PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Novel Ethanol Fermentations from Sugar Cane and Straw

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Novel ethanol fermentations from sugar cane and straw

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Most agree that it is ultimately desirable to produce bulk chemicals such as ethanol from renewable resources; the questions focus on 'where', 'when' and 'how'. For developing counties with abundant biomass, growing needs for bulk chemicals and energy, and balance of exchange problems, the time is now. Sugar cane is an obvious feedstock but the economics depend on whole-crop utilization and capital and energy input. The latter is currently met by burning the cane straw (bagasse), but hydrolysis of this and fermentation and the resulting sugars would increase ethanol yields. A high-temperature fermentation would reduce energy input and capital costs.

A mutant strain of *Bacillus stearothermophilus* (LLD-15) can make ethanol from sucrose at 70 °C in yields and at rates equivalent to yeasts at 30 °C. Aerobically, the wild type grows rapidly on many sugars, and equally rapidly anaerobically producing mainly L-lactate and traces of acetate, formate and ethanol via pyruvate–formate lyase. In the mutant, the latter anaerobic pathway normally predominates. But under certain stresses pyruvate flux can be diverted exclusively via pyruvate dehydrogenase, resulting in quantitative conversion of sucrose to ethanol and CO_2 without growth. Hence the mutant offers potential for novel fermentation processes at high temperature, with high yield, high productivity and broad substrate range.

Why produce ethanol by fermentation?

At a time when oil prices have slumped to below U.S. $10 per barrel, it may seem academic to discuss the production of fermentation ethanol for use as a fuel additive. But the interest aroused by this meeting in the series 'Technology in the 1990s' shows that many people are convinced that this situation may be historically temporary, and that we should use the respite provided by low oil prices to develop new technology targeted towards renewable fuel resources.

Fermentation ethanol is attractive for this purpose because it has been extensively demonstrated that a 5–10% (by volume) mixture with gasoline is equivalent in octane value to leaded fuels. The environmental pressures to replace the latter are now huge. Moreover, the scale on which fermentation ethanol could be produced would undoubtedly satisfy such a world demand.

The other extraordinary factor for the 1990s is the impact of the revolution in agricultural technology that has quietly taken place over the last few decades. The World is likely to have a superabundance of food unless land is taken out of production. Sad stories of famine in certain developing countries cannot mask this fact. The surplus would remain even if the political and economic barriers to useful distribution of this food were lifted. Hence production of energy crops is logically and morally acceptable.

So we return to price. Everyone agrees that the cost of the substrate is the major element in the price of fermentation ethanol, but there is no consensus on what that cost should be. The laws of supply and demand simply do not work when governments are planning their

agricultural policies and food prices. They will not, and should not, abandon their rural population to the vagaries of world trade, particularly the erratic price of oil. Hence a decision to opt for fuel ethanol can make great sense in certain locations, even at this very moment.

Agricultural surpluses will invariably be the chief motivating factor, with shortage of indigenous liquid fuels and balance of payments problems following closely behind. Transport of fuels is another factor that could swing policy towards ethanol fermentations. Because these are most efficiently carried out close to the crop source, the fuel could be produced where it is most needed.

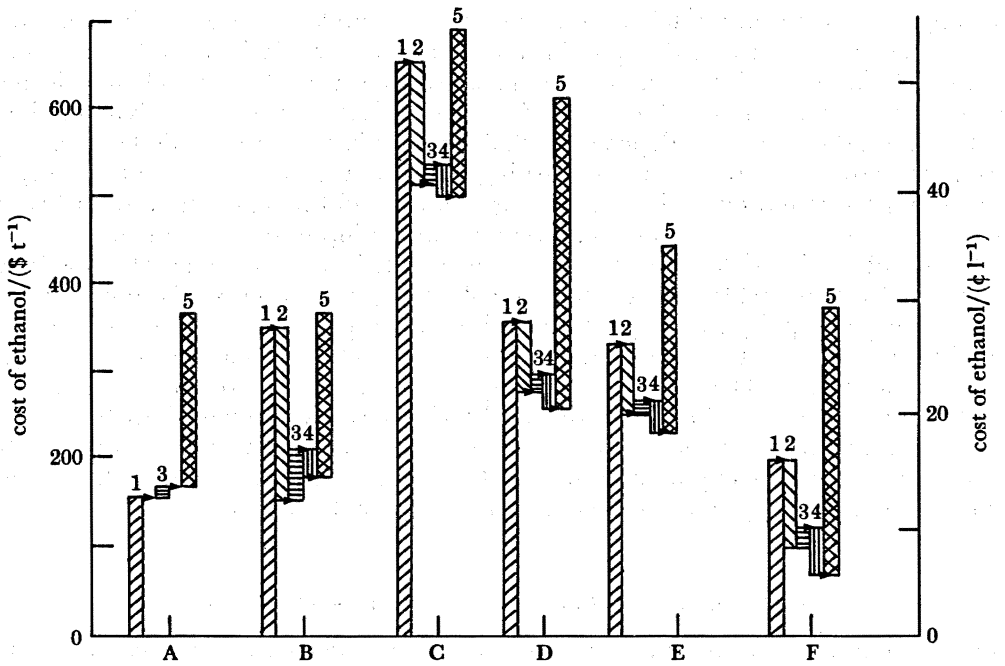


FIGURE 1. Production costs of fuel ethanol from various substrates (Martin 1982), based on 1982 prices and a capacity of 10 t h^{-1}. Fixed costs estimated at 30% of total capital, include depreciation, maintenance, labour, supervision and overheads. Cases A (sugar cane, Brazil) and B (maize, U.S.A.) are for operating plants. Cases C (wheat, Europe) and D (sugarbeet, Europe) assume conversion of straw or beet 'tops' and stillage to methane and animal feed. Case E (sugar beet and green crops, Europe) assumes as feed a succession of sugar beet and sugar-rich crops (e.g. sweet sorghum). Case F (wood waste, Europe) is very speculative in guessing both processing costs and significant by-product value for the hemicellulose and lignin fractions. Column numbers: 1, feed; 2, by-product (credit); 3, water, chemicals, fuel; 4, export electrical energy (credit); 5, fixed costs.

Figure 1 (Martin 1982) shows the relative distribution of ethanol production costs for various substrates at that time. The high prices for European wheat and sugar beet are a reflection of EEC agricultural policy, but these have now been reduced to below World prices for use in industrial fermentations. However, it is doubtful whether these substrates would be economic for ethanol production at current World prices and current oil costs.

Nevertheless, a debate is currently raging within the EEC as to whether to further subsidize current grain surpluses for fuel-ethanol production as an alternative to leaving them to rot in store.

Maize is more attractive for ethanol fermentation, largely because of the high by-product value (corn oil and dried distiller's grain). Hence, in the U.S.A., 5 Mt of maize per annum

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

are currently converted to 'gasohol' blends, which meet 4–5% of the U.S. gasoline market. However, subsidies estimated at $114M are involved, although a reduction in grain subsidies of $129M counterbalances this. As in Europe, debate about whether such subsidies are justified prejudices the future of the U.S. gasohol programme. Moreover gasohol is threatened by another success of modern biotechnology, the enzymic conversion of corn starch to high fructose syrups, which have displaced a major part of U.S. sugar imports. One can predict that this industry will grow.

This is bad news for the World's sugar-cane producers, but the good news is that sugar cane is the best current substrate for ethanol fermentation. It has the highest annual yield of biomass per hectare of any commercial crop and the cane juice, some 45% of the total dry mass, it readily fermentable by the current yeast technology. Moreover, cane refineries are ideal places in which to conduct ethanol fermentations, because production of crystalline sugar produces an almost equivalent yield of molasses. More cane producers have an unsaleable surplus of molasses and it is often poured into rivers (creating huge pollution) or spread back on the cane fields (by using precious transport and creating negative value). For these surpluses, the major costs of ethanol production are capital and running costs, so new technology can offer prospect of significant profit.

But the biggest challenge to new technology is to use lignocellulosic agricultural wastes. In Europe, wheat straw is now burned in the fields; to plough it in would mean that the land must lie fallow while natural lignocellulose degradation proceeds. There is strong environmental pressure to prohibit such burning, so can we technologists find a new use for it? The farmer might accept a price of £10 per tonne, equivalent to £20 per tonne for the constituent sugars or £50 per tonne ($0.04 per litre) of ethanol. If hydrolysis, fermentation and recovery costs could be contained within £300 per tonne this substrate would compete with sugar cane.

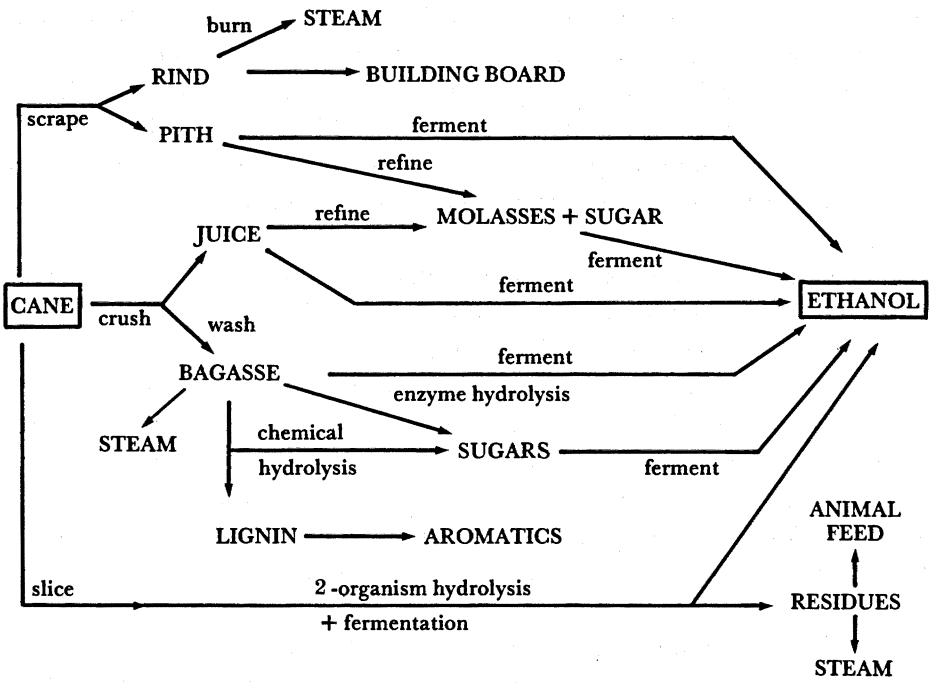
An equally low or lower price might be estimated for sugar-cane bagasse, because it is produced *in situ* at the cane refinery and has value only as an inefficient boiler fuel. Hence processing, fermentation and recovery costs will dominate ethanol production costs for these feedstocks.

Ethanol from sugar cane

Scheme 1 illustrates some of the options available for converting sugar cane to ethanol and some of the challenges offered for new technology. It is clear that the economics will depend on efficient use of all the by-products, so integrated processes are essential.

Almost without exception, preprocessing is currently carried out at cane refineries by crushing the cane and washing the bagasse to extract the juice. The bagasse is left to dry and then burned inefficiently to produce steam to evaporate the juice for successive crystallizations of sugar. It would be intelligent to ferment the molasses to ethanol by yeast batch fermentations, because ample steam should be available for distillation and markets for molasses are limited. However, the quality of molasses is variable and yeasts are finicky in fermenting it, so these fermentations demand microbiological skills and capital investment that may be rare in rural cane-growing areas. An ideal plant would operate continuously (to minimize capital costs) and with automated sensors and controls (to maximize yield and productivity) on cane juice during the harvesting season and molasses thereafter. There is less pressure to maximize energy conservation because the plants can operate on waste bagasse.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES



SCHEME 1. Possible routes to production of ethanol from sugar cane.

However, if methods can be found to upgrade the value of bagasse beyond its fuel value, energy conservation becomes justified. Hence utilization of this lignocellulosic waste becomes a key factor in the economics of ethanol fermentation from sugar cane. The ICI chemical hydrolysis process described by Ragg & Fields (this symposium) first produces a crude xylose and lignin-rich waste stream that might be fermentable. Prior removal of the hemicellulose appears to remove the 'glue' that binds the waterproofing provided by lignin from the crystalline cellulose fibres. This makes delignification easier. The resulting fibres may have value for making paper, but the second-stage hydrolysis produces a fairly clean glucose syrup that could certainly be fermented. The effective cost of these two sugar streams is only the processing cost, because waste bagasse would already be available at the plant. On this basis, the lignin extract is the only real waste, because one assumes that the microbial mass will have value as animal feed. To find added value for the lignin would make the whole process even more attractive.

Would enzymic hydrolysis compete with chemical hydrolysis of the bagasse? The process would be simpler, but the cost of the enzyme and the speed of the conversion may well make enzymic processes more costly. Lignin protects cellulose from water, so the cellulases ought, ideally, to be produced *in situ* within the fibres, as in the natural degradation process. Moreover, product inhibition of cellulases sets in when sugar concentrations rise above about 20 g l^{-1}.

Hence the attraction of a two-organism process combining a cellulase producer with an efficient ethanol producer. The process of Wang *et al.* (1983) combining *Clostridium thermocellum* and *C. thermosaccharolyticum* produced ethanol (25.3 g l^{-1}) from pure cellulose (solka floc) in 6 days, by using up 75% of the pure cellulose, equivalent to 1.52 moles of ethanol per mole of glucose residue. However, a similar fermentation with corn stover gave an ethanol concentration of only 9.7 g l^{-1}, equivalent to 0.92 moles per mole of glucose residue and only 37%

of the corn stover was consumed in 6 days. Nevertheless, such a process might be feasible with the delignified cellulose obtained from the ICI process as an alternative to their catalytic step.

Alternative strategies for ethanol production might affect the conventional preprocessing of cane crushing and washing. The Ex-Ferm process (Rolz 1980) envisages a 'solid-state' fermentation in which cane is separated into rind and pith by the Tilby process (Miller 1969). Either the chipped rind or the pith are packed directly into batch fermenters and fermented *in situ* with yeast plus minimum added water. The resulting ethanol is filtered off and distilled.

Such preprocessing could have advantages for a two-organism process, such as that of Wang *et al.* (1983). The pith fraction remaining after fermentation of the juice will be rich in hemicellulose and amorphous cellulose, but low in lignin and crystalline cellulose (table 1). Hence it might be a good substrate for *C. thermocellum*. The rind fraction could be washed to remove residual sugars and burned or converted to building board.

TABLE 1. COMPOSITION OF SUGAR CANE

(Units given in kilograms.)

	cane	rind	pith	juice	fibre
sugars	130	18	112	90	22
cellulose	67	34	33	—	33
pentosans	55	22	33	—	33
lignin	34	24	10	—	10
ash	4	2	2	1	1
H_2O	710	100	610	492	118
total	**1000**	**200**	**800**	**583**	**217**

HIGH-TEMPERATURE ETHANOL FERMENTATIONS

Our own approach is to exploit the advantages of an ethanol fermentation at 70 °C or higher. The Vacuferm process (Ramalingham & Finn 1977) demonstrated the advantages of a continuous yeast fermentation of molasses in which the ethanol vapour is continuously removed under vacuum. High sugar feed concentrations can be used, giving high ethanol concentrations, and the heat of fermentation is used directly to distil the ethanol (providing 7% of the total latent heat). However, the vacuum required is high (32 mmHg) for yeasts operating at 30 °C, so the process is probably uneconomic.

To demonstrate the advantages of a 70 °C fermentation, Hartley *et al.* (1983) modelled continuous processes for their organism, *Bacillus stearothermophilus* LLD-15 (table 2), based on cane juice, and designed this to be as close as possible to the classical yeast process modelled by Cysewski & Wilke (1978) and by using identical cane, fuel, chemical and capital costs. The comparison is unfair to the thermophile because the ethanol in the vapour (400 g l^{-1} at 70 °C) is washed out with water and then redistilled to facilitate the process comparison. This is clearly redundant in any novel process. The comparison is also unfair in that the yeast organism and process have been optimized while that for LLD-15 is based only on preliminary data. Nevertheless, savings of about U.S. $0.05 per litre are indicated, as shown in table 2. This represents a 25–40% saving in processing costs. The major savings are in the cooling of fermenters, which is in practice a serious problem in tropical countries. Heat input into the

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TABLE 2. COSTS FOR CONTINUOUS ETHANOL PRODUCTION (42000 m^3 a^{-1}) FROM SUGAR CANE BY YEAST (30 °C) OR *B. STEAROTHERMOPHILUS* LLD-15 (70 °C)

(From Hartley *et al.* (1983))

	production costs (10^6 U.S. $) (or U.S. cents per litre: 1982 rates)				
	yeast	*B. stearothermophilus* LLD-15			optimized organism
breakdown	A: Cysewski & Wilke (1978)	B: as for yeast	C: with vacuum	D: with CO_2 recycling	E: with CO_2 recycling
cane ($15 per tonne)	11.23 (25.6)	10.58 (24.1)	9.97 (22.7)	9.96 (22.9)	9.05 (20.7)
steam ($0.35 per kg)	1.50 (3.4)	1.16 (2.6)	1.23 (2.8)	1.23 (2.8)	1.10 (2.5)
water, chemicals, stillage treatment	1.95 (4.4)	0.90 (2.1)	0.74 (1.7)	0.74 (1.7)	0.55 (1.3)
labour, taxes, insurance	0.30 (0.7)	0.26 (0.6)	0.37 (0.8)	0.37 (0.8)	0.33 (0.7)
capital (15% per annum)	0.51 (1.2)	0.42 (1.0)	0.62 (1.4)	0.50 (1.2)	0.47 (1.1)
total	**15.48 (35.3)**	**13.32 (30.4)**	**12.93 (29.5)**	**12.80 (29.4)**	**11.51 (26.3)**

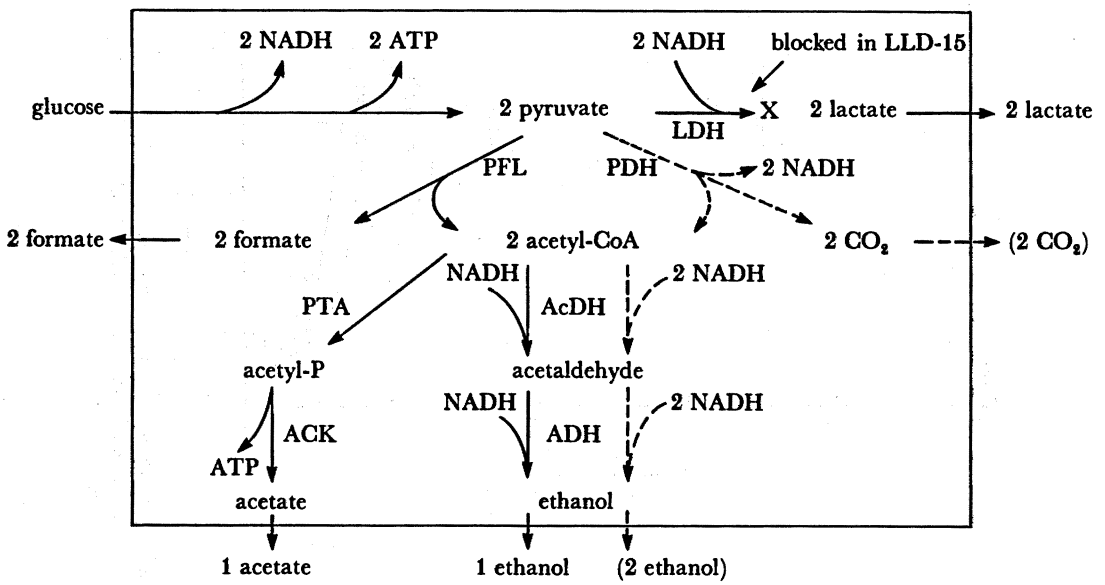
Process A is the cell-recycling model of Cysewski & Wilke (1978), and B is an almost identical model for LLD-15 at 70 °C in which ethanol is scrubbed from the vapour with water and added back to the beer before distillation. Process C uses mild vacuum, and D and E use sparging with recycled CO_2 to remove ethanol from the beer so that sugar feeds can be 150 g l^{-1} against 100 g l^{-1} in A and B. Properties of LLD-15 (ethanol yields 0.44–0.45 g per gram of sucrose; productivity on 1.3 g per gram of sucrose; tolerance 40 g l^{-1}) are those observed in the best batch fermentations. Process E assumes improved properties (yield, 0.49; productivity, 3.0; tolerance, 60 g l^{-1}). Each model uses identical costs, e.g. fixed capital is 4 × installed equipment; labour, taxes and insurance are 10% of fixed capital.

fermenter would ultimately be incorporated into any practical design, so the fermenter or still might be painted white and exposed to the full fury of the sun!

But substrate costs will always dominate any ethanol fermentation, so the ability to produce high yields of ethanol from crude molasses, crude hydrolysates of bagasse, straw, sawdust or waste paper or from paper pulping or dairy-waste streams will be a feature of any organism that might challenge yeast for ethanol production.

BACILLUS STEAROTHERMOPHILUS LLD-15

Our own approach to this challenge was to try to manipulate metabolism towards ethanol production in a facultative anaerobe that grows rapidly at high temperatures. After screening a range of these we selected *B. stearothermophilus* because of its high growth rates ($t_{\frac{1}{2}}$ at 60 °C = 25 min both aerobically and anaerobically) and temperature tolerance (aerobic $t_{\frac{1}{2}}$ at 70 °C = 30 min). However, L-lactate was the major anaerobic produce (scheme 2). If the L-lactate dehydrogenase (LLD) were inactivated by mutation, we reasoned that the anaerobic flux should be directed exclusively through the pyruvate–formate lyase (PFL) pathway resulting in 1 mole of ethanol, 1 mole of acetate and 2 moles of formate per mole of glucose. We selected such a mutant (Payton & Hartley 1985) and its properties appeared to confirm this speculation. But the key to our strategy was to make a second mutation in pyruvate–formate lyase. We were convinced that such a double mutant would not grow anaerobically because there would be no pathway for the energy flux and no route to produce acetyl-CoA. But pyruvate dehydrogenase (PDH) acts aerobically to produce acetyl-CoA + CO_2, and we hoped that a third mutation, which caused it to become constitutive anaerobically, would establish the new pathway shown in scheme 2, leading to 2 moles of ethanol and 2 CO_2 per mole of glucose.



SCHEME 2. The major anaerobic energy pathway in *B. stearothermophilus*. Glucose enters the cell (box) and is converted to pyruvate by glycolysis. Lactate dehydrogenase (LDH) is absent in the mutant strain LLD-15, so the main energy flux is via pyruvate–formate lyase (PFL) giving formate, acetate (via phosphotransacetylase, PTA, and acetate kinase, ACK) and ethanol (via acetaldehyde dehydrogenase, AcDH, and alcohol dehydrogenase, ADH). Under certain conditions, pyruvate dehydrogenase (PDH) is induced anaerobically to yield ethanol and CO_2.

We failed to select PFL$^-$ mutants (perhaps, in retrospect, because our assumptions were false) so decided to study the microbial physiology of strain LLD-15 in more detail. The first surprise (figure 2) was that while the final ratio of fermentation products on 23.5 g l^{-1} sucrose at 60 °C was 1.8 ethanol: 1.8 acetate: 3.2 formate per mole of sucrose at high pH, it changed to 2.9 ethanol: 0.6 acetate: 1.3 formate at pH 6.2. These ethanol yields were higher than possible by the PFL pathway; hence a new pathway must be switched on at low pH leading to ethanol $+CO_2$. This might be our hypothetical 'PDH pathway'.

The second clue came from anaerobic batch fermentations on glucose of concentrations 50 g l^{-1} or higher. Figure 3 shows a run at pH7, 60 °C on sucrose (50 g l^{-1}), tryptone (20 g l^{-1}), yeast extract (10 g l^{-1}). The fermentation proceeds in four stages.

(*a*) *A lag phase* (0–3 h), probably because an aerobic inoculum was used and the cells must become adapted to anaerobic conditions.

(*b*) *Rapid exponential growth* (3–10 h) with $t_{\frac{1}{2}} = 1.38$ h. Up to this point about half of the pyruvate flux appears to be via the 'PDH pathway' and half via PFL, and small quantities of pyruvate are secreted. Ethanol yields are about 1.4 moles per mole of glucose, and productivity is 0.6 g per gram of cells per hour.

(*c*) *A diauxic growth phase* (10–20 h) with $t_{\frac{1}{2}} = 2.04$ h. During this phase, 53% of the pyruvate flux is through PDH, 36% through PFL and 11% is excreted. Ethanol yields are unchanged but productivity has risen to 0.9 g per gram of cells per hour.

(*d*) '*Stationary phase*' (20–25 h). Biomass and acetate levels actually decline slightly, and pyruvate and formate secretion ceases. But ethanol production continues at 0.52 g per gram of cells per hour, giving quantitative yields of 2 moles per mole of glucose.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

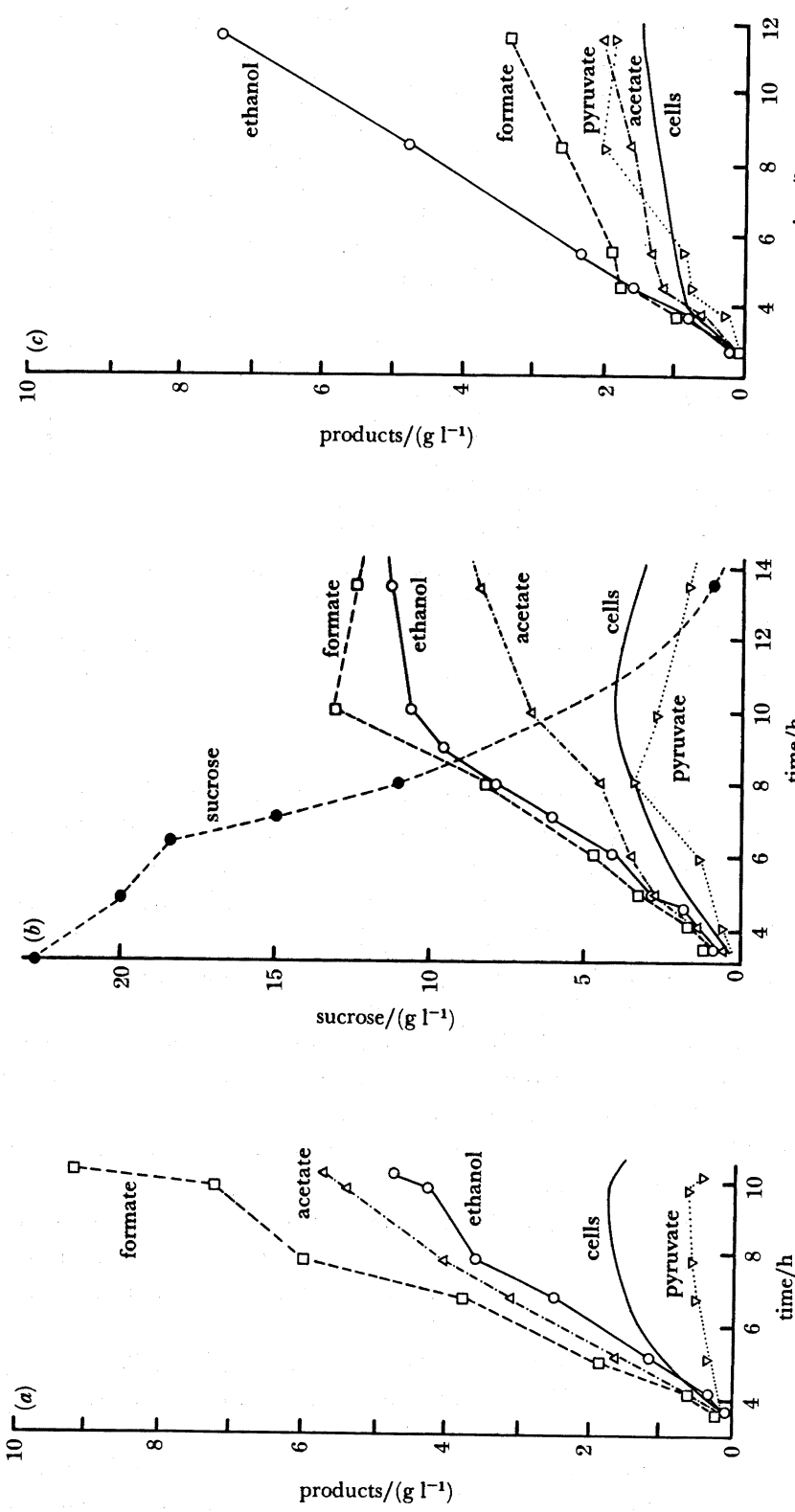


FIGURE 2. Anaerobic batch fermentations (3.4 l) by strain LLD-15 at 60 °C on sucrose (2.35 g l^{-1}), tryptone (20 g l^{-1}), yeast extract (10 g l^{-1}) with pH control. pH values: (*a*) 7.7; (*b*) 7.0; (*c*) 6.2. Sucrose uptake (●) was measured only at pH 7. Cell dry masses (solid line) are from A_{600} measurements with calibration curves. Ethanol (○), formate (□), acetate (△) and pyruvate (▽) are in g l^{-1}.

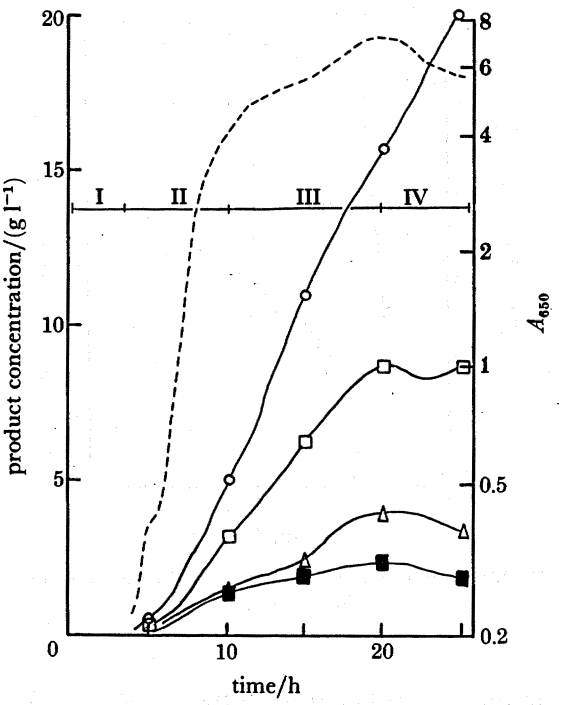


FIGURE 3. Anaerobic batch fermentations (3.4 l) by strain LLD-15 at 60 °C on sucrose (50 g l^{-1}), tryptone (20 g l^{-1}), yeast extract (10 g l^{-1}). Cell densities are shown both as A_{600} (broken line) and dry mass (g l^{-1}) (■). Ethanol (○), formate (□), and acetate (△) concentrations are in g l^{-1}.

Hence the whole fermentation has produced ethanol (21.8 g l^{-1}) from sucrose (44.5 g l^{-1}) in 20 h, with a final concentration of cells of 2 g l^{-1}. This averages at 3.64 moles per mole of sucrose (91% of theoretical) at a rate of about 0.55 g per gram of cells per hour, or 1.09 g l^{-1} h^{-1}, which is almost as good as yeast.

MICROBIAL PHYSIOLOGY OF STRAIN LLD-15

The above experiments were very encouraging, but raised several questions. The final yields of ethanol from the series of batch fermentations on sucrose (2.35 g l^{-1}), tryptone (2 g l^{-1}), yeast extract (1 g l^{-1}) at 60 °C were very erratic, as shown in table 3. Nevertheless certain tendencies are apparent. The high ethanol yields were obtained at lower pH, at high sucrose concentrations and at higher temperatures and appear to correlate with relatively high levels of pyruvate secretion. Ethanol productivity is at a minimum at pH7 and rises with temperature.

Although CO_2 was not measured in these experiments, later results have confirmed that the increased ethanol yields are indeed related to CO_2 production. Although there are high levels of tryptone and yeast extract present, radioactive-labelling experiments showed that almost all of the fermentation products arise from sucrose.

The reason for these erratic yields can be seen when we analyse the ethanol productivity at different stages of batch fermentation. We have already seen this in the analysis of a fermentation on 50 g l^{-1} sucrose (figure 3). A similar phenomenon is seen in the fermentation at pH 6.2 shown in figure 2*c*. The ratio of ethanol to formate signals the relative flux through the PFL pathway: a ratio of 0.5 is expected. However, we see from figure 4 that there is first

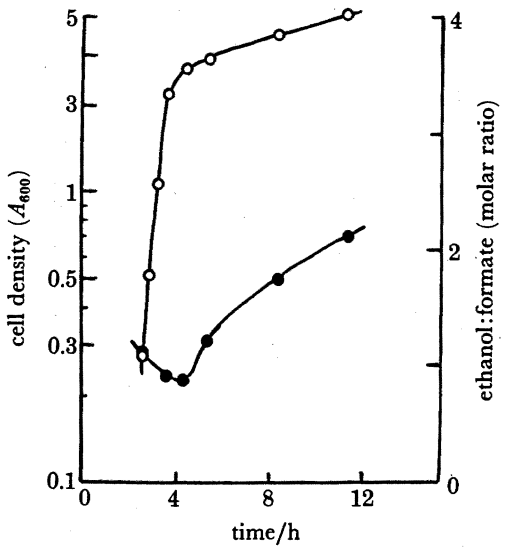


FIGURE 4. Cell density and ratio of ethanol: formate in the batch fermentation (60 °C, pH 6.2) shown in figure 2*c*.

TABLE 3. FINAL PRODUCTS FROM BATCH FERMENTATIONS OF LLD-15 ON SUCROSE[1]

temperature °C	pH	initial sucrose g l^{-1}	time h	sucrose uptake g l^{-1}	product/(g l^{-1})(moles per mole sucrose) ethanol	formate	acetate	pyruvate	cells	Et OH production[2]
50	8.0	26.0	8	17.0	3.2 (1.1)	6.4 (2.8)	3.2 (1.1)	0.1 (0.0)	2.2	0.2
50	7.0	23.5	8	20.3	4.7 (1.7)	4.8 (1.8)	3.8 (1.1)	0.1 (0.0)	2.4	0.2
60	8.0	26.6	6	24.8	4.7 (1.4)	9.2 (2.8)	5.7 (1.3)	0.3 (0.0)	1.7	0.8
60	7.0	23.9	6	23.0	5.6 (1.8)	6.2 (2.0)	4.2 (1.0)	0.7 (0.1)	1.8	0.4
60	7.0	26.3	7	25.2	6.4 (1.9)	6.8 (2.0)	3.2 (0.7)	2.6 (0.4)	2.0	0.5
60	7.0	22.1	8	21.5	7.6 (2.6)	6.0 (2.1)	3.1 (0.8)	1.7 (0.3)	2.0	0.6
60	7.0	50.0	14	49.2	14.0 (2.1)	11.2 (1.7)	5.6 (0.6)	6.6 (0.5)	2.5	0.4
60	7.0	50.0	19	44.1	21.8 (3.7)	9.1 (1.5)	3.5 (0.5)	3.8 (0.3)	2.1	0.6
60	7.0	109.5	11	39.3	12.0 (2.3)	4.5 (0.9)	5.4 (0.8)	2.3 (0.2)	1.9	0.6
60	6.6	24.5	8	23.9	7.9 (2.5)	5.0 (1.6)	3.0 (0.7)	2.0 (0.3)	1.7	0.6
60	6.2	22.9	8	21.1	8.1 (2.9)	3.3 (1.2)	2.0 (0.5)	1.9 (0.3)	1.5	0.6
70	7.7	24.4	4	22.8	6.4 (2.1)	7.2 (2.3)	4.4 (1.1)	1.6 (0.3)	1.6	1.3
70	7.0	21.9	5	15.1	6.0 (3.0)	3.8 (1.9)	1.6 (0.6)	2.4 (0.6)	1.1	0.9

[1] The time from end of lag phase to maximum ethanol concentration is shown. Products at that time are indicated.

[2] Ethanol productivity is the final concentration (in grams) per final dry mass of cells (in grams) per hour of fermentation time.

a rapid exponential growth in which the ratio is about 1 and then this rises steadily to above 2 throughout a slower 'diauxic' growth phase. Clearly there is a switch from the PFL-pathway to our putative 'PDH-pathway', and the final yields will reflect the balance between these two routes over the whole batch fermentation.

What controls the switch to the 'PDH-pathway'? The following factors appear to favour it.

(*a*) *Temperature.* The final yields in batch fermentations at 70 °C were higher than at 60 °C, but the effect is best seen in the rates of ethanol production shown in table 3.

(*b*) *Low pH*. This is demonstrated in figure 2 and also in table 3.

(*c*) *High sugar concentrations*. The results with a sucrose concentration of 50 g l^{-1} (figure 3), rather than a concentration of 23.5 g l^{-1} present another question. Why does exponential growth stop after 7 h, and the 'diauxic growth' after 20 h, when there is still plenty of sucrose left? One obvious solution would be that some other component of the medium becomes growth limiting. This is not the case, because the levels of tryptone, yeast extract and salts are very high and can be reduced several-fold, or largely replaced by ammonium salts without significant change in the fermentation profiles.

Alternatively, some fermentation products might inhibit growth. One would suspect ethanol, but our early shake-flask experiments indicated an ethanol tolerance of 40 g l^{-1} and figure 5*a* shows that ethanol is not the culprit. If ethanol is added in mid-exponential phase to give a concentration equal to that in the final stationary phase, the growth rate and sucrose uptake are unaffected.

However, if acetate and formate are added at a similar point, sucrose uptake ceases and the culture density declines (figure 5*b*). This gives a strong clue to the nature of the metabolic switch. Like many bacilli, this strain can sporulate. There is a precedent for a switch to ethanol production by presporulating cells of *Clostridium thermosaccharolyticum* (Hsu & Ordal 1970), although in that strict anaerobe it is improbable that pyruvate dehydrogenase is involved.

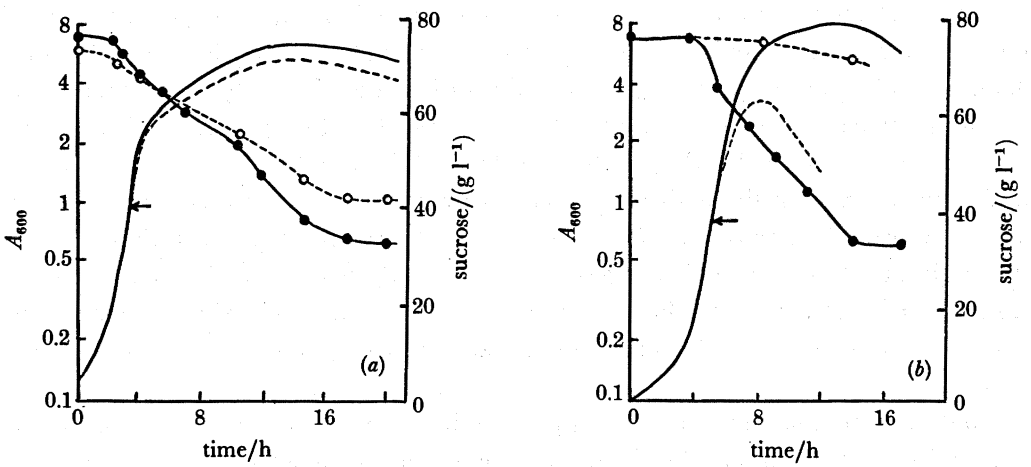


FIGURE 5. Effect of ethanol (*a*) or acetate+formate (*b*) on growth (A_{600}, ---) and sucrose uptake (○) by LLD-15 in batch fermentations on sucrose (75 g l^{-1}), 2% tryptone (20 g l^{-1}), yeast extract (10 g l^{-1}) at 60 °C, pH 7. The ethanol (4 g l^{-1}) or acetate (2 g l^{-1})+formate (4 g l^{-1}) were added in mid-exponential phase (arrows) to give the maximum concentrations expected in stationary phase. Identical control fermentations (—— and ●) with no additions were conducted in parallel.

Could this acetate formate switch be responsible for the stationary-phase ethanol production by *B. stearothermophilus* LLD-15 shown in figure 3? When acetate and formate were added more slowly to an exponential phase culture, we were able to induce a prolonged stationary phase like that shown in figure 3 in which sucrose uptake continues steadily.

Finally, one should question whether pyruvate dehydrogenase is truly responsible for the CO_2 production that accompanies the increased ethanol production? A very plausible alternative route might be the induction of an NAD-linked formate-dehydrogenase activity yielding CO_2 and NADH, which would then be utilized to reduce acetyl-CoA to acetaldehyde

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

and ethanol. We were unable to detect any such activity in anaerobic LLD-15 cell extracts. However, anaerobically grown LLD-15 cells contained higher levels of pyruvate dehydrogenase than either LLD-15 or wild-type grown aerobically. In contrast, PDH levels were low in anaerobic cells of wild-type or of a spontaneous revertant of LLD-15 that arose in continuous culture at 60 °C. This revertant appeared to be identical to wild-type in producing L-lactate rather than acetate, formate and ethanol. Hence the high levels of PDH are a consequence of the mutation in L-lactate dehydrogenase and not merely an adventitious secondary mutation.

Control of the pyruvate flux in strain LLD-15

These results suggest the following hypothesis. Wild-type *B. stearothermophilus* grows very rapidly on sucrose, either aerobically or anaerobically, so it must have an efficient sugar-uptake system. The main aerobic energy flux is through glycolysis followed by conversion of pyruvate to L-lactate (scheme 2) and the PFL-pathway is presumably used to produce the acetyl-CoA necessary for growth, with ethanol and acetate as minor products of a secondary energy-flux pathway.

But when the LDH-pathway is knocked out by the mutation in LLD-15, the flux through this secondary pathway increases. What then controls the growth rate? Our observations suggest that excretion of acetic acid and formic acid become the rate-limiting step in energy metabolism. Excretion of protons against a pH gradient might limit this flux at acid pH, and excretion of acetate and formate ions against a concentration gradient would add to the problem. In consequence, pyruvate would pile up because the acetyl-CoA concentration is limited by the size of the CoA pool, and a high sucrose flux through glycolysis continues.

It is not unreasonable that high pyruvate levels might induce pyruvate dehydrogenase synthesis. However, even the high enzyme levels observed anaerobically cannot be sufficient to reduce the size of the pyruvate pool, because we observe pyruvate secretion whenever the flux through PDH is high. This suggests that the total energy flux is then limited by the maximum PDH activity. This flux may be inadequate to maintain the extremely high growth-rate characteristic of this organism, resulting in the 'diauxic growth' that we have observed.

The final stage of ethanol production appears to be closely associated with sporulation. Like many bacilli this organism can form spores. A precedent for a switch in metabolic pathways before sporulation is seen in the studies of Hsu & Ordal (1970) on *Clostridium thermosaccharolyticum*, where slow glucose feed increased ethanol production by presporulating cells. We do not suggest that PDH activity would be involved in this strict anaerobe; in their case induction of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and alcohol dehydrogenase was demonstrated. Nevertheless there is analogy to our case.

One can extrapolate this hypothesis into a scenario for the lifestyle of *B. stearothermophilus*. It is common in compost heaps, some of which contain pockets up to 70–80 °C. Cellulolytic fungi produce more sugars than they can use. Our *Bacillus* would avidly consume these sugars aerobically, producing more heat and reducing oxygen tension. This would cause it to switch to anaerobic lactate production while still outgrowing competitors. But as the environment becomes more acid, as temperatures rise, and as sugars become less available, it begins to form spores. Such spores are ideally suited to survive until new hot pockets, rich in sugar, can be colonized. Hence there is some excuse for including this organism in a discussion on the utilization of lignocellulosic wastes.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Comparison of ethanol-producing microorganisms

There are now realistic alternatives to yeasts for producing ethanol from various sugars. Table 4 shows that *Zymomonas mobilis* has higher yield and higher productivity on glucose or fructose, but yields on sucrose are much lower (Dawes *et al.* 1966), and it cannot utilize pentoses, cellobiose, maltose or starch (Swings & de Ley 1977). Several thermophiles can utilize a broad

Table 4. Comparison of thermophilic ethanol producers

	yeast[1]	*zymo.*[2]	LLD-15[3]	*Cl.t-s.*[4]	*Cl.t-h.*[5]	*Th.eth.*[6]
Et OH/(g g^{-1} sugar)	0.44	0.47	0.43	0.36	0.36	0.44
Et OH/(g g^{-1} cells h^{-1})	0.87	2.5	1.01	0.15	?	0.29
optimum temperature/°C	30	30	70	60	68	69
growth rate/h^{-1}	0.06	0.13	1.66	0.02	0.57	0.20
Et OH tolerance/(g l^{-1})	12%	16%	4%	3%	?	0.4%
sugar tolerance/(g l^{-1})	25%	25%	>10%	?	?	2%
O_2 tolerance	++	++	++	−	−	−
ferments:						
sucrose	++	+	++	++	++	++
xylose	−	−	++	++	++	++
cellobiose	−	−	++	++	++	++

Notes: [1] *Saccharomyces uvarum* (Rogers *et al.* 1980); [2] *Zymomonas mobilis* (Rogers *et al.* 1980); [3] *B. stearothermophilus* LLD-15 (Hartley *et al.* 1983); [4] *Clostridium thermosaccharolyticum* (Wang *et al.* 1983); [5] *Cl. thermohydrosulfuricum* (Wiegel *et al.* 1979); [6] *Thermoanaerobacter ethanolicus* (Wiegel & Ljungdahl 1981; Kannan & Mutharasan 1985).

range of sugars including the products of lignocellulose hydrolysis, and the advantages of high-temperature fermentations have been discussed above and elsewhere (Zeikus 1979; Zeikus *et al.* 1981). The properties of some that might compete with *B. stearothermophilus* LLD-15 for this purpose are shown in table 4. Because ethanol yield is generally all-important, only *Thermoanaerobium ethanolicus* seems a serious competitor. However, the growth rate and ethanol productivity of this strain are much lower, and it suffers from low ethanol and sugar tolerance. Moreover it is a strict anaerobe whereas strain LLD-15 has high growth rates both aerobically and anaerobically. Further genetic improvement and control of its microbial physiology would make it the organism of choice for a wide range of fuel-ethanol fermentations.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES