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Effects of process errors on the production of ethanol by *Escherichia coli* KO11

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***Escherichia coli* KO11 was previously constructed for the production of ethanol from both hexose and pentose sugars in hemicellulose hydrolysates by inserting the *Zymomonas mobilis* genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*). This biocatalyst appears relatively resistant to potential process errors during fermentation. Antibiotics were not required to maintain the maximum catabolic activity of KO11 even after deliberate contamination with up to 10% soil. Fermentations exposed to extremes of temperature (2 h at 5°C or 50°C) or pH (2 h at pH 3 or pH 10) recovered after re-adjustment to optimal fermentation conditions (35°C, pH6) although longer times were required for completion in most cases. Ethanol yields were not altered by exposure to extremes in temperature but were reduced by exposure to extremes in pH. Re-inoculation with 5% (by volume) from control fermentors reduced this delay after exposure to pH extremes.**

Keywords: lignocellulose; biomass; fermentation; ethanol; *E. coli* KO11; xylose; process errors; process upsets

Introduction

The commercial viability of biological processes for the production of bulk chemicals is critically dependent upon the development of robust engineering designs and microbial catalysts. Species of *Saccharomyces* have proven to be excellent biocatalysts for ethanol production from grain-derived and cane-derived hexose sugars [14–17,19]. Genetically engineered strains of *Escherichia coli* and other bacteria have been used for the large-scale production of higher value products such as amino acids [8,12,22] and recombinant proteins [9,13,21,23]. Although extensive industrial experience has been gained with both systems, *E. coli* is generally regarded by bioprocess engineers as the most desirable microbial platform for the development of new products (Engineering Foundation Conference, rDNA Biotechnology: Metabolic Engineering, Danvers, MA, October 1996).

Over the past 10 years, our laboratory has focussed on process development and the genetic engineering of enteric bacteria for fuel ethanol production from lignocellulose [4,10,11,24]. Genes encoding the ethanol pathway from *Zymomonas mobilis* were chromosomally integrated into *E. coli* to redirect central metabolism and produce ethanol and CO_2 as primary products of fermentation [20]. Although ethanol tolerance of the recombinant *E. coli* is only half that of conventional yeasts, the sugar content of most hemicellulose-derived syrups is limited to 80–100 g L^{-1} and serves to limit expected product concentrations to below 50 g ethanol L^{-1} [11]. Under optimal conditions (pH 6–7 and 32–37°C), recombinant *E. coli* efficiently ferment all hexose and pentose sugar components of lignocellulose (after hydrolysis) to ethanol [1,3]. Information concerning the industrial hardiness of large-scale processes using *E. coli* has been gathered by industry but little is available in the public domain.

Process errors are likely to occur in large-scale fermentations which cause swings in pH or temperature, or result in the accidental contamination of fermentations with other organisms. In this study we have determined the consequences of potential process errors during the fermentation of xylose to ethanol by recombinant *E. coli* and examined the potential benefit of re-inoculation as a method to accelerate recovery.

Materials and methods

Bacterial strains and media

E. coli KO11 [20] was used in all fermentation studies. Chloramphenicol acyl transferase (*cat*) and the *Z. mobilis* genes for ethanol production (*pdc, adhB*) are integrated into the chromosome of this strain. Stock cultures were maintained on modified Luria-Bertani (LB) [2] agar containing (per liter): 5 g NaCl, 5 g yeast extract, 10 g tryptone, 20 g xylose, 15 g agar, and 600 mg chloramphenicol. Although chloramphenicol was included in solid medium, none was added to seed cultures or to fermentations.

Batch fermentations by E. coli KO11 using laboratory ingredients

Fermentations (350 ml working volume) were conducted in 500-ml beakers (Fleakers) fitted with a rubber cap and portals for sampling, gas escape, and a pH probe [3]. Unless stated otherwise, fermentations were conducted using modified LB containing 90 g xylose L^{-1} but omitting agar and chloramphenicol [1,3,18]. Sugars and complex nutrients were autoclaved separately and mixed after cooling just before inoculation. Seed cultures were grown without pH control in unshaken flasks containing a similar medium but with a lower concentration of xylose (50 g L^{-1}) and

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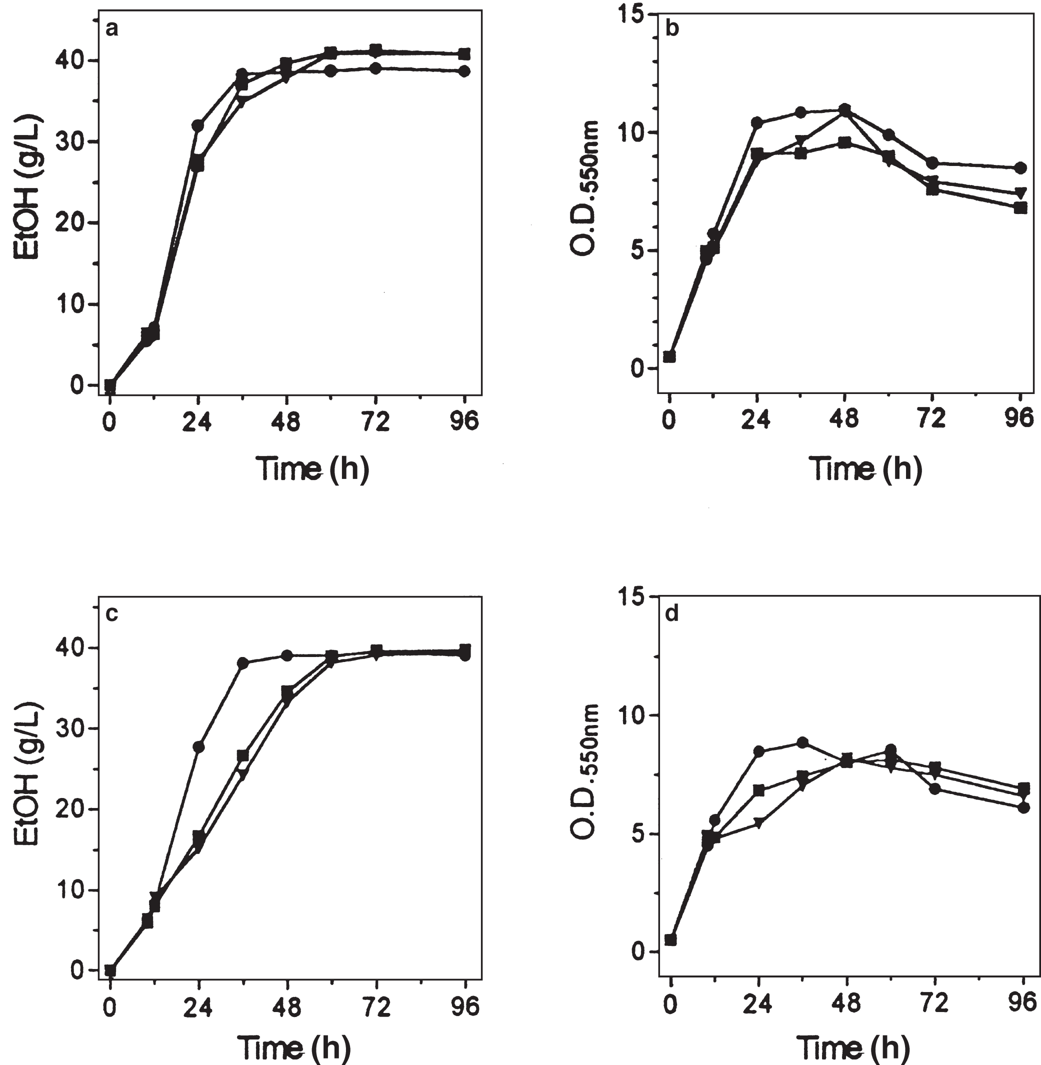

Figure 1 Effect of a 2-h exposure to temperature extremes on fermentations. Shifts in temperature were carried out 10 h after inoculation. Fermentors were returned to 35°C water baths and fermentation continued. Symbols for all graphs: ●, control maintained at 35°C; ▼, shifted to an alternative temperature for 2 h, ■, shifted to an alternative temperature for 2 h, returned to 35°C, and re-inoculated with 5% by volume from the control fermentation. (a) Ethanol production after exposure to 5°C. (b) Growth after exposure to 5°C. (c) Ethanol production after exposure to 50°C. (d) Growth after exposure to 50°C.

without agar. Seed cultures were harvested by centrifugation (5000× *g*, 5 min, 5°C) and used to inoculate fermentors to an initial density of 0.5 OD at 550 nm, approximately 0.16 gdw L^{-1}. KOH (2 N) was automatically added to prevent the pH from declining below pH 6.0 (35°C). The temperature was controlled using a water bath with circulating pump. This water bath was placed above a multiplace, magnetic stirrer which served to agitate each of the fermentors (100 rpm). No control was provided to prevent an increase in pH. Shifts in temperature or pH were initiated after 10 h of fermentation and lasted for 2 h prior to readjustment to optimal conditions. Additions of fresh soil as a source of contamination were made at the beginning of fermentation.

Batch fermentations using hemicellulose hydrolysate

To investigate resistance to contamination further, fermentations were also conducted without sterilization using a dilute sulfuric acid hydrolysate of sugar cane bagasse hemicellulose. This hydrolysate was kindly provided by BC International (Hingham, MA, USA) and was reported to contain per liter: 14 g glucose, 72 g xylose, 4 g arabinose, and 1 g mannose in dilute sulfuric acid (91 g total sugar L^{-1}). Toxins were removed by adjusting to pH 10 with $Ca(OH)_2$ followed by decanting to eliminate the larger gypsum particles. The resulting hydrolysate was neutralized by the addition of HCl. Dry LB nutrients (tryptone and yeast extract) were dissolved in lime-treated hydrolysate immediately before inoculation. Seed cultures were prepared as

Table 1 Effect of simulated process errors on cell growth and ethanol production[a]

Treatment	Maximum cell density[b] (gdw L^{-1})	Base added[c] (mmoles L^{-1})	Maximum ethanol[d]		Ethanol yield[e] (% theoretical)
			Time (h)	Conc. (g L^{-1})	
None (*n* = 14) control	3.3 ± 0.2	45.7 ± 8.2	48	42.6 ± 1.9	93 ± 4
50°C, 2 h	2.9	65.7	60	39.3	89
+ 5% inoculum	2.7	62.9	60	39.5	89
5°C, 2 h	3.6	57.4	60	40.8	91
+ 5% inoculum	3.2	48.6	60	41.2	92
pH 10, 2 h	3.6	97.1	96	34.4	79
+ 5% inoculum	4.0	103	48	37.5	86
pH 3, 2 h	3.6	117	96	36.0	83
+ 5% inoculum	3.7	109	72	36.9	85
1 gdw soil L^{-1}	nd[f]	47.1	48	44.3	99
10 gdw soil L^{-1}	nd	42.9	48	43.7	97
50 gdw soil L^{-1}	nd	38.6	48	42.3	94
100 gdw soil L^{-1}	nd	40.2	48	41.2	92
Hydrolysate[g]	nd	8.6	96	41.0	92

[a]Results represent an average of two or more fermentations with 90 g xylose L^{-1}. Control values are averages of 14 fermentations with standard deviations (12 fermentations for cell mass).
[b]Maximum cell density in grams (dry weight) per liter.
[c]Base (2 N KOH) added to maintain fermentation at pH 6 or above.
[d]Values represent total ethanol per liter of original fermentation broth and have been adjusted for dilution by added base.
[e]The theoretical yield from 90 g xylose is 45.9 g of ethanol.
[f]nd, not determined.
[g]Hydrolysate and nutrients were not sterilized. The maximum theoretical yield from 86.9 g sugar is 44.3 g ethanol.

described above. Fermentations were inoculated by adding 10% seed (by volume) and maintained at pH 6 by the automatic addition of 2 N KOH. Since seed was grown in medium containing 50 g L^{-1} xylose, the total sugar input in the fermentation broth was calculated as follows:

$$0.1\ L\ (50\ g\ L^{-1}) + 0.9\ L\ (91\ g\ L^{-1}) = 86.9\ g\ L^{-1}\ \text{total sugar.}$$

Analytical methods

Ethanol was measured by gas chromatography [3,18]. Cell mass was estimated by converting optical density at 550 nm to cell dry weight (3.3 gdw L^{-1} = 10 OD at 550 nm) based on a standard curve. Data presented represent an average of two or more fermentations. With the glycolytic pathways in *E. coli*, the maximum theoretical yield from 1 kg xylose is 510 g of ethanol and 490 g of CO_2.

Results

Effects of temperature shifts on fermentation

Previous studies have identified 35°C as the optimal temperature for ethanol production using *E. coli* B containing ethanol genes from *Z. mobilis* [1,3]. During commercial operation, it is likely that errors will occur sporadically which result in large shifts in temperature. We have simulated these process errors by shifting fermentors from a 35°C water bath to an adjacent 50°C water bath or to an adjacent 5°C water bath. Temperature shifts were conducted 10 h after inoculation and lasted for 2 h, after which fermentors were returned to 35°C. For comparison, control fermentations were maintained at 35°C.

A shift to 50°C was more disturbing to fermentation than a shift to 5°C (Figure 1). During the 12-h period immediately after a 2-h shift to 50°C, growth was reduced by 80% and ethanol production was reduced by 50% in comparison to the control fermentation maintained at 35°C. The time required to complete fermentation was increased by 24 h (Figure 1c) after exposure to 50°C although the final yields of ethanol and cell mass after thermal stress (high and low temperature) were equivalent to those of the control (Table 1). More base was required to maintain pH 6 after exposure to 50°C than was required in the control or after exposure to 5°C.

Re-inoculation (5% by volume from control fermentors) after exposure to high or low temperature had no effect on performance after either thermal stress.

Effect of pH shifts on fermentation

Although ethanologenic derivatives of *E. coli* B ferment efficiently between pH 5.8 and pH 7.5 [3], pH 6.0 has been used as a practical optimum which minimizes solubilization of CO_2. To examine the effects of pH stress, fermentors (10 h after inoculation) were adjusted to pH 3 with 12 N HCl (*ca* 2 ml) or to pH 10 with 10 N KOH (*ca* 3 ml), held for 2 h, and re-adjusted to pH 6.0 with 10 N KOH or 12 N HCl, respectively.

Exposure to high or low pH was quite detrimental to growth and ethanol production (Figure 2). Cell growth was arrested and did not resume for 12 h after exposure to pH 3 or for 22 h after exposure to pH 10. The time required to complete fermentation increased by 36 h after exposure to pH 3 but was increased by over 60 h after exposure to pH 10. Ethanol yields were lower after a 2-h exposure to either pH 3 or pH 10 (Table 1). After re-adjustment to pH 6, pH-stressed fermentations required approximately twice as much base to prevent acidification as control fermentations.

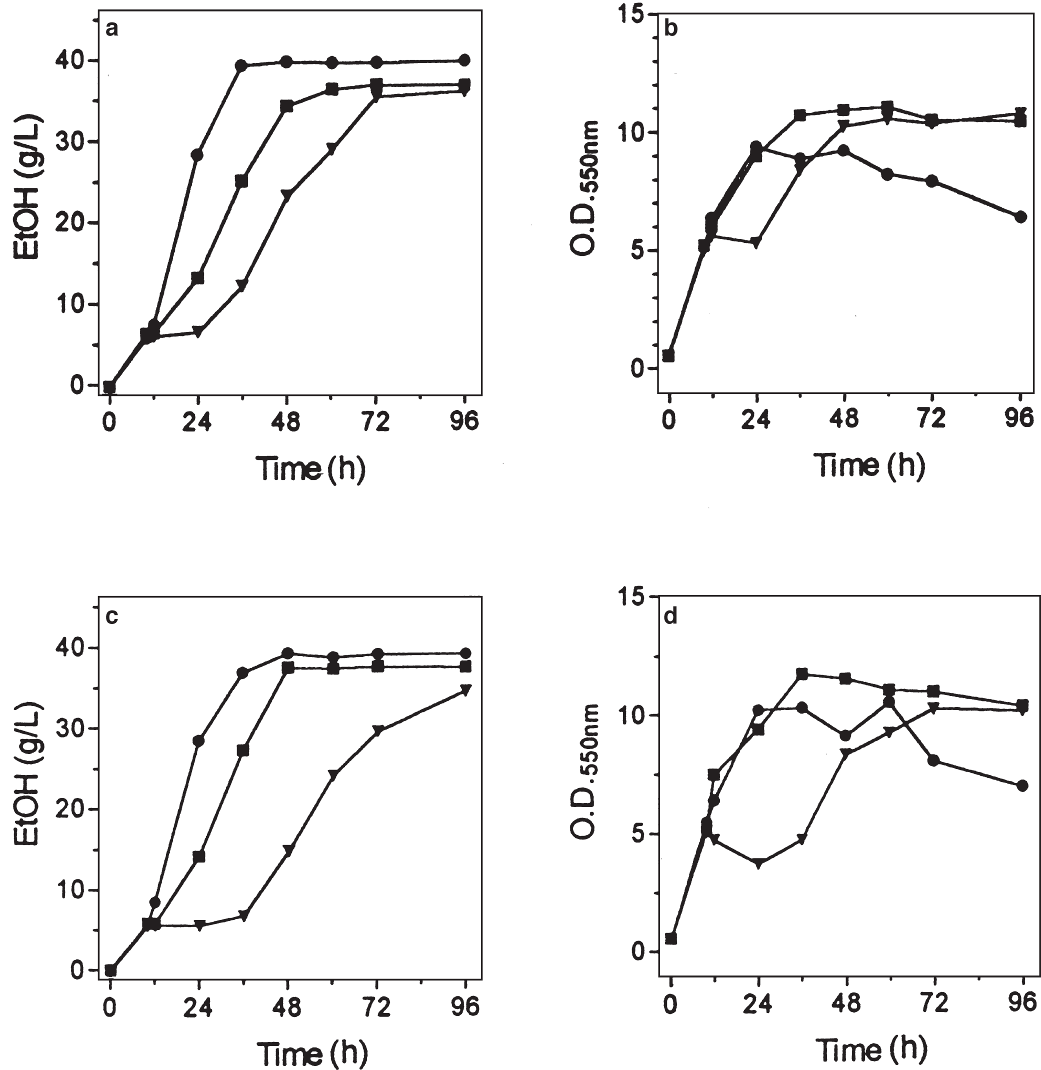

Figure 2 Effect of a 2-h exposure to extremes of pH on fermentation (pH 6). Shifts in pH were carried out 10 h after inoculation followed by re-adjustment to pH 6. Symbols for all graphs: ●, control maintained at pH 6 or above; ▼, shifted to an alternative pH for 2 h; ■, shifted to an alternative pH for 2 h, adjusted to pH 6, and re-inoculated with 5% by volume from the control fermentation. (a) Ethanol production after exposure to pH 3. (b) Growth after exposure to pH 3. (c) Ethanol production after exposure to pH 10. (d) Growth after exposure to pH 10.

Fermentations stressed by exposure to pH 3 or pH 10 were improved by re-inoculation with cell suspensions (5% by volume) from the control fermentor (Figure 2). Cell growth resumed immediately upon re-adjustment to pH 6 and re-inoculation. After re-inoculation, pH-stressed fermentations required approximately 12 h longer to reach completion than the unstressed control maintained at pH 6 (Figure 2). However, ethanol yields remained lower in pH-stressed fermentations than in the control. Taken as a group, the modest reduction in ethanol yield after pH stress is significant ($P < 0.05$) when compared to the control. This may be due in part to the reactivity of reducing sugars with amino acids or peptides which is promoted by extremes in pH [6,7]. Interestingly, cell growth in fermentors exposed to pH shifts consistently exceeded that of the pH 6 control.

Effects of contamination with soil on fermentation

Accidental contamination of fermentors with soil organisms associated with lignocellulosic biomass is inevitable in a large-scale bioconversion facility. To investigate this problem, we have added freshly collected soil from an adjacent field at the time of inoculation with *E. coli* KO11 (Figure 3a). No detrimental effects were observed with soil concentrations from 1–100 gdw L^{-1}. Indeed, ethanol yields with 1–10 gdw L^{-1} were slightly higher than the average yield without soil. Growth was not monitored due to turbidity resulting from added soil.

Additional studies were also conducted to investigate the lack of an apparent requirement for sterility. LB nutrients were added directly to lime-treated hemicellulose hydrolysate and fermented directly without sterilization (Figure 3b). Although this fermentation required longer to complete than fermentations with pure laboratory sugars, the ethanol yields for both were equivalent.

Discussion

Antibiotics were not required to maintain the dominance of *E. coli* KO11 even when fermentations were deliberately

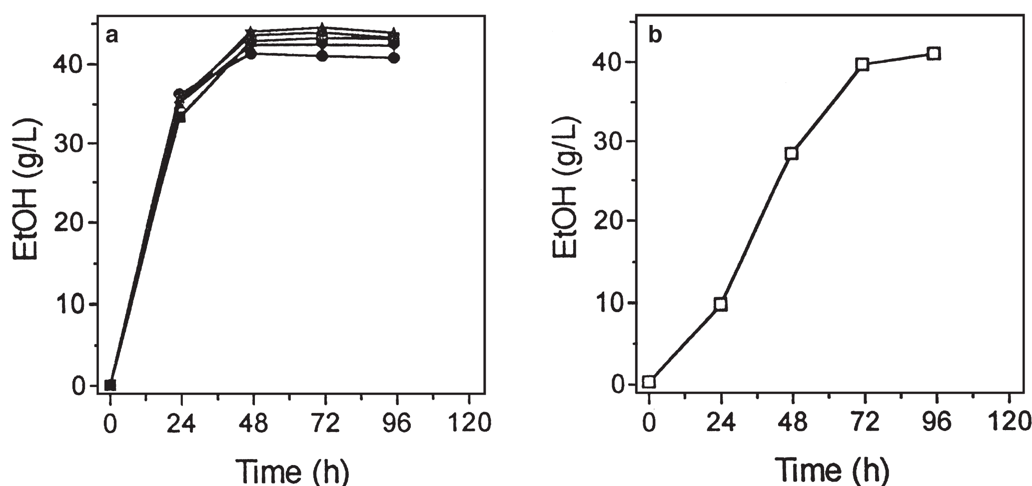

Figure 3 Effect of microbial contamination. Symbols: –■–, no dirt (control); –▲–, 0.1% dirt; –▼–, 1.0% dirt; –◆–, 5.0% dirt; –●–, 10.0% dirt; –□–, unsterilized hemicellulose hydrolysate. (a) Addition of fresh, unsterilized soil to fermentations. Soil was addeded at the time of inoculation. (b) Fermentation of unsterilized hemicellulose hydrolysate containing LB nutrients.

contaminated with up to 10% fresh soil (by weight) or when hemicellulose hydrolysate with LB nutrients was fermented without sterilization (Figure 3). Ethanol production by *E. coli* KO11 was surprisingly resistant to errors in temperature control (Figure 1). A 2-h exposure to 5°C had little effect on the rate of ethanol production or ethanol yield. Re-inoculation with 5% (by volume) from control fermentors was not required. Exposure to 50°C delayed the completion of fermentation regardless of re-inoculation. In contrast, exposure to extremes of pH was very detrimental, reducing the rate of ethanol production and ethanol yield. Exposure to extremes in pH (Figure 2) caused long delays in growth indicating damage to the biocatalyst *E. coli* KO11. These delays in growth appear responsible for the increased time required to complete fermentation after exposure to pH 3 or pH 10. Re-inoculation with 5% by volume from a control fermentor partially restored growth which in turn accelerated the completion of fermentation. Even with re-inoculation, pH-stressed fermentations required an additional 12 h to reach completion.

Ethanol yield was significantly reduced by a 2-h exposure to high or low pH (Table 1). It is likely that this decrease in yield is due in part to sugar destruction. Fermentation broth was considerably darkened by exposure to high or low pH (and re-adjustment to pH 6) indicating that both treatments caused chemical changes. High pH causes sugar destruction [5]. Other possible sources of sugar destruction include the formation of Maillard reaction products from xylose and amino acids or peptides [6,7]. Loss of sugar via either mechanism would lead to a corresponding decrease in sugar in ethanol yield which could not be reversed by re-inoculation.

Our results indicate that fermentations need not be discarded after process errors in pH or temperature control but can be salvaged by re-adjustment to optimal pH and temperature with only a modest loss in ethanol yield. While no more than an additional 2 h was required to allow completion of fermentation after brief exposure to 5°C, an additional 12 h would be required for fermentations stressed at 50°C or pH extremes even with re-inoculation. Sufficient nutrients remained after exposure to different temperatures, high pH, and low pH to allow further growth of the biocatalyst. No microbial toxins appear to be generated by these treatments which prevent the growth of *E. coli* KO11. It is likely that the increase in fermentation times after pH or temperature stress (Table 1) could be further reduced by adding larger amounts of catalysts from control fermentations or by blending the broth from stressed fermentations with fresh substrates and nutrients. However, all approaches will cause a temporary reduction in the volumetric productivity of the plant.

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References

1 Asghari A, RJ Bothast, JB Doran and LO Ingram. 1996. Ethanol production from hemicellulose hydrolysates of agricultural residues using genetically engineered *Escherichia coli* KO11. J Ind Microbiol 16: 42–47.
2 Atlas RM and LC Parks (eds). 1993. Handbook of Microbiological Media. CRC Press, Boca Raton, FL.
3 Beall DS, K Ohta and LO Ingram. 1991. Parametric studies of ethanol production from xylose and other sugars by recombinant *Escherichia coli*. Biotechnol Bioeng 38: 296–303.
4 Brooks TA and LO Ingram. 1995. Conversion of mixed waste office waste paper to ethanol by genetically engineered *Klebsiella oxytoca* strain P2. Biotechnol Prog 11: 619–625.
5 Bruijn JM de, APG Kieboom, H van Bekkum and PW van der Poel. 1986. Reactions of monosaccharides in aqueous alkaline solutions. Sugar Technol Rev 13: 21–52.
6 Chang CH and J Hong. 1984. The effects of Browning reaction products on the yeast (*S. cerevisiae*) fermentation. Biotechnol Bioeng 26: 797–799.
7 Einarsson H, BG Snygg and C Eriksson. 1983. Inhibition of bacterial growth by Maillard reaction products. J Agric Food Chem 31: 1043–1047.

8 Furukawa S, A Ozaki, T Nakanishi, Y Kotani and M Sugimoto. 1991. Process for producing l-threonine. US Patent No. 5017483.
9 Glick BR and JJ Pasternak. 1994. Molecular Biotechnology. Principles and Applications of Recombinant DNA. American Society for Microbiology, Washington, DC.
10 Ingram LO, T Conway, DP Clark, GW Sewell and JF Preston. 1987. Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol 53: 2420–2425.
11 Ingram LO, X Lai, M Moniruzzaman, BE Wood and SW York. 1997. Fuel ethanol production from lignocellulose using genetically engineered bacteria. In: Fuels and Chemicals from Biomass, ACS Symposium Series 666 (BC Saha and J Woodward, eds), pp 57–73, American Chemical Society, Washington, DC.
12 Jetten MSM and AJ Sinskey. 1995. Recent advances in the physiology and genetics of amino acid-producing bacteria. Crit Rev Biotechnol 15: 73–103.
13 Kanjino T, Y Saito, O Asami, Y Yamada, M Hirai and S Udata. 1997. Extracellular production of an intact and biologically active human growth hormone by *Bacillus brevis* system. J Ind Microbiol Biotechnol 19: 227–231.
14 Katzen R and DE Fowler. 1994. Ethanol from lignocellulosic wastes with utilization of recombinant bacteria. Appl Biochem Biotechnol 45/46: 697–707.
15 Keim CR 1995. Wet milling of grain for alcohol production. In: The Alcohol Textbook (TP Lyons, DR Kelsall and JE Murtagh, eds), pp 1–10, Nottingham University Press, Nottingham, UK.
16 Kelsel DR. 1995. Grain dry milling and cooking for alcohol production. In: The Alcohol Textbook (TP Lyons, DR Kelsall and JE Murtagh, eds), pp 11–26, Nottingham University Press, Nottingham, UK.
17 Lewis SM. 1996. Fermentation alcohol. In: Industrial Enzymology (T Godfrey and S West, eds), 2nd edn, pp 11–48, MacMillan Press, London, UK.
18 Moniruzzaman M, X Lai, SW York and LO Ingram. 1997. Extracellular melibiose and fructose are intermediates in raffinose catabolism during fermentation to ethanol by engineered enteric bacteria. J Bacteriol 179: 1880–1886.
19 Murtagh JE. 1995. Molasses as a feedstock for alcohol production. In: The Alcohol Textbook (TP Lyons, DR Kelsall and JE Murtagh, eds), pp 27–34, Nottingham University Press, Nottingham, UK.
20 Ohta K, DS Beall, JP Mejia, KT Shanmugam and LO Ingram. 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.
21 Thomas JG, A Ayling and F Baneyx. 1997. Molecular chaperones, folding catalysts, and the recovery of active recombinant proteins from *E. coli*. To fold or to refold. Appl Biochem Biotechnol 66: 197–238.
22 Tsuchida T and K Sano. 1983. Method for producing l-phenylalanine by fermentation. US Patent No. 4407952.
23 Yabuta M, Y Suzuki, K Ohsuye, T Oshima, S Onai, K Magota and S Tanaka. 1997. Process for producing peptides in *E. coli*. US Patent No. 560340.
24 Wood BE and LO Ingram. 1992. Ethanol production from cellobiose, amorphous cellulose, and crystalline cellulose by recombinant *Klebsiella oxytoca* containing chromosomally integrated *Zymomonas mobilis* genes for ethanol production and plasmids expressing thermostable cellulase genes from *Clostridium thermocellum*. Appl Environ Microbiol 58: 2103–2110.