Pathways of ethanol production from sucrose by a mutant thermophilic *Bacillus* in continuous culture

RICARDO SAN MARTIN,[†] DES BUSHELL, DAVID J. LEAK* and BRIAN S. HARTLEY

Centre for Biotechnology, Imperial College of Science, Technology and Medicine, London SW7 2AZ, UK

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A mutant (strain LLD-15) of *Bacillus stearothermophilus* strain LLD-R, that lacks L-lactate dehydrogenase activity was studied in anaerobic single-stage continuous culture using a defined minimal medium for its capacity to produce ethanol from sucrose at 70 °C. Cultures were stable at neutral pH, dilution rates below 0.25 h⁻¹ and sucrose concentrations below 15 g l⁻¹, producing mainly ethanol, CO₂, formate and acetate plus a little succinate. The data obtained were used to estimate the maintenance energy coefficient at 70 °C and showed that the relative fluxes through the pyruvate-formate lyase (PFL) pathway and an anaerobic pyruvate dehydrogenase (PDH) pathway were about equal, though the latter predominates at low dilution rates. At higher dilution rates or at acid pH, L-lactate became the major anaerobic product due to takeover by wild-type revertants. Reversion occurred only above a critical specific sucrose consumption rate of 4.2 g sucrose h⁻¹ (g cells)⁻¹ at pH 7, at which point pyruvate production was also seen. This can be attributed to saturation of the PFL pathway and PDH pathways by increasing glycolytic flux and consequent accumulation of intracellular pyruvate. The growth rate then declines, but a small population of revertant LLD-R cells would always be present in the bioreactor since the mutation in the L-lactate dehydrogenase gene has a significant reversion rate. These revertants have no selective advantage below the critical sugar uptake rate, but at higher rates they divert excess pyruvate to L-lactate, so their growth is unimpaired and they take over in the culture. At lower pH, growth restriction and reversion occur at low sucrose consumption rates due to difficulty in excreting acetic and formic acids against a pH gradient.

Introduction

The use of thermophilic bacteria for production of fuel-grade ethanol offers important advantages over traditional yeast processes. In a process operated above 65 °C, ethanol is easily removed in the vapour by sparging or mild vacuum (Maiorella *et al.*, 1984), decreasing end-product inhibition and lowering separation costs. Moreover, many thermophilic bacteria can ferment a wide variety of sugars including pentoses, and so could be used to produce ethanol from low-cost lignocellulosic hydrolysates. However, unlike yeasts and *Zymomonas* species, most thermophiles produce little ethanol; lactate, formate and acetate are normally the major anaerobic products.

To construct an ethanol-producing thermophile, Payton & Hartley (1985) screened for a thermophilic *Bacillus* that grew rapidly anaerobically at 70 °C. This strain was originally believed to be the well-known *B. stearothermophilus* strain NCA 1503, but further characterization (Hartley, 1988; Amartei *et al.*, 1991) showed that it was a novel organism, deposited as *Bacillus stearothermophilus* strain LLD-R (NCIB 12403). By screening for fluoropyruvate resistance, a spontaneous mutant of this thermophile was selected that lacked L-lactate dehydrogenase (strain LLD-15; NCIB 12428). The rationale was that the pyruvate arising from glycolysis would then be diverted via pyruvate-formate lyase (PFL) to produce acetate, ethanol and formate in a 1:1:2 ratio (per mol of hexose). The ethanol yield would still be uncompetitive with yeasts, but further physiological or genetic manipulations to decrease PFL flux might cause the accumulating intracellular pyruvate to be diverted via pyruvate dehydrogenase (PDH; then believed to be active only aerobically) to give 2 ethanol + 2 CO₂ per mol of hexose, like yeast.

Hartley & Shama (1987) showed the above speculation

* Author for correspondence. Tel. (071) 589 5111; fax (071) 225 8942.

[†] Present address: Universidad Católica, Departamento de Ingeniería Química, Vicuña Mackenna 4860, Casilla 6177, Santiago, Chile.

Abbreviations: PDH, pyruvate dehydrogenase; PFL, pyruvate-formate lyase.

to be correct. The PFL pathway predominates in anaerobic batch cultures on sucrose at 70 °C at high pH, but at acid pH high yields of ethanol and CO₂ were seen, consistent with flux through PDH (which appeared to have unchanged levels in both aerobic and anaerobic cells). The switch to the PDH pathway appeared to be due to increasing levels of acetate and formate in the broth and coincided with a slowing in growth rate. When growth eventually stopped, sucrose continued to be metabolized entirely to ethanol+CO₂ by the PDH pathway, suggesting that this was the sole route for maintenance metabolism under these conditions.

Continuous culture is an obvious tool to unravel these phenomena, but this had to await development of a defined minimal medium for the anaerobic cultivation of strain LLD-15 on 10 g sucrose l⁻¹ (San Martin *et al.*, 1992). In the present work, this has allowed determination of detailed material and energy balances that can be related to growth and maintenance metabolism. However, the continuous cultures were stable only at dilution rates below 0.3 h⁻¹; above this value, lactate was detected in the broth, due to take-over by the revertant strain LLD-R. This suggests that the lactate pathway confers little growth advantage at low dilution rates, but is an important pathway of 'overflow metabolism' when sugar utilization rates increase above a critical threshold.

Methods

Strains and media. The mutant strain *Bacillus stearothermophilus* strain LLD-15 (NCIB 12428) and the revertant strain LLD-R (NCIB 12403) have been described previously (San Martin *et al.*, 1992). The defined minimal medium BST-AMM developed by San Martin *et al.* (1992) for 10 g input sucrose l⁻¹ was the basis for developing the media for 15 and 20 g input sucrose l⁻¹ (Table 1). Batch experiments were performed to detect possible growth inhibition due to increased concentrations of salts, amino acids and vitamins. In all cases, complete sucrose utilization occurred and growth inhibition due to media composition was not detected.

For batch and continuous cultures, the salt solution was sterilized separately and after cooling to room temperature the amino acids and vitamins were added using 0.2 µm sterile filters (Gelman). In all cases, sucrose was autoclaved separately.

All amino acids and vitamins were 'cell culture tested' grade (Sigma). Each amino acid was dissolved separately and filter-sterilized before being added to the basic salt medium. In the case of methionine and isoleucine, it was necessary to heat to approximately 50 °C to achieve complete dissolution. All of the vitamins were dissolved together in approximately 100 ml of distilled water and filter-sterilized. All salts were analytical grade (BDH) and distilled water was used throughout.

Bioreactor operation and analyses. All experiments were carried out at 70 °C in a 2 litre bioreactor with 1.5 litre working volume (Life Science Laboratories), prepared and operated as described by San Martin *et al.* (1992). Periodic analyses of biomass, sucrose, ethanol, lactic, formic, acetic and succinic acids were performed as previously described (San Martin *et al.*, 1992).

In experiments at 10 and 15 g input sucrose l⁻¹, the bioreactor was sparged with 50 ml oxygen-free nitrogen min⁻¹ to maintain anaerobic

conditions. To monitor CO₂ production, the output gases were passed through a stainless steel condenser located on top of the bioreactor, kept between -2 to -5 °C using a Grant FC 25 chiller and FH 15A heater/circulator in combination (Grant Instruments) for the refrigerant (40%, v/v, ethylene glycol). The gases were then filtered through 0.3 µm filters (Gamma 12, Whatman), and dried by passing through containers of anhydrous calcium sulphate (Hammond Drierite). Gas analysis was performed on-line using a quadrupole mass spectrometer (model 200 F, VG Gas Analysis) equipped with a 16-way multipoint stream selector. The spectrometer was calibrated every 24 h using the following standard gas mixtures (BOC): calibration gas 1, N₂ (85.1%), O₂ (9.3%), CO₂ (1.98%), CH₄ (2.59%) and Ar (1.03%); calibration gas 2, He; calibration gas 3, compressed air.

In experiments at 20 g input sucrose l⁻¹, an increased nitrogen flow (200 ml min⁻¹) was used to improve ethanol removal. The exiting gases were passed through a vertical condenser located outside the bioreactor, maintained at -2 to -5 °C as above, and the distillate was collected in a separate reservoir.

To prevent take-over by revertants (see Results and Discussion), continuous culture experiments at 20 g sucrose l⁻¹ were initiated by starting the culture at 10 g input sucrose l⁻¹/BST-AMM and steady-state was attained at *D* = 0.2 h⁻¹ at a substrate input rate of 2 g sucrose l⁻¹ h⁻¹. Then the reservoir was changed to 20 g input sucrose l⁻¹/BST-AMM, and the dilution rate was reduced to 0.1 h⁻¹ to attain the same sucrose input rate. Simultaneously, the nitrogen flow was increased from 50 ml min⁻¹ to 200 ml min⁻¹ and the ethanol removal system was connected.

Carbon and redox balance equations. (A list detailing the nomenclature used is given in an appendix following Results and

Table 1. *Defined BST-AMM media used for continuous cultures*

Component	Concentration			
<i>Main components (g l⁻¹)</i>				
Sucrose	5.0	10.0	15.0	20.0
Citric acid	0.32	0.32	0.32	0.32
MgSO ₄ ·7H ₂ O	0.27	0.27	0.30	0.50
Na ₂ HPO ₄ ·2H ₂ O	0.13	0.13	0.26	0.50
K ₂ SO ₄	0.50	0.50	0.50	0.50
NH ₄ Cl	2.0	2.0	2.0	3.0
<i>Other minerals (mg l⁻¹)</i>				
CaCl ₂ ·2H ₂ O	2.5	2.5	5.0	5.0
MnCl ₂ ·4H ₂ O	3.0	3.0	3.6	4.8
<i>Trace element solution (ml l⁻¹)*</i>	0.25	0.25	0.35	0.5
<i>Amino acids (mg l⁻¹)</i>				
Isoleucine	100	150	150	300
Serine	150	150	150	300
Glutamic acid	400	400	400	600
Methionine	100	150	150	300
<i>Vitamins (µg l⁻¹)</i>				
Thiamin	1000	1000	1000	2000
Riboflavin	450	450	450	900
Nicotinic acid	1500	1500	1500	3000
Pyridoxal	450	450	450	900
Biotin	1000	1000	1000	2000
C/N molar ratio	4.7	9.4	14.1	12.5

* Trace element solution (mg l⁻¹): ZnSO₄·H₂O (320); H₃BO₃ (80); CoCl₂·6H₂O (400); CuSO₄·5H₂O (1600); NiCl₂·6H₂O (80); FeCl₃·6H₂O (4300); EDTA (2000).

Discussion.) The carbon balance (C-bal) was calculated assuming an elemental composition for biomass of $C_{1.0}H_{1.8}O_{0.5}N_{0.2}$ with a formula weight of 24.6 (Roels, 1980).

$$C\text{-bal} = [(\sum Y_{pi/S} n_{C/pi}) + n_{C/x} Y_{x/S}] / n_{C/S} 100\% \quad (1)$$

The redox balance was calculated from the yields of products and biomass according to Roels (1980), where the generalized degree of reduction (γ_i) for a compound of composition $C_aH_bO_cN_d$ is calculated assuming $\gamma_C = 4$, $\gamma_H = 1$, $\gamma_O = -2$ and $\gamma_N = -3$ in the equation

$$\gamma_i = (4a + b - 2c - 3d)/a$$

The generalized reduction balance (γ -bal) can then be calculated as

$$\gamma\text{-bal} = [(\sum n_{C/pi} Y_{pi/S} \gamma_{pi}) + n_{C/x} Y_{x/S} \gamma_x] / n_{C/S} \gamma_S 100\% \quad (2)$$

Correction for vapour ethanol removal and dissolved CO_2 . Experimentally determined ethanol concentrations refer only to ethanol in the broth, including that returned in the condensate. Vapour ethanol losses due to incomplete condensation were estimated experimentally by passing a synthetic mixture through the system that mimicked the fermentation products at 10 g input sucrose l^{-1} , i.e. 3 g ethanol l^{-1} , 3 g formate l^{-1} , 1.7 g acetate l^{-1} and 0.7 g succinate l^{-1} . This mixture was passed through the system at dilution rates of 0.11 and 0.18 h^{-1} under conditions similar to those in the fermentation experiments, i.e. 70 °C, pH 7, sparging with 50 ml nitrogen min^{-1} and identical agitation rate and condenser operation. The ethanol losses were estimated from the difference between the ethanol in the input stream and the exit stream.

Low nitrogen sparging rates (50 ml min^{-1}) were used to prevent excessive ethanol vapour losses, but this prevented accurate CO_2 readings because the mass spectrometer ideally requires a minimum nitrogen flow rate of 70–100 ml min^{-1} . Additionally, CO_2 and bicarbonate dissolved in the broth [$CO_2(l)$] removed from the bioreactor via the liquid outflow, were not determined. Hence, the CO_2 measurements were likely to be underestimates of the true productivity. Dissolved CO_2 [$CO_2(l)$] was estimated experimentally by sparging the bioreactor with 5% $CO_2/95\%$ N_2 (v/v) at 150 ml min^{-1} while operating continuously under the standard conditions at $D = 0.4 h^{-1}$ with either medium BST-AMM or the synthetic product mixture described above. The pH was maintained at 7 throughout by automatic pH control. The amount of dissolved CO_2 was estimated by the difference between input CO_2 and exit CO_2 , as determined by the mass spectrometer. It should be recognized that this is a combined value which incorporates the bicarbonate that would be present at this pH. As all of the experimental calculations referred to pH 7, it was not necessary to calculate bicarbonate separately. However, at other pH values appropriate corrections should be made.

Theoretical correction for ethanol and CO_2 losses. A better correction for these can be made by considering the probable pathways of product formation. The major products were ethanol and CO_2 produced by the PDH pathway, and formate, acetate and ethanol produced by the PFL pathway, together with a little succinate that probably arises from phosphoenolpyruvate by CO_2 fixation. Since redox balance must be reached in steady-state continuous culture, one can set γ -bal in equation (2) to 100% (total product recovery). Since the degree of reduction of CO_2 is equal to zero, this equation then provides the theoretical ethanol yield

$$(Y_{EtOH/S})_{theor} = [n_{C/S} \gamma_{C/S} - K] / [\gamma_{EtOH} n_{C/EtOH}] \quad (3)$$

where $K = \sum (n_{C/pi} Y_{pi/S} \gamma_{pi})$ for all products but ethanol.

The rate of formate production is a good estimate of flux through the PFL pathway. Since one ethanol is produced for every two formates, the ethanol yield via the PFL pathway is

$$(Y_{EtOH/S})_{PFL\text{ pathway}} = (Y_{formate/S})/2$$

Since the rest of the ethanol is produced via the PDH pathway, its yield is

$$(Y_{EtOH/S})_{PDH\text{ pathway}} = (Y_{EtOH/S})_{theor} - (Y_{EtOH/S})_{PFL\text{ pathway}}$$

The theoretical CO_2 molar yield is the yield of ethanol produced via the PDH pathway less the CO_2 utilized in succinate production, i.e.

$$(Y_{CO_2/S})_{theor} = (Y_{EtOH/S})_{PDH\text{ pathway}} - (Y_{succinate/S})$$

Finally, the consistency of the theoretical values was evaluated by checking that the carbon balance did not exceed 100%.

Reproducibility of results. All chemical analyses were done in duplicate and are presented as the mean values. Much of the continuous culture work was repeated at least once to confirm the trends obtained. However, the results presented here are those from representative runs.

Results and Discussion

Continuous cultures on 10 g sucrose l^{-1}

Table 2 shows the kinetic parameters and molar yields for strain LLD-15 grown on 10 g input sucrose l^{-1} . The culture remained stable at dilution rates below 0.3 h^{-1} . Above this value, lactate was detected in the broth, due to take over by the revertant strain LLD-R as discussed by San Martin *et al.* (1992). Within the stable region (i.e. 0.1–0.25 h^{-1}), the molar ratio of formate to acetate remained relatively constant at approximately 2.3:1, in fair agreement with the balance predicted by the PFL pathway. Observed yields of ethanol higher than those predicted by the PFL pathway (0.7–0.8 mol per mol of fermented sucrose), together with significant CO_2 production, suggested anaerobic operation of the PDH pathway.

Table 2 shows that experimental values for carbon and reduction balances were lower than expected. Formate, acetate, succinate and lactate levels are probably reliable, since acetate values were in good agreement by different assays with or without derivatization, and extensive analyses failed to reveal any other products. Hence significant losses in vapour ethanol and dissolved CO_2 are indicated. The model experiments showed that about 15% of the ethanol was lost in the exit vapours when the nitrogen sparging rate was only 50 ml min^{-1} . In real experiments, the losses were probably even higher, depending on dilution rate, nitrogen sparging and condenser operation.

Significant amounts of CO_2 are likely to be present in the culture broth as dissolved CO_2 in equilibrium with bicarbonate. Very little CO_2 was evolved when the influent BST-AMM medium was acidified, but CO_2 equivalent to 4.4 mmol l^{-1} was released on acidification of the effluent broth. This represents approximately 15% of the total CO_2 produced under those conditions. These results agree with observations for mesophilic anaerobic systems, where significant amounts of CO_2 remain dissolved in the broth even after gas sparging (van

Table 2. Kinetic data for the continuous culture of *B. stearothermophilus* LLD-15 under anaerobic conditions at 10 g input sucrose l⁻¹ in defined BST-AMM media, pH 7.0, 70 °C

<i>D</i> (h ⁻¹)	Sucrose [g l ⁻¹ ; (% used)]		Cells (g l ⁻¹)	<i>Y</i> _{x/s} *	<i>q</i> _s †	<i>q</i> _{EtOH} ‡		
	Uptake	Catabolized						
0.10	10.3 (98 %)	9.9 (96 %)	0.38	0.037	2.60	0.79 (1.05)		
0.15	10.7 (98 %)	10.2 (95 %)	0.51	0.048	3.00	0.85 (1.11)		
0.20	10.6 (98 %)	10.0 (94 %)	0.61	0.057	3.28	0.83 (1.18)		
0.25	10.2 (97 %)	9.6 (97 %)	0.60	0.059	4.00	1.06 (1.48)		
0.30	10.2 (97 %)	9.5 (93 %)	0.70	0.069	4.03	1.06 (-)		

<i>D</i> (h ⁻¹)	Products (mm)							
	EtOH	CO ₂	Formate	Acetate	Succinate	Lactate	C-bal (corr.)	γ-bal (corr.)
0.10	65 (87)	33 (54)	46	20	7.5	0.3	83 % (99 %)	85 % (100 %)
0.15	63 (82)	34 (47)	50	22	10.0	0.3	84 % (98 %)	84 % (100 %)
0.20	57 (78)	28 (47)	52	21	9.4	0.3	82 % (98 %)	82 % (100 %)
0.25	57 (77)	24 (42)	52	22	9.0	0.3	82 % (98 %)	83 % (100 %)
0.30	54 (-)	24 (-)	44	17	8.2	6.3	-	-

<i>D</i> (h ⁻¹)	Molar yields [mol product (mol sucrose uptake) ⁻¹						
	EtOH	CO ₂	Formate	Acetate	Succinate	Lactate	Cells
0.10	2.2 (2.9)	1.1 (1.8)	1.5	0.7	0.3	0.01	0.5
0.15	2.0 (2.6)	1.1 (1.5)	1.6	0.7	0.3	0.01	0.7
0.20	1.8 (2.4)	0.9 (1.5)	1.7	0.7	0.3	0.01	0.8
0.25	1.9 (2.6)	0.8 (1.4)	1.7	0.7	0.3	0.01	0.8
0.30	1.8 (-)	0.8 (-)	1.5	0.6	0.2	0.27	0.9

<i>D</i> (h ⁻¹)	Catabolic pathway fluxes [g product (g cells) ⁻¹ h ⁻¹ ; (% used)]							
	PFL pathway				PDH pathway			Succinate pathway
	<i>q</i> _{EtOH}	<i>q</i> _{Formate}	<i>q</i> _{Acetate}	<i>q</i> _{PFL}	<i>q</i> _{EtOH}	<i>q</i> _{CO₂}	<i>q</i> _{PDH}	<i>q</i> _{Succinate}
0.10	0.28	0.56	0.31	1.15 (41 %)	0.77	0.65	1.42 (51 %)	0.23 (9 %)
0.15	0.34	0.68	0.39	1.41 (44 %)	0.77	0.64	1.41 (44 %)	0.35 (11 %)
0.20	0.38	0.79	0.42	1.59 (46 %)	0.80	0.71	1.51 (44 %)	0.36 (11 %)
0.25	0.50	1.00	0.56	2.06 (49 %)	0.98	0.81	1.79 (42 %)	0.44 (10 %)

* *Y*_{x/s}, g cells (g sucrose uptake)⁻¹.† *q*_s, g sucrose catabolized (g cells)⁻¹ h⁻¹.‡ *q*_{EtOH}, g aqueous ethanol (g cells)⁻¹ h⁻¹ (corrected for vapour ethanol losses).

|| Values in parentheses are corrected theoretically for losses as described in the text.

Beusekom *et al.*, 1981). The losses are comparable to the saturation values predicted from the theoretical relations presented by van Beusekom *et al.* (1981) for the system CO₂/water.

The experimental determination of ethanol and CO₂ losses gives an approximate correction, but a better estimate of the true ethanol production can be obtained by calculating theoretical ethanol and CO₂ molar yields, as described in Methods. The results summarized in Table 2 show that the experimental ethanol and CO₂ measurements were underestimated by approximately 25 % and 40 % respectively.

Table 2 also shows the specific rates of product formation in continuous cultures at four dilution rates, using the theoretical molar yields correction. The specific

ethanol productivity (*q*_p) for strain LLD-15, corrected for vapour ethanol losses, is 1.5 g EtOH (g cells)⁻¹ h⁻¹ at 0.25 h⁻¹. This is higher than values reported for other ethanologenic thermophiles (Wiegel *et al.*, 1979; Wiegel & Ljungdahl, 1981; Kannan & Mutharasan, 1985) and even mesophilic yeasts (0.87 g g⁻¹ h⁻¹), but lower than the 2.5 g g⁻¹ h⁻¹ reported for the mesophilic *Zymomonas mobilis* grown on glucose (Rogers *et al.*, 1980).

The volumetric ethanol productivity was within the range 0.55–0.89 g l⁻¹ h⁻¹ when corrected for ethanol vapour losses, which is significantly higher than those reported for other thermophiles. This is probably due to the successful establishment of continuous culture; e.g. a volumetric productivity of 0.045 g EtOH l⁻¹ h⁻¹ can be calculated from the data of Kannan & Mutharasan

Table 3. Kinetic data for the continuous culture of *B. stearothermophilus* LLD-15 under anaerobic conditions at 15 g input sucrose l⁻¹ in defined BST-AMM media, pH 7.0, 70 °C

<i>D</i> (h ⁻¹)	Sucrose [g l ⁻¹ ; (% used)]		Cells (g l ⁻¹)	<i>Y</i> _{x/s} * †	<i>q</i> _s †	<i>q</i> _{EtOH} ‡		
	Uptake	Catabolized						
0.10	13.7 (95 %)	13.2 (96 %)	0.53	0.030	2.6	0.76 (1.03)		
0.15	13.8 (95 %)	13.1 (95 %)	0.74	0.054	2.8	0.78 (1.10)		
0.20	14.0 (97 %)	13.3 (95 %)	0.67	0.048	4.2	0.81 (1.03)		
0.25	13.8 (96 %)	13.1 (95 %)	0.68	0.049	5.1	0.91 (1.11)		

<i>D</i> (h ⁻¹)	Products (mm)							
	EtOH	CO ₂	Formate	Acetate	Succinate	Lactate	C-bal (corr.)	γ-bal (corr.)
0.10	89 (119)	40 (80)	55	22	6.3	0	78 % (99 %)	82 % (100 %)
0.15	87 (118)	40 (76)	74	27	4.5	0	80 % (99 %)	82 % (100 %)
0.20	60 (77)	33 (58)	37	12	5.5	58	88 % (99 %)	90 % (100 %)
0.25	55 (67)	20 (48)	33	10	4.4	63	88 % (99 %)	92 % (100 %)

<i>D</i> (h ⁻¹)	Molar yields [mol product (mol sucrose uptake) ⁻¹						
	EtOH	CO ₂	Formate	Acetate	Succinate	Lactate	Cells
0.10	2.2 (2.9)	1.1 (1.8)	1.4	0.6	0.2	0	0.5
0.15	2.1 (2.9)	1.1 (1.5)	1.8	0.7	0.1	0	0.7
0.20	1.5 (1.9)	0.9 (1.5)	0.9	0.3	0.1	1.4	0.7
0.25	1.4 (1.7)	0.8 (1.4)	0.8	0.3	0.1	1.6	0.7

<i>D</i> (h ⁻¹)	Catabolic pathway fluxes [g product (g cells) ⁻¹ h ⁻¹ ; (% used)]							
	PFL pathway				PDH pathway			Succinate pathway
	<i>q</i> _{EtOH}	<i>q</i> _{Formate}	<i>q</i> _{Acetate}	<i>q</i> _{PFL}	<i>q</i> _{EtOH}	<i>q</i> _{CO₂}	<i>q</i> _{PDH}	<i>q</i> _{Succinate}
0.10	0.24	0.48	0.25	0.97 (38 %)	0.79	0.66	1.47 (57 %)	0.14 (5 %)
0.15	0.34	0.69	0.33	1.36 (47 %)	0.76	0.68	1.44 (49 %)	0.11 (4 %)

* *Y*_{x/s}, g cells (g sucrose uptake)⁻¹.† *q*_s, g sucrose catabolized (g cells)⁻¹ h⁻¹.‡ *q*_{EtOH}, g aqueous ethanol (g cells)⁻¹ h⁻¹ (corrected for vapour ethanol losses).

|| Values in parentheses are corrected theoretically for losses as described in the text.

(1985) for batch cultivation of *Thermoanaerobacter ethanolicus* on 20 g glucose l⁻¹. Attempts to use continuous culture with other ethanol-producing thermophiles have been less successful. Low ethanol yields were reported for continuous cultures of *T. ethanolicus* on 60 g soluble corn starch l⁻¹ (Wiegel *et al.*, 1983) or for *Thermoanaerobium brockii* (Sonnleitner *et al.*, 1984), even when high amounts of yeast extract were used.

Continuous cultures on 15 and 20 g sucrose l⁻¹

Table 3 shows the kinetic parameters and molar yields for strain LLD-15 grown under similar conditions on 15 g input sucrose l⁻¹. At dilution rates of 0.1 and 0.15 h⁻¹, strain LLD-15 was stable and all kinetic parameters and molar yields compared well with those obtained at 10 g input sucrose l⁻¹. However, above this dilution rate, lactate was detected in the broth indicating

take-over by the revertant. Confirmation that this represented reversion rather than the induction of an alternative pathway for lactate synthesis was obtained by (a) demonstrating the presence of NAD⁺-linked lactate dehydrogenase activity, which was indistinguishable from that present in the wild-type strain in extracts of cells taken from the culture, and (b) demonstrating that the process was irreversible, i.e. high levels of lactate production remained after returning to the lower dilution rates.

Fig. 1 shows *q*_s as a function of dilution rate for the experiments at 10 and 15 g input sucrose l⁻¹. It is evident from the change in slope of *q*_s/*D* and the appearance of lactate that take-over occurs above a *q*_s value of 4.2 g g⁻¹ h⁻¹, although the switch point with 15 g input sucrose l⁻¹ appears slightly lower. This could be artifactual, due to a transient increase in *q*_s above 4.2 g g⁻¹ h⁻¹ when the dilution rate was increased from

Fig. 1. Specific sucrose consumption rate [q_s , g sucrose (g cells) $^{-1}$ h $^{-1}$] for strain LLD-15 at 10 g (○) and 15 g (△) input sucrose l $^{-1}$, 70 °C, pH 7.0. The hatched areas under the q_s line (arrowed) represent the proportion of the total flux from pyruvate which goes via the PDH pathway, PFL pathway and to succinate. When q_s exceeds about 4.2 g sucrose (g cells) $^{-1}$ h $^{-1}$, LLD-R revertants take over the cultures. The proportion of the pyruvate flux that proceeds through the three catabolic pathways is calculated from the data of Table 2.

0.15 to 0.2 h $^{-1}$. Alternatively, it could be due to reduced PFL pathway flux, caused by higher extracellular concentrations of acetate and formate that cause problems with export of these acids (Hartley & Shama, 1987).

These results support the hypothesis that growth of strain LLD-15 is inhibited at high q_s because flux through both the PFL and PDH pathways becomes saturated. At pH 8.5, the carbon metabolic flux in LLD-15 at moderate growth rates is almost entirely through the PFL pathway (Hartley & Shama, 1987). However, flux through PDH increases at more acid pH. Therefore it has been proposed that the PDH pathway is induced because flux through PFL becomes limiting as a result of end-product inhibition, which would be exacerbated at lower pH because of diffusion of acetic and formic acids (protonated) back into the cells (Hartley & Shama, 1987). Evidence in Tables 2 and 3 that the relative flux shifts from PDH to the (already restricted) PFL pathway with increasing growth rate indicates that flux through both pathways is becoming increasingly restricted. It is also significant that pyruvate excretion is seen just before production of lactate is observed. Under these conditions, strain LLD-15 approaches its $\mu_{\max}$, but the revertant strain LLD-R has a higher $\mu_{\max}$ because it can divert the excess pyruvate to L-lactate.

The molar yields obtained in batch experiments using 20 g input sucrose l $^{-1}$ were comparable to the experiments with 10 and 15 g input sucrose l $^{-1}$. However, shortly after continuous operation was started ($D = 0.1$ – 0.15 h $^{-1}$), cell density dropped and cell lysis was detected in the broth. This was followed by take-over by a lactate-producing revertant.

Several experiments were performed to determine the cause of growth inhibition at 20 g sucrose l $^{-1}$. These included: increase and decrease of medium components, change of base to NH $_4$ OH to check possible inhibition by K $^+$ as reported by Atkinson *et al.* (1975), and different protocols for initiating continuous culture (e.g. exponential growth vs stationary phase). However, all these experiments gave similar results, and it was therefore considered that accumulated ethanol or acids were the cause, as observed in batch cultures by Shama & Hartley (1987).

To test this hypothesis, continuous culture was performed with 20 g input sucrose l $^{-1}$ at a high nitrogen sparging rate to aid removal of ethanol from the broth. This allowed establishment of a steady state at 0.1 h $^{-1}$, in which the residual sucrose, CO $_2$ levels and acid concentrations were comparable to those obtained at 10 g input sucrose l $^{-1}$, indicating similar theoretical ethanol yields though vapour ethanol losses were now severe.

Effect of pH

Although ethanol removal allowed for operation at higher substrate input concentrations, ethanol yields remained low, at approximately 70% of the maximum theoretical (i.e. 4 mols EtOH per mol of sucrose). Hartley & Shama (1987), working with batch cultures, found that the production of ethanol by strain LLD-15 was improved at acid pH (pH 6.2). A plausible explanation is that excretion of acetic and formic acid becomes increasingly difficult with reduction in pH, inducing a relatively higher flux through the PDH pathway to metabolize the resulting excess pyruvate.

To achieve further process improvement, a series of experiments were performed at pH 6.5, both at 10 and 15 g input sucrose l $^{-1}$. Unfortunately, the system was highly unstable, and both cell density and sucrose consumption were significantly lower than at pH 7.0. After 10–15 h, lactate was detected in the broth, indicating take-over by LLD-R.

Estimation of maintenance energy at 1% (w/v) input sucrose

Maintenance energy is defined as the energy consumed for purposes other than the production of new cell biomass, e.g. to balance decay in intracellular proteins and metabolites, osmotic regulation, maintenance of intracellular pH and motility (Dawes, 1986). Generally, the maintenance energy coefficient, m_s , is estimated from continuous culture experiments, using the relation between growth yield, $Y_{x/s}$, and specific growth rate, μ , introduced by Pirt (1965).

$$1/Y_{x/s} = m_s/\mu + 1/Y_{x/s\max}$$

However, this expression is only valid when the amount of energy derived from the growth substrate is constant at all dilution rates (De Vries *et al.*, 1970). In the case of strain LLD-15, the flux through acetate kinase, which generates one ATP more than the PDH flux, depends on the dilution rate (see Table 2). However, the following relation still holds.

$$1/Y_{\text{ATP}} = m_s/\mu + 1/Y_{\text{ATP(max)}}$$

where $Y_{\text{ATP(max)}}$ is the growth yield per mol of ATP, corrected for maintenance energy, m_s is the amount of ATP required for maintenance [mmol ATP (g cells)⁻¹ h⁻¹] and Y_{ATP} is the apparent growth yield per mol of ATP (De Vries *et al.*, 1970).

A plot of $1/Y_{\text{ATP}}$ vs $1/D$ was made for strain LLD-15 at 10 g input sucrose l⁻¹, within the stable range (i.e. 0.1–0.25 h⁻¹). The calculation of Y_{ATP} assumed that sucrose is converted to two hexose phosphates via a sucrose phosphorylase (Gottschalk, 1979), although this remains to be established. Hence 5 ATPs would be generated in converting sucrose to pyruvate and 1 ATP would be produced in the acetate kinase step of the PFL pathway. Using linear regression analysis, and considering that in continuous culture $\mu = D$, the following relation was obtained.

$$1/Y_{\text{ATP}} = 29.6/\mu + 152.1 \quad R = 0.99$$

This gives $m_s = 29.6$ mmol ATP (g cells)⁻¹ h⁻¹ and $Y_{\text{ATP(max)}} = 6.6$ g cells (mol ATP)⁻¹. A similar analysis, assuming that only 4 mols of ATP are generated per mol of sucrose, yields $m_s = 23.0$ mmol ATP (g cells)⁻¹ h⁻¹ and $Y_{\text{ATP(max)}} = 7.4$ g cells (mol ATP)⁻¹. Compared to anaerobic mesophilic micro-organisms, this value of $Y_{\text{ATP(max)}}$ is low while m_s is relatively high (Esenert *et al.*, 1981), supporting the argument of Zeikus (1979) that high maintenance energy is an inherent characteristic of extreme thermophiles. High maintenance requirements have also been reported for aerobic non-ethanologenic thermophilic bacteria such as *Thermus thermophilus* (Sonnleitner *et al.*, 1982). The maintenance energy for *Thermoanaerobium brockii* in continuous culture at 70 °C was recently estimated as 0.5 g glucose (g cells)⁻¹ h⁻¹ (Sonnleitner *et al.*, 1984), compared with the 1.5 g sucrose (g cells)⁻¹ h⁻¹ derived from Fig. 1. However more detailed comparison with this work is not possible, since the end-product compositions for *T. brockii* were not reported.

Relative flux through the PFL, PDH and succinate pathways

The above data make it possible to estimate the relative flux through the PDH, PFL and succinate pathways at

different growth rates, as shown in Tables 2 and 3, and the fluxes at 10 g input sucrose l⁻¹ at pH 7 are illustrated in Fig. 1. At low dilution rates, the PDH pathway predominates, but at higher growth rates the PDH flux declines and the relative amount of flux through the PFL and succinate pathways increases. For example, of the total increase in pyruvate flux to products between $D = 0.1$ h⁻¹ and $D = 0.25$ h⁻¹, 60% was diverted through the PFL pathway, 27% through the PDH pathway and 13% through the succinate pathway. As discussed, this suggests that the PDH pathway was becoming saturated under the conditions studied, and that increased carbon flow was diverted preferentially through the PFL pathway at a higher q_s .

Previous results in batch cultures have shown that both growth and PFL flux decline at acid pH and/or when acetate and formate accumulate in the medium (Hartley & Shama, 1987), which has been ascribed to difficulty in excreting acetic and formic acids against a concentration and pH gradient. The consequent decline in flux through the acetate pathway would slow up the regeneration of free CoA, which is a co-substrate for PDH. Hence flux through that pathway may also decline through shortage of free CoA, so growth inhibition and pyruvate accumulation would be enhanced.

These considerations make it clear that single-stage continuous culture is not a suitable mode for commercial production of ethanol by this mutant thermophile, even if non-reverting strains were selected, since maximum ethanol yields via the PDH pathway are shown by non-growing cells operating under conditions of high maintenance metabolism. Hence dilution rates should be very low to avoid wash-out.

Appendix: Nomenclature

The following abbreviations are used throughout the paper.

C_s	Concentration of limiting carbon source (g l ⁻¹)
C_{So}	Incoming concentration of limiting carbon source (g l ⁻¹)
C_x	Concentration of cells (g l ⁻¹)
C_p	Concentration of product (g l ⁻¹)
D	Dilution rate (h ⁻¹)
μ	Specific growth rate (h ⁻¹)
m_s	Maintenance factor based on substrate requirements [g substrate consumed (g cells) ⁻¹ h ⁻¹]
$n_{\text{C/S}}$	C-mols per mol of sugar
$n_{\text{C/pi}}$	C-mol per mol of product i
$n_{\text{C/x}}$	C-mols per mol of cells
$Y_{\text{pi/S}}$	Mols of product i per mol of sugar fermented
$Y_{\text{x/S}}$	Mols of cells per mol of sugar fermented

- $Y_{x/s}$ Yield factor of biomass on substrate [g cells (g substrate consumed)⁻¹]
 $Y_{x/p}$ Yield factor of biomass on product [g cells (g product produced)⁻¹]

References

- AMARTEY, S., LEAK, D. J. & HARTLEY, B. S. (1991). Development and optimization of a defined medium for aerobic growth of *Bacillus stearothermophilus* LLD-15. *Biotechnology Letters* **13**, 621–626.
- ATKINSON, A., EVANS, C. G. T. & YEO, R. G. (1975). Behaviour of *Bacillus stearothermophilus* grown in different media. *Journal of Applied Bacteriology* **38**, 301–304.
- DAWES, E. A. (1986). *Microbial Energetics*. Glasgow: Blackie and Son.
- DE VRIES, W., KAPTEYN, W. M. C., VAN DER BEEK, E. G. & STOUTHAMER, A. H. (1970). Molar growth yields and fermentation balances of *Lactobacillus casei* L3 in batch cultures and in continuous cultures. *Journal of General Microbiology* **63**, 333–345.
- ESENERT, A. A., ROELS, J. A. & KOSSEN, N. W. F. (1981). Comments on the description of maintenance metabolism during anaerobic growth with product formation. *Biotechnology Letters* **3**, 15–20.
- GOTTSCHALK, G. (1979). *Bacterial Metabolism*, p. 114. New York: Springer-Verlag.
- HARTLEY, B. S. (1988). Thermophilic ethanol production. *International Patent Application UK 8712410*.
- HARTLEY, B. S. & SHAMA, G. (1987). Novel ethanol fermentations from sugar cane and straw. *Philosophical Transactions of the Royal Society A* **321**, 555–568.
- KANNAN, V. & MUTHARASAN, R. (1985). Ethanol fermentation characteristics of *Thermoanaerobacter ethanolicus*. *Enzyme and Microbial Technology* **7**, 87–89.
- MAIORELLA, B., BLANCH, H. & WILKE, C. (1984). Economic evaluation of alternative ethanol fermentation processes. *Biotechnology and Bioengineering* **26**, 1003–1025.
- PAYTON, M. A. & HARTLEY, B. S. (1985). Mutants of *Bacillus stearothermophilus* lacking NAD-linked L-lactate dehydrogenase. *FEMS Microbiology Letters* **26**, 333–336.
- PIRT, S. J. (1965). *Principles of Microbe and Cell Cultivation*. Oxford: Blackwell Scientific.
- ROELS, J. A. (1980). Application of macroscopic principles to microbial metabolism. *Biotechnology and Bioengineering* **22**, 2457–2514.
- ROGERS, P. L., LEE, K. J. & TRIBE, D. E. (1980). High productivity ethanol fermentations with *Zymomonas mobilis*. *Process Biochemistry* (August/September), 7–11.
- SAN MARTIN, R., BUSHILL, D., LEAK, D. J. & HARTLEY, B. S. (1992). Development of a synthetic medium for the continuous cultivation of a mutant of *Bacillus stearothermophilus* lacking L-lactate dehydrogenase. *Journal of General Microbiology* **138**, 987–996.
- SONNLEITNER, B., COMETTA, S. & FIECHTER, A. (1982). Growth kinetics of *Thermus thermophilus*. *European Journal of Applied Microbiology and Biotechnology* **15**, 75–82.
- SONNLEITNER, B., FIECHTER, A. & GIOVANNINI, F. (1984). Growth of *Thermoanaerobium brockii* in batch and continuous cultures at supraoptimal temperatures. *Applied Microbiology and Biotechnology* **19**, 326–332.
- VAN BEUSEKOM, C. N., DE ROK, H. E. & ROELS, J. A. (1981). A method for the determination of dissolved carbon dioxide. *Biotechnology and Bioengineering* **23**, 2397–2401.
- WIEGEL, J. & LJUNDAHL, L. G. (1981). *Thermoanaerobacter ethanolicus* gen. nov. spec. nov., a new extreme thermophilic, anaerobic bacterium. *Archives of Microbiology* **128**, 343–348.
- WIEGEL, J., LJUNDAHL, L. G. & RAWSON, J. R. (1979). Isolation from soil and properties of extreme thermophile *Clostridium thermo-hydrosulfuricum*. *Journal of Bacteriology* **139**, 800–810.
- WIEGEL, J., CARREIRA, L. H., MOTHERSHED, CH. P & PULS, J. (1983). Production of ethanol from biopolymers by anaerobic, thermophilic and extreme thermophilic bacteria. II. *Thermoanaerobacter ethanolicus* JW 200 and its mutants in batch cultures and resting cell experiments. *Biotechnology and Bioengineering Symposium* no. 13, 193–205.
- ZEIKUS, J. G. (1979). Thermophilic bacteria: ecology, physiology and technology. *Enzyme and Microbial Technology* **1**, 243–252.