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Increased Phenotypic Stability and Ethanol Tolerance of Recombinant *Escherichia coli* KO11 When Immobilized in Continuous Fluidized Bed Culture

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ABSTRACT: The recombinant *Escherichia coli* B strain KO11, containing chromosomally-integrated genes for ethanol production, was developed for use in lignocellulose-to-ethanol bioconversion processes but suffers from instability in continuous culture and a low ethanol tolerance compared to yeast. Here we report the ability cell immobilization to improve its phenotypic stability and ethanol tolerance during continuous culture on a 50 g/L xylose feed. Experiments conducted in a vertical tubular fermentor operated as a liquid-fluidized bed with the cells immobilized on porous glass microspheres were compared to control experiments in the same reactor operated as a chemostat without the support particles. Without cell immobilization the ethanol yield fell sharply following start-up, declining to 60% of theoretical after only 8–9 days of continuous fermentation. While immobilizing the cells did not prevent this decline, it delayed its onset and slowed its rate. With immobilization, a stable high ethanol yield (>85%) was maintained for at least 10 days, thereafter declining slowly, but remaining above 70% even after up to 40 days of fermentation. The ethanol tolerance of *E. coli* KO11 cells was substantially increased by immobilization on the glass microspheres. In ethanol tolerance tests, immobilized cells released from the microspheres had survival rates 2.3- to 15-fold higher than those of free cells isolated from the same broth. Immobilization is concluded to be an effective means of increasing ethanol tolerance in *E. coli* KO11. While immobilization was only partially effective in combating its phenotypic instability, further improvements can be expected following optimization of the immobilization conditions.

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KEYWORDS: ethanol tolerance; immobilization; recombinant; *E. coli* KO11; fluidized bed; stability

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Introduction

The production of ethanol from renewable lignocellulosic biomass including energy crops and crop wastes has the potential to contribute substantially to world liquid fuel requirements, while mitigating greenhouse gas production (Zaldivar et al., 2001). Until now, lignocellulose-derived ethanol has been unable to compete commercially with oil due to the expense and complexity of the steps involved in its manufacture and, particularly, the inability of conventional yeast to ferment hemicellulose. It is important for an economic conversion that all hemicellulosic sugars, including the pentoses xylose and arabinose, can be efficiently fermented to ethanol (Hahn-Hägerdal et al., 2006). Recently, various investigators have constructed recombinant microorganisms that can ferment efficiently some or all of the hemicellulose sugars, representing a major step toward the development of a commercial lignocellulose-to-ethanol conversion technology (Becker and Boles, 2003; Dien et al., 2003; Hahn-Hägerdal and Pamment, 2004; Karhumaa et al., 2005; Kuyper et al., 2004). Among these constructs, *Escherichia coli* KO11, from Ingram's laboratory (Ohta et al., 1991), produces high ethanol yields from all the hemicellulose sugars. This strain contains chromosomally-integrated genes from *Zymomonas mobilis* encoding for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) (Ohta et al., 1991) which, when expressed at high levels, divert pyruvate metabolism from acid production to ethanol production.

Since its initial development, *E. coli* KO11 has been studied extensively for its performance in batch fermentation with the results showing promise for its commercial use (Dien et al., 2003; Hahn-Hägerdal et al., 1994; Lima et al., 2002; Moniruzzaman and Ingram, 1998; Rao et al., 2007). However, to decrease operating and capital costs, continuous fermentation would be preferable to batch operations.

Continuous fermentation is carried out industrially in many yeast-based ethanol production processes, but its feasibility with recombinant ethanologens remains to be adequately established. In particular, the genetic stability of the recombinant strains, while not so problematic for batch processes, is a major issue for long-term continuous cultures if there is the possibility of loss or diminishing expression of the foreign genes.

Chemostat cultures of *E. coli* KO11 are phenotypically stable (with respect to their hyperethanologenicity) when grown on glucose as the sole fermentable sugar, without addition of antibiotic. However, when grown on xylose (the dominant sugar in most hardwood and agricultural residue hydrolysates) they progressively and irreversibly lose their hyperethanologenicity, suggesting an underlying genetic instability (Dumsday et al., 1999). The genetic stability of a recombinant strain depends on the nature of the genetic manipulation in combination with environmental variables such as medium composition, temperature, pH and the mode of cultivation. One mode of fermentation that has shown particular promise in enhancing genetic stability has been the use of cell immobilization (Zhang et al., 1996). This can be achieved by entrapment within a porous matrix, attachment to a carrier, self-aggregation, or containment behind a barrier such as a membrane, and can be operated in various reactor systems (e.g., packed bed, air-lift reactor, or fluidized bed) (Karel et al., 1985).

Although there is no fully accepted explanation of why immobilization improves genetic stability, many investigators have shown that it increases plasmid stability in continuous cultures (Barbotin et al., 1998; Chau et al., 2000; Chen et al., 2006; de Taxis du Poët et al., 1987; Zhang et al., 1996). This is most likely due to its restriction of cell growth (as can alternatively be achieved by other means including nutrient limitation and manipulation of the environmental conditions). A similar mechanism might be expected to apply with immobilized strains of organisms such as *E. coli* KO11, in which the genes are chromosomally integrated; however there is an absence of information about whether immobilization has a stabilizing effect on such strains.

Also important for long-term continuous ethanol fermentation is the ethanol tolerance of the strain. Continuous culture places higher demands on an organism's ethanol tolerance than batch culture, since the culture must be capable of remaining viable for long periods in the presence of ethanol. While cell immobilization has been shown to improve significantly the ethanol tolerance of yeast strains (Ciesarova et al., 1998; Desimone et al., 2002; Jirku, 1999; Krisch and Szajáni, 1997), this effect has not yet been reported in bacteria.

In this paper we investigate the effects of immobilization on the phenotypic stability and ethanol tolerance of *E. coli* KO11 during long-term continuous fermentations on xylose. Our study appears to be the first attempt to study the effect of immobilization on the ethanol tolerance of a bacterial strain, and the first report of the effect of immobilization on strain stability in a chromosomal integrant.

Materials and Methods

Strain

E. coli KO11 was kindly supplied by Professor L. O. Ingram (University of Florida, Gainesville, FL) and was stored in freeze-dried ampoules.

Media

The base medium was modified Luria broth which contained: tryptone (Oxoid, Adelaide, Australia), 10 g/L; yeast extract (Oxoid), 5 g/L; and NaCl, 5 g/L. The carbon source was 50 g/L D-xylose (Sigma-Aldrich, Castle Hill, Australia). While this medium is too expensive for commercial alcohol production it was used for consistency with the majority of preceding work on *E. coli* KO11. The organism is currently being modified for use with simple mineral salts medium (Martinez et al., 2007). Plating medium contained Luria broth ingredients at the above concentrations, xylose (20 g/L) and agar (Oxoid) (15 g/L). Feed medium for the continuous fermentation experiments was filter-sterilized. All other media were sterilized by autoclaving at 121°C for 20 min. No antibiotics were used in the fermentation experiments.

Inoculum Preparation

Freeze-dried cells were transferred from ampoules into a Malcony bottle (20 mL) containing 10 mL modified Luria broth supplemented with 20 g/L xylose. These cultures were incubated without shaking for 8 h at 30°C. Two milliliters of this culture were then inoculated into 500 mL Erlenmeyer flasks containing 200 mL of medium of the above composition. The flasks were incubated overnight (16 h) in an orbital shaker at 100 rpm and 30°C. The cells for the inoculum were then harvested by centrifugation (10 min at 5,000 rpm [7650 G]) in a Beckman model J2-21centrifuge and resuspended in 50 mL of medium having the same composition as the feed.

Recirculating Reactor

Experiments were performed in a laboratory-scale vertical tubular fermenter (working volume 610 mL) that was kept mixed by rapid, continuous recirculation of the culture broth using a pump-around system as previously described (Dumsday et al., 1997; Martin et al., 2006). It was operated either as a continuous fluidized bed reactor or a chemostat. For fluidized bed operation the cells were immobilized on open-pore sintered glass beads (SIKUG/041/02/120A, Schott AG, Mainz, Germany) that were retained in the fluidized bed section and not recirculated. For chemostat operation the same reactor was used without any immobilizing microspheres present.

The pH was maintained at 6.0 by automatic addition of 3.0 M KOH and the temperature was controlled at 30°C using a heating coil of flexible tubing wrapped around the reactor. Sterilized antifoam solution [10 mL Dow Corning 1520 (Dow Corning, Midland, MI) 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 free cell population density was ca. 2 g dry weight/L), after which the feed pump was switched on.

Ethanol Tolerance

The ethanol tolerance of free and immobilized cells in the fluidized bed reactor was determined by comparing their survival rates after 2 h exposure to solutions containing 15% or 0% (v/v) ethanol and 20 g/L xylose. Free cells were collected by centrifugation, washed with modified Luria broth, centrifuged again and finally resuspended in 1 mL of Luria broth. 0.1 mL of the suspension was then added to 1 mL of each of the control and ethanol-containing treatment solutions. After a 2-h incubation at 30°C, the free cells were harvested and washed, resuspended in modified Luria broth and the total viable numbers determined from plate counts (28 h at 30°C). Glass beads containing immobilized cells were rinsed in Luria broth, and about 50 beads added to 1 mL of the treatment solutions. After 2 h at 30°C, the beads were rinsed in modified Luria broth, the immobilized cells released by vortexing (this released a substantial proportion of the cells without affecting cell viability) and the suspension plated. The ethanol tolerance was calculated as the number of viable cells that survived exposure to ethanol for 2 h as a percentage of those that survived exposure to the ethanol-free solution for the same period (Krisch and Szajáni, 1997).

Analytical Methods

Ethanol was analyzed by headspace gas chromatography as previously described (Hobley and Pamment, 1997). Total reducing sugars were measured using an adaptation of the DNS assay (Lindsay, 1973). Acetic, lactic, and succinic acids were measured by HPLC as previously described (Dumsday et al., 1999). Biomass was estimated by measuring the optical density at 550 nm.

Results

Chemostat Cultures Conducted in the Recirculating Reactor Without Cell Immobilization

Previously we studied the stability of unimmobilized *E. coli* KO11 in chemostat culture using a 20 g/L xylose feed and a stirred-tank reactor [CSTR] (Dumsday et al., 1999). To study the effects of immobilization in the present work we first performed duplicate control fermentations using a 50 g/L xylose feed with unimmobilized cells. This was performed by operating the recirculating reactor as a chemostat, under the same conditions as in subsequent experiments with immobilized cells, but with the support particles omitted.

For both experiments (Fig. 1), the ethanol concentration was relatively high (ca. 20 g/L) only for the first 5 days, before dropping to below 15 g/L over the next 3 days, and then remaining between 9 and 13 g/L for the rest of the experiment. A similar rapid fall in ethanol concentration was observed in our earlier CSTR experiments using a 20 g/L xylose feed (Dumsday et al., 1999). Residual xylose was initially low (ca. 4 g/L) but increased during the experiment reaching concentrations of up to 20 g/L. The pattern of organic acid production (only measured for the first experiment) was similar to that of the previous CSTR

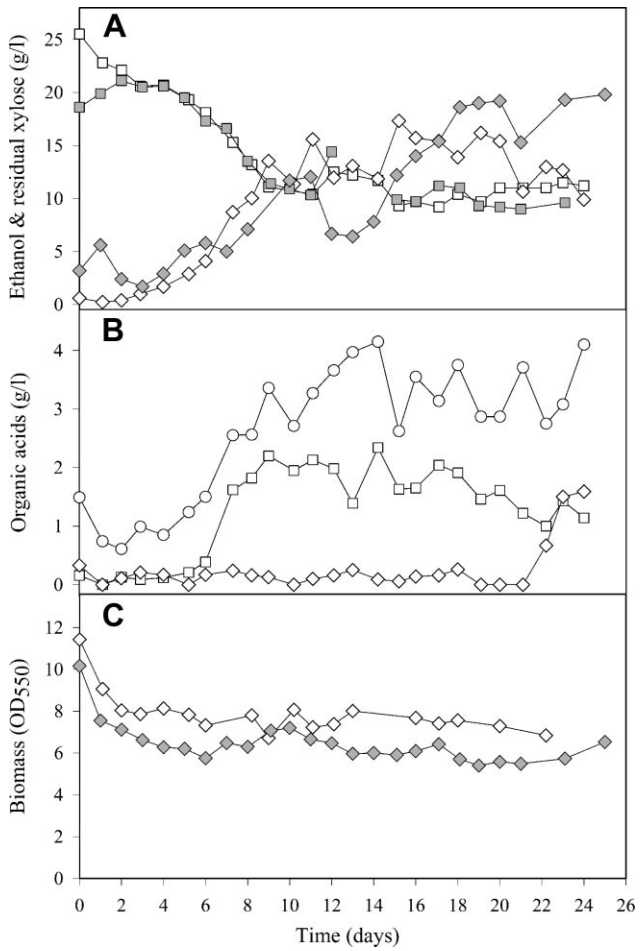

Figure 1. Fermentation kinetics of *E. coli* KO11 in chemostat culture, as conducted in the recirculating reactor, without cell immobilization. The medium was modified Luria broth containing 50 g/L xylose. The dilution rate was 0.046/h for experiment 1 and 0.050/h for experiment 2. The recirculation rate was 42.6 L/h for both experiments. Open and gray-shaded symbols represent experiments 1 and 2, respectively. **A**: Ethanol (□, ■) and residual sugar (◇, ◆); (**B**) acetic acid (○), lactic acid (□), and succinic acid (◇); (**C**) Biomass (◇, ◆).

experiment with 20 g/L xylose as feed (Dumsday et al., 1999), rising concomitantly with the drop in ethanol yields, indicating loss of hyperexpression of the transposed genes. The acetic acid concentration began at less than 1 g/L before progressing to between 3 and 4 g/L by the end of the experiment. Lactic acid was produced in appreciable quantities (up to 2.3 g/L) as early as day 6, whereas succinic acid production did not occur until day 22.

Continuous Fluidized Bed Culture

Three continuous fluidized bed experiments lasting 40, 18, and 28 days were conducted with 50 g/L xylose in the feed (Fig. 2). The first and second experiments included 50 g of immobilizing microspheres (SIKUG41) and the third experiment had 60 g.

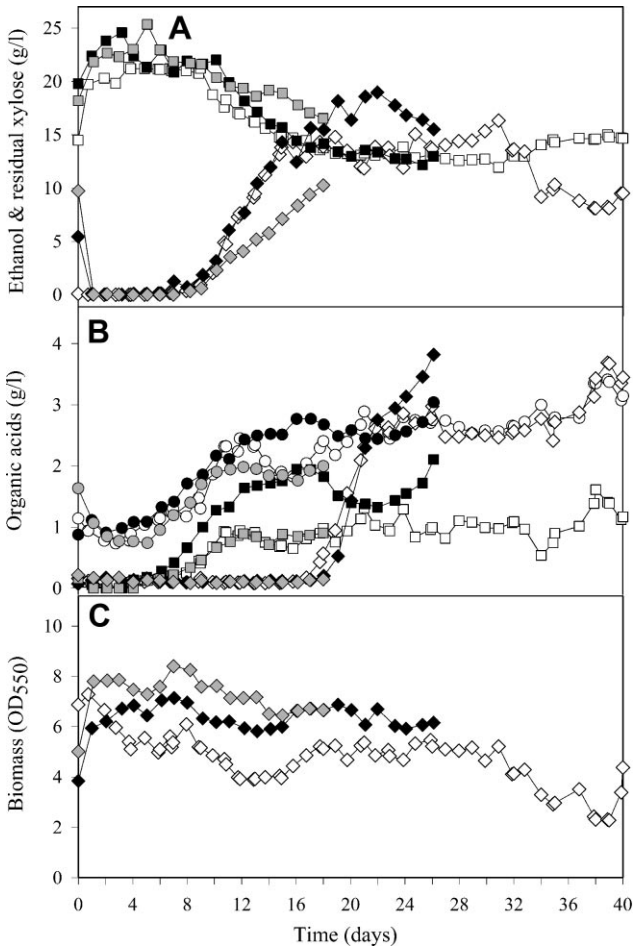

Figure 2. Fermentation kinetics of *E. coli* KO11 in fluidized bed culture with cells immobilized on glass microspheres (SIKUG41 50 g in experiment 1 and 2, 60 g in experiment 3). The medium was modified Luria broth containing 50 g/L xylose. The dilution rate was 0.050/h for all experiments. The recirculation rate was 42.6 L/h for experiments 1 and 2, and 36.1 L/h for experiment 3. Open, gray-shaded and filled symbols represent experiments 1, 2, and 3 respectively. **A**: Ethanol (□,■,■) and residual sugar (◇,◆,◆); (**B**) acetic acid (○,●,●), lactic acid (□,■,■), and succinic acid (◇,◆,◆); (**C**) Biomass (◇,◆,◆).

In the two experiments with 50 g of microspheres an ethanol concentration of 20–25 g/L was maintained for the first 9 days, before gradually falling to an ostensibly stable level of 14–15 g/L. While immobilization failed to ultimately prevent a decline in ethanologenicity, the rate of fall was much slower, and the final ethanol concentration higher, than in the control experiments performed without immobilization. Increased organic acid production again occurred concomitantly with the decreases in ethanol yield. Both experiments revealed sharp rises in lactic, acetic and succinic acids after approximately 9, 10, and 15 days, respectively. Xylose utilization also decreased, after being complete for the first 9 days. In the third experiment, with more glass beads, only slight differences were observed, with the ethanol yield remaining stable for slightly longer and the maximum concentrations of succinic and lactic acids somewhat higher (Fig. 2).

For all experiments the ethanol yield was calculated as a percentage of the theoretical maximum (Fig. 3). All of the experiments achieved yields of 80–100% for the first few days. However, in the two chemostat experiments a sharp decline in yield to about 60% of theoretical occurred within 8–9 days of start-up. In contrast, in the fluidized bed experiments, the decline was more gradual and the final stable yield considerably higher (approximately 70% of theoretical).

Ethanol Tolerance of Free and Immobilized *E. coli* KO11 Grown on Xylose

The table shows the results of ethanol tolerance tests performed on both immobilized and free cells on selected days of experiment 3 in Figure 2 above. The data show that, for every sample, the ethanol tolerance of *E. coli* KO11 cells

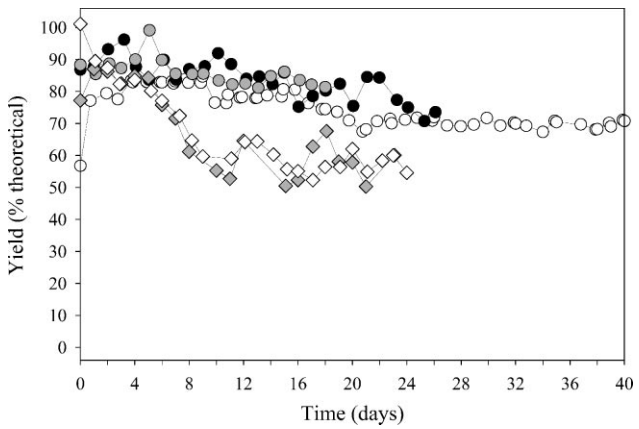

Figure 3. Comparison of ethanol yields obtained in continuous culture with and without cell immobilization. Open and gray-shaded diamonds (◇,◆) represent experiments 1 and 2, respectively, from Figure 1. Open, gray-shaded and filled circles (○,●,●) represent experiments 1, 2, and 3, respectively, from Figure 2.

was substantially increased by immobilization on the glass microspheres, the proportion of survivors exceeding those in the control by from 2.3 to 15-fold, depending upon the sample. The reason for the difference in the extent of improvement between samples of different age is unclear.

Discussion

Enhanced Phenotypic Stability of Ethanol Production

E. coli KO11 has previously been shown to be phenotypically unstable when grown on 20 g/L xylose in continuous culture in a stirred tank chemostat (Dumsday et al., 1999). This was confirmed in this work in experiments using 50 g/L xylose in the recirculating reactor operated as a chemostat without cell immobilization. Under these conditions, ethanol yields above 80% were sustained for no more than 5 days and fell rapidly thereafter (Fig. 3). It was suggested previously that the loss of hyperethanologenicity of *E. coli* KO11 during long-term chemostat culture is due to its reversion to its wild host strain *E. coli* B ATCC 11303, reflecting a genetic change, rather than a physiological adaptation to chemostat culture (Dumsday et al., 1999). This was supported by the failure of cells grown in the chemostat on xylose to regain high ethanologenicity in batch culture and the similarity between the final ethanol yield (0.21) obtained after cultivation of strain KO11 for 48 days on xylose (Dumsday et al., 1999) and that reported for batch cultures of the host strain (0.22) (Lawford and Rousseau, 1996).

When immobilized and cultivated in the fluidized bed fermenter on 50 g/L xylose, *E. coli* KO11 demonstrated better phenotypic stability (as measured by ethanol yield) than when grown in chemostat culture in the same reactor without the support particles. Ethanol yields remained above 82% of the theoretical for 9–10 days, approximately twice as long as in the chemostats (Fig. 3). Additionally, the final stable ethanol yield in the fluidized bed reactor was considerably higher than that achieved in chemostat culture (Fig. 3). Despite this, loss of hyperethanologenicity was not prevented entirely by cell immobilization. This could be partly because not all cells in the reactor are immobilized, free cells being inevitably present in the culture broth. While no attempt was made to optimize the ratio of bound to unbound cells in this work, this would be a worthwhile direction for future work.

These results suggest that cell immobilization can significantly improve the operational stability of poorly-stable chromosomally-integrated recombinant strains. In previous studies immobilization has been shown to lead to improved operational stability with strains in which the recombinant genes are plasmid-borne (Barbotin et al., 1998; Chau et al., 2000; Chen et al., 2006; de Taxis du Poët et al., 1987; Zhang et al., 1996). This has been ascribed to various causes, including a reduced growth rate of the cells when immobilized (which would slow the rate of plasmid loss) and a reduced overall competition between P^+ and P^- cells due to their compartmentalization within the immobilizing agent (de Taxis du Poët et al., 1987). The reason for the greater stability of the chromosomal integrant *E. coli* KO11 when immobilized is as yet unknown but may be due to the presence of slowly- or non-dividing genetically stable cells on the support particles, with the slower growth rates of the immobilized cells attributable to mass transfer limitation of the uptake of one or more growth nutrients. In our view, resolution of this question would best be handled in conjunction with an investigation of the underlying genetic reasons for the loss in stability of strain KO11 during long-term culture on xylose, which to date remain unclear.

Enhanced Ethanol Tolerance

The observed increase in the ethanol tolerance of immobilized *E. coli* KO11 cells (Table I), while consistent with findings on *Saccharomyces cerevisiae* (see Introduction Section), appears to be the first report of such an effect in bacteria, including the native ethanologen *Zymomonas mobilis*. In *S. cerevisiae*, it has been suggested that immobilization would provide protection by enhancing the stability of the hydration layer around the cells (Desimone et al., 2002). However, the improved tolerance in our results was maintained after the cells had been released from the protective environment, suggesting that the improvement is due to a more permanent adaptation of the immobilized cells. Mass transfer limitations may contribute to an increase in ethanol concentration around the immobilized cells

Table I. Ethanol tolerance of immobilized and free cells of *E. coli* KO11 derived from experiment 3 of Figure 2.

Sample age[a] (days)	Free cells (% of population surviving)[b]	Immobilized cells[c] (% of population surviving)[b]	Relative tolerance (immobilized:free)[d]
0.2	0.44	1.69	3.8
2.8	0.13	0.30	2.3
5.3	0.06	0.22	3.7
13.3	0.04	0.60	15.0
23.0	0.25	1.08	7.2

[a]The number of days after the commencement of continuous culture.
[b]Average of triplicate plate counts from cells exposed to 0% or 15% (w/v) ethanol for 2 h.
[c]Based on cells released after vortexing.
[d]Ratio of % survivors (immobilized cells)/% survivors (free cells).

(compared to the broth). This might cause the immobilized cells to be better adapted to high ethanol concentrations, which could explain their better performance in the ethanol tolerance tests. It has been shown that in response to the presence of ethanol, *E. coli* can increase its unsaturated fatty acid content, thereby enhancing tolerance (Ingram et al., 1980). Work with *S. cerevisiae* has also attributed the increased ethanol tolerance of immobilized cells to similar changes in fatty acid content (Jirku, 1999).

Immobilization for Industrial Ethanol Production

Whole cell immobilization has been advocated widely as a means of reducing the fermentation time (Najafpour et al., 2004; Rebros et al., 2005) in industrial and fuel alcohol fermentations, but has yet to be taken up commercially, presumably because the production cost savings are currently marginal. It was not our aim here to evaluate the economics of immobilization but rather to highlight the benefits of increased stability, ethanol tolerance, and alcohol productivity that may tilt the economic balance in favor of the technique when used with recombinant organisms. Immobilization has been successfully adopted commercially at the production scale to reduce the maturation time in secondary beer fermentations, and immobilization of the primary beer fermentation is under active commercial development in Europe (Willaert and Nedovic, 2006). Immobilization for the production of non-beverage alcohol may become more viable with the development of a lignocellulose-based fuel ethanol industry having production volumes large enough to substitute for a substantial fraction of present-day gasoline consumption. Under such circumstances the pressure to reduce costs at all stages of production would be intense (Hahn-Hägerdal et al., 2006).

Conclusion

Immobilization appears to offer an effective means of improving the ethanol tolerance of recombinant *E. coli* KO11 and is partially effective in improving the operational stability of ethanol production in this chromosomal integrant. Further work to optimize the ratio of immobilized to free cells, and experimentation with alternative immobilizing agents, may well lead to additional gains in tolerance and stability.

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