

Ethanol production from lignocellulosic biomass by recombinant *Escherichia coli* strain FBR5

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Lignocellulosic biomass, upon pretreatment and enzymatic hydrolysis, generates a mixture of hexose and pentose sugars such as glucose, xylose, arabinose and galactose. While *Escherichia coli* utilizes all these sugars it lacks the ability to produce ethanol from them. Recombinant ethanologenic *E. coli* strains have been created with a goal to produce ethanol from both hexose and pentose sugars. Herein, we review the current state of the art on the production of ethanol from lignocellulosic hydrolyzates by an ethanologenic recombinant *E. coli* strain (FBR5). The bacterium is stable without antibiotics and can tolerate ethanol up to 50 gL⁻¹. It produces up to 45 g ethanol per L and has the potential to be used for industrial production of ethanol from lignocellulosic hydrolyzates.

Introduction

Ethanol is a renewable oxygenated fuel. In the USA, about 13.23 billion gallons of fuel ethanol was produced in 2010, replacing the gasoline produced from some 445 million barrels of imported oil.¹ Various agricultural residues (corn stover, wheat straw, barley straw, rice straw and sugarcane bagasse), processing byproducts (corn fiber and rice hulls), and energy crops (switchgrass and miscanthus) are available as low cost feedstocks for conversion to fuel ethanol. The production of ethanol from lignocellulosic biomass generally involves four steps—feedstock pretreatment, enzymatic saccharification, fermentation and product recovery. The pretreatment of any lignocellulosic biomass is crucial before enzymatic hydrolysis.² Furthermore, any lignocellulosic biomass, upon pretreatment and enzymatic saccharification, produces a mixture of sugars such as glucose, xylose, arabinose and galactose.

The utilization of all the sugars generated from lignocellulosic biomass is essential for the economic production of ethanol.³ The conventional ethanol fermenting yeast (*Saccharomyces cerevisiae*) or bacterium (*Zymomonas mobilis*) cannot ferment pentose sugars to ethanol.⁴ One major technical hurdle to converting any lignocellulosic feedstock to ethanol is the development of an appropriate microorganism for fermentation of both hexose and pentose sugars. A number of recombinant microorganisms such as *Escherichia coli*, *Klebsiella oxytoca*, *Z. mobilis* and *S. cerevisiae*

have been developed over the past 25 y with the express goal of fermenting both hexose and pentose sugars to ethanol simultaneously.⁴ Herein, we review the production of ethanol by a recombinant *E. coli* strain (FBR5) from various lignocellulosic biomass substrates.

Construction of Recombinant *E. coli* Strain FBR5

The ethanol pathway in *S. cerevisiae* and *Z. mobilis* requires only two enzymes, pyruvate decarboxylase and alcohol dehydrogenase. Ingram et al.⁵ inserted the genes encoding these two enzymes from *Z. mobilis* into *E. coli* under the control of a single enteric promoter to produce an artificial operon, designated the *pet* operon, for the production of ethanol. The resultant recombinant *E. coli* strain produced high levels of ethanol from glucose. Later Ohta et al.⁶ integrated the pyruvate decarboxylase (*pdh*) and alcohol dehydrogenase II (*adhB*) genes into the *E. coli* chromosome within or near the pyruvate formate-lyase gene (*pfl*). Strains carrying the *pet* operon in plasmid (e.g., *E. coli* B/pLOI297) or in chromosomal sites (e.g., *E. coli* KO11) require antibiotics in the medium to maintain genetic stability and high ethanol productivity.⁷ To overcome this requirement, Hespell et al.⁸ used the conditionally lethal *E. coli* strain FMJ39, which carries mutations for lactate dehydrogenase and pyruvate formate lyase and grows aerobically but is incapable of anaerobic growth unless these mutations are complemented.⁹ *E. coli* strains FBR1 and FBR2 were created by transforming *E. coli* FMJ39 with the *pet* (production of ethanol) operon plasmids pLOI295 and pLOI297, respectively. Both strains continued to produce high levels of ethanol from glucose in the absence of antibiotics. However, the strain FMJ39 does not metabolize xylose. Dien et al.¹⁰ was able to isolate FMJ39 mutants that do metabolize xylose and then transformed one of these mutants with plasmid pLOI297 which resulted in the new *E. coli* strain FBR3. The strain FBR3 selectively maintained the plasmid pLOI297 when grown anaerobically. Following 10 serial transfers of this strain in aerobic and anaerobic cultures containing either glucose or xylose with no selective antibiotics led to loss of the plasmid from the aerobic cultures. An average of 97.4 ± 3.5% of the cells maintained the plasmid in anaerobic cultures. The strain utilized 10% (w/v) each of glucose, xylose, arabinose or a mixture of these sugars [4% (w/v) glucose, 4% (w/v) xylose and 2% (w/v) arabinose] and produced 4.38–4.66% (w/v) ethanol. Dien

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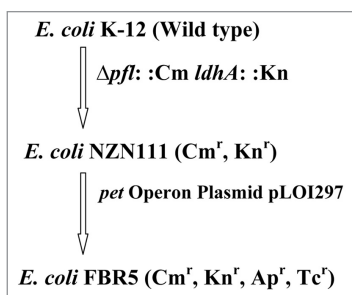


Figure 1. Construction of ethanologenic *Escherichia coli* strain FBR5. Details are available in references 12 and 11. Cm, chloramphenicol; Kn, kanamycin; Ap, ampicillin; Tc, tetracycline.

et al.¹¹ used this approach to construct two additional *E. coli* strains with different genetic backgrounds. They introduced the plasmid pLOI297 into two new *E. coli* strains (DC1368 and NZN111) that also carry *pfl* and *ldhA* mutations.¹² Unlike the parent strain (FMJ39x) for FBR3, the *pfl* mutation in these strains was introduced by genetic recombination and included an antibiotic resistance marker which is the gene for chloramphenicol acetyl transferase (*cat*) responsible for conferring resistance to chloramphenicol (Cm). These two recombinant *E. coli* strains were named FBR4 and FBR5, respectively. Serial transfer experiments demonstrated that the ethanol plasmid was stably maintained in the absence of antibiotics.¹¹ *E. coli* FBR5 performed better than FBR4 in ethanol production from corn fiber hydrolyzate. **Figure 1** shows an illustration outlining the construction of the recombinant *E. coli* strain FBR5. Nichols et al.¹³ constructed ethanologenic *E. coli* strains with the glucose phospho-transferase (*ptsG*) mutation. The mutants (FBR14, FBR16) had an altered pattern of mixed sugar utilization—xylose and arabinose were simultaneously consumed as fast as glucose, suggesting that the catabolic pathways for xylose and arabinose were fully activated despite the presence of glucose in the medium (FBR16). The fumarate dehydrogenase gene (Δ *frdABCD*) was further deleted in the mutant FBR16, preventing the production of succinic acid to minimize byproduct formation. However, *ptsG*- also disables active glucose transport in *E. coli* strain FBR16. For this reason, the mutants grew slower on glucose and were more sensitive to inhibitors present in corn fiber hydrolyzate than FBR5.¹⁴

Fermentation of Mixed Sugars

Dien et al.¹¹ investigated the fermentation of xylose (7.62%, w/v) and mixed sugars (7.62%, w/v) containing glucose (3.05%), xylose (3.05%) and arabinose (1.52%) by the recombinant *E. coli* FBR5; revealing that it produced 3.34 ± 0.04 and $3.40 \pm 0.05\%$ (w/v) ethanol from xylose and mixed sugars with the ethanol productivity of 0.66 ± 0.01 and 0.92 ± 0.04 gL⁻¹h⁻¹, respectively. The strain produced 39.2 to 41.5 g ethanol from 95 g xylose per L with ethanol productivity of 0.59 gL⁻¹h⁻¹. Qureshi et al.¹⁵ investigated substrate and product inhibition and kinetic parameters for ethanol production from xylose by the strain. The culture could tolerate very high xylose concentrations (up to 250 gL⁻¹) in the medium. The maximum ethanol produced from xylose was

43.5 gL⁻¹ and the bacterium tolerated a maximum of 50 g ethanol per L. In pH controlled experiments, the maximum ethanol productivity of 0.90 gL⁻¹h⁻¹ was obtained from xylose (90 gL⁻¹). The strain tolerated up to a maximum 40 g salt (sodium chloride) per L of fermentation broth but showed inhibition of growth at above 10 g salt per L.¹⁵

Fermentation of Lignocellulosic Hydrolyzates

The ethanol production from wheat straw by various pretreatments (hydrothermal, dilute acid, concentrated acid, lime, alkaline peroxide and microwave), enzymatic saccharification and batch fermentations of both non-detoxified and detoxified (by overliming) hydrolyzates by both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) using the recombinant *E. coli* strain FBR5 has been investigated in detail.¹⁶⁻¹⁹ Integration of process steps is important in lowering the cost of the production of ethanol from any lignocellulosic feedstock.³ SSF is an option that is considered a necessary step in this regard.²⁰ The optimal pH and temperature for the enzymatic hydrolysis of pretreated wheat straw and rice hulls were at 5.0 and 45–50°C.^{17,21} However, the recombinant *E. coli* FBR5 performed well at pH 6.5 and 35°C.¹¹ In SSF experiments, a compromised pH of 6.0 and temperature at 35°C were used. The minimum and maximum ethanol produced from pretreated wheat straw (86 gL⁻¹) in these studies were 13.0 ± 2.0 and 22.5 ± 0.6 gL⁻¹, respectively. The ethanol yields varied between 0.37 to 0.50 gg⁻¹ available sugars depending on the type of pretreatment used. The fermentation time also varied greatly from 17 to 136 h which was also highly dependent of the type of pretreatment and the inhibitory compounds present in the hydrolyzate. **Figure 2** shows the patterns of utilization of glucose, xylose, arabinose and total sugars and production of ethanol by the strain from alkaline peroxide pretreated and enzymatically saccharified wheat straw hydrolyzate.¹⁸ It is evident that the strain utilized glucose first, then arabinose and finally xylose at a slower rate, even though it did completely utilize xylose. Similar patterns of mixed sugar utilization were also observed in other pretreated hydrolyzates^{16,17,19,21-24} and with another ethanologenic recombinant *E. coli* ATCC 11,303 carrying plasmid pLOI297.²⁵ Saha et al.²¹⁻²⁴ obtained a maximum ethanol production of 18.7 ± 0.6 gL⁻¹ in 64 h from dilute acid pretreated rice hulls (150 gL⁻¹) and 11.9 ± 0.0 g ethanol per L in 17 h from alkaline peroxide pretreated barley straw (100 gL⁻¹) by SHF. Recently, Saha et al.²⁶ studied ethanol production by recombinant *E. coli* strain FBR5 from dilute acid pretreated wheat straw by SHF and SSF in detail. The yield of total sugars from dilute acid (0.5% H₂SO₄) pretreated (160°C, 10 min) and enzymatically saccharified (pH 5.0, 45°C, 72 h) wheat straw (86 gL⁻¹) was 50.0 ± 1.4 gL⁻¹. The hydrolyzate contained 1,184 \pm 19 mg furfural and 161 \pm 1 mg hydroxymethyl furfural (HMF) per L which are toxic byproducts. The recombinant *E. coli* FBR5 showed no growth at pH controlled at 4.5 to 6.5 in the non-abated wheat straw hydrolyzate at 35°C. However, it produced 21.9 ± 0.3 g ethanol per L from non-abated wheat straw hydrolyzate (total sugars, 44.1 ± 0.4 gL⁻¹) in 90 h including the lag time of 24 h when pH was controlled

at pH 7.0 and 35°C (SHF). It appears that this *E. coli* strain was able to metabolize furfural, HMF and acetic acid at pH 7.0. This finding is both important and encouraging because it provides an option of running the fermentation at pH 7.0 for SHF process without detoxifying the hydrolyzate. The bacterium produced 21.6 ± 0.5 g ethanol per L in 40 h from the bioabated and enzymatically saccharified wheat straw hydrolyzate (total sugars, 44.1 ± 0.4 g) at pH 6.0 and 35°C. The bioabatement of wheat straw was performed by growing *Coniochaeta ligniaria* NRRL 30616,²⁷ in the liquid portion of the pretreated wheat straw aerobically at pH 6.5 and 30°C for 15 h. The recombinant *E. coli* strain FBR5 produced 24.9 ± 0.3 g ethanol per L in 96 h and 26.7 ± 0.0 g ethanol per L in 72 h from bioabated wheat straw hydrolyzate by batch and fed-batch SSF, respectively. SSF offered a distinct advantage over SHF with respect to reducing the total time required to produce ethanol from the bioabated wheat straw by the recombinant *E. coli* strain FBR5. However, mixing is one of the major problems of using SSF for ethanol production from lignocellulosic feedstocks. By feeding the pretreated wheat straw 3 times in the reactor (fed-batch approach), Saha et al.²⁶ were able to complete the fermentation of bioabated wheat straw within 72 h. Fed-batch SSF performed better than the batch SSF with respect to shortening the time requirement and increase in ethanol yield.

Dien et al.¹¹ reported that the recombinant *E. coli* strain FBR5 produced a maximum of 30–42 g ethanol per L in 60 h from xylose (95 g L^{-1}) with ethanol yields of 86–92% of theoretical in batch culture. Qureshi et al.¹⁵ showed that the maximum ethanol that could be produced by the strain from xylose in batch culture was 43.5 g L^{-1} . Increasing the ethanol titer in the fermentation broth is crucially important for cost reduction of cellulosic ethanol production due to the high energy demand for ethanol recovery by distillation.²⁸ An ethanol concentration of 40 g L^{-1} or above in the fermentation broth could be considered as a bench mark for economically viable distillation.²⁹ Saha et al.³⁰ studied the ethanol production by the recombinant bacterium from wheat straw at high solids loading. The yield of total sugars from dilute acid (0.75% H_2SO_4 , v/v) pretreated (160°C, 10 min) wheat straw (150 g L^{-1}) after enzymatic saccharification at pH 5.0 and 45°C for 72 h was $86.3 \pm 1.5 \text{ g L}^{-1}$. The pretreated wheat straw was bio-abated by growing *C. ligniaria* NRRL 30616,²⁷ aerobically in the liquid portion for 16 h. The recombinant *E. coli* strain FBR5 produced 41.1 ± 1.1 g ethanol per L from non-abated wheat straw hydrolyzate (total sugars, $86.6 \pm 0.3 \text{ g L}^{-1}$) in 168 h at pH 7.0 and 35°C (SHF). The bacterium produced 41.8 ± 0.0 g ethanol per L in 120 h from the bioabated wheat straw by SHF. It produced 41.6 ± 0.7 g ethanol per L in 120 h from bioabated wheat straw by fed-batch SSF. The fed-batch SSF was started with one-fourth of the pretreated solid material in the fermentation broth and the remaining three portions were added at 16, 21 and 24 h. This approach helped significantly with the high solids (150 g L^{-1}) mixing problem. This is the first report of the production of > 40 g ethanol per L from a lignocellulosic hydrolyzate by this recombinant bacterium. A summary of the fermentation activity of recombinant *E. coli* FBR5 from dilute acid pretreated wheat straw hydrolyzate is presented in Table 1.

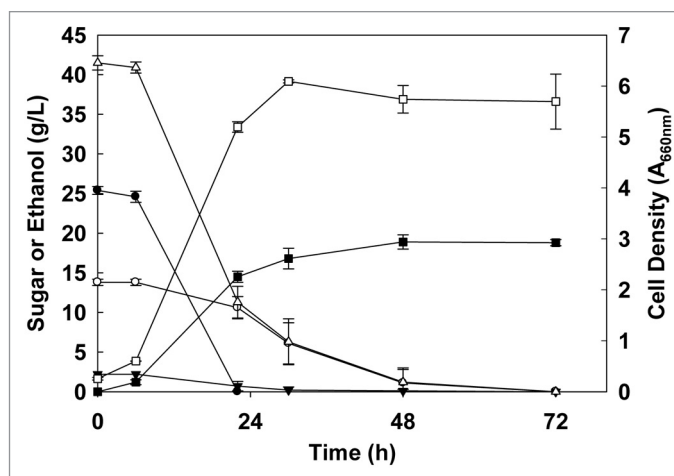


Figure 2. Time course of ethanol production by recombinant *Escherichia coli* FBR5 from alkaline H_2O_2 pretreated (2.15%, w/v, pH 11.5, 3 h) and enzymatically saccharified (45°C, pH 5.0, 120 h) wheat straw (8.6%, w/v) hydrolyzate at pH 6.5 and 35°C. The data presented are averages of two individual experiments. Symbols: ●, glucose; ○, xylose; ▼, arabinose; ▲, total sugars; ■, ethanol; □, cell density. From reference 18.

Liu et al.^{31,32} evaluated the performance of *E. coli* FBR5 for ethanol production from dilute acid hydrolyzate of hot water wood extract. Its growth was strongly inhibited in the dilute acid hydrolyzate of hot-water wood extract. The strain was then challenged by hot water wood extract (containing 59.1 g reducing sugars per L). After repeated strain adaptation, an improved strain FBHW was obtained. This *E. coli* strain FBHW was resistant to toxicity of the hydrolyzate in the fermentation medium containing concentrated hydrolyzate and xylose was completely utilized by the strain to produce ethanol. The strain FBHW was grown in the concentrated hydrolyzate without any detoxification. The yield of ethanol was 36.8 g L^{-1} of fermentation broth in 96 h. Sanny et al.³³ further engineered *E. coli* FBR5 using three different constructs, to contain and express *Vitreoscilla* hemoglobin gene (*vgh*). The three resulting strains expressed *Vitreoscilla* hemoglobin (VHb) at various levels, and the production of ethanol was inversely proportional to the expressed VHb level. High levels of VHb were correlated with an inhibition of ethanol production. However, the strain with the lowest VHb expression (approximately the normal induced level in *Vitreoscilla*), produced more ethanol than the parental strain FBR5 with glucose, xylose or corn stover hydrolyzate as the predominant carbon source under microaerobic condition in shake flasks.

Stability of Recombinant *E. coli* FBR5

Martin et al.³⁴ studied the stability of the ethanologenic *E. coli* strain FBR5 during continuous culture on glucose or xylose. They obtained stable ethanol yields of about 80–85% of the theoretical on glucose (50 g L^{-1}) or xylose (50 g L^{-1}) over 26 d at dilution rates of 0.075 h^{-1} for glucose and 0.045 h^{-1} for xylose under chemostat conditions using this strain. Recently, Saha and Cotta³⁵ studied the long-term performance of this recombinant bacterium in a series of continuous culture runs (16–105 d)

Table 1. Summary of fermentation of dilute acid pretreated wheat straw by recombinant *Escherichia coli* FBR5 at 35°C^{26,30}

Fermentation type	Fermentation time (h)	Total sugars (gL ⁻¹)	Ethanol (gL ⁻¹)	Ethanol productivity (gL ⁻¹ h ⁻¹)	Ethanol yield (gg ⁻¹ sugar)	Ethanol (gg ⁻¹ straw)
Wheat straw (86 gL⁻¹)						
Non-abated						
SHF (pH 7.0)	90	44.1 ± 0.4	21.9 ± 0.3	0.24	0.50	0.25
SSF (pH 7.0)	96	-	17.4 ± 1.8	0.18	0.39	0.20
Bioabated						
SHF (pH 6.5)	66	44.1 ± 0.4	21.6 ± 0.5	0.33	0.49	0.25
SSF (pH 6.0)	96	-	24.9 ± 0.3	0.26	-	0.29
Fed-batch SSF (pH 6.0)	72	-	26.7 ± 0.0	0.37	-	0.31
Washed solids						
SSF (pH 6.0)	96	-	12.2 ± 0.3	0.13	-	0.14
Wheat straw (150 gL⁻¹)						
SHF						
Non-abated (pH 7.0)	168	86.6 ± 0.3	41.1 ± 1.1	0.24	0.47	0.27
Bioabated (pH 6.5)	120	86.6 ± 0.3	41.8 ± 0.0	0.35	0.48	0.28
SSF						
Non-abated (pH 7.0)	-	-	0.0	-	-	-
Bioabated (pH 6.0)	104	-	41.6 ± 0.7	0.40	-	0.28

The dilute acid pretreatment of wheat straw (86 gL⁻¹, 0.5% H₂SO₄, v/v; 150 gL⁻¹, 0.75% H₂SO₄, v/v) was performed at 160°C for 10 min. Enzymatic saccharification was performed at pH 5.0 and 45°C for 72 h with a cocktail of 3 commercial enzyme (cellulase, β-glucosidase, and hemicellulase) preparations. For wheat straw (86 g/L), fed-batch SSF was performed by adding the substrate 3 times (0, 16 and 24 h) in 3 equal portions. Washed solid was prepared by separating the liquid portion of pretreated wheat straw and washing the residue with water. For wheat straw (150 gL⁻¹), fed-batch SSF was performed by adding the substrate 4 times (0, 16, 21 and 24 h) in 4 equal portions. SHF, separate hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation.

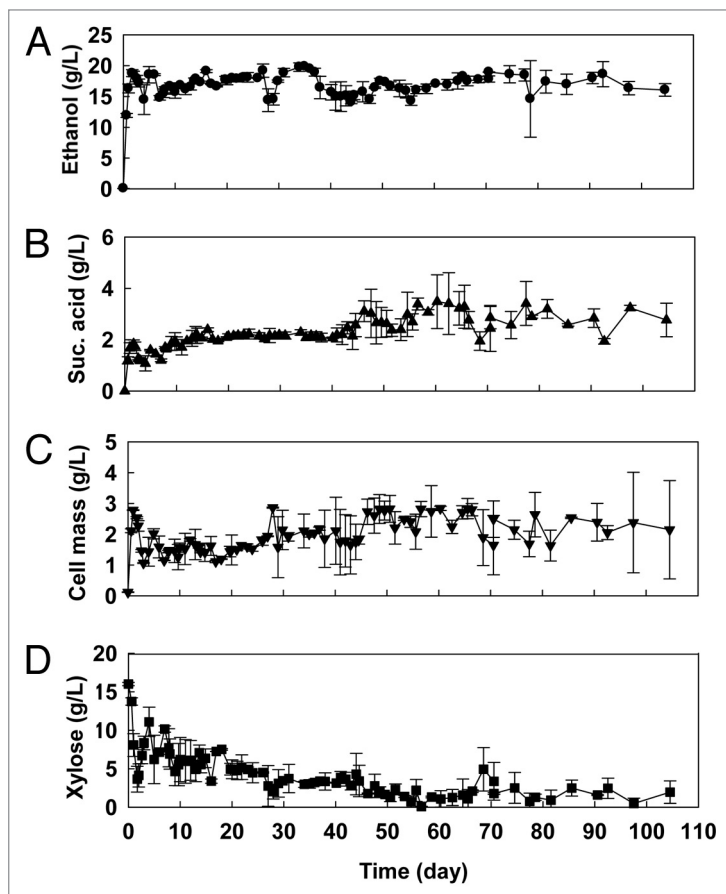


Figure 3. pH-Controlled continuous culture of recombinant *Escherichia coli* FBR5 on alkaline H₂O₂ pretreated (2.15%, w/v, pH 11.5, 3 h) and enzymatically saccharified (45°C, pH 5.0, 120 h) wheat straw hydrolyzate at pH 6.5 and 35°C. Dilution rate, 0.04 h⁻¹. Total sugars in feedstock, 44.1 gL⁻¹. Reactor volume, 240 ml. The data presented are averages of two parallel experiments. (A) Ethanol produced; (B) succinic acid produced; (C) cell mass; (D) residual xylose.³⁵

using alkaline peroxide pretreated and enzymatically saccharified wheat straw hydrolyzate as a feedstock without using any antibiotics. The average ethanol produced from the available sugars (21.9 to 47.8 gL⁻¹) ranged from 8.8 to 17.3 gL⁻¹ (0.28 to 0.45 gg⁻¹ available sugars, 0.31 to 0.48 gg⁻¹ sugar consumed) with ethanol productivity of 0.27 to 0.78 gL⁻¹h⁻¹ in a set of 14 continuous culture runs (16–105 d). During these studies, no loss of ethanol productivity was observed which indicates that the strain showed robustness in performance. The time courses of the production of ethanol and succinic acid, growth (cell mass) and residual xylose concentration are shown in Figure 3 for a set of two continuous cultures run up to 105 d. On average, about 11.2% of total sugars (22.8% of xylose) were left unutilized. Glucose was almost completely utilized. A disadvantage of recombinant *E. coli* strain FBR5 is that the fumarate reductase enzyme apparently functions in strain FBR5, since none of the four subunit genes were

disrupted allowing the production of considerable quantities of succinic acid.^{11,34} The fumarate reductase is only expressed anaerobically. Relatively stable succinic acid production (1.4–3.1 gL⁻¹) was observed in all these continuous culture experiments performed. Similar quantities (1.0–3.0 gL⁻¹) of succinic acid were produced by the recombinant *E. coli* FBR5 over the course of continuous culture experiments using 50 g glucose or xylose per L as feedstock.³⁴

Martin et al.³⁴ reported that the maximum ethanol concentration achievable in continuous cultures is frequently limited by the ethanol tolerance of the recombinant bacterium. Qureshi et al.¹⁵ reported that the maximum ethanol tolerance of the strain FBR5 was 50 gL⁻¹. Asghari et al.³⁶ reported that recombinant *E. coli* strain K011 produced about 45 gL⁻¹ ethanol from hemicellulose hydrolyzates of agricultural residues (bagasse, corn stover and corn hulls). The ethanol productivity by the recombinant *E. coli* FBR5 was decreased by 36% when the additional ethanol concentration was increased from 1.5 to 2.5%.³⁵

Lawford and Rousseau⁷ investigated the factors contributing to the loss of ethanologenicity of *E. coli* B recombinants pLOI297 and K011 employing glucose- or xylose limited chemostat cultures. Both recombinant strains carry markers (*cat* gene) for antibiotic resistance. Strain K011 expressed high levels of *cat* and was a spontaneous mutant that was selected to high levels (600 µg mL⁻¹) of chloroamphenicol. However, both recombinant strains exhibited rapid loss of ethanologenicity in chemostat cultures with glucose even when the selection pressure was imposed by the inclusion of antibiotics in the feed medium. Under xylose limitation, the plasmid-bearing recombinant *E. coli* strains appeared to be stabilized by antibiotics. These authors concluded that based on an average cost for large bulk quantities of antibiotics at \$55 kg⁻¹ and an inclusion level of 40 mgL⁻¹ of fermentation medium, the estimated economic impact regarding the potential stabilization by antibiotics in a plant operating in batch mode, the antibiotics cost can be \$0.29 gal⁻¹ of ethanol for antibiotic addition in all fermentation media. Thus, the stable nature of *E. coli* strain FBR5 without antibiotics is advantageous for industrial production of ethanol from a lignocellulosic hydrolyzate as the antibiotic use will not only add extra cost but also create environmental problems.

Martin et al.³⁴ suggests that if the *E. coli* FBR5 would have lost the plasmid, the revertant strain would simply be the host strain NZN111. This host strain does not produce ethanol.^{11,12} In conclusion, the recombinant *E. coli* FBR5 performed very well over the periods studied with respect to stability and viability without any antibiotics. The information is very

important for the use of the recombinant bacterium for continuous production of ethanol from lignocellulosic hydrolyzates. Based on the results of the continuous culture experiments, we have studied the production of ethanol by the recombinant strain *E. coli* FBR5 at the pilot scale (100 L) level using dilute acid pretreated wheat straw as a feedstock in order to demonstrate the performance of the strain for large scale ethanol production from lignocellulosic hydrolyzates. The results have not yet published.

Concluding Remarks and Future Directions for Research

The recombinant *E. coli* strain FBR5 performs well in the absence of antibiotics. Under proper conditions, it can ferment all sugars including xylose and arabinose to ethanol quantitatively and is also somewhat tolerant to common fermentation inhibitors such as furfural, HMF and acetic acid. It works better at neutral or near neutral pH range which makes the SSF process using the recombinant bacterium to run at a compromising pH 6.0. In comparison, the conventional yeast performs optimally at pH 4.5–5.0 which is also the optimum pH for action of cellulase and hemicellulase enzymes. The cost of enzymes remains a concern. Approaches can be taken to clone and express cellulase enzymes in the ethanologenic recombinant *E. coli* strain. The maximum ethanol tolerance of recombinant *E. coli* strain FBR5 is 50 gL⁻¹. Research needs to be performed to increase the ethanol tolerance of the bacterium. Even though considerable research has been done on the production of ethanol by the recombinant bacterial strain, no pilot plant demonstration of the fermentation process has been performed. To our knowledge, no studies have been done on the optimization of fermentation medium components which is also an essential part to lower the ethanol production cost. Moreover, there is no data available on the utilization of the post fermentation left-over materials including the *E. coli* cells for use as animal feed or for generating electricity after the product ethanol has been removed from the fermentation broth by distillation. These issues need to be addressed not only for ethanologenic recombinant *E. coli* strains but also for recombinant *S. cerevisiae* before their use industrially.

Note

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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