0273-2289/02/98-100/0327/$13.50

Reduction of Furfural to Furfuryl Alcohol by Ethanologenic Strains of Bacteria and Its Effect on Ethanol Production from Xylose

Tony Gutiérrez, Marian L. Buszko, Lonnie O. Ingram, and James F. Preston*

University of Florida,
Institute of Food and Agricultural Science,
Department of Microbiology and Cell Science,
PO Box 110700, Gainesville, FL 32611-0700,
E-mail: jpreston@ufl.edu

Abstract

The ethanologenic bacteria *Escherichia coli* strains KO11 and LYO1, and *Klebsiella oxytoca* strain P2, were investigated for their ability to metabolize furfural. Using high performance liquid chromatography and ^{13}C-nuclear magnetic resonance spectroscopy, furfural was found to be completely biotransformed into furfuryl alcohol by each of the three strains with tryptone and yeast extract as sole carbon sources. This reduction appears to be constitutive with NAD(P)H acting as electron donor. Glucose was shown to be an effective source of reducing power. Succinate inhibited furfural reduction, indicating that flavins are unlikely participants in this process. Furfural at concentrations >10 m*M* decreased the rate of ethanol formation but did not affect the final yield. Insight into the biochemical nature of this furfural reduction process may help efforts to mitigate furfural toxicity during ethanol production by ethanologenic bacteria.

Index Entries: Furfural detoxification; furfuryl alcohol; ethanol fermentation; *Escherichia coli; Klebsiella oxytoca*.

Introduction

Furfural is the most widely distributed simple furan in nature and is found as a component in plant oils, fruit juices, alcoholic beverages, and cooked foods *(1,2)*. It is an aldehyde with an aromatic nature, first described by Dobereiner in 1832 *(3)*, with large-scale production initiated in 1922 *(4)*.

*Author to whom all correspondence and reprint requests should be addressed.

Industrially, it is formed by the acid hydrolysis of pentosan-containing lignocellulosics such as corncobs, sugarcane bagasse, cottonseed hulls, and oat or rice hulls *(3,5)*. Small amounts of furfural (3–15 m*M*) are also formed during the dilute-acid hydrolysis of biomass for production of hemicellulose-derived syrups *(6)*. Although beneficial as a flavoring agent in the food industry *(7)*, furfural is recognized as an important inhibitor of ethanol production from hemicellulose hydrolysate *(8)*. Rates of ethanol production and final ethanol yields can be adversely affected by the presence of furfural, thus creating an unwanted limitation in ethanol production processes that can be attributed to furfural's antimicrobial activity *(9–14)*.

Within the past 10 yr numerous bacteria and yeast have been reported to transform furfural to either furfuryl alcohol or furoic acid, or a combination of both. *Saccharomyces* spp. have been shown to reduce furfural to furfuryl alcohol by a two-electron reduction *(15)*. Boopathy et al. *(16)* demonstrated that various enteric bacteria can cometabolize furfural into alcohol under both aerobic and anaerobic conditions. Several groups have reported the anaerobic degradation of furfural by *Desulfovibrio* spp. under sulfate-reducing conditions *(17–19)*. By using ^{1}H nuclear magnetic resonance (NMR) spectroscopy, Schoberth et al. *(20)* were able to not only follow the formation of furfuryl alcohol, furoic acid, and acetate from furfural by *Desulfovibrio furfuralis*, but were also able to detect trace amounts of ethanol that had previously escaped detection by gas chromatographic analysis. This indicates a potential for the development of ethanologenic organisms to metabolize furfural into ethanol. Other organisms reported to transform furfural include various strains of *Escherichia coli (16–21)*, *Rhodococcus erythropolis (21)*, and *Pseudomonas* spp. *(21–23)*.

The ethanologenic derivatives of *E. coli B*, strains KO11 and LYO1, and that of *Klebsiella oxytoca* M5A1, strain P2, were engineered by Ingram et al. *(24–26)* for fuel ethanol production. Previous studies with these *E. coli* strains have shown that inhibitors derived from the dilute-acid hydrolysis of lignocellulosics, such as furfural, are detrimental to ethanol production from glucose *(9,14,27,28)*. Insight into the physiological and biochemical processes affecting furfural's fate should assist our efforts toward mitigating limitations in ethanol production that are caused by furfural. We determined the furfural-biotransformation potential of each strain using high-performance liquid chromatography (HPLC) and ^{13}C NMR spectroscopy.

Materials and Methods

Organisms and Media

E. coli strains KO11 *(25)*, LYO1 *(26)*, and *K. oxytoca* strain P2 *(24)* were used. Strains KO11 and LYO1 are derivatives of *E. coli* B and contain chromosomally integrated *Zymomonas mobilis* genes (*pdc* and *adhB*) for ethanol production. Stock cultures were maintained at 4°C on Luria-Bertani (LB)

agar medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per L of water). Inocula were prepared in LB broth, supplemented with or without furfural as required, and grown to the mid- to late exponential phase prior to use for experimentation. Seed cultures were prepared by inoculating 50 mL of LB broth with colonies from a fresh plate and incubating for approx 5 h (37°C, 190 rpm). These cultures were used as inocula at a final level of 1% (v/v).

Biotransformation Potential of Furfural for Each Strain

Four types of experiments were performed to investigate furfural metabolism and its effects on fermentation. Experiments were performed in screw-capped 125-mL Erlenmyer flasks containing 50 mL of LB broth with the 1% cell inoculum (37°C, 190 rpm). Each strain was inoculated into 50 mL of LB medium supplemented with furfural (4–4.5 m*M*). For experiments 1–3, HPLC was used to monitor furfural metabolism. Samples for HPLC analysis and turbidity measurements (OD_{600}) were taken at the time of inoculation and periodically thereafter.

For experiment 1, furfural was added to LB broth prior to inoculation. Experiments 2 and 3 both contained furfural and 0.5% xylose in LB broth; however, with the latter experiment, the furfural was added 5 h after inoculation rather than initially. An LB broth culture without furfural served as control.

Experiment 4 used NMR spectroscopy to monitor the bioconversion of $^{13}C_1$-furfural. For this experiment, cells were inoculated (1%) into a 12-mm NMR tube (Wilmad Glass, Buena, NJ) containing 3.5 mL of LB medium supplemented with $^{13}C_1$-furfural to approx 5 m*M* final concentration. Acquisitions were made every 30 min during incubation at 37°C in the NMR tube. $^{13}C_1$-Propionic acid, with a chemical shift of 186 ppm for the C1 carbon, was used as the external standard.

Preparation of Cell-Free Extracts and Dialysates from Strain LYO1

Cell-free extracts were prepared from 1 L of cells grown to the late exponential phase in LB medium. The cells were harvested, washed twice with 25 m*M* potassium phosphate buffer (pH 7.0) and resuspended in the same buffer (total volume of 12 mL). The concentrated cell suspension was disrupted by two passages through an Aminco French pressure cell at approx 16,000 lb/in^{-2} (5°C). A portion of the cell lysate was dialyzed through a 7-kDa pleated SnakeSkin dialysis membrane (Pierce, IL) in a 2-L solution of 25 m*M* phosphate buffer (pH 7.0, 4°C, 2 h). Both the dialyzed and nondialyzed extracts were centrifuged and filtered (0.45 µm) to remove undisrupted cells and particulate matter prior to immediate use.

Effect of Coenzymes and Metabolites on Furfural Reduction In Vitro

For furfural reduction each reaction mixture contained 25 m*M* potassium phosphate buffer (pH 7.0), 200 µL of a freshly prepared cell-free

extract, 3.4 m*M* furfural, and either NAD(P)H (3.34 m*M*), glucose, or succinate (6.68 m*M*), in a final volume of 1 mL. A fifth reaction mixture was also included, omitting the addition of any NAD(P)H, glucose, or succinate, to act as an untreated control. Furan concentrations were determined initially and after 4 and 21 h of incubation (37°C, no agitation) by HPLC analysis.

To determine the stoichiometry of furfural reduction, in vitro reactions were carried out using dialyzed cell-free extracts as follows: 25 m*M* potassium phosphate buffer (pH 7.0), 50 µL of a freshly prepared dialyzed extract, 4.2 m*M* furfural, in a final volume of 1 mL. Samples were removed at various times during incubation (37°C, no agitation). Three reaction conditions were investigated. After 2.25 h of incubation, NADH, NADPH, or glucose (2 m*M* each) was added to each of the three reaction mixtures. Sampling was continued for another 1.75 h.

Analytical Techniques

The concentrations of furfural, furfuryl alcohol, xylose, and ethanol were determined by HPLC using a Waters Millenium HPLC system equipped with a Model 712 WISP injector, a dual-wavelength detector (Model HM/HPLC holochrome), and a Waters 410 refractometer. A 300 × 7.8 mm Aminex HPX-87H column (Bio-Rad, Hercules, CA) was used at 35°C. The eluent was 0.01 *N* H_2SO_4 containing 20% acetonitrile *(29)*, and the flow rate was 0.4 mL/min^{-1}. Samples were diluted as required prior to injection (25 µL).

For NMR spectroscopy, an NT300 spectrometer, adapted for data analysis over the Internet *(30)*, was operated in the Fourier Transform mode. Instrument conditions were as follows: ^{13}C observed frequency, 75.460 MHz; excitation pulse width, 20 µs; pulse repetition delay, 3 s; acquisition time per spectrum, 30 min; spectral width, 20 kHz; sample tube, 12 mm. A $^{1}H/^{13}C$ dual probe was used with ^{1}H broadband decoupling and NOE enhancement.

Total protein concentration was determined using the BCA protein assay kit (Sigma, St. Louis, MO) with bovine serum albumin as the standard. Total carbohydrate was assayed as described previously by Dubois et al. *(31)* with glucose as the standard. Absorbance and cell turbidity measurements (OD_{600}) were performed using a Hewlett Packard 845A diode array spectrophotometer.

Chemicals

Xylose, succinate, NADH, and NADPH were obtained from Sigma; glucose from Fisher (Fair Lawn, NJ); furfural and furfuryl alcohol from Aldrich (Milwaukee, WI); and $^{13}C_1$-propionic acid and $^{13}C_1$-xylose from Cambridge Isotope Laboratories (Andover, MA). $^{13}C_1$-Furfural was prepared by distillation of a reaction mixture containing $^{13}C_1$-xylose in 6 N HC1, as described in TAPPI Test Methods (1994–1995).

Results and Discussion

Reduction of Furfural During Growth

We used HPLC and NMR spectroscopy to investigate the fate of furfural during the growth of *E. coli* strains KO11 and LYO1, and *K. oxytoca* strain P2. Results presented in Fig. 1 show the reduction of furfural (4–5 m*M*) in LB medium with no added xylose or other carbon source. Furfural was completely reduced by each strain with an almost stoichiometric amount of furfuryl alcohol produced as the sole product of this biotransformation. This one-step conversion is beneficial for both cell growth and ethanol production because furfuryl alcohol is less toxic, by approximately six-fold to KO11 and LYO1, as compared with furfural *(14,28)*. It has been suggested that the aldehyde group on the furan ring inhibits, or reduces, the rate of ring opening *(5)*, which is why most organisms with the ability to transform furfural do not often metabolize it any further than furfuryl alcohol or furoic acid. With each strain, the transformation of furfural to the alcohol was concurrent with bacterial growth, thus indicating that biotransformation of furfural was a function of cellular metabolism. In the absence of cells or cell extracts, the reduction of furfural to the alcohol did not occur, and the initial concentration of the furfural was totally quantifiable and constant after a period that ranged the full length of our experiments (results not shown). The reduction in each case commenced after 1–2 h of incubation coinciding with the beginning of the exponential phase of growth. This initial lag can be explained by the low concentration of the enzyme(s) catalyzing this reduction, which is thus a direct consequence of the low concentration of cells present in the inoculum. *E. coli* strains KO11 and LYO1 and *K. oxytoca* strain P2 reduced >90% of the furfural to furfuryl alcohol in 4–5 h.

Furthermore, none of the strains performed this biotransformation when furfural was supplied as the sole carbon source in a minimal medium (results not shown). As previously observed with various enteric bacteria *(16)*, the biotransformation of furfural in minimal medium was shown to occur only cometabolically when an alternative carbon source such as glucose was present. This suggests that an alternative carbon source was required for growth and also as a source of reducing potential for the reduction of the furfural. In our experiments using LB medium (without xylose), reducing potential may have been provided by the metabolism of compounds in tryptone and/or yeast extract.

Figure 2 displays the time-lapse NMR spectra acquired during growth of strain KO11 in LB medium (without xylose) supplemented with $^{13}C_1$-labeled furfural. The signal of the labeled aldehyde group of furfural, with a chemical shift at 182 ppm, decreased over time and coincided with the appearance of only one signal identified to be that of the C1 of furfuryl alcohol, with a chemical shift at 57 ppm. *E. coli* strain LYO1 and *K. oxytoca* P2 produced very similar spectra (results not shown) to those of strain

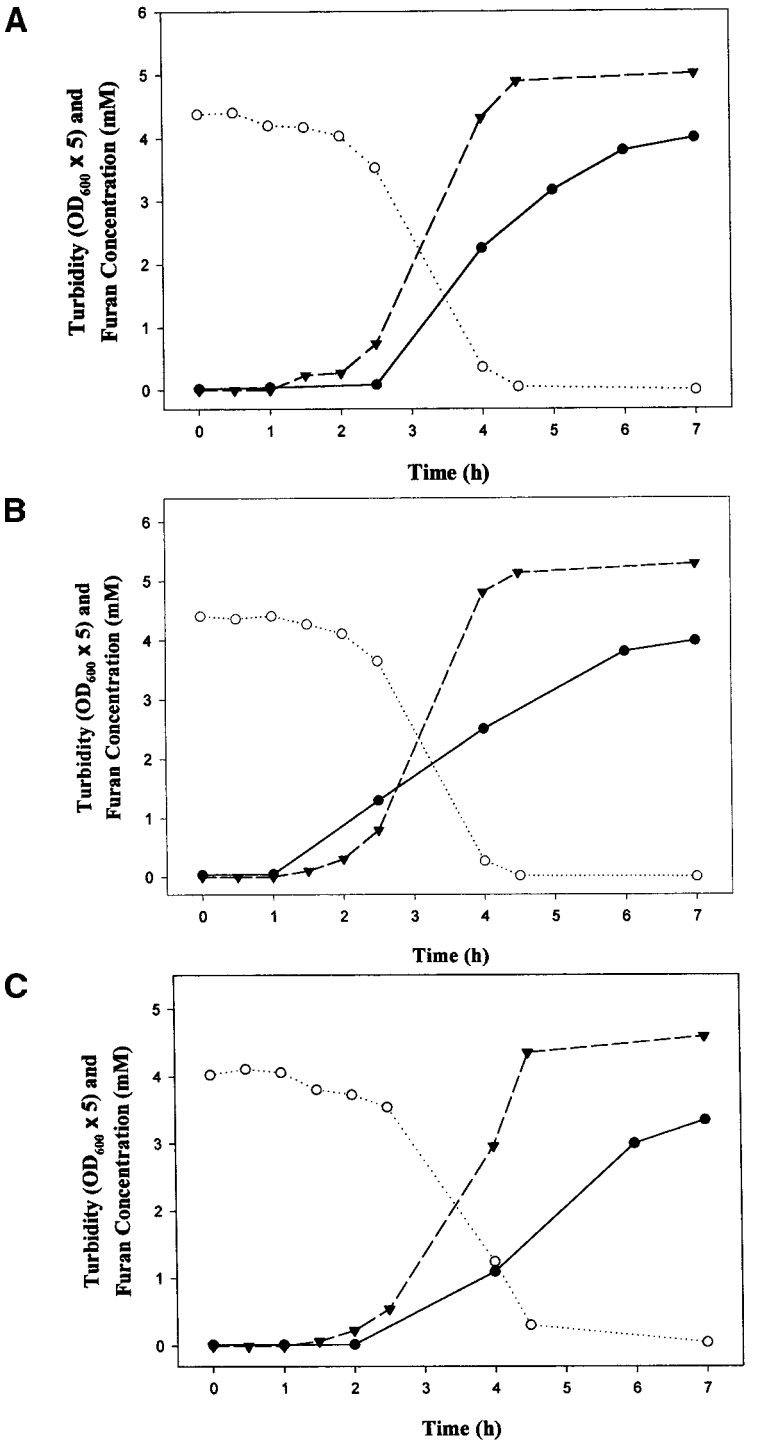

Fig. 1. Formation of furfuryl alcohol from furfural by *E. coli* strain KO11 **(A)**, LYO1 **(B)**, and *K. oxytoca* strain P2 **(C)** during growth in LB medium (no sugar added) supplemented with furfural added prior to inoculation. ●, Cell turbidity; ○, furfural; ▼, furfuryl alcohol.

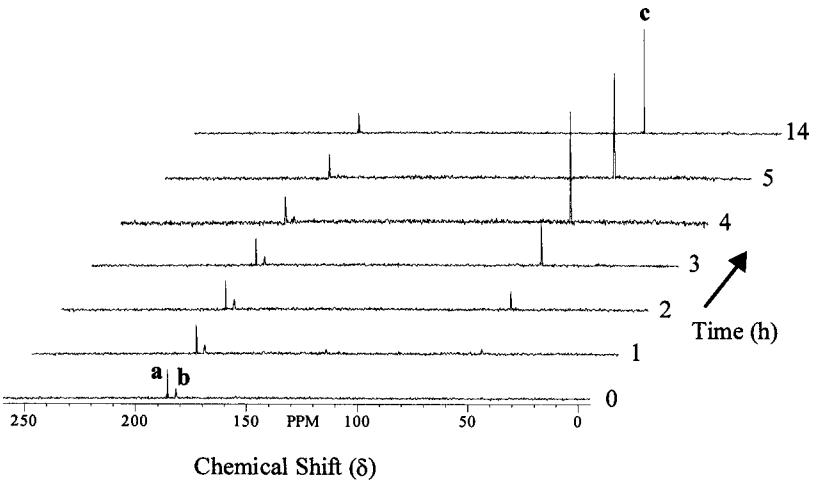

Fig. 2. Time sequence of NMR spectra acquired during growth of *E. coli* strain KO11 with C1-labeled ^{13}C-furfural in LB medium (no sugar added). The chemical shift, relative to propionic acid at 186 ppm (a), was 182 ppm for furfural (b) and 57 ppm for furfuryl alcohol (c).

KO11. Furthermore, furfuryl alcohol was detected within the first hour of incubation, suggesting that the genes encoding a reductase(s) catalyzing this reduction may be constitutively expressed. This constitutive nature for furfural metabolism has also been shown with the yeast *Candida guillermondii (13)*. The appearance of a significantly stronger furfuryl alcohol signal, compared with that of the furfural, is due to a nuclear Overhauser effect for the aldehyde group *(32)*.

Biotransformation of Furfural During Ethanol Production

As shown in Fig. 3, the transformation of furfural to furfuryl alcohol by strain KO11, growing in LB medium containing 0.5% xylose, commenced prior to the onset of xylose metabolism and ethanol production, with 38% transformed before a detectable decrease in xylose was observed. Similar results were obtained with strain LYO1, a derivative of KO11 selected for tolerance to a higher concentration of ethanol (results not shown). By the time both strains began to metabolize xylose and produce ethanol at maximal rates, a total of 85–90% of the furfural was transformed. Furthermore, the lag preceding the onset of xylose metabolism and ethanol production was 4 h, compared with 3 h when the strains were grown under identical conditions in the absence of furfural. Similar results were reported by Palmqvist et al. *(15)*, who showed furfural to lengthen the lag phase in acetate production by 4 h, as well as to inhibit the excretion of glycerol by *Saccharomyces cerevisiae* cells during furfural reduction. In addition, they showed acetaldehyde and pyruvate to be excreted during the furfural reduction phase, which they suggested to be the result of furfural acting as a redox sink, redirecting the pool of reducing power in the form of either

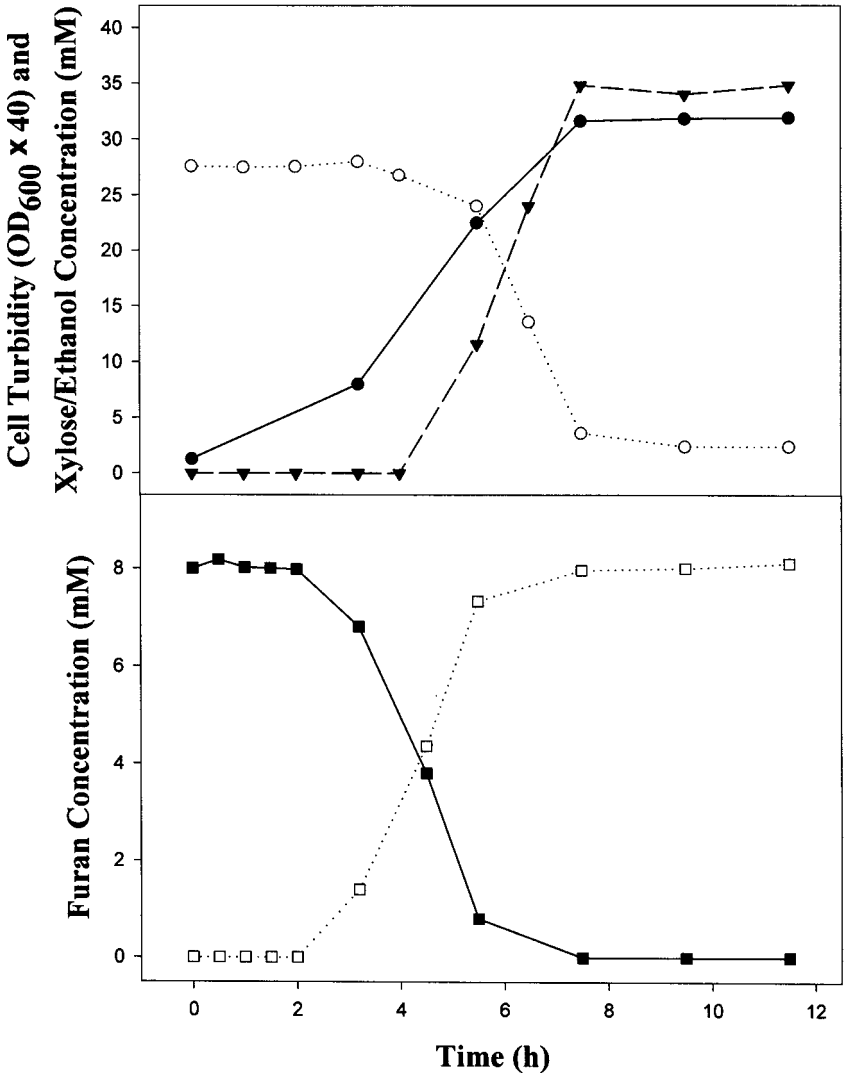

Fig. 3. Transformation of furfural to furfuryl alcohol by *E. coli* strain KO11 during early stages of growth and production of ethanol from xylose (0.5%) in LB medium. ●, Cell turbidity; ○, xylose; ▼, ethanol; ■, furfural; □, furfuryl alcohol.

NADH or NADPH and oxidizing it for its own reduction to furfuryl alcohol. Since furfural produced a lag in ethanol production with strains KO11 and LYO1, reducing power may have been redirected away from reduction of acetaldehyde to ethanol, at least during this early stage in growth. This is supported by the results of other researchers who have shown furfural to interfere with electron transport *(33)* and inhibit glycolytic enzymes *(34)*. It was only after most of the furfural had been reduced (>85%) by KO11 and LYO1 that both xylose metabolism and ethanol production proceeded at a maximal rate.

On the other hand, furfural may have had a direct inhibitory effect on the activity of an enzyme(s) in the ethanol pathway of these strains. Compared to a furfural-untreated control, concentrations of furfural, within the limits of those used in our experiments, have not been shown to reduce the final yield of ethanol from xylose by strains KO11 and LYO1 *(9)*. Hence, by using conditions for ethanol production with a supplemented medium (LB), similar to those of an industrial system employing supplemented hydrolysate material, the bioconversion of furfural to the alcohol may increase the lag in fermentative growth and onset of ethanol production, but it has little effect on the final ethanol yield. This suggests that the reducing power, which is initially redirected away from ethanol production and used for furfural reduction, is at a later stage in growth replaced by a resource(s) other than xylose, possibly from the yeast extract or tryptone present in the supplemented (LB) medium.

Results presented in Fig. 4 provide further support for the constitutive nature of the furfural reductase since no detectable lag in the reduction of furfural to furfuryl alcohol was observed when furfural was added directly into a growing culture of *E. coli* strain LYO1. Furfural was reduced so rapidly that its initial concentration—i.e., at the time when it was added into the medium—could only be estimated on the basis of the amount added. The final concentration of furfuryl alcohol (10.5 m*M*) formed from this reduction is essentially equivalent to the estimated 10.0 m*M* initial furfural concentration. At these concentrations, we have shown furfural to have no effect on the growth yield of either of these *E. coli* strains on LB medium (results not shown). However, as shown in Fig. 4, the addition of furfural at a time point during maximal metabolism of xylose and ethanol production caused a detectable decrease in both of these rates from 0.137 to 0.099 m*M*/min for xylose, and 0.176 to 0.069 m*M*/min for ethanol. These rates remained constant during this time range in the furfural-untreated control (results not shown). Hence, the addition of 10.0 m*M* furfural to a growing culture of strain LYO1 produced a 28 and 61% decrease in the rates of xylose metabolism and ethanol production, respectively. Although the ethanol production rate appeared to continue nonuniformally thereafter, the final ethanol yield was unaffected compared with that of a furfural-untreated control (results not shown). The results suggest that furfural either acts as a redox sink to redirect reducing power from ethanol production, or directly inhibits an enzyme(s) involved in the conversion of sugar to ethanol. However, since the final ethanol yield was unaffected, this once again points to the presence of another resource of reducing power in the supplemented medium, other than the xylose, that can replenish that used for furfural reduction.

Effect of NAD(P)H, Glucose, and Succinate on Furfural Reduction in Cell-Free Extracts

As shown in Fig. 5, the coenzymes NADH and NADPH increased the reduction of furfural significantly in cell-free extracts of LYO1. NADH was

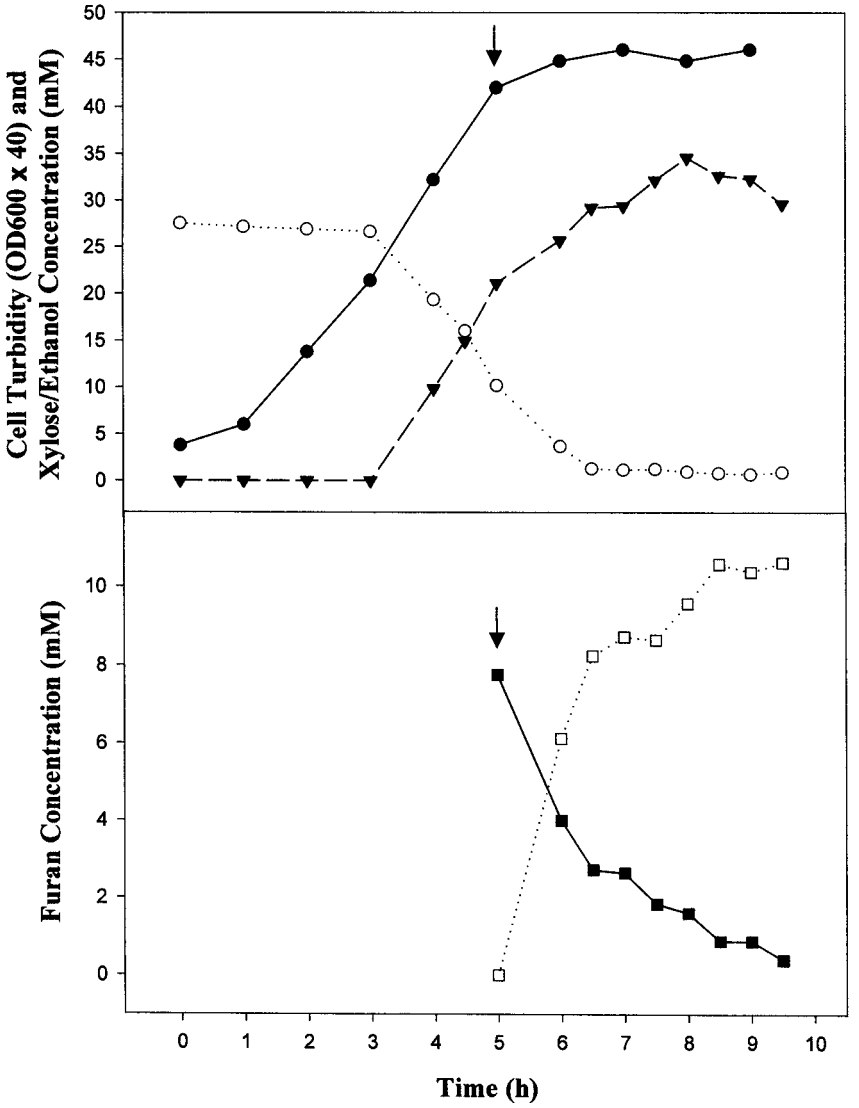

Fig. 4. Immediate effect of furfural addition on xylose metabolism and ethanol production by *E. coli* strain LYO1. Furfural (10 mM) was added to the growing culture at 5 h of incubation. Arrow indicates time of furfural addition. ●, Cell turbidity; ○, xylose; ▼, ethanol; ■, furfural; □, furfuryl alcohol.

able to reduce 10.6 and 2.2% more of the furfural after 4 and 21 h, respectively, compared to NADPH. This dual specificity is not surprising considering that aldehyde reductases, which are recognized as cytosolic enzymes belonging to the aldose-ketose oxidoreductase superfamily, are known to have a dependency for either or both coenzymes NAD(P)H *(35)*. Glucose was also able to support the reduction of furfural in these crude extracts, with 100% reduction accomplished within the first 4 h (Fig. 5). Sugars such as glucose and fructose have been shown to perform similar effects in other

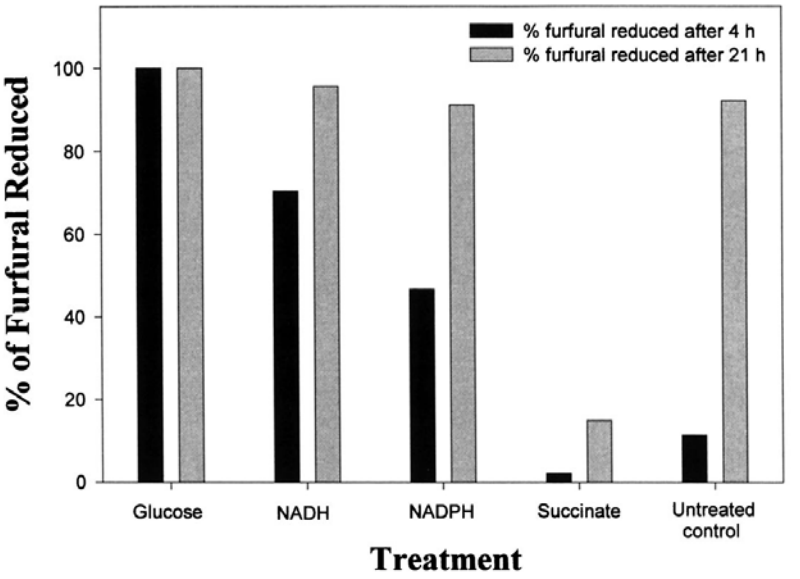

Fig. 5. Effect of NAD(P)H (3.34 m*M*), glucose (6.68 m*M*) and succinate (6.68 m*M*) on reduction of furfural by a nondialyzed cell-free extract of *E. coli* strain LYO1 in reaction mixtures containing 200 µL of the extract, 3.4 m*M* furfural, and 25 m*M* phosphate buffer (pH 7.0) in total volume of 1 mL.

microbial systems by acting as regenerators of reducing power *(36–39)*. Succinate was evaluated to determine whether flavins, such as $FADH_2$, could serve to reduce furfural. In fact, the addition of succinate caused an 11.1 and a 77.4% decrease in the reduction of the furfural after 4 and 21 h, as compared to the control without any added coenzymes or organics (Fig. 5). In spite of its partial aromatic nature, the reduction of furfural appears to be favored by hydride ion–mediated reduction with reduced adenine dinucleotides rather than unpaired electrons that would be derived from flavins.

Furthermore, the untreated reaction mixture displayed furfural-reducing ability, with 13.3% of the furfural being reduced and converted to furfuryl alcohol in 4 h. Although this shows a much slower rate of reduction compared to when NAD(P)H or glucose were present, most of the furfural (92.3%) was completely reduced in 21 h. The most plausible explanation to account for this complete reduction of the furfural is that this sample of crude extract contained a total of 2.68 mg of glucose equivalents/mL (or 110 µg/mg of protein), as determined by a total carbohydrate analysis. A candidate source for this glucose is glycogen, a large molecular weight polymer common to *E. coli* as well as many other bacteria, which on phosphorolysis could provide 14.9 m*M* glucose equivalents as glucose-1-phosphate. Assuming each mole equivalent of glucose could be oxidized to generate at least 1 mole equivalent of NAD(P)H, even with the possibility of side reactions involving oxidation of NAD(P)H, this endogenous carbohydrate should be more than adequate to supply the reducing power

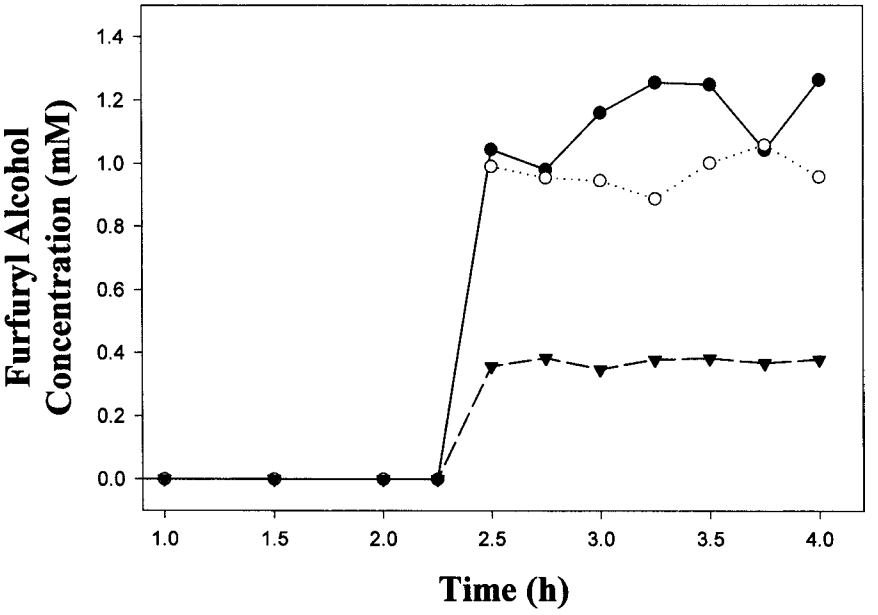

Fig. 6. Stoichiometry of 2 m*M* NADH, NADPH, or glucose to production of furfuryl alcohol from furfural by a dialyzed cell-free extract of *E. coli* strain LYO1 in reaction mixtures containing 50 µL of the dialyzed extract, 4.2 m*M* furfural, and 25 m*M* phosphate buffer (pH 7.0) in total volume of 1 mL. Furfuryl alcohol concentrations when NADH (●), NADPH (○), and glucose (▼) were tested are shown.

needed to reduce 3.4 m*M* furfural to furfuryl alcohol. To support this hypothesis, we have shown the addition of glycogen to increase the rate of furfural reduction in these reaction mixtures (results not shown). Since these extracts were dialyzed prior to use in these experiments, the presence of an endogeneous soluble pool of reducing power taking part in this reduction can be ruled out. Furthermore, since the extracts were derived from cells grown in the absence of furfural, the fact that they displayed the ability to reduce furfural further confirms that the enzyme(s) system catalyzing this reduction is of a constitutive nature.

Effect of NAD(P)H and Glucose on Furfural Reduction in Dialyzed Extracts

On a molar basis, both NADH and NADPH were more effective as a source of reductant than glucose (Fig. 6). A complete stoichiometric reduction of furfural, however, was not obtained. This may be because of the presence of other components in the system that also oxidized the NAD(P)H. This was confirmed spectrophotometrically at 340 nm, where dialyzed and cell-free extracts were able to solely, and in the absence of any added furfural, oxidize NAD(P)H (results not shown). Therefore, reducing potential may have been lost to side reactions, accounting for

the incomplete reduction of 2 m*M* furfural to 2 m*M* furfuryl alcohol by 2 m*M* NAD(P)H.

Concentrations of furfural (>10 m*M*) higher than those used in our study have been shown to be toxic to strains KO11 and LYO1, as well as to reduce final ethanol yields *(14)*. Based on our results and the constitutive nature of the furfural reduction, one has two options to mitigate furfural inhibition under circumstances in which the feedstock material used for ethanol production contains concentrations of >10 m*M* furfural. The first is to increase the level of enzyme(s) that reduces the furfural. Alternatively, we could attempt to make the cells intrinsically more resistant to furfural. To explore the former option, it would be important to identify the gene(s) encoding activities for furfural reduction, and to increase their level of expression. Although at this point the furfural-reducing activity may not be assigned to a specific enzyme, it may be similar to alcohol dehydrogenase enzymes since some organisms, primarily yeast, have been reported to carry out the reduction of furfural utilizing an alcohol dehydrogenase *(40,41)*. Using micro-array technology, we have recently identified the expression levels of the many genes in *E. coli* strains KO11 and LYO1 (42). A total of 20 putative dehydrogenases and 40 oxidoreductases were observed to be constitutively expressed at high levels during the growth of these strains under similar conditions used in the present study in which furfural was not present in the medium. It is these genes that will be investigated for their role in furfural reduction.

Acknowledgments

We appreciate the insightful comments made by K. T. Shanmugam on the manuscript.

This work was supported by US Department of Agriculture grant 9804921; US Department of Energy grant FG02-96ER20222; and the Institute of Food and Agricultural Sciences, University of Florida Experiment Station, as CRIS Project MCS 03763. This article is Journal Series No. R-08243 of the Florida Agricultural Experiment Station.

References

1. Dean, F. M. (1963), *Naturally Occurring Ring Compounds*, Butterworths, London.
2. Trudgill, P. W. (1984), in *Microbial Degradation of Organic Compounds*, Gibson, D. T., ed., Marcel Dekker, NY, pp. 295–307.
3. Dobereiner, W. (1832), *Ann. der Chem.* **3,** 141–146.
4. QO Chemicals (1989), Bulletin 203-D, QO Chemicals, West Lafayette, IN.
5. Dunlop, A. P. (1948), *Ind. Eng. Chem.* **40**, 204–209.
6. Martinez, A., Rodriguez, M. E., Wells, M. L., York, S. W., Preston, J. F., and Ingram, L. O. (2001), *Biotechnol. Prog.* **17**, 287–293.
7. Bauer, K., Garbe, D., and Surburg, H. (1990), *Common Fragrance and Flavor Materials*, VCH, NY, p. 111.
8. Azhar, A. F., Bery, M. K., Colcord, A. R., Roberts, R. S., and Corbitt, G. V. (1981), *Biotechnol. Bioeng. Symp.* **11**, 293–300.
9. Beall, D. S., Ohta, K., and Ingram, L. O. (1991), *Biotechnol. Bioeng.* **38**, 296–303.

10. Ranatunga, T. D., Jervis, J., Helm, R. F., McMillan, J. D., and Hatzis, C. (1997), *Appl. Biochem. Biotechnol.* **67**, 185–195.
11. Taherzadeh, M. J., Niklasson, C., and Liden, G. (1999), *Bioresour. Technol.* **69**, 59–66.
12. Taherzadeh, M. J., Gustafsson, L., Niklasson, C., and Liden, G. (1999), *J. Biosci. Bioeng.* **87**, 169–174.
13. Sanchez, B., and Bautista, J. (1988), *Enzyme Microb. Technol.* **10**, 315–318.
14. Zaldivar, J., Martinez, A., and Ingram, L. O. (1999), *Biotechnol. Bioeng.* **65**, 24–33.
15. Palmqvist, E., Almeida, J. S., and Hahn-Hagerdal, B. (1999), *Biotechnol. Bioeng.* **62**, 447–454.
16. Boopathy, R., Bokang, H., and Daniels, L. (1993), *J. Ind. Microbiol.* **11**, 147–150.
17. Boopathy, R., and Daniels, L. (1991), *Curr. Microbiol.* **23**, 327–332.
18. Brune, G., Schoberth, S. M., and Sahm, H. (1983), *Appl. Environ. Microbiol.* **46**, 1187–1192.
19. Folkerts, M., Ney, U., Kneifel, H., Stackebrandt, E., Witte, E. G., Forstel, H., Schoberth, S. M., and Sahm, H. (1989), *Syst. Appl. Microbiol.* **11**, 161–169.
20. Schoberth, S. M., Bubb, W. A., Chapman, B. E., and Kuchel, P. W. (1993), *J. Microbiol. Methods* **17**, 85–90.
21. Wang, P., Brenchley, J. E., and Humphrey, A. E. (1994), *Biotechnol. Lett.* **16**, 977–982.
22. Hong, S. W., Han, H. E., and Chae, K. S. (1981), *J. Liquid Chromatogr.* **4**, 285–292.
23. Lee, B. U., Yu, B. S., Lee, K. J., and Hah, Y. C. (1985), *Korea J. Microbiol.* **23**, 241–247.
24. Ohta, K., Beall, D. S., Mejia, J. P., Shanmugan, K. T., and Ingram, L. O. (1991), *Appl. Environ. Microbiol.* **57**, 2810–2815.
25. Ohta, K., Beall, D. S., Shanmugan, K. T., and Ingram, L. O. (1991), *Appl. Environ. Microbiol.* **57**, 893–900.
26. Yomano, L. P., York, S. W., and Ingram, L. O. (1998), *J. Ind. Microbiol. Biotechnol.* **20**, 132–138.
27. Zaldivar, J., and Ingram, L. O. (1999), *Biotechnol. Bioeng.* **66**, 203–210.
28. Zaldivar, J., Martinez, A., and Ingram, L. O. (2000), *Biotechnol. Bioeng.* **68**, 524–530.
29. Yuan, J.-P., and Chen, F. (1999), *Food Chem.* **64**, 423–427.
30. Buszko, M. L., Buszko, D., and Wang, D. C. (1998), *J. Magn. Reson.* **131**, 362–366.
31. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Analy. Chem.* **28**, 350–356.
32. Neuhaus, D. and Williamson, M. (1989), *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, VCH, NY.
33. Vitrinskaya, A. M. and Soboleva, G. A. (1975), *Appl. Biochem. Microbiol.* **11**, 579–585.
34. Banerjee, N., Bhatnagar, R., and Viswanathan, L. (1981), *Appl. Microbiol. Biotechnol.* **11**, 226–228.
35. Neuhauser, W., Haltrich, D., Kulbe, K. D., and Nidetzky, B. (1997), *Biochem. J.* **326**, 683–692.
36. Hata, H., Shimizu, S., and Yamada, H. (1987), *Agric. Biol. Chem.* **51**, 3011–3016.
37. Kataoka, M., Rohani, L. P. S., Wada, M., Kita, K., Yanase, H., Urabe, I., and Shimizu, S. (1998), *Biosci. Biotechnol. Biochem.* **62**, 167–169.
38. Kataoka, M., Rohani, L. P. S., Yamamoto, K., Wada, M., Kawabata, H., Kita, K., Yanase, H., and Shimizu, S. (1997), *Appl. Microbiol. Biotechnol.* **48**, 699–703.
39. Shimizu, S., Kataoka, M., Katoh, M., Morikawa, T., Miyoshi, T., and Yamada, H. (1990), *Appl. Environ. Microbiol.* **56**, 2374–2377.
40. Diaz de Villegas, M. E., Villa, P., Guerra, M., Rodriguez, E., Redondo, D., and Martinez, A. (1992), *Acta Biotechnol.* **12**, 351–354.
41. Weigert, B., Klein, K., Rizzi, M., Lauterbach, C., and Dellweg, H. (1988), *Biotechnol. Lett.* **10**, 895–900.
42. Han, T., Gonzales, R., Martinez, A., Rodriguez, M., Ingram, L. O., Preston, J. F., and Shanmugam, K. T. (2001), *J. Bacteriol.* **183**, 2979–2988.