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Comparative stability of ethanol production by *Escherichia coli* KO11 in batch and chemostat culture

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Differing claims regarding the stability of the recombinant ethanologen *E. coli* KO11 are addressed here in batch and chemostat culture. In repeat batch culture, the organism was stable on glucose, mannose, xylose and galactose for at least three serial transfers, even in the absence of a selective antibiotic. Chemostat cultures on glucose were remarkably stable, but on mannose, xylose and a xylose/glucose mixture, they progressively lost their hyperethanologenicity. On xylose, the loss was irreversible, indicating genetic instability. The loss of hyperethanologenicity was accompanied by the production of high concentrations of acetic acid and by increasing biomass yields, suggesting that the higher ATP yield associated with acetate production may foster the growth of acetate-producing revertant strains. Plate counts on high chloramphenicol-containing medium, whether directly, or following preliminary growth on non-selective medium, were not a reliable indicator of high ethanologenicity during chemostat culture. In batch culture, the organism appeared to retain its promise for ethanol production from lignocellulosics and concerns that antibiotics may need to be included in all media appear unfounded.

Keywords: ethanol; recombinant; *E. coli* KO11; lignocellulosic; chemostat; stability

Introduction

Ethanol derived from the fermentation of cheap renewable lignocellulosic materials has the potential to make a significant contribution to world liquid fuel requirements, while reducing net carbon dioxide emissions. For economic production of ethanol, utilisation of the hemicellulose component of the feedstock is essential: in lignocellulose pretreatment processes this component is commonly hydrolysed separately to produce a solution containing the pentose sugars, xylose and arabinose, along with hexoses including mannose, glucose and galactose. Hardwoods and crop residues generally yield hydrolysates containing a high proportion of xylose; in softwoods, mannose is a major component [28].

While *Saccharomyces cerevisiae* has traditionally been used to produce ethanol from sugars, its usefulness for fermenting hemicellulosic hydrolysates is limited due to its inability to metabolise pentoses. One approach to this problem has been the insertion of genes for the assimilation and metabolism of specific pentose sugars into either *S. cerevisiae* itself [6,23,29] or the bacterial ethanologen *Zymomonas mobilis* [7,8,31]. The alternative approach, initiated by Ingram and colleagues [1,14,26] is the insertion of genes for ethanol production into organisms such as *Escherichia coli*, which can naturally metabolise all the sugars present in hemicellulosic hydrolysates but which normally produce only small amounts of ethanol. Ohta *et al* [26] produced an ethanologenic strain (KO3) of *E. coli* ATCC 11303 containing chromosomally integrated genes for alcohol production from *Z. mobilis* (the *pdc* and *adhII* genes) and the chloramphenicol acetyl transferase (*cat*) gene conferring resistance to chloramphenicol (Cm). Mutants of this strain possessing both enhanced ethanologenicity and high chloramphenicol resistance were obtained following overnight culture on plates containing 600 μg ml^{-1} chloramphenicol. One isolate (strain KO4) was subsequently modified by deletion of the *frd* gene, resulting in the reduction of succinate production by 95% [26]. In batch culture, the resulting strain (designated KO11) produced high yields of ethanol from individual hemicellulose sugars and sugar mixtures, including a variety of native hemicellulose hydrolysates [3–5]. The KO11 strain was tested successfully on various hydrolysates under semi-industrial conditions in fermentations up to 10 000-L capacity [3,15]. In an interlaboratory comparison of the performance of various bacterial and fungal ethanologens on a xylose-rich corn cob hydrolysate, *E. coli* KO11 gave the highest ethanol yield and was considered to be the most promising ethanol producer [12].

The economics of ethanol production can be improved by recycling a portion of the microbial population from batch to batch, thereby avoiding the production costs and waste substrate associated with growing fresh cells for each fermentation. Even further savings are possible through the use of continuous culture. However, both these techniques require a genetically stable strain. There are differing claims regarding the stability of *E. coli* KO11. Lawford and Rousseau [19] reported that, when grown on glucose and mannose in serial batch culture, the strain lost much or all of its ability to produce ethanol in less than 12 generations. Even when 40 μg ml^{-1} chloramphenicol was included in the medium, the strain was unable to retain the ability to produce high yields of ethanol. Further, when grown in continuous culture on 25 g L^{-1} glucose or xylose, *E. coli*

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KO11 rapidly lost its ethanologenicity and diverted a substantial portion of the carbon consumed into the production of organic acids, notably lactate [22]. It was suggested that, for batch fermentations, antibiotics might need to be included in all media, raising the cost of ethanol production by as much as 29 cents per gallon [22] and that, overall, the results called into question the utility of the organism for large-scale ethanol production [19].

The stability of the *E. coli* KO11 construct was not specifically tested by its originators: however, the parent strain, *E. coli* KO4, was serially transferred in glucose/Luria broth medium lacking antibiotics for more than 60 generations without loss of ethanologenicity (as measured by ADHII activity) [26]. Strain KO11 differs from KO4 only in the introduction of the *frd* mutation [26]. In our own laboratory, *E. coli* KO11 was grown continuously on glucose/Luria broth medium without antibiotics for up to 27 days in both a chemostat with cell recycle and a fluidised bed fermenter: in contrast to the results of Lawford and Rousseau [22] no loss of ethanologenicity was observed [11].

While a strain of *E. coli* has recently been constructed [10] which ferments glucose, xylose and arabinose to ethanol with good yields and maintains the plasmid-borne ethanologenic genes selectively under anaerobic conditions, its ethanol productivities so far remain below those of *E. coli* KO11 and its utility for ethanol production under practical conditions has to date been much less extensively demonstrated. Strain KO11 possesses good resistance to inhibitors present in lignocellulosic hydrolysates (notably acetate) and has recently been shown to be relatively insensitive to process errors associated with commercial ethanol production, including sudden contamination and exposure to extremes of temperature and pH [25]. A commercial scale plant which will employ the strain has recently been announced [2]. Given the lack of agreement between published studies concerning its stability, we have carried out an extensive investigation of the phenotypic stability of strain KO11. In this paper we report findings on the behaviour of the strain in repeat batch cultures under the conditions employed by Lawford and Rousseau [19], as well as in long-term continuous culture on glucose, mannose and xylose and a glucose/xylose mixture. We also show the ineffectiveness of plate counting on chloramphenicol-containing medium as an indicator of hyperethanologenicity in this strain.

Materials and methods

Strain and growth conditions

E. coli KO11 was obtained from Professor LO Ingram (University of Florida) and was stored freeze-dried in ampoules. Cells for the ampoules were grown on medium containing 600 μg ml^{-1} Cm. Prior to use, lyophilised cells were transferred to 10 ml of a modified Luria broth (LB) containing 20 g L^{-1} of the appropriate sugar and incubated for 16 h at 30°C without shaking. The modified Luria broth contained (g L^{-1}): tryptone 10; yeast extract 5; NaCl 5; sugar (glucose, mannose or xylose) 20. All media, except the feed for the chemostat, were sterilised by autoclaving them (the sugars were autoclaved separately from the other LB components). The chemostat feed was sterilised by filtration through a 0.2-μm pore size cartridge filter. All fermentation experiments were performed in the absence of antibiotics.

Culture conditions

All cultures were grown at 30°C. Serial batch cultures were carried out in static test tubes according to the protocol of Lawford and Rousseau [19]. Chemostat cultures were grown at a dilution rate of 0.06 h^{-1} in 860 ml of medium in a New Brunswick model 19 fermenter with a 2.0-litre glass vessel. The vessel was stirred at *ca* 200 rpm and the pH was maintained at 6.0 by automatic addition of 3.0 M KOH. A low bleed-rate of nitrogen was fed to the headspace and antifoam was added manually as required. The chemostat was inoculated with a 10-ml LB culture prepared as described above and operated as a batch fermentation for the first 8 h, after which the feed pump was started. The cultures were examined daily for contamination.

Monitoring of chloramphenicol resistance

Samples were diluted in LB and spread plated on LB agar plates containing Cm (0, 40 or 600 μg ml^{-1}) and 20 g L^{-1} of the sugar used in the particular experiment. The plates were incubated at 30°C for 28 h. In some experiments (see text), plates were also prepared by transferring colonies grown on non-selective plates to plates containing 600 μg ml^{-1} Cm; at least 60 randomly-selected colonies were transferred for each plate.

Analytical methods

Ethanol was analysed by gas chromatography of headspace gases using a Perkin Elmer HS40 automatic headspace sampler connected to a Hewlett Packard 5890A gas chromatograph with a megabore capillary column (30 m × 0.53 mm, Econo-Cap™, EC-WAX, 1.2 μm film thickness, Alltech Associates Inc, Australia). Headspace vials containing 2 ml of diluted sample were equilibrated at 30°C for 30 min before injection. The column was operated isothermally at 50°C, with the injector and detector both at 200°C. Propanol was used as an internal standard.

Residual sugars and organic acids were measured using a Varian Star Liquid Chromatography system with a refractive index detector and a Bio-Rad Aminex HPX-87H organic acid column (Bio-Rad, Hercules, CA, USA). The column was operated at 65°C with 5 mM H_2SO_4 (0.6 ml min^{-1}) as the mobile phase. Lactose was used as an internal standard.

Biomass was estimated by measuring the optical density of an appropriately diluted sample at 550 nm.

Results

Sugar fermentation in serial batch culture

The organism was grown without antibiotics through three successive batch cultures in static test tube culture at 30°C, following the procedure of Lawford and Rousseau [19] (Figure 1). The medium was phosphate-buffered Luria broth containing either glucose, galactose, mannose or xylose as the carbon source. The experiment was repeated four times for each sugar, each experiment having been started from independent inocula. While overall yields were

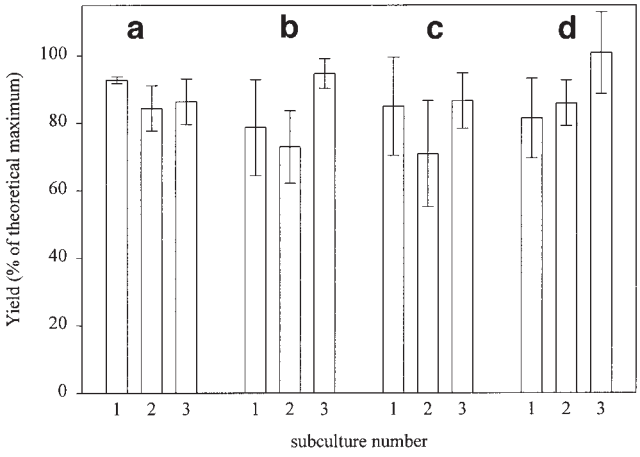

Figure 1 Ethanol production by *E. coli* KO11 in serial batch culture without antibiotics using (a) glucose, (b) galactose, (c) mannose, and (d) xylose as the carbon source. Data are the means of four separate experiments. Error bars indicate standard deviations.

relatively low, consistent with the sub-optimal fermentation conditions used in the experiment, the results show no significant loss of ethanologenicity over the course of the three subcultures (approximately 12 generations) on any of the sugars tested.

Sugar fermentation in continuous culture

Glucose fermentation: *E. coli* KO11 was grown in glucose-limited continuous culture with little or no loss in ethanologenicity (Figure 2). Data are shown for two separate experiments lasting 16 and 22 days. The average ethanol yield was 0.42 g g^{-1} of sugar consumed (82% of the theoretical maximum). Only trace amounts of lactic acid and succinic acid were detected and on average approximately 0.8 g L^{-1} of acetic acid was produced (Figure 2). While all the cells were resistant to 40 μg ml^{-1} Cm, the proportion of cells capable of growth on plates containing 600 μg ml^{-1} Cm declined from 100% at the beginning of the experiment to 2% at day 8, after which it remained constant.

Mannose fermentation

Repeat experiments lasting 27 and 29 days were conducted with 20 g L^{-1} mannose in the feed (Figure 3). In both experiments, the ethanol concentration peaked at day 3 and then declined significantly, stabilising after about 17 days at an average 6.0 g L^{-1} (59% of the theoretical maximum). The ethanol yield in experiment 2 was slightly lower than in experiment 1, with concomitantly higher organic acid production. The residual mannose concentration in both experiments was close to zero.

The decline in ethanol production during growth on mannose was associated with a general increase in production of organic acids and an increase in biomass concentration. Acetate stabilized at about 3 g L^{-1} after 15–20 days. The maximum concentration of lactate produced was much higher in experiment 2 (*ca* 4 g L^{-1}) than in experiment 1 (*ca* 1 g L^{-1}). Succinate was not produced until relatively late in the experiments, but subsequently accumulated,

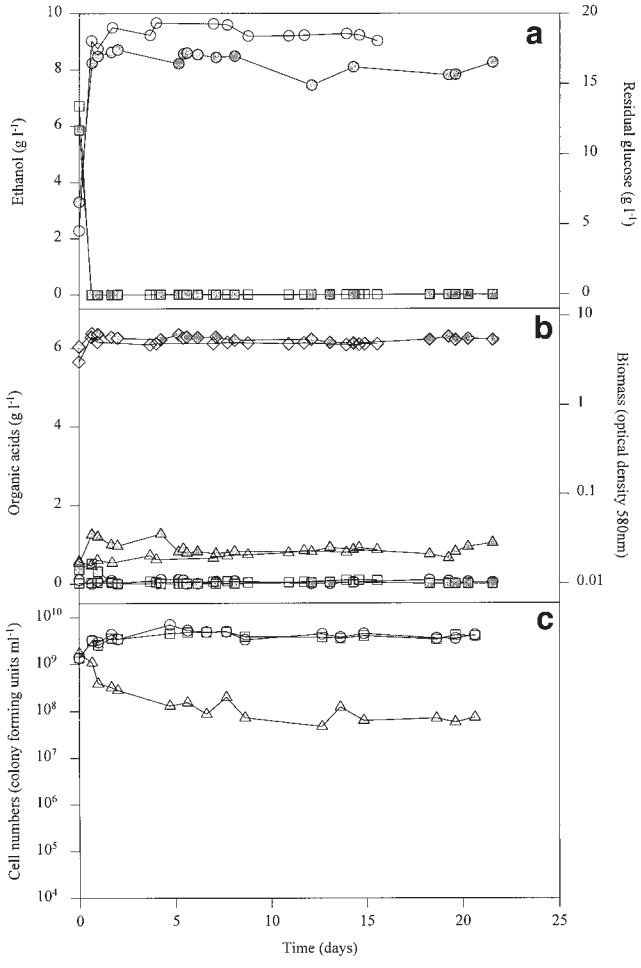

Figure 2 Fermentation kinetics of *E. coli* KO11 in chemostat culture on 20 g L^{-1} glucose. (a) (○, ●) ethanol, (□, ■) residual glucose; (b) (◇, ◆) biomass, (△, ▲) acetic acid, (□, ■) lactic acid, (○, ●) succinic acid; (c) colony forming units in the presence of (○) zero, (□) 40 μg ml^{-1} Cm and (△) 600 μg ml^{-1} Cm. Open symbols, experiment 1; closed symbols, experiment 2.

again reaching a particularly high concentration in experiment 2.

The proportion of cells capable of direct growth on 600 μg ml^{-1} Cm-containing plates fell much more rapidly than on glucose, eventually stabilising at about 0.1% of the total population. All cells remained resistant to 40 μg ml^{-1} Cm (Figure 3d).

Xylose fermentation

With 20 g L^{-1}xylose in the feed (Figure 4) the ethanol concentration declined from close to 9.0 g L^{-1} at the beginning of the experiments to 4.5–5.5 g L^{-1} at day 14, and thereafter remained approximately stable. (A slight recovery in ethanol concentration was observed in experiment 2.) As observed with mannose, the overall decline in ethanol concentration was matched by large increases in the concentrations of organic acids, notably acetate. Lactate production was relatively low. As with mannose, succinate production did not commence until midway through the experiment but then rose substantially. Periods of incom-

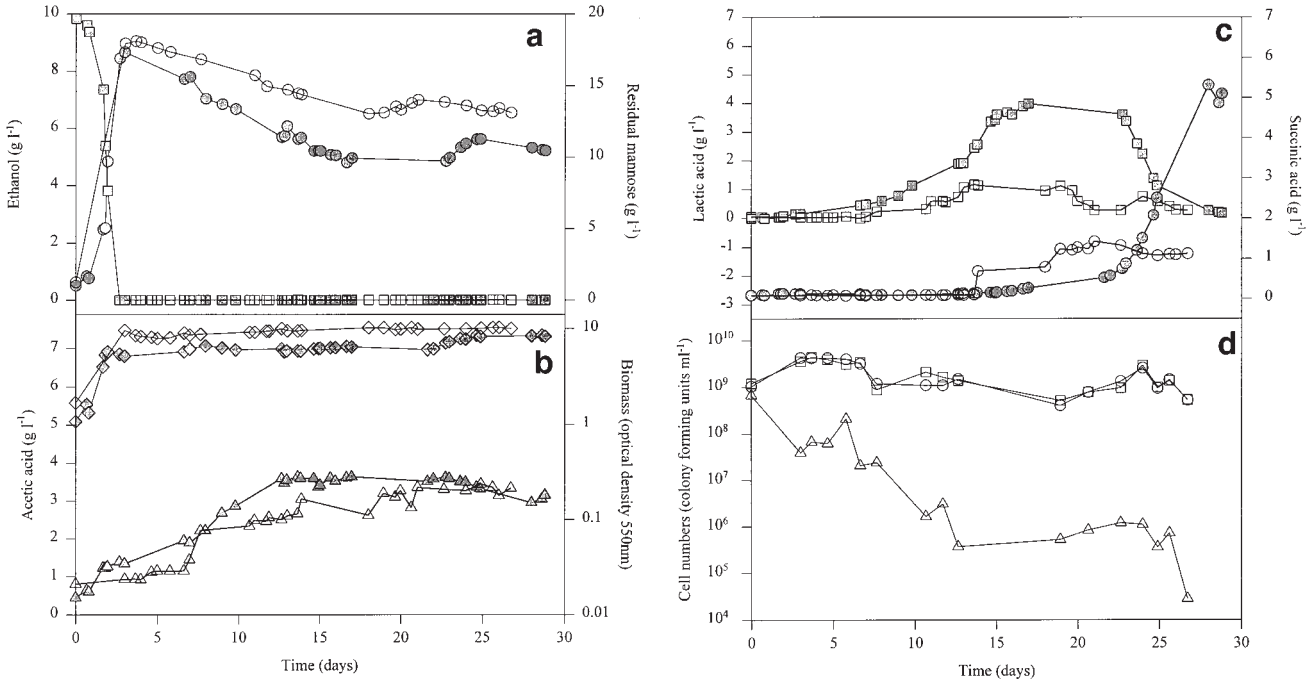

Figure 3 Fermentation kinetics of *E. coli* KO11 in chemostat culture on 20 g L^{-1} mannose. (a) (○, ●) ethanol, (□, ■) residual mannose; (b) (◇, ◆) biomass, (△, ▲) acetic acid; (c) (□, ■) lactic acid, (○, ●) succinic acid; (d) colony forming units in the presence of (○) zero, (□) 40 μg ml^{-1} Cm and (△) 600 μg ml^{-1} Cm. Open symbols, experiment 1; closed symbols, experiment 2.

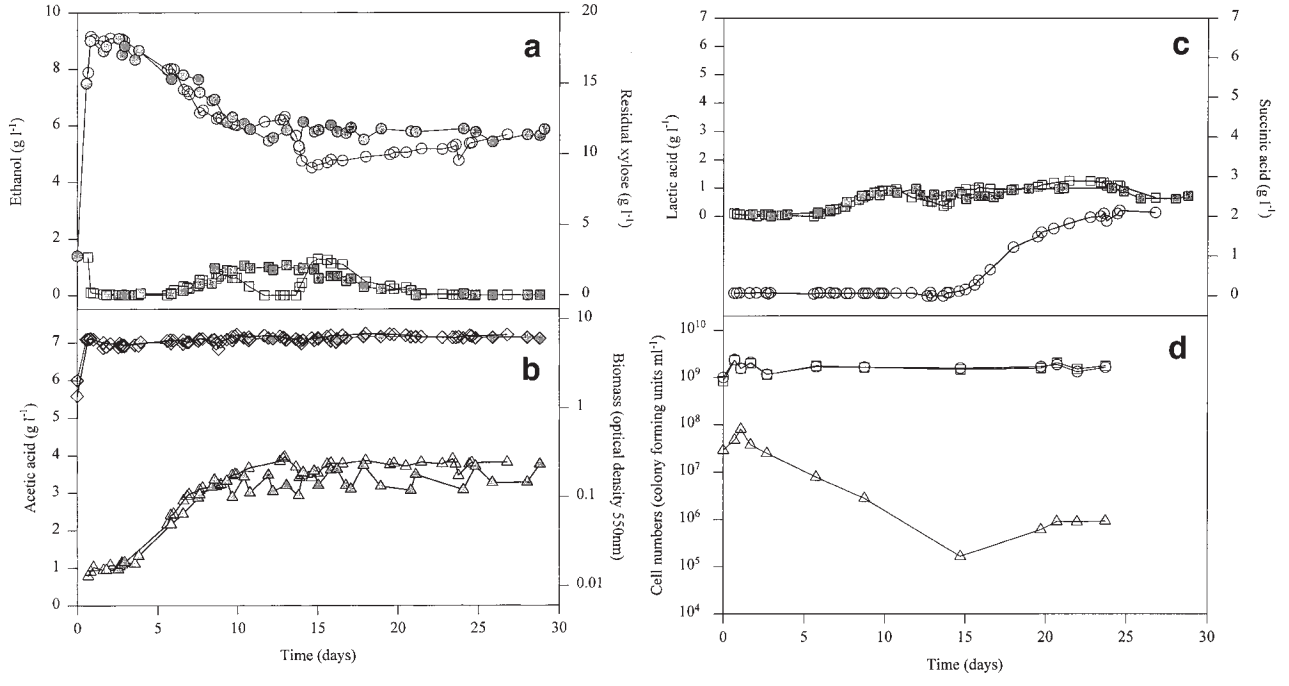

Figure 4 Fermentation kinetics of *E. coli* KO11 in chemostat culture on 20 g L^{-1} xylose. (a) (○, ●) ethanol, (□, ■) residual xylose; (b) (◇, ◆) biomass, (△, ▲) acetic acid; (c) (□, ■) lactic acid, (○, ●) succinic acid; (d) colony forming units in the presence of (○) zero, (□) 40 μg ml^{-1} Cm and (△) 600 μg ml^{-1} Cm. Open symbols, experiment 1; closed symbols, experiment 2.

plete sugar utilization were observed during both replicate experiments on xylose.

Sugar mixtures: To determine whether the presence of glucose in hemicellulosic mixtures might help to stabilise ethanol production from the mixture as a whole, the organism was grown on a mixture of xylose (13.3 g L^{-1}) and glucose (6.7 g L^{-1}) (Figure 5). While the fall in ethanologenicity was slower under these conditions, it was not prevented. Acetate was again the dominant byproduct. The fall

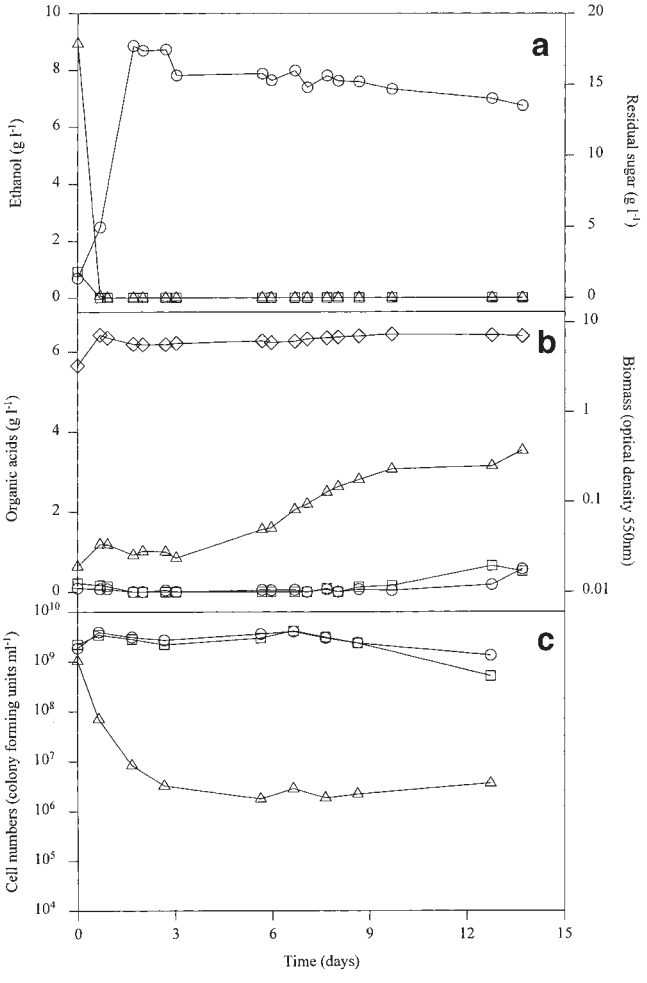

Figure 5 Fermentation kinetics of *E. coli* KO11 in chemostat culture on 6.7 g L^{-1} glucose and 13.3 g L^{-1} xylose. (a) (○) ethanol, (□) residual glucose, (△) residual xylose; (b) (◇) biomass, (△) acetic acid, (□) lactic acid, (○) succinic acid; (c) colony forming units in the presence of (○) zero, (□) 40 μg ml^{-1} Cm and (△) 600 μg ml^{-1} Cm.

in the proportion of cells incapable of direct growth on 600 μg ml^{-1} Cm plates was comparable to that observed with xylose alone.

Plate counts on high Cm medium are ineffective as indicators of hyperethanologenicity in chemostat culture: Plate count data on Cm-containing medium have not been previously reported for chemostat cultures of *E. coli* KO11. Since the strain was selected on the basis of both hyperethanologenicity and hyperresistance to chloramphenicol [26] and expression of the two traits is considered to be linked [26,30], the extent of correlation between the two phenotypes in chemostat culture is of interest. In our experiments, the proportion of colonies resistant to 600 μg ml^{-1} Cm during chemostat culture on glucose appears anomalously low given the stability of ethanol production (Figure 2). In a repeat chemostat experiment on glucose, the proportion of cells capable of growth following direct plating on 600 μg ml^{-1} Cm medium was again very low. Colonies allowed to grow initially on non-selective medium grew without exception when transferred to 600 μg ml^{-1} Cm plates (data not shown), suggesting either that chemostat-grown cells lack the vigour to grow directly on high Cm medium (perhaps due to injury by ethanol and other fermentation products) or that re-selection for high Cm resistance occurred on the plates (a high frequency of mutation for high chloramphenicol resistance appears to have been observed during the isolation of strain KO4 [26]). During chemostat culture on xylose (Figure 6), the proportion of colonies resistant to 600 μg ml^{-1} Cm following transfer from non-selective plates was 100% for nearly 30 days, during which time the ethanol yield declined to about 0.25 g g^{-1}, only slightly higher than that reported for the host strain in batch culture on this sugar (0.21 g g^{-1}) [21]. Whether this result represents re-selection for chloramphenicol resistance on the plates or differential retention of the high chloramphenicol resistance and hyperethanologenicity traits during chemostat culture is uncertain. Whichever explanation is correct, the data show that neither direct nor transfer plate counting is effective as a measure of hyperethanologenicity for chemostat cultures of this strain. The possible occurrence of re-selection for high Cm resistance during plating also needs to be considered as an alternative explanation of the apparent long-term retention of this trait during serial batch culture of *E. coli* KO4 [26].

Loss of hyperresistance to Cm commenced after approximately 30 days, with less than 40% of colonies possessing this trait after 48 days of chemostat culture on xylose (Figure 6). Between days 28 and 48 the proportion of cultures resistant to 40 μg ml^{-1} Cm fell from *ca* 90% to *ca* 50% (data not shown) suggesting that the *cat* gene is ultimately lost.

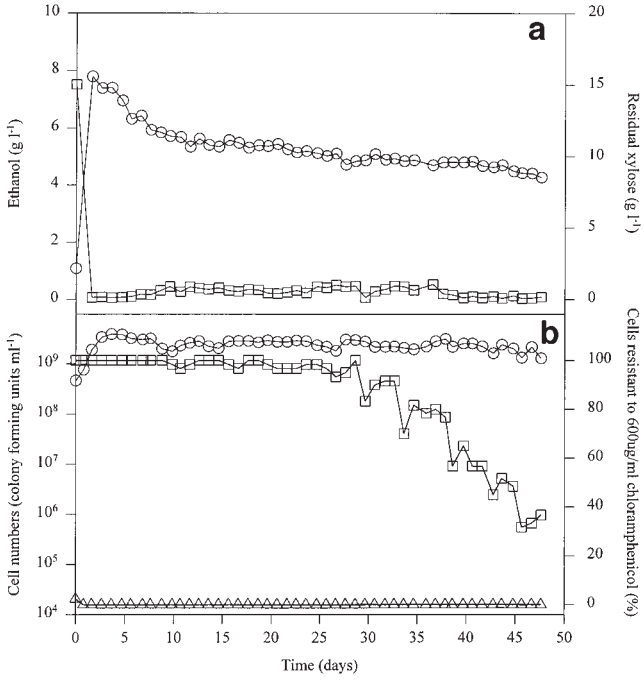

Figure 6 Chloramphenicol resistance of *E. coli* KO11 during long-term cultivation on 20 g L^{-1} xylose. (a) (○) ethanol, (□) residual xylose; (b) (○) colony forming units in the absence of Cm; percentage of resistant cells upon (△) direct plating on 600 μg ml^{-1} Cm and (□) following transfer of colonies to 600 μg ml^{-1} Cm after initial growth on non-selective plates.

Hyperethanologenicity lost during chemostat culture is not regained during subsequent batch cultivation: After 32 days of chemostat cultivation on xylose (Figure 6), three colonies resistant to 600 μg ml^{-1} Cm were each compared to the stock culture of *E. coli* KO11 for their ability to produce ethanol in 46-h pH-uncontrolled shake flask cultures on 50 g L^{-1} xylose. Both the chemostat-derived and stock culture cells were grown through three prior 16-h subcultures in Luria Broth containing (successively) 20 g L^{-1}, 40 g L^{-1} and 50 g L^{-1} xylose to allow re-acclimatisation of the chemostat-derived cells to 50 g L^{-1} xylose. Ethanol production from the chemostat-derived colonies was only 3.7 g L^{-1} (0.1) (mean and standard deviation of three cultures) compared to 24.8 g L^{-1} (0.3) for the stock culture (the latter represents 97.3% of the theoretical yield (0.51) based on the initial sugar concentration). The result confirms the loss of hyperethanologenicity by cells retaining high Cm resistance and suggests that the loss is permanent.

Discussion

Our investigation confirms the occurrence of phenotypic instability in *E. coli* KO11 as reported by Lawford and Rousseau [19,22]. The performance of the strain was, however, generally better than observed in their studies. In serial batch cultures, we observed no significant loss in ethanologenicity on any of the sugars in the absence of antibiotics. In the corresponding experiment of Lawford and Rousseau [19], the yields of ethanol from glucose and mannose fell to 49% and 0% in the third subculture, while the yields on galactose and xylose remained approximately constant. The mean yields on all four sugars in our experiments (glucose 87%, galactose 85%, mannose 81%, xylose 89%) were lower than has generally been reported for *E. coli* KO11 [9,26]; this is likely due to the sub-optimal fermentation conditions, ie the use of unstirred test-tube cultures lacking effective pH control (the pH fell as low as 5 during the experiments) and the choice of an arbitrary time-length for the fermentations. Our data suggest that recycling of *E. coli* KO11 over at least three batches is possible, with corresponding savings in substrate and operating costs. The effect of extending further the number of recycles was not studied: more extensive recycling may be impractical in large-scale batch cultures because of the likelihood of eventual contamination. Our data do not support the suggestion [19] that antibiotics might need to be included in all media, prohibitively increasing production costs.

The relative stability of *E. coli* KO11 during chemostat culture on glucose confirms our earlier report [11] but contrasts with data reported by Lawford and Rousseau [22]. At a similar dilution rate (0.07 h^{-1}) and glucose concentration (25 g L^{-1}) to that used in the present experiments and in the presence of 40 μg ml^{-1} Cm, the ethanol yield in Lawford and Rousseau's experiments fell to only 0.12 g g^{-1} in as little as 48 h of continuous cultivation: the addition of 300 μg ml^{-1} chloramphenicol to the medium slightly retarded the loss of ethanologenicity, but did not prevent it. The decline in ethanol production was accompanied by the production of high levels of lactate (up to 18 g L^{-1}) with lesser quantities of acetate and succinate. The long-term stability of the strain on glucose (but not other sugars) as revealed in our experiments helps to reconcile the reported stability of strain KO4 [26] with subsequent findings, the original work having been based on serial transfers in glucose medium [26].

During growth on 20 g L^{-1} xylose, the fall in ethanol production during our experiments was similar to that reported by Lawford and Rousseau [22] using 25 g L^{-1} xylose at a similar dilution rate (0.07 h^{-1}). Residual xylose concentrations were not reported for the above authors' experiment, however the presence of residual xylose during a portion of both our experiments indicates that the dilution rate employed is close to the critical one for complete utilisation of xylose at this feed concentration. The average concentration of acetate was slightly lower than reported by Lawford and Rousseau [22] and the pattern of succinate production was different, with succinate production commencing only after about 15 days cultivation, and then increasing relatively rapidly, rather than increasing progressively throughout the experiment. Lactate concentrations were not reported for Lawford and Rousseau's experiment.

The continuous growth of *E. coli* KO11 on mannose has not been previously investigated. After the attainment of the maximum population density, residual mannose concentrations remained close to zero throughout both experiments. This contrasts with the behaviour of the culture on xylose (Figure 4) and demonstrates that, at this dilution rate, mannose is metabolised slightly more rapidly than xylose by *E. coli* KO11 (and/or derivatives of it arising by mutation during the cultivation). This contrasts with data obtained in batch culture with *E. coli* B strain ATCC 11303 pLO1297 containing plasmid-borne genes for ethanol production, in which volumetric and specific sugar consumption rates on xylose were 2–3 times higher than on mannose [17,18]. In our experiments, the initial (immediately post start-up) biomass concentrations reached on mannose and xylose were similar, indicating that the specific uptake rates of the two sugars by *E. coli* KO11 would have been approximately equal. Ethanol production declined over the course of continuous culture on mannose at a rate slightly higher than observed on xylose, when both replicate experiments are taken into account.

This appears to be the first report of conversion of sugar mixtures to ethanol by *E. coli* KO11 in continuous culture, the specific rate of xylose consumption being double that of glucose consumption as a consequence of its higher concentration in the mixture. Both sugars were completely consumed. Simultaneous utilisation of glucose and xylose was earlier reported for *E. coli* KO11 in batch cultures containing a similar preponderance of xylose [27]; however xylose utilisation rates were much lower than glucose utilisation rates as long as glucose remained in the medium [27]. Our data show no such inhibition effect in continuous culture, presumably due to the lower ambient glucose concentration. Complete utilisation of a 21.4 g L^{-1} glucose/12.0 g L^{-1} xylose mixture in chemostat culture at a dilution rate of 0.1 h^{-1} was earlier reported by Lawford and Rousseau in studies with *E. coli* B ATCC 11303 pLOI297 [16]. In batch culture, this strain also exhibits inhibition of xylose utilisation by glucose when the latter exceeds about 40%

of the sugars present in the medium [18]. The presence of glucose in the glucose/xylose mixture appeared to retard loss of ethanologenicity in comparison to growth on xylose alone.

Acetate was the dominant fermentation by-product formed in our experiments. In the host strain, acetate derives from the conversion of pyruvate to acetyl CoA and formate by pyruvate formate lyase (pfl). In the construction of *E. coli* KO11, *pfl* was the target gene for insertion of the ethanologenic genes; however *pfl* was subsequently found not to have been completely inactivated [26]. The conversion of acetyl CoA to acetate (via acetyl phosphate) results in the formation of a mole of ATP. Hence in the anaerobic metabolism of this recombinant *E. coli*, the production of a mole of acetate yields a net two moles of ATP per mole of end-product, compared to only one mole for ethanol, lactate or succinate production [20]. The production of high levels of acetate during chemostat culture on mannose and xylose in our experiments may thus account in part for the high biomass yields on these sugars in comparison with glucose. Biomass yields on glucose, mannose and xylose averaged over the whole course of the fermentations were 0.26, 0.33 and 0.30 OD units/(g L^{-1} sugar consumed). Final values for mannose and xylose were significantly higher. This contrasts with batch culture data for *E. coli* KO11 grown on the same medium (LB) in which biomass yields on glucose and mannose were similar, both significantly exceeding those observed with xylose, with acetate production remaining at relatively low levels [21]. Our data appear to reflect the higher energetic yield associated with acetate production.

It has been suggested that the decline in functional stability of *E. coli* KO11 in chemostat culture is due to genetic instability [19], although no direct evidence for this interpretation has been produced. Our data, demonstrating the failure of cells grown in the chemostat on xylose to regain high ethanologenicity in batch culture, along with their eventual loss of high Cm resistance (Figure 6) provide strong indirect evidence for the mutation hypothesis. Lawford and Rousseau [22] suggested that strain KO11 may revert to KO3 and that strain KO3 may compete with KO11 in continuous culture. Our data are not inconsistent with this hypothesis and moreover suggest that, in the absence of antibiotic feed, the reversion may proceed further, with half the cells having lost resistance to low levels of Cm (40 μg ml^{-1}) after 48 days continuous cultivation on xylose. The final ethanol yield (0.21) from xylose in our experiment (Figure 6) is similar to that reported for batch cultures of the host strain [21]. The mutation in the *frd* gene appears relatively more stable than that conferring hyperethanologenicity; however the eventual appearance of succinate in our experiments with mannose and xylose (Figures 3 and 4) suggests that this modification is also ultimately lost.

The apparent ability of revertant strains to outcompete *E. coli* KO11 in chemostat culture on sugars other than glucose may reflect the greater metabolic load associated with high levels of expression of the ethanol-producing genes. However the high concentrations of acetate observed in all our experiments on these sugars suggests that an additional mechanism may be the higher ATP yields associated with acetate production. The relative stability of ethanol production on glucose remains unexplained; an understanding of this behaviour may prove valuable in the future construction of fully stable chromosomally integrated strains.

In simple chemostat culture, *E. coli* KO11 appears to be unsuitable for continuous ethanol production from hemicellulose sugars and sugar mixtures. Recently developed *E. coli* recombinants with positive selection pressure for the ethanologenic genes under anaerobic conditions [10,13] currently appear to offer more potential in this regard. The stability of *E. coli* KO11 is likely to be greater in continuous cultures employing cell recycle or immobilisation, in which cell division is reduced. In continuous immobilised whole cell culture, the organism produces ethanol stably and at high yield from hemicellulose mixtures for periods of at least 28 days (Zhou, unpublished data).

E. coli KO11 remains the most extensively tested of recent genetic constructs developed for the fermentation of hemicellulosic mixtures. In recent developments, it has been shown to be comparatively resistant to the effects of process errors arising during ethanol production [25] and its ethanol tolerance has been increased by mutation, resulting in ethanol concentrations and yields from xylose that exceed those reported for recombinant strains of *Saccharomyces* and *Zymomonas* [30]. The organism has also been re-engineered to permit its use in the simultaneous saccharification and fermentation of cellulose, thus permitting the use of a single organism for the complete conversion of lignocellulose to ethanol [24]. It appears to remain a serious candidate organism for use in ethanol production from lignocellulosic materials, at least in batch culture.

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