
16. Pirt, S.J. 1965. The maintenance energy of bacteria in growing cultures. Proc. R. Soc. Series B **163**: 224–231.
17. San Martin, R. 1989. A high temperature continuous two-stage ethanol fermentation, Ph.D. thesis, Centre for Biotechnology, Imperial College for Science, Technology and Medicine, London, UK.
18. San Martin, R., Bushell, D., Leak, D., Hartley, B.S. 1992. Development of a synthetic medium for continuous anaerobic growth and ethanol production with a lactate dehydrogenase mutant of *Bacillus stearothermophilus*, J. Gen. Microbiol. **138**: 987–996.
19. San Martin, R., Bushell, D., Leak, D., Hartley, B.S. 1993. Pathways of ethanol production from sucrose by a mutant thermophilic *Bacillus* in continuous culture. J. Gen. Microbiol. **139**: 1033–1040.
20. Skoog K., Hahn-Hagerdal, B. 1988. Xylose fermentation. Enzyme Microb. Technol. **10**: 66–80.
21. Slapack, G.E., Russell, I., Stewart, G.G. (eds). 1987. Thermophilic microbes in ethanol production. CRC Press, Boca Raton, FL.
22. Sonnleitner, B., Fiechter, A., Giovannini, F. 1984. Growth of *Thermoanaerobium brockii* in batch and continuous cultures at supraoptimal temperatures. Appl. Microbiol. Biotechnol. **19**: 326–334.