J Ind Microbiol Biotechnol (2006) 33: 834–844
DOI 10.1007/s10295-006-0129-9

ORIGINAL PAPER

Gregory J. O. Martin · Andreas Knepper
Bin Zhou · Neville B. Pamment

Performance and stability of ethanologenic *Escherichia coli* strain FBR5 during continuous culture on xylose and glucose

Received: 19 October 2005 / Accepted: 3 April 2006 / Published online: 6 May 2006

Abstract *Escherichia coli* FBR5 containing recombinant genes for ethanol production on plasmids that are also required for anaerobic growth was cultivated continuously on 50 g/l xylose or glucose in the absence of antibiotics and without the use of special measures to limit the entry of oxygen into the fermenter. Under chemostat conditions, stable ethanol yields of ca. 80–85% of the theoretical were obtained on both sugars over 26 days at dilution rates of 0.045/h (xylose) and 0.075/h (glucose), with average plasmid retention rates of 96% (xylose) and 97% (glucose). In a continuous fluidized bed fermenter, with the cells immobilized on porous glass beads, the extent of plasmid retention by the free cells fell rapidly, while that of the immobilized cells remained constant. This was shown to be due to diffusion of oxygen through the tubing used to recirculate the medium and free cells. A change to oxygen-impermeable tubing led to a stable high rate of plasmid retention (more than 96% of both the free and immobilized cells) with ethanol yields of ca. 80% on a 50 g/l xylose feed. The maximum permissible level of oxygen availability consistent with high plasmid retention by the strain appears to be of the order of 0.1 mmol per hour per gram dry biomass, based on measurements of the rate of oxygen penetration into the fermenters. Revertant colonies lacking the ethanologenic plasmid were easily detectable by their morphology which correlated well with their lack of ampicillin resistance upon transfer plating.

Keywords FBR5 · *Escherichia coli* · Lignocellulose · Continuous culture · Ethanol

G. J. O. Martin · A. Knepper · B. Zhou · N. B. Pamment (✉)
Department of Chemical and Biomolecular Engineering,
University of Melbourne, Melbourne, VIC, Australia 3010
E-mail: nbp@unimelb.edu.au
Tel.: +61-3-83446627
Fax: +61-3-83444153

Introduction

The production of ethanol from low-cost lignocellulosic materials such as wood and crop wastes has considerable promise as a future source of liquid transport fuel. Substantial advances in lignocellulose-to-ethanol conversion technology have occurred in recent times, with perhaps the most important being the development of microorganisms capable of fermenting efficiently the hemicellulose component of the feedstock [16]. While hemicellulose comprises up to 40% of the carbohydrate content of lignocellulose, it cannot be fermented by conventional *Saccharomyces cerevisiae* strains. Ingram and colleagues developed a series of recombinant *Escherichia coli* B strains capable of producing ethanol at close to theoretical yields from all the common hemicellulose sugars, including xylose, mannose, arabinose, and galactose in addition to glucose. In particular, the chromosomal integrant strain *E. coli* KO11 [18] was trialled extensively in large-scale batch fermentations under industrial conditions with considerable success and was selected for commercial development [1].

For lignocellulose-derived ethanol to compete economically with oil, cost reductions need to be made at all stages of the production process, including fermentation. The use of continuous fermentation, as currently applied extensively in the grain-based ethanol industry in the United States and elsewhere, offers one such avenue for reducing production costs. By eliminating the batch turn-around time and permitting operation at a high, fixed biomass concentration, continuous culture increases ethanol productivity and allows the use of smaller fermenters [2]. In molasses-to-ethanol fermentations, continuous culture was estimated to result in a 57% reduction in fixed capital investment and a 37% reduction in production costs when raw materials were excluded [6]. However, for successful long-term continuous fermentation, a high degree of stability of the recombinant strain is essential. *E. coli* KO11, while stable for extended periods in chemostat culture on glucose

feeds, has been found to lose its ethanologenicity when cultivated on other sugars found in hemicellulose, presumably due either to loss of its chromosomally-integrated ethanologenic genes, or the loss of the ability to hyper-express them [11].

To address this problem, the Fermentation Biotechnology Research Unit of the U.S. Department of Agriculture developed a series of ethanologenic *E. coli* K12 strains starting from conditionally lethal mutants in which the lactate dehydrogenase (*ldh*) and pyruvate formate lyase (*pfl*) genes are deleted [12]. These strains are unable to grow anaerobically because they cannot regenerate the NAD^+ required for the continuation of glycolysis. Transformation of these hosts with plasmids containing genes for ethanol production allowed anaerobic growth to occur due to the regeneration of NAD^+ at the alcohol dehydrogenase step. This also provided selection pressure for retention of the ethanol-producing genes under anaerobic conditions without a requirement for antibiotic supplementation. Transformation was accomplished with the plasmids pLOI295 and pLOI297 bearing the PET operon developed earlier by Ingram and colleagues, consisting of the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase genes (*adh*) from *Zymomonas mobilis* and genes conferring resistance to ampicillin (pLOI295 and pLOI297) and tetracycline (pLOI297) [14].

The strains initially developed by the group shared the inability of the host strain, FMJ39, to metabolise xylose, the dominant sugar in hardwood and crop-waste hemicellulose. Dien et al. [8] subsequently isolated spontaneous mutants of FMJ39 that do metabolise xylose, four of which were transformed with pLOI297. The best of the resulting constructs, designated *E. coli* FBR3, produced high yields of ethanol (ca. 90% of theoretical) when grown on glucose, xylose or corn fibre hydrolysate in batch culture and demonstrated greater than 97% plasmid maintenance for 10 serial transfers [8].

Strain FBR3 shares the disadvantage of its host strain FMJ39 in requiring amino acids for growth, an impediment to the development of an economic industrial medium. Further, any reversion of the critical *pfl* mutation cannot be tested for in individual cells because it was introduced into FMJ39 by chemical mutagenesis [8]. Dien et al. [9] therefore produced an improved xylose-fermenting strain (FBR5) based on the host NZN111 [4]. This host does not require amino acids for growth and its *pfl* mutation, which was introduced by genetic recombination, is associated with a chloramphenicol resistance marker. As with strain FBR3, the ethanol-producing genes were contained on pLOI297, which also carries genes for ampicillin and tetracycline resistance. In anaerobic batch cultures, strain FBR5 was shown to produce ethanol in high yields on glucose, xylose, and arabinose [17] and on corn fibre hydrolysates [9], with confirmed retention of the plasmid over at least 10 serial transfers [9]. The strain was also more robust than FBR3 (requiring less acclimatization to corn fibre hydrolysate) and showed higher ethanol productivities when fermenting this feedstock [9]. Strain FBR5 remains the best-performing strain so far developed in the FBR series and has been recently used to successfully ferment hydrolysates of rice hulls [20] and wheat straw [19] producing respectively 0.43 and 0.46 g ethanol/g sugar in batch culture, further confirming its potential for use in industrial processing.

Despite the evident potential of the FBR series of strains for use in continuous ethanol production, relatively little has been done to test them under these conditions. The only strain (FBR2) to have been tested continuously was produced early in the development of the series and does not ferment xylose. It was grown successfully in chemostat culture on a low (5 g/l) glucose feed in the absence of antibiotics for 28 generations [12]. No loss of antibiotic resistance was observed (indicating retention of the ethanologenic plasmid), and batch cultures started from chemostat-derived samples produced high levels of ethanol [12]. However, the above study employed continuous nitrogen sparging of the fermenter to exclude oxygen, thus providing an artificial selection pressure for plasmid retention. Under aerobic conditions, the above [12] and a subsequent [8] study showed the plasmid to be rapidly lost in the absence of antibiotic supplementation. The need to ensure strict anaerobiosis or to include antibiotics in the medium would be a strong disincentive to the use of the organisms under industrial conditions. The stability of the strains in continuous culture therefore requires investigation under conditions more typical of industrial processing, in which exposure of the organisms to oxygen cannot normally be prevented. There is a particular need to study the stability of the xylose-fermenting strains, especially in the light of our earlier finding that *E. coli* KO11 is unstable on this sugar, though stable on glucose [11].

The aims of the present work were to study the stability and ethanologenicity of *E. coli* FBR5 in continuous culture on xylose and glucose. We further investigate for the first time the relationship between specific oxygen availability and the retention of the ethanologenic plasmid in the FBR series of strains. The organism was grown under typical self-anaerobic conditions in either continuous stirred tank culture or in fluidized bed culture with the cells immobilized on porous glass beads.

Materials and methods

Microorganism

Escherichia coli FBR5 was sourced from the Fermentation Biotechnology Research Unit, National Center for Agricultural Utilization Research, USDA, Peoria, IL, USA. It was maintained at −80°C in 50% (v/v) glycerol containing ampicillin (100 μg/ml).

Media

The base medium was a modified Luria broth which contained: tryptone 10 g/l; yeast extract 5 g/l; sodium chloride 5 g/l and sodium acetate 1 g/l. This medium, supplemented with 50 g/l D-glucose or D-xylose, was used as the feed for the continuous culture experiments. Solid media contained modified Luria broth ingredients as above with glucose or xylose (20 g/l) and agar (15 g/l). Feed medium for continuous fermentation experiments was filter sterilised. All other media were sterilized by autoclaving for 20 min at 121°C.

Inoculum preparation

The culture was transferred from glycerol stocks onto selective plates (100 μg/ml ampicillin) and incubated at 37°C for 24 h. Colonies were then transferred to 500 ml Erlenmeyer flasks containing 200 ml modified Luria broth supplemented with 15 g/l xylose, 1 g/l sodium acetate, and 100 μg/ml ampicillin. Nitrogen was bubbled through the medium for 30 min to remove oxygen, after which the flasks were incubated overnight (16 h) in an orbital shaker at 100 rpm and 37°C. The cells for the inoculum were then harvested by centrifugation (10 min at 7,650*g*) and resuspended in 50 ml of medium having the same composition as the feed.

Continuous stirred tank reactor fermentations

Continuous stirred tank reactor (CSTR) experiments were performed in a New Brunswick model 19 fermenter (New Brunswick, Edison, NJ, USA) with a 2.0 l glass vessel. Cultures were grown in 860 ml of modified Luria broth containing 50 g/l glucose or xylose at a fixed dilution rate between 0.045 and 0.082 h^{-1} and were stirred at ca. 200 rpm. The pH was maintained at 6.5 by automatic addition of 3.0 M KOH and the temperature was controlled at 35°C. Sterilized antifoam solution [10 ml Dow Corning 1520 (Dow Corning, Midland, MI, USA) in 400 ml distilled water] was added manually as required. The fermenter was inoculated with 10 ml of the inoculum culture and the fermentation was allowed to proceed as a batch for 8 h (at which time the population density was ca. 2 g dry weight/l), after which the feed pump was switched on.

Fluidized bed reactor fermentations

The fluidized bed reactor [10] consisted of a 25 mm internal diameter × 850 mm long vertical glass column (fluidized bed section), a pump-around system to recirculate the culture broth and fluidize the support particles, and a continuous feed arrangement. The total working volume was approximately 610 ml. The cells were immobilized on open-pore sintered glass beads (SIKUG/041/02/120A, Schott AG, Mainz, Germany) of diameter 0.4–1 mm, pore volume 55–60% and pore diameter, 60–300 μm. The glass beads (80 g) were retained between fine stainless steel mesh placed at either end of the fluidized bed section and were not recirculated. The free liquid was recirculated at approximately 42.3 l/h corresponding to a linear flow rate in the column of 2.4 cm/s. Sterile feed medium was pumped into the reactor via a mixing chamber to achieve dilution rates between 0.04 and 0.08 h^{-1}. Antifoam was added from the top of the reactor in 4 ml aliquots every 4 h. The pH was maintained at 6.5 by the addition of 3.0 M KOH using a gravity-fed system monitored by a pH/ORP Controller model 3671 (Jenco, San Diego, CA, USA). The temperature was maintained at 35°C (± 0.5°C) using a heating coil of flexible Tygon® tubing (Masterflex, Cole-Parmer, Vernon Hills, IL, USA) wrapped around the reactor. The fermentation was initially conducted as a batch for 16–24 h to achieve an adequate population (ca. 3 g dry wt/l) of immobilized cells. Samples of fermentation broth containing free cells were taken daily as for the CSTR. Small samples of glass beads were taken every 4–5 days to allow sampling of the immobilized cells. Once removed, the glass beads were gently rinsed with distilled water, added to fresh Luria broth and vortexed for 5 min to liberate the immobilized cells.

Determination of oxygen transfer rate

The rate of oxygen transfer into the CSTR and fluidized bed fermenters was estimated by monitoring the rate of oxidation of a sodium sulphite solution based on the method of Cooper et al. [5]. The reactors were operated under the same conditions as for the fermentations except that no cells were present, the pH was not controlled after being set to the initial value of 6.5, and the flow of feed was stopped. The contents of the fluidized bed reactor were recirculated as described above.

Calculation of oxygen availabilities

Specific oxygen availabilities (Fig. 6) were calculated by dividing the measured oxygen uptake for each reactor system (Table 2) by the dry cell weights from Figs. 1d, 2d, 4d and 5d. The dry weight of the free cells was taken as the time-averaged value for each experiment (cf Figs. 1d, 2d, 4d, 5d). The dry weight of the immobilized cells was read from a straight line of best fit of the data in Figs. 4d and 5d. Curves e and f (Fig. 6) were calculated by dividing the measured oxygen uptakes of the fluidized bed fermenter with silicone and Pharmed tubing respectively by the sum of the dry weights of the free and immobilized cell populations from Figs. 4d and 5d, respectively. For

curves c and d (Fig. 6), the rate of oxygen uptake through the recirculation tubing was assumed to be equal to the difference between the oxygen uptake rates of the fluidized bed reactor with Pharmed and silicone rubber tubing respectively. The oxygen uptake of the remainder of the reactor (i.e. excluding the tubing) was assumed to be the same as that for the reactor with impermeable Pharmed tubing. The oxygen availability to the immobilized cells (curve d) was determined by dividing the oxygen uptake of the reactor (excluding the recirculation tubing) by the sum of the dry weights of the free and immobilized cells from Fig 4d. The oxygen availability to the free cells (curve c) was assumed to be the same as that available to the immobilized cells, plus an additional amount equal to the rate of oxygen uptake through the recirculation tubing per dry weight of free cells.

Analytical methods

Ethanol was analyzed by headspace gas chromatography as previously described [13]. Total reducing sugars were measured using an adaptation of the DNS assay of Lindsay [15]. Acetic, lactic, and succinic acids were measured by HPLC as described by Dumsday et al. [11]. Biomass was estimated by measuring the optical density at 550 nm. The dry weight of the free cells was calculated from the OD_{550} based on the correlation that an OD_{550} of 1.0 corresponds to 330 mg/ml dry weight [3, 21]. The dry weight of cells immobilized on the glass beads was calculated from the difference in mass of the sampled beads (with adsorbed cells) dried in an oven at 80°C for 24 h and the same sample after combustion in a muffle furnace for 6 h at 550°C. The percentage of cells which had lost the ethanologenic plasmids was determined on the basis of either colony morphology or resistance to ampicillin, with both methods correlating closely (see Results). Colony morphology was assayed by counting the proportion of aberrant small colonies on triplicate, non-selective plates which had been incubated for 24–48 h at 35°C. Antibiotic resistance was tested by transfer plating of colonies from non-selective to selective plates containing either ampicillin (100 μg/ml), kanamycin (50 μg/ml), tetracycline (20 μg/ml) or chloramphenicol (10 μg/ml) and incubating for 24 h at 35°C. Anaerobic selective and non-selective plates were prepared by feeding nitrogen into the plate headspace for one minute, sealing with Parafilm and incubating for 24 h at 35°C. For anaerobic batch mini-fermentations, 10 ml of modified Luria broth containing 40 g/l xylose was transferred to sterile Malcony bottles and sparged with nitrogen for 5 min prior to inoculation. Nitrogen was subsequently fed into the headspace and the bottles sealed and incubated for 48 h at 35°C. Aerobic batch mini-fermentations were performed as above but were not sparged with nitrogen and were shaken vigorously while exposed to air.

Results

Continuous fermentation on glucose and on xylose

Two chemostat (fixed feed-rate) experiments were conducted in the CSTR, one with feed containing 50 g/l glucose and the other 50 g/l xylose. To determine whether the strain was capable of maintaining its ethanologenicity under natural fermentation conditions without the application of artificial oxygen-excluding procedures, oxygen scavengers were not included in the medium, the feed was not deoxygenated, and the connections between the reactor system and the outside air were not water-sealed to prevent back-diffusion of oxygen.

With a 50 g/l glucose feed, a stable ethanol yield averaging ca. 82% of the theoretical maximum was achieved at a dilution rate of 0.075 h^{-1} (Fig. 1). Residual glucose declined from ca. 4 g/l at the beginning of the experiment to negligible levels after 6–7 days of operation. Succinic acid (1–3 g/l) and, to a lesser extent, acetic acid (0–1.5 g/l), were produced over the course of the experiment. No lactic acid production was observed, as expected given the deletion of the *ldh* gene.

With a 50 g/l xylose feed, complete substrate utilization could not be achieved at the original dilution rate of 0.082 h^{-1} (Fig. 2). The dilution rate was lowered to 0.045 h^{-1} after sampling on day 4 whereupon the residual xylose concentration fell rapidly to ca. 8 g/l, before declining to a negligible amount after 17 days. An increase in the ethanol yield (from 75 to 85% of the theoretical maximum) also occurred after the lowering of the dilution rate. Organic acid production was low throughout the experiment. Succinic acid was produced in the greatest quantities (ca. 1–2 g/l), its concentration slowly rising over the course of the experiment. Acetic acid was produced at a near constant concentration of ca. 1 g/l.

The proportion of cells lacking the ethanologenic plasmid was determined by counting the proportion of atypical colonies on triplicate non-selective plates; as discussed below, the atypical colonies are revertants of strain FBR5 that have shed the introduced plasmid. For both fermentations, the proportion of cells experiencing plasmid loss averaged less than 5%. An increase in the proportion of atypical colonies occurred towards the end of the glucose-fed experiment, however there was no detectable decrease in the ethanol yield (Fig. 1). After about 22 days of experimentation on glucose, growth of both the typical and atypical colonies on the non-selective plates was too slow to allow morphological identification or transfer of the colonies to selective plates, even after extended incubation (48 h), preventing the determination of plasmid maintenance after this time. Sugar uptake, total cell concentration and the ethanologenicity of the cultures remained unaffected until at least day 27. This suggests that, while the cells became progressively enfeebled (i.e. lost metabolic vigor) after

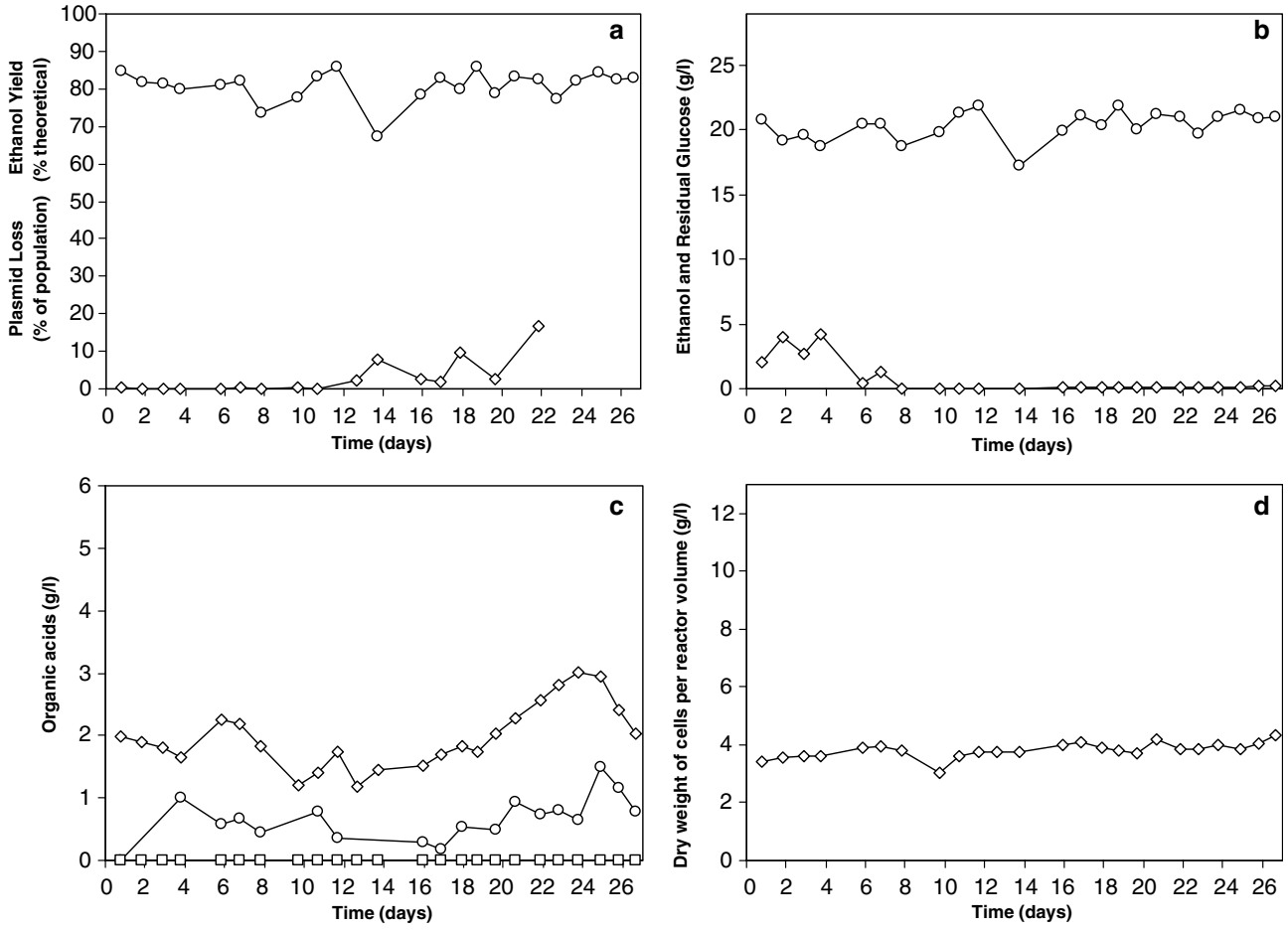

Fig. 1 Fermentation kinetics of *E. coli* FBR5 in chemostat (*CSTR*) culture on modified Luria broth containing 50 g/l glucose. The dilution rate was 0.075 h^{-1}. **a** *Circle* ethanol yield, **b** *diamond* plasmid loss; **b** *circle* ethanol, residual glucose; **c** *diamond* succinic acid, *circle* acetic acid, **d** *square* lactic acid; *diamond* dry weight of cells

prolonged continuous cultivation, restricting their ability to grow in the relatively harsh environment of agar plates, they were still able to grow and metabolise effectively in the reactor.

Plasmid maintenance, ampicillin resistance and colony morphology

As discussed above, all experiments revealed the existence of a small proportion of atypical colonies on non-selective plates. The normal colonies were smoother, shinier, more highly raised and more opaque than the atypical colonies, allowing easy differentiation (Fig. 3). When transferred onto ampicillin-containing plates, the normal colonies grew rapidly while the atypical colonies failed to grow. This suggested that the atypical colonies represented cells that had lost the plasmid (pLOI297) that confers ethanologenicity and ampicillin resistance. However, as the possibility of an ampicillin-sensitive contaminant could not be excluded, further work was done to confirm the identity of the atypical cells.

If strain FBR5 were to shed the plasmid, the revertant strain would simply be the host strain NZN111. This strain does not produce ethanol, has chromosomally-integrated genes encoding resistance to chloramphenicol and kanamycin, and cannot grow anaerobically [9]. Cells from typical and atypical colonies were tested for resistance to ampicillin, tetracycline, and kanamycin, and for their ability to produce ethanol and to grow anaerobically (Table 1). The atypical cells possessed all the characteristics of the host strain, including resistance to kanamycin and chloramphenicol, and an inability to grow anaerobically, supporting the conclusion that they were revertants and not contaminants.

Fluidized bed experimentation

Strain FBR5 was next cultivated in a fluidized bed reactor with the cells immobilized on microporous glass beads. In earlier studies in our laboratory, immobilization was shown to improve both the phenotypic stability and ethanol tolerance of ethanologenic *E. coli* KO11 (B. Zhou, Department of Chemical and Biomolecular

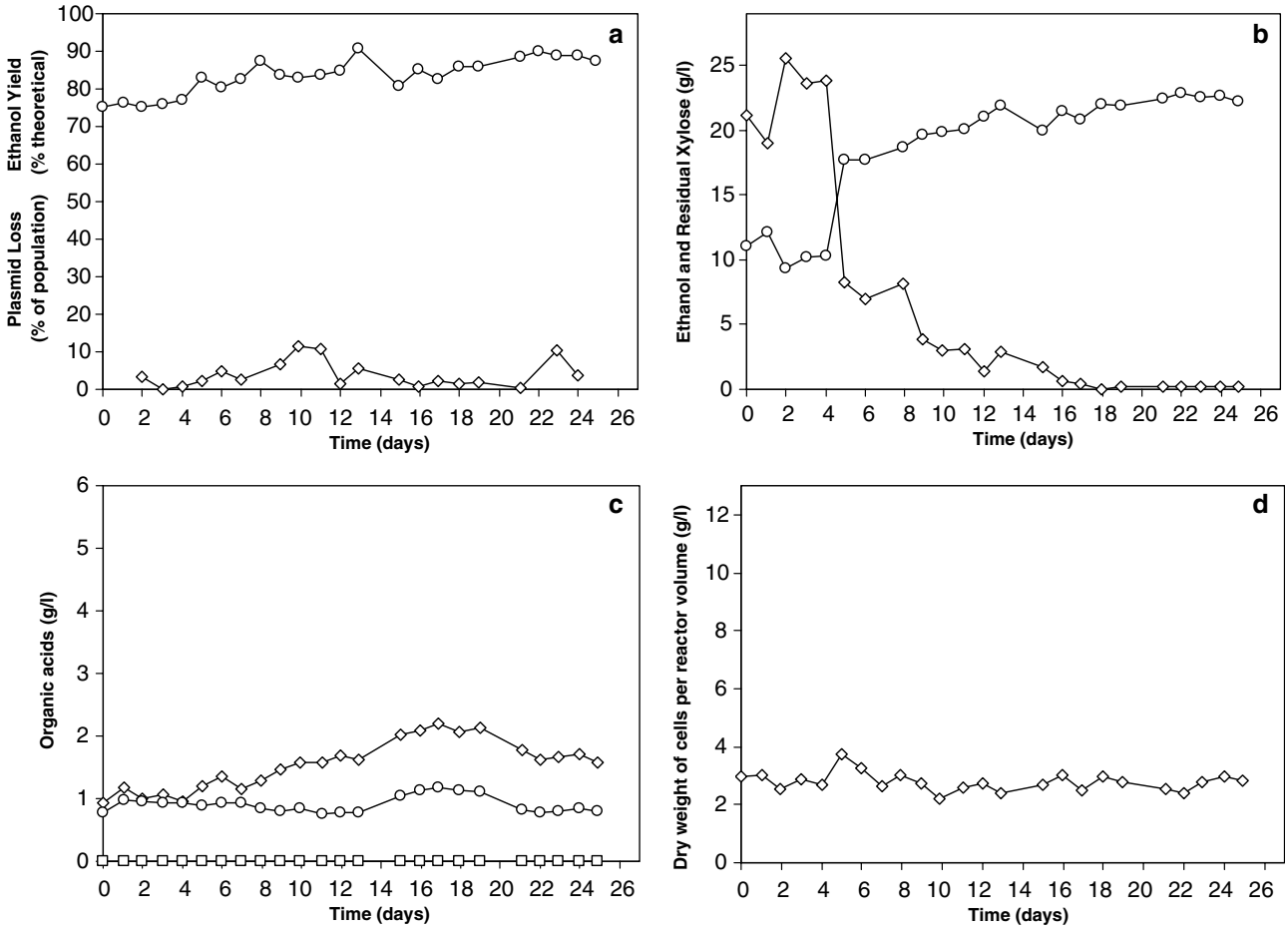

Fig. 2 Fermentation kinetics of *E. coli* FBR5 in chemostat (*CSTR*) culture on modified Luria broth containing 50 g/l xylose. The dilution rate was 0.082 h^{-1} (day 0–4) to 0.045 h^{-1} (day 5–25). **a** *Circle* ethanol yield, *diamond* plasmid loss; **b** *circle* ethanol, *diamond* residual xylose; **c** *diamond* succinic acid, *circle* acetic acid, *square* lactic acid; **d** *diamond* dry weight of cells

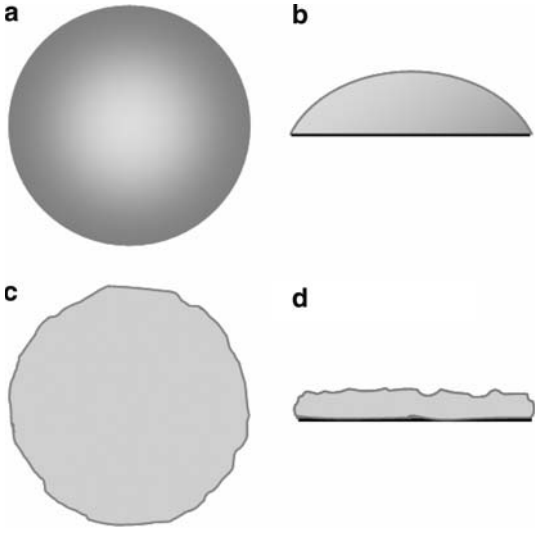

Fig. 3 Normal and atypical colonies as seen on agar plates after approximately 24 h growth. *Top* Normal colony as viewed **a** from above and **b** from the side. *Bottom* Atypical colony as viewed **c** from above and **d** from the side

Engineering, University of Melbourne, personal communication 2005). Xylose (50 g/l) was used as the substrate, due to the relative difficulty of fermenting it compared to glucose. At a constant dilution rate of 0.05 h^{-1} (Fig. 4), ethanol yields of between 70 and 80%

Table 1 Characteristics of normal and atypical colonies observed in this work compared to data of Dien et al. [9] for *E. coli* NZN111

Characteristic/test	Normal cells	Atypical cells	*E. coli* NZN111 [9]
Gram stain test	Negative	Negative	Negative
Microscopic appearance	Rods	Rods	Rods
Ampicillin resistance	Yes	No	No
Tetracycline resistance	Yes	No	No
Kanamycin resistance	Yes	Yes	Yes
Chloramphenicol resistance	Yes	Yes	Yes
Growth in presence of O_2	Yes	Yes	Yes
Anaerobic growth	Yes[a]	No	No
Ethanol production	Yes[b]	No	No

[a]Determined from both anaerobic mini batch fermentations and by plating on anaerobic non-selective plates

[b]As detected by gas chromatography of broth from mini batch fermentations conducted without exclusion of oxygen

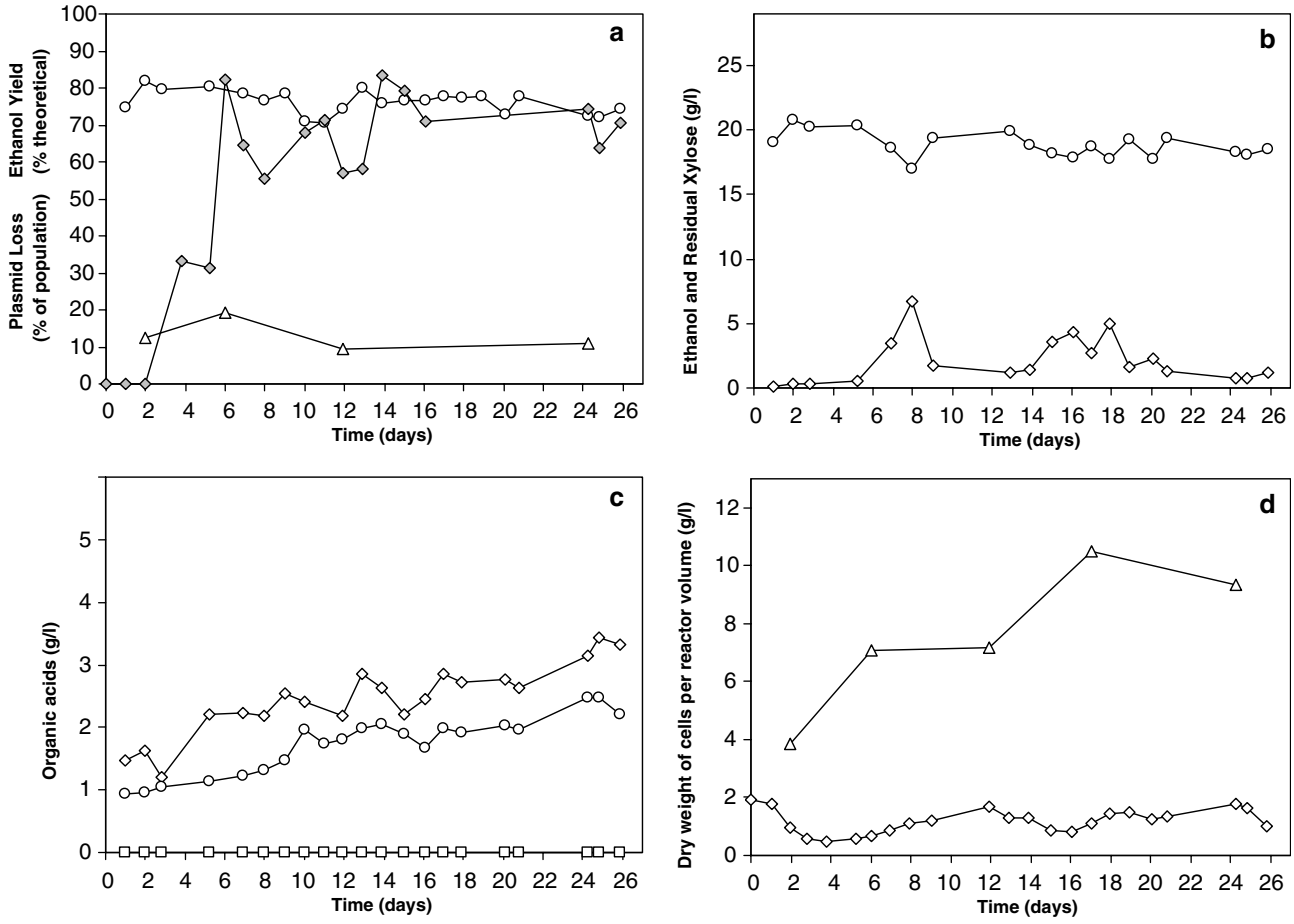

Fig. 4 Fermentation kinetics of *E. coli* FBR5 in fluidized bed culture on modified Luria broth containing 50 g/l xylose at a dilution rate of 0.05 h^{-1}. The cells were immobilized on SIKUG/041/02/120A microporous glass beads (80 g total loading). Silicone rubber tubing was used in the recirculation lines. **a** *Circle* ethanol yield, *filled diamond* plasmid loss from free cells, *triangle* plasmid loss from immobilized cells; **b** *circle* ethanol, *diamond* residual xylose; **c** *diamond* succinic acid, *circle* acetic acid, *square* lactic acid; **d** *diamond* dry weight of free cells, *triangle* dry weight of immobilized cells

of the theoretical maximum were maintained over 26 days, with a slight decline evident over time. The average residual xylose concentration was ca. 2.5 g/l. Organic acid production gradually increased during the experiment, with the succinic acid concentration increasing from 1.5 to 3.5 g/l and acetic acid from 1.0 to over 2 g/l after 24 days.

The most prominent feature of this fermentation was the rapid loss of the recombinant plasmid by the free cell population (Fig. 4a). After a week of operation, the proportion of free cells having shed the plasmid averaged close to 70%. In contrast, plasmid loss by the immobilized cell population averaged 13% over the whole course of the experiment and did not increase with time.

The ability of revertant cells to maintain a stable population in the reactor appeared only explicable if sufficient oxygen were entering the fermenter to permit sustained respiratory growth, eliminating the selection pressure for plasmid maintenance. This initially appeared unlikely, given that the system was under positive pressure due to carbon dioxide evolution and would have a natural tendency to self-anaerobiosis in the absence of an air supply. Despite this, comparison of the designs of the stirred-tank and fluidized bed suggested that a possible source of oxygen entry in the latter could be diffusion through the silicone-rubber tubing used for the liquid-recirculation loop, especially as the rapid recirculation rate would tend to increase the driving force for oxygen transfer and the liquid-phase oxygen mass transfer coefficient. This might also explain the increased plasmid loss in the free cell population, as only the free cells were re-circulated.

The previous experiment was repeated using relatively oxygen-impermeable Pharmed tubing (Masterflex, Cole-Parmer) for the recirculation loop (Fig. 5). The oxygen permeability of the replacement tubing was stated to be 200 cm^3 mm/(cm^2 s cm Hg) $\times 10^{-10}$ compared to 7,961 cm^3 mm/(cm^2 s cm Hg) $\times 10^{-10}$ for a silicone rubber tubing similar to the original (Cole-Parmer catalogue, Cole-Parmer, USA), a reduction of 40-fold. Under these conditions, plasmid pLOI297 was maintained almost entirely by both the free and immobilized cell populations (Fig. 5). The proportion of cells

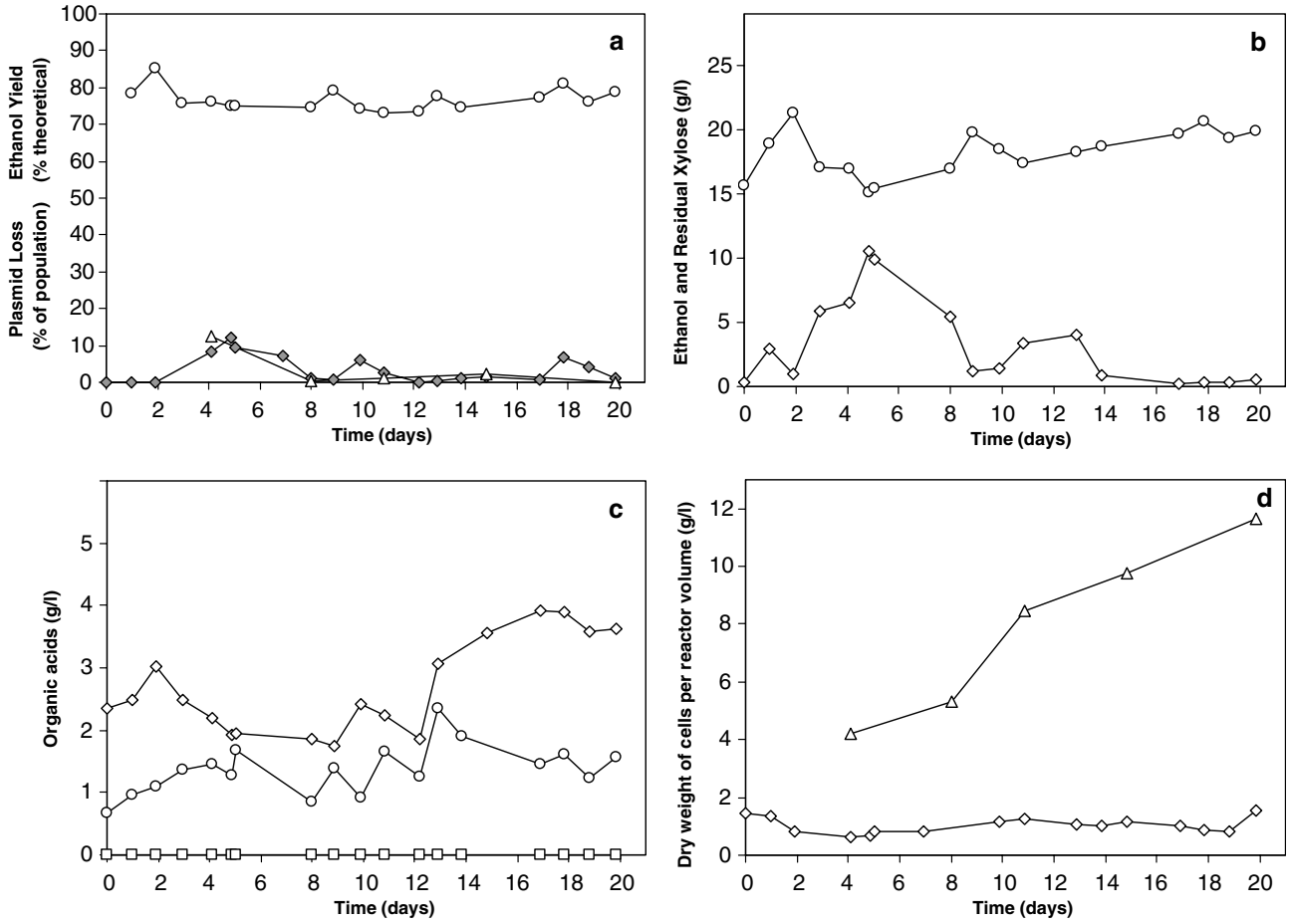

Fig. 5 Repeat of the experiment of Fig. 4 using low oxygen permeability Pharmed tubing in place of silicone rubber tubing for the recirculation line. All other conditions were as in Fig. 4. **a** *Circle* ethanol yield, *filled diamond* plasmid loss from free cells, *triangle* plasmid loss from immobilized cells; **b** *circle* ethanol, *diamond* residual xylose; **c** *diamond* succinic acid, *circle* acetic acid, *square* lactic acid; **d** *diamond* dry weight of free cells, *triangle* dry weight of immobilized cells

shedding the plasmid increased above 10% on only one occasion (day 4–5) and, at the end of the experiment, was close to zero in both the free and immobilized populations. The ethanol yield was again close to constant during the experiment, averaging 78% of the theoretical maximum (Fig. 5).

The organic acid profiles were similar to those found in the experiment with the original tubing. Succinic acid concentrations increased from around 2.5 to 3.5 g/l and were slightly higher than in the previous experiment. Acetic acid production was somewhat lower than during the first experiment, increasing from 1 to 1.5 g/l, possibly reflecting a diminished respiration rate.

Determination of rate of oxygen transfer into reactors

To test the proposal that the observed differences in plasmid loss resulted from the presence of oxygen, the rate of oxygen transfer into the fluidized bed reactor with either silicone rubber or Pharmed® tubing was measured by the sulphite oxidation technique under conditions similar to those used in the fermentations (see Materials and methods). All the tests showed linear rates of oxygen uptake with time (r^2 = 0.978 or better) with replicate tests. Replacement of the recirculation tubing approximately halved the oxygen transfer rate (OTR) into the fluidized bed reactor (Table 2). The rate of oxygen entry into the CSTR was also measured and was found to be about half that of the fluidized bed reactor with Pharmed® tubing (Table 2).

The specific oxygen availability [in mmol O_2 h^{-1} g^{-1} dry weight of biomass (mmol/(h. g-biomass))] was estimated for each reactor system (Fig. 6). For the fluidized bed reactor with silicone rubber tubing, the specific oxygen availability was calculated according to two assumptions: either that all the entering oxygen was equally available to both the free and immobilized cells (Fig. 6e), or that the oxygen entering through the recirculation tubing was consumed only by the free cell population (with oxygen entering from other sources assumed to be equally available to both populations) (Fig. 6c, d). Oxygen entry through the Pharmed tubing was assumed to be negligible, with oxygen entering from

Table 2 Measured oxygen transfer rates of various reactor systems

Reactor system	Oxygen uptake rate (mmol O_2 transferred l^{-1} h^{-1})
Fluidised bed–silicone rubber tubing	1.11 (± 0.01)[a]
Fluidised bed–Pharmed® tubing	0.59 (± 0.05)[a]
Chemostat	0.30 (± 0.05)[a]

[a]Deviation from the mean of duplicate experiments

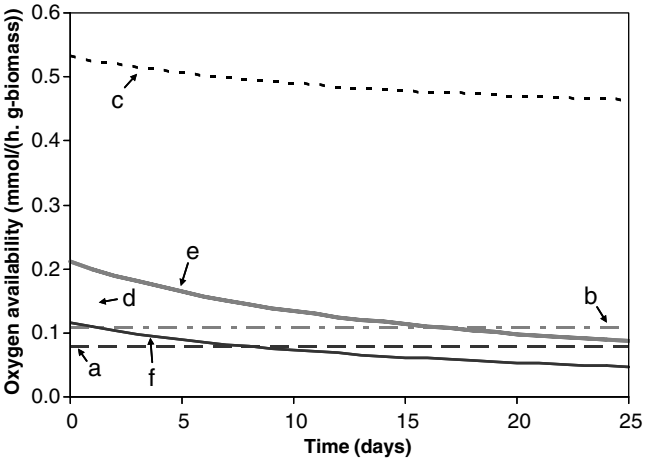

Fig. 6 Calculated oxygen availability per mass of cells for the different reactor systems. **a** Chemostat with glucose (experiment of Fig. 1), **b** chemostat with xylose (experiment of Fig. 2), **c** free cells and **d** immobilized cells in fluidized bed reactor with silicone rubber tubing assuming increased oxygen availability for free cell population (experiment of Fig. 4), **e** free cells and immobilized cells in fluidized bed reactor with silicone rubber tubing assuming identical oxygen availability for free and immobilized populations (experiment of Fig. 4), **f** free cells and immobilized cells in fluidized bed reactor with Pharmed tubing assuming identical oxygen availability for free and immobilized populations (experiment of Fig. 5)

elsewhere assumed to be available equally to both populations. Cells in the fluidized bed with Pharmed tubing (Fig. 6f) had the lowest specific oxygen availabilities [declining from 0.1 to 0.05 mmol/(h. g-biomass)], consistent with their very high rate of plasmid retention (Fig. 5). Cells in the chemostat had oxygen availabilities of about 0.1 mmol/(h. g-biomass) (Fig. 6a, b) and experienced slightly higher plasmid loss (Figs. 1a, 2a), suggesting that this is close to the maximum permissible oxygen availability consistent with high plasmid retention for this organism. In the fluidized bed with silicone tubing, the conservative assumption that the available oxygen is shared equally between the free and immobilized populations leads to a predicted specific oxygen availability of 0.1–0.2 mmol/(h. g-biomass), which is consistent with the overall higher rate of plasmid loss under these conditions, but fails to explain the disproportionate losses by the free cells. Since only the free cells were recirculated, they can reasonably be assumed to have consumed a disproportionate quantity of the oxygen entering through the tubing. In the extreme case where all such oxygen is available only to the free cells, a specific oxygen availability of 0.5–0.53 mmol/(h. g-biomass) is predicted (Fig. 6c), which is broadly consistent with their observed rate of plasmid loss (Fig. 4a). This interpretation also helps to explain the similar rates of plasmid retention by the immobilized cells, regardless of the tubing employed and the nearly equal rates of plasmid retention by the free and immobilized cells when oxygen-impermeable tubing was employed.

Discussion

This work is the first to investigate the stability and ethanologenicity of the xylose-fermenting strains of the FBR series in continuous culture. The high, essentially constant ethanol yields achieved by strain FBR5 in both chemostat and continuous fluidized bed culture, together with the generally high rate of retention of the ethanologenic plasmid are encouraging evidence of the value of the approach taken by the strain's originators to construct a stable organism for use in continuous culture. While recent comparative studies conducted in our laboratory (X. Hong, Deparment of Chemical and Biomolecular Engineering, University of Melbourne, personal communication 2005) show that Ingram's extensively tested *E. coli* KO11 strain remains a more efficient fermenter of xylose in batch culture, in continuous culture on this sugar, strain FBR5 was clearly superior. In similar chemostat experiments to those conducted in this work, *E. coli* KO11, while stable on a glucose feed, rapidly lost ethanologenicity on xylose, the ethanol yield declining to 44–54% of the theoretical over 14 days, with a similar decline observed on mannose [11]. This decline was attributed to the progressive loss of the chromosomally integrated ethanologenic genes, or at least of the ability to hyper-express them [11]. Xylose is the principal sugar present in hemicellulose hydrolysates produced from hardwoods and crop wastes and its efficient utilization is essential for economic ethanol production from these feedstocks [16].

While our earlier experiments [11] with *E. coli* KO11 employed continuous nitrogen flushing of the reactor headspace, this and other rigorous measures to exclude oxygen were not employed in the present work, in keeping with the conditions generally encountered industrially. The construction of strain FBR5 permits reversion to occur in the presence of significant amounts of oxygen, since under these conditions the organism may be able to make enough ATP to grow by respiration alone, removing the selection pressure for plasmid maintenance. Respiring cells may either be able to outgrow cells with the plasmid because maintaining the plasmid places an extra metabolic load on the organism or there could be a simultaneous gradual loss of the plasmid from all the cells. While our data do not allow a precise determination of the maximum oxygen uptake

rate consistent with high plasmid retention, they suggest (Fig. 6) that it is of the order of 0.1 mmol O_2/(h. g-biomass) or less. Our work shows that revertant cells of FBR5 can be readily detected and enumerated during continuous culture, based on the morphology of their colonies on non-selective plates, which correlates closely with their ampicillin resistance as measured by the more time-consuming method of transfer plating.

The ethanol yield obtained when oxygen-permeable recirculation tubing was employed in the fluidized bed barely declined over the course of the experiment, in spite of the marked rise in the proportion of revertants among the free cells (Fig. 4). This is due to the high proportion of immobilized cells, which constituted more than 80% of the total biomass and retained between 85 and 95% of the plasmids over the whole course of the experiment. While there could be various explanations of the higher stability of the immobilized cells, their reduced exposure to the available oxygen appears likely to have been the major determining factor in this system.

It was not our aim to compare directly the productivity advantages of continuous over batch culture using strain FBR5 (or the comparative economics), since no attempt was made in this study to optimize either the operating conditions or the reactor configuration for continuous culture (for example, it would be possible to increase substantially the microbial population in the fermenters, either by cell recycle, or by modifying the design of the fluidized bed to increase the number of immobilized cells). However it is significant that the ethanol productivity of strain FBR5 in the fluidized bed reactor (based on the data of Fig. 5) was ca. 0.9 g/(l h) compared to 0.51–0.59 g/(l h) in published batch experiments with the same strain on modified Luria broth medium containing 95 g/l xylose [9]. The true productivity in batch culture would be considerably less, since neither the lag phase, nor the turn-around time between batches was accounted for. The comparative economics of continuous and batch ethanol production are also affected by distillation costs, which are higher for continuous culture, due to the lower ethanol concentrations achieved. In batch culture, *E. coli* FBR5 produced ca. 40 g/l ethanol [9], compared to 18–22 g/l in continuous culture in this work. Further work to determine the maximum ethanol concentration achievable in continuous culture with this strain is desirable. The maximum ethanol concentration achievable in continuous culture is frequently limited by the organism's ethanol tolerance. Ingram and co-workers have been able to obtain mutants of their ethanol-producing *E. coli* B strains possessing substantially increased ethanol tolerance by serially transferring them for extended periods in ethanol-containing medium [22]. The use of a similar approach with *E. coli* FBR5 and related strains is recommended to increase further their attractiveness for use in continuous ethanol production.

A disadvantage of strain FBR5 is that the fumarate reductase (*frd*) gene was not deleted, [9], allowing the production of considerable quantities of succinic acid. In a subsequently developed strain (FBR16) this deletion was performed, with a glucose phosphotransferase (*ptsG*) mutation also being inserted, allowing the mutants to ferment glucose, xylose and arabinose simultaneously [17]. However, the *ptsG* mutation disables active glucose transport, slowing glucose consumption and increasing the susceptibility of the organism to inhibitors in batch fermentations [7]. In our earlier continuous culture work with *E. coli* KO11 [11], simultaneous utilization of glucose and other sugars occurred spontaneously (i.e. without the need for genetic intervention to reduce catabolite repression) because the much faster rate of uptake of glucose compared to other sugars ensured that its concentration in the fermenter normally remained close to zero. This suggests that, for continuous culture, it may be worthwhile deleting the *frd* gene from strain FBR5 without inserting the *ptsG* mutation.

Acknowledgements We are grateful to Dr BS Dien for the gift of the *E. coli* FBR5 strain, to Associate Professor PH Janssen, Dr AR Barber and Dr DE Tribe for helpful discussions and to Dr GA Stanley and Mr J Pelle for assistance with the HPLC assays.

References

1. Anon (1998) The Chemical Engineer Nov. 5th:15
2. Bailey JE, Ollis DF (1986) Biochemical engineering fundamentals, 2nd edn. McGraw-Hill Book Company, New York
3. Beall DS, Ohta K, Ingram LO (1991) Parametric studies of ethanol production from xylose and other sugars by recombinant *Escherichia coli*. Biotechnol Bioeng 38:296–303
4. Bunch PK, Mat Jan F, Lee N, Clark DP (1997) The *ldhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*. Microbiol-UK 143:187–195
5. Cooper CM, Fernstrom GA, Miller SA (1944) Performance of agitated gas-liquid contactors. Ind Eng Chem 36:504–508
6. Cysewski GR, Wilke CR (1978) Process design and economic studies of alternative fermentation methods for the production of ethanol. Biotechnol Bioeng 20:1421–1444
7. Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 63:258–266
8. Dien BS, Hespell RB, Wyckoff HA, Bothast RJ (1998) Fermentation of hexose and pentose sugars using a novel ethanologenic *Escherichia coli* strain. Enzyme Microb Technol 23:366–371
9. Dien BS, Nichols NN, O'Bryan PJ, Bothast RJ (2000) Development of new ethanologenic *Escherichia coli* strains for fermentation of lignocellulosic biomass. Appl Biochem Biotechnol 84–86:181–196
10. Dumsday GJ, Zhou B, Buhmann S, Stanley GA, Pamment NB (1997) Continuous ethanol production by *Escherichia coli* KO11 in continuous stirred tank and fluidized bed fermenters. Aust Biotechnol 7:300–303
11. Dumsday GJ, Zhou B, Yaqin W, Stanley GA, Pamment NB (1999) Comparative stability of ethanol production by *Escherichia coli* KO11 in batch and chemostat culture. J Ind Microbiol Biotechnol 23:701–708
12. Hespell RB, Wyckoff HA, Dien BS, Bothast RJ (1996) Stabilization of *pet* operon plasmids and ethanol production in *Escherichia coli* strains lacking lactate dehydrogenase and pyruvate formate-lyase activities. Appl Environ Microbiol 62:4594–4597
13. Hobley TJ, Pamment NB (1997) Liquid injection gas chromatography and the ALDH assay overestimate free acetaldehyde in complex fermentation media. Biotechnol Tech 11(1):39–42

14. Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF (1987) Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol 53:2420–2425
15. Lindsay H (1973) A calorimetric estimation of reducing sugars in potatoes with 3,5-dinitrosalicylic acid. Potato Res 16:176–179
16. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–577
17. Nichols NN, Dien BS, Bothast RJ (2001) Use of catabolite repression mutants for fermentation of sugar mixtures to ethanol. Appl Microbiol Biotechnol 56:120–125
18. Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO (1991) Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl Environ Microbiol 57:893–900
19. Saha BC, Cotta MA (2006) Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw. Biotechnol Bioeng (in press)
20. Saha BC, Iten LB, Cotta MA, Wu YV (2005) Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. Biotechnol Prog 21:816–822
21. Wood BE, Beall DS, Ingram LO (1997) Production of recombinant bacterial endoglucanase as a co-product with ethanol during fermentation using derivatives of *Escherichia coli* KO11. Biotechnol Bioeng 55:547–555
22. Yomano LP, York SW, Ingram LO (1998) Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. J Ind Microbiol Biotechnol 20:132–138