ENGINEERING AND CHARACTERIZATION OF HEMICELLULOSE HYDROLYSATE STRESS RESISTANCE IN *ESCHERICHIA COLI*

By

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To the efforts of those that came before, on which my accomplishments are founded, and to all that tread thereafter.

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Carbohydrate polymers that comprise the lignocellulosic matrix of plants are an abundant and renewable source of sugar for biocatalytic conversion into a range of commercially viable products. However, these carbohydrates must first be depolymerized to monomeric sugars prior to fermentation. Depolymerization of hemicellulose by treatment with dilute mineral acid is quite effective but also results in the formation of inhibitors such as furfural that retard growth and fermentation. To address this issue a series of studies were conducted focusing on osmotic and chemical stress found in hemicellulose syrups formed from dilute acid hydrolysis.

To increase osmotic stress tolerance, the effect of two osmoprotectants, trehalose and betaine, were investigated. Trehalose was produced internally in high concentrations in *Escherichia coli* by expression of the *otsAB* operon through transposon insertion into the chromosome. In addition, the media was supplemented with 1 mM betaine and additive effects of the two osmoprotectants were investigated in the presence of osmotic stress agents. Individually, each osmoprotectant conferred increased tolerance to osmotic stress from sodium chloride, sodium lactate, glucose, xylose, and succinate, but not ethanol. In the cases of sodium chloride, sodium lactate, glucose, or xylose, the combination of trehalose over-production and betaine addition increased tolerance more than either osmoprotectant could alone.

Three *E. coli* strains with increased trehalose production, EM2P (an ethanol producing derivative of KO11), EM2L (an ethanol producing derivative of LY163), and EM2T (a lactate producing derivative of TG106) were tested for desiccation survival. All strains with increased trehalose production had higher survival levels than the control. The growth sugar also impacted desiccation survival, with xylose yielding lowest survival, glucose providing intermediate survival, and sucrose providing the highest survival. Desiccating the cells in mid-log growth phase provided the highest survival levels compared to other growth phases. The highest overall survival rate (up to 80%) was achieved by over-producing trehalose while growing in sucrose and harvesting in mid-log phase of growth.

In addition to osmotic stress, chemical stress caused by furfural was addressed. Ethanologenic *E. coli*, strain LY180, was transferred in fermentation vessels containing minimal salts medium AM1 with xylose as a carbon source, and furfural in order to obtain a furfural resistant mutant. Changes in global mRNA levels in response to furfural were compared in the mutant, EMFR9, and the parent, LY180. These studies revealed 8 genes encoding oxidoreductases with at least 2 fold increased expression and 4 genes with at least 2 fold decreased expression in EMFR9 relative to LY180. Expression from plasmid in LY180 of the 8 genes did not increase furfural tolerance. However, expression by plasmid in EMFR9 of the 4 genes conferred a decrease in furfural tolerance in the cases of *yqhD*, *dkgA*, and *yqfA*. YqhD and DkgA exhibited the most pronounced effects. These two enzymes were purified and shown to have NADPH dependent furfural reductase activity, with a low Km for NADPH (8 μM and 23 μM, respectively). Deletion of these two genes in LY180 increased furfural tolerance, supporting the idea that furfural reduction competes for NADPH needed for growth. Plasmid

based expression of two native transhydrogenases, *sthA* and *pntAB*, in LY180 led to an increase in furfural tolerance with regard to *pntAB,* but *sthA* had no effect.

Further analysis of microarray data revealed an increase in cysteine and methionine pathway mRNA levels in LY180 upon furfural addition. In order for sulfate, the only source of sulfur in AM1 medium, to be incorporated into cysteine, 4 NADPH are required. Supplementation with 0.5 mM L-cysteine, D-cysteine, or sodium thiosulfate increased furfural tolerance in LY180 but not in EMFR9, presumably by alleviating NADPH demand for sulfate incorporation. Supplementation with taurine, an alternative source that requires almost as much NADPH as sulfate to be incorporated, provided no benefit to furfural tolerance. Together, the data indicates that conversion of furfural to furfuryl alcohol by YqhD limits available NADPH required for biosynthesis of molecules such as cysteine, preventing growth.

CHAPTER 1
INTRODUCTION

Hydrolysis of Hemicellulose

Plants are perhaps the most abundant renewable resource on earth, covering its entire surface and providing us with wood to build homes, chemicals from which herbal remedies can be derived, and even oxygen that we as a species require to survive. Furthermore, the basic materials necessary for the accumulation of flora are equally abundant; solar energy for photosynthesis, minerals from the soil, and carbon dioxide from the atmosphere. Since this natural commodity is so widespread it is no wonder that decades of scientific research have been employed to make the most of its potential, with one resulting outlet being the formation of hydrolysate.

Hydrolysate in general is simply a product of hydrolysis, and can be produced from any number of materials, including fish (120), yeast (26), soy (107), and many others (21, 68, 122). Hemicellulose hydrolysate is formed as a result of hydrolysis of the hemicellulose portion of the lignocellulose that comprises a large portion of the plant (132). The lignocellulose itself can be divided into three primary components: cellulose, hemicellulose, and lignin (132). The cellulose is composed of repeating units of glucose that are linked in a 1,4-beta fashion to make them inaccessible to digestion (40). The hemicellulose is composed of a variety of pentose and hexose sugars, including xylose, arabinose, mannose, glucose, and galactose (21). Lignin is a polymer of various aromatic compounds (syringic acid, hydroxybenzaldehyde, catechol, etc.) and aids in giving the plant rigidity . In addition to these polymers, the lignocellulose contains pectin, which accounts for approximately 2-20% of the lignocellulosic content and is composed of galacturonic acid and rhamnose. The hemicellulose sugars along with the glucose released from hydrolysis of the cellulose provide a suitable set of substrates for microorganisms to metabolize into useful

products. However, before this can occur the sugars must be made accessible through a pretreatment process.

Pretreatment of lignocellulose opens the crystalline structure, separating tightly packaged lignin, hemicellulose, and cellulose polymers from each other, as well as in certain instances partially hydrolyzing the cellulose and hemicellulose bonds (131). There are a number of pretreatment approaches that can be taken, each with benefits and pitfalls (132). They include dilute sulfuric acid pretreatment, dilute phosphoric acid pretreatment, flowthrough pretreatment, pH controlled water pretreatment, ammonia fiber explosion pretreatment, ammonia recycle percolation pretreatment, N-methylmorpholine-N-oxide pretreatment, and lime pretreatment.

Pretreatment by dilute sulfuric acid in conjunction with high temperatures and pressures leads to the recovery of most of the hemicellulose as dissolved sugars along with hydrolysis of part of the cellulose (21, 122, 131, 132). In addition, part of the lignin structure is disrupted, allowing attack on the cellulose by enzymes (122, 131). The use of phosphoric acid pretreatment also promotes cellulose hydrolysis, although not to the same degree as with sulfuric acid (122) and the remaining phosphoric acid can be used as a source of phosphate for growth. Pressurized liquid forced through biomass allows for disruption of the lignocellulosic structure, even without acid addition (132), but unfortunately the high amounts of water and energy required make the process difficult to use commercially. Maintaining the pH at 4-7 using water or stillage and implementing high pressure and temperature can dissolve a large portion of the lignin and hemicellulose, and cleave hemiacetyl linkages, thus freeing acids that can further degrade the biomass (132).

In addition to acidic pretreatment methods, alkaline pretreatment has been shown to be effective. Ammonia fiber explosion, or AFEX, decrystallizes cellulose so that it is more

accessible to enzymatic degradation, the ammonia is volatile enough that it can be quickly recycled for further use, and remaining ammonia promotes fermentation (132). Ammonia recycle percolation, or ARP, pushes aqueous ammonia though biomass at high temperatures, breaking apart the lignin as well as the lignin-hemicellulose bonds for enzymatic digestion of the hemicellulose (132). N-methylmorpholine N-oxide pretreatment, or NMMO pretreatment, uses NMMO to dissolve the cellulose so it is susceptible to enzymatic attack, and most of the NMMO can be readily recovered (61). Finally, addition of lime can be used for lignin removal, with the added benefit of temperature versatility (132), meaning that the reaction can occur at temperatures as low as 25 degrees Celsius so long as the pretreatment time is increased accordingly.

After the biomass has been pretreated by one of the above techniques the remaining cellulose and hemicellulose polymers, not completely digested but now readily accessible, need to be broken into their component sugars so that they may be metabolized. To do this enzymes such as cellulases and xylanases are employed. Cellulases hydrolyze the 1,4 linkages of cellulose that join glucose monomers together, and are implemented by a variety of organisms (27, 68, 76, 117). This often results in the formation of cellobiose that needs to be further broken down by beta-glucosidases to form two glucose molecules (106). In addition, the hemicellulose can be hydrolyzed by xylanases that release xylose present in the polymer (16, 27, 102).

In addition to hemicellulose hydolysate, hydrolysate from starch is commonly used in industrial biocatalytic product formation (114). It is advantageous to use starch in that the glucose that comprises the starch polymer is much more easily hydrolyzed to metabolizable sugars than lignocellulose and so complex pretreatment processes are unnecessary. This means that the cost of preparing the hydrolysate is lower, and the concentration of inhibitors formed and

released is much less. The starch, however, comes from materials such as corn that require significant costs in nutrients to grow and are competed for commercially as a food commodity.

Fermentation of Hemicellulose Sugars

After the hydrolysate has been prepared it can used as a source of sugar for microorganisms to grow and produce various chemical compounds. The microorganisms that make these valuable commodities range in classification from bacteria, to eukaryotes, and even archaeal species. Each of these domains have the ability to grow under a unique set of conditions regarding media, temperature, aeration, substrate preference, and each can produce a different but overlapping set of products. Furthermore, even within each domain organisms differ with respect to the above criteria.

Bacteria can produce a wide range of commercially important products, including ethanol, propanol, butanol, lactate, succinate, acetate, and pyruvate. Ethanol, an alternative fuel source (40), is made as a product naturally in many bacteria fermentatively in order to achieve redox balance (130). However, generally speaking, other products are also produced that lower overall yield and complicate purification strategies. To alleviate this issue bacteria have been developed though genetic engineering and directed evolution to make ethanol as their primary product, with two of the most prominent being *Escherichia coli* and *Klebsiella oxytoca* (8, 51, 133). 1-butanol is made in *Clostridium* using acetone-butanol-ethanol fermentation (31, 67), and certain species of *Clostridium* can make 1-propanol in low concentrations using threonine catabolism (50). Heterologous expression of genes in *E. coli* has allowed it to produce measurable quantities of both 1-butanol and 1-propanol (110). Genetic engineering of *E. coli* has led to its ability to produce 1.2 M lactate--an acid useful in the formation of plastics and pharmaceuticals (2, 44)--in minimal media, with high chiral purity (142). A number of other bacteria, particularly those of the genera *Bacillus*, have been engineered to produce lactate

industrially (94). In addition, a derivative of *E. coli* C was engineered to make 0.7 M succinate in minimal media (53). Other bacteria can make succinate effectively, including *Actinobacillus succinogenes* and *Enterecoccus flavescens* (135). *E. coli* W3110 was engineered to make 0.9 M acetate (18), and *Acetobacter* is a well-known acetate forming bacteria that uses ethanol as a substrate under aerobic conditions. Pyruvate can be made in *E. coli* to a final yield of 0.8 M (17), and can be used as a precursor in the formation of a number of amino acids.

Eukaryotic microorganisms that are engineered to make commercial products from sugars found in hemicellulose hydrolysate are generally yeast. *Saccharomyces cerevisiae* is perhaps the most well studied and implemented of these yeasts. It can use glucose, mannose, and fructose via the Embden-Meyerhoff pathway of glycolysis, galactose using the Leloir pathway, and xylose through metabolic engineering to express a xylose isomerase (126). These and other strategies allow *S. cerevisiae* to make significantly higher concentrations of products, particularly ethanol, than the parent strains (55). In addition, a number of other fungi have been known to convert both glucose and xylose into ethanol, including *Fusarium*, *Mucor*, *Rhizopus*, and *Monila* (96). *S. cerevisiae* has also been engineered to make optically pure D-Lactate from glucose during batch fermentations (135).

Archaea are perhaps the least studied of the three groups, due in part to their lower degree of prevalence, difficulty to cultivate, and absence of genetic manipulation tools compared to bacteria and yeast. However, these microorganisms are interesting potential vessels for commercial product formation from biomass for a number of reasons. Like members of the other domains, archaea have the ability to implement a wide range of carbohydrates. Marine archaea have been shown to be able to digest both alpha and beta linked glucans, including starch, barley glucan, laminarin and chitin (12). As fascinating is their ability to grow at

temperatures that are permissible to enzymes which degrade lignocellulosic polymers but are not desirable to growth of contaminanting microbes.

Toxicity of Hemicellulose Hydrolysate

Pretreatment of plant biomass hydrolyses a portion of the lignocellulose, freeing sugars that can then be readily metabolized, and opens the crystalline structure to enzymatic attack for further degradation. However, this process often leads to the release and formation of numerous inhibitors, which can be broken into two major categories: osmotic inhibitors and chemical inhibitors. These compounds, which retard cell growth and product formation (123, 136-138), can be dealt with, either by their removal from the media prior to addition of the inoculum or through the development of tolerant strains.

Types of Inhibitors

Osmotic inhibition results from hypertonic conditions imposed by components of the hydrolysate, including sugars released from hydrolysis of the cellulose and hemicellulose, salts formed from acid treatment as well as those released from the plant itself, and acids cleaved from the lignocellulosic polymers. The resulting hypertonicity draws water out of the cell, leading to plasmolysis of the membrane and disruption of cellular processes (23).

Chemical inhibitors affect the biocatalyst in a multitude of ways, with patterns of inhibition linked to the class of inhibitor involved. The three major categories of inhibitors are alcohols, acids, and aldehydes. Alcohols in hydrolysate result primarily from the release of aromatic alcohols from lignin, as well as through the formation of furfuryl alcohol via conversion of pentose sugars during pretreatment (64, 137). They include vanillyl alcohol, methylcatechol, guaiacol, coniferyl alcohol, hydroquinone, furfuryl alcohol, and catechol (137). Alcohols have been shown to act by solubilization of the cell membrane, and as such toxicity can be directly correlated to hydrophobicity of the compound (137). Of the above alcohols,

methylcatechol was shown to be the most inhibitory to an ethanologenic strain of *E. coli*, with a minimal inhibitory concentration, or MIC, of 1.5 g liter^{-1} (137). Interestingly, inhibition of alcohols present in pairs was fairly additive (137).

Organic acids present in hemicellulose hydrolysate come from numerous sites including lignin, acetyl-xylan, and conversion of hydrolyzed sugars (121). Specific acids include ferulic, gallic, furoic, formic, levulinic, and acetic acid (136). Acetate is thought to consistently be the most abundant hemicellulose hydrolysate acid (136). Acids act by crossing the cell membrane in neutral form and then disassociating, releasing a hydrogen ion and in turn collapsing the proton motive force (136). Increasing the initial pH of the medium decreases toxicity in most instances, most likely because a greater portion of the acids disassociate outside of the cell and thus are unable to diffuse across the membrane (136). As with aldehydes, toxicity is related to hydrophobicity, although acids do not appear to disrupt membrane integrity (136).

The effect of aldehydes on cell function appears to be more complicated than that of either acids or alcohols and is not yet fully understood. Aldehydes present in hemicellulose hydrolysate include soluble aromatic aldehydes released from lignin, hydroxymethyl furfural from conversion of hexose sugars, and furfural from conversion of pentose sugars. Aromatic aldehydes appear to be more toxic on a weight basis than furfural or hydroxymethyl furfural (138). However, furfural generally exists in higher concentrations than any individual aromatic aldehyde, its toxicity is uniquely synergistic, and it is the only tested aldehyde to strongly inhibit ethanol production in KO11 and LY01 (138); these serve as indications that furfural is perhaps the most important of inhibitory aldehydes. The inhibitory effects of furfural have been extensively studied. Furfural has been shown to mutate DNA at and below 20 mM furfural (59), can cause strand breaks in duplex DNA (39), and can react with the amino group of adenine to

form N-6-furfuryladenine (9). Furfural also effects enzyme function, reducing the activity of alcohol dehydrogenase (82), aldehyde dehydrogenase (82), pyruvate dehydrogenase (82) at a concentration of less than 2 mM furfural, and glucose phosphate isomerase (48), and glucose-6P-dehydrogenase (48) at a concentration of 4 mM furfural. At high temperatures both 10 and 30 mM furfural were shown to interact chemically with lysine to form furpipate (83). Hydroxymethyl furfural, while not as toxic as furfural, can also inhibit growth (138) and has been shown to affect mammalian DNA polymerase λ and the terminal deoxynucleotidyltransferase, with an IC_{50} of 26.1 and 5.5 μM, respectively, for these two enxymes (81).

Addressing Inhibitors

Microorganisms respond to osmotic stress by the accumulation of various compatible solutes, neutral molecules that can exist in the cytoplasm in high concentrations without harming cell function (58). In *E. coli*, high concentrations of external osmolytes led to the uptake of potassium ions, which in turn initiates the formation of the compatible solute glutamate (58). Other compatible solutes, such as proline, are either made or taken into the cell through a set of transporters (58). The non-reducing sugar trehalose is naturally produced in both prokaryotic and eukaryotic organisms during osmotic stress using trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase (47, 58); increasing trehalose production in *E. coli* by expressing the *otsAB* operon on a plasmid led to enhanced osmotic stress resistance (103). In addition, *E. coli* can take up the compatible solute betaine from the medium (19, 58, 98) or produce it oxidatively in the presence of choline (58). Gram-positive bacteria have a similar response mechanism, taking up and producing proline and betaine upon onset of hypertonicity (58).

Chemical inhibitors present in hemicellulose hydrolysate can be managed either by detoxification prior to culture addition or by engineering and selection of resistant strains. A number of approaches have been taken to remove chemical inhibitors prior to inoculum addition, including overliming, resin filtration, charcoal filtration, and selective hydrogenation. Overliming, or the addition of calcium hydroxide to hot hydrolysate, leads to a significant reduction in total furans and phenolic compounds (32, 73, 74), but also reduces overall sugar concentrations (73). Treatment of hydrolysate with IRA-400 ion exchange resin removes a portion of the acetate, furfural, sulfate, and phenolic compounds (32). Activated charcoal functions in a similar fashion but removes less furfural (32) and less nitrogen (105). In both cases, treatment allowed for an increase in carbohydrate utilized by *Klebsiella pneumoniae* to make 2,3-butanediol (32). Increasing treatment temperature during implementation of charcoal increases its effectiveness (79). While charcoal and resin provide an opportunity for removal of inhibitors, costs of both are a factor to be taken into consideration. Additionally, although not directly connected to hydrolysate remediation, conversion of furfural to the less toxic furfuryl alcohol can be performed by selective hydrogenation using a copper oxide catalyst (42).

In addition to removing inhibitors from hydrolysate prior to culture addition, growth conditions can be modified to allow for improved tolerance in the presence of inhibitors. One of the primary means by which this is accomplished is through microaeration. Growth of *E. coli* KO11 in waste house wood hydrolysate, or WHW, in the presence of small quantities of oxygen led to an increase in xylose consumption and ethanol production (91). Other methods of this nature include encapsulation of *Saccharomyces cerevisae* in an alginate matrix for protection against inhibitors as well as an increase in capacity for in situ detoxification (119).

Alteration of the hydrolysate to become suitable for biocatalytic growth is a general strategy that has been adopted in many instances, but adaptation of the organism itself to hydrolysate inhibitors, either through genetic engineering or directed evolution, can be more beneficial in that it reduces complication of hydrolysate preparation and sidesteps costs associated with resin, alginate, and so forth. One means by which this can be achieved is through selection of resistant strains by screening and transferring in media containing the appropriate inhibitors. *S. cerevisae*, *Pachysolen tannophilus*, *Brettanomyces custersii*, *Candida shehatae*, and *Candida acidothermophilum* have been screened for growth in dilute acid softwood prehydrolysate (56), and various yeast have been adapted to hydrolysate inhibitors by continuous cultivation in prehydrolysate and hydrolysate (56, 70, 71). *S. cervisiae* was adapted to sugarcane bagasse hydrolysate by continuous cultivation in increasing concentrations of phenolic compounds, furaldehydes, and aliphatic acids (71). Two yeast species, *Pichia stipitis* and *Trichoderma reesi*, were adapted to grow in high concentrations of acetate (43, 88), a common hemicellulose hydrolysate inhibitor. *P. stipitis* is particularly interesting as an ethanol producing yeast due to its ability to utilize xylose (1).

With knowledge of the mechanisms by which hydrolysate inhibitors act, and the means by which each biocatalyst attempts to tolerate them, genetic engineering of the biocatalyst can be implemented to further promote growth and product formation. Laccase, an extracellular fungal enzyme, has been demonstrated to oxidize phenolic compounds (63). Expression of laccase from *Trametes versicolor* in *S. cerevisae* led to increased tolerance to coniferyl aldehyde when oxygen is present (63). A mutation in the ABC transporter *aatA* in *Acetobacter aceti* was shown to convey acetic acid, formic acid, propionic acid, and lactic acid sensitivity; expression of *aatA* in the mutant restored acetate resistance, and expression of *A. aceti aatA* in *E. coli* increased

resistance to acetate (86). Furthermore, expression of aconitase in *Acetobacter aceti* has been shown to increase acetate resistance (85). Multiple enzymes in *S. cerevisiae* have been shown to reduce 5-hydroxymethyl furfural, including a mutated alcohol dehydrogenase 1 and alcohol dehydrogenase 6 (5, 62, 99). In *E. coli*, a furfural reductase was purified that reduced furfural to furfuryl alcohol using NADPH specifically as a co-factor (36). In *S. cerevisiae*, mutations in four genes within the pentose phosphate pathway--*zwf*, *gnd*, *rpe*, *tkl*—led to furfural sensitivity, and overexpression of *zwf* allowed for increased tolerance to furfural (34).

Objectives

The primary goal of this research project is to develop and analyze mechanisms in *E. coli* that increase tolerance to inhibitors commonly found in hydrolysate. Doing so will lend a basic scientific understanding of the inhibitors' action on the cell, as well as lead to a commercial platform for overcoming the toxicity of these moieties. This approach will seperately target osmotic stess and chemical stress factors.

Engineering Resistance to Osmotic Stress

Osmotic stress is imposed upon the cell by salts and sugars found in hemicellulose hydrolysate (23, 57, 100), in addition to products which accumulate during fermentation (103). This study will test the ability of osmotic stress to be overcome in *E. coli* using two known osmoprotectants, betaine and trehalose. Addition of 1mM betaine to media containing seperate osmotic stress agents will be used to gauge its effect on osmotolerance through growth measurements. The same approach will be implemented to test improved osmotic tolerance through increased expression of the *otsBA* operon, genes necessary for trehalose production, by random insertion in the host chromosome using a transposon. Finally, the conjoined effect of betaine addition and increased trehalose production will be measured to test the ability of two osmoprotectants to work synergistically.

Engineering Resistance to Desiccation

The ability of cultures to survive anhydrobiosis has been long studied (22, 101), and stress factors that prevent survival are separate, but related, to those observed with omotic stress. *S. cerevisiae* and other yeast exhibit increased survival by producing intracellular trehalose (22), the same disaccaride used as an osmoprotectant, and drying in added trehalose or sucrose also increases desiccation survival (65, 101). However, not all osmoprotectants serve as anhydrobiotic protectants, as is the case with betaine (129). Using ethanol producing and lactate producing *E. coli* strains with integrated *otsBA* operons under control of a tac promotor, this study will determine if engineered intracellular production of trehalose leads to increases in desiccation survival. The ability of added xylose, glucose, fructose, arabinose, lactose or mannose will also be tested for its effect on desiccation tolerance, both alone and in conjunction with increased intracellular trehalose production. In addition, the growth phase of harvested cells with relation to survival will be assessed. Finally, the effect of desiccation on fermentation of subsequently rehydrated cells will be determined.

Engineering Resistance to Chemical Stress

One of the primary chemical stress agents observed in hydrolysate is furfural, a dehydration product of the pentose sugar xylose (64, 73, 138). This study will develop a furfural resistant *E. coli* through selective evolution, growing batch cultures in minimal media containing furfural and transferring to new media when growth is observed. The furfural resistant *E. coli* will subsequently be subjected to a microarray comparison to the parent strain in order to delineate evolved resistance mechanisms. *E. coli* has previously been demonstrated to contain a furfural reductase (36), but no corresponding gene had been assigned. To attempt identification of furfural reductases, putative and known oxidoreductases with perturbed mRNA expression will be cloned and tested, both for furfural reductase activity as well as for their effect on furfural

tolerance. Oxidoreducatases with furfural reducing activity will subsequently be histidine tagged by gene cloning into plasmid vector pET15b and purified on a nickel column to be subjected to kinetic analysis. Finally, global gene expression analysis through microarray studies will be used to group genes perturbed by furfural addition into functional catagories. Together, the gathered data will allow development of a model outlining furfural's inhibition mechanism.

CHAPTER 2
INCREASING TOLERANCE TO OSMOTIC INHIBITORS

Introduction

Current high costs of petroleum and petroleum-derived chemicals provide an opportunity for the expansion of renewable microbial products from carbohydrate feedstocks. To be competitive, microbial processes for bulk chemicals must be simple, robust, rapid, efficient, and inexpensive. High product titers are desirable to minimize costs associated with water handling, purification, and waste treatment. To achieve these titers in simple fermentations, microbial biocatalysts must be able to tolerate osmotic and chemical stress from high concentrations of carbohydrate substrates in the hydrolysate as well as accumulated products released from the microorganism. Although the mechanism of osmotolerance is not fully understood, plants, animals, and microbes utilize intracellular compatible osmolytes such as glutamate, betaine, proline, and trehalose to counter osmotic stress from extracellular solutes (22, 23, 103). *E. coli* can synthesize all of these compounds during oxidative metabolism provided choline is available, but biosynthesis is limited to glutamate and trehalose during anaerobic growth. Trehalose accumulation during fermentative growth is hindered by expression of catabolic enzymes such as trehalase (14, 45).

Betaine has generally been regarded as a superior protective osmolyte for *E. coli* and has been shown to increase tolerance to sugars, salts, and organic acids (23, 30, 58, 123, 141). This compound is actively concentrated from the environment by a stress-inducible uptake system (98, 124) and serves as a protective intracellular osmolyte. In *E. coli*, biosynthesis of trehalose is also induced as part of the osmotic stress response (23, 58, 103). Uptake of betaine has been shown to reduce the intracellular levels of trehalose, suggesting that these protective osmolytes may be biologically interchangeable (19, 20, 100). Other studies have shown that over-

expression of trehalose biosynthetic genes can be used to increase trehalose levels above that of wild type strains and increase osmotolerance to both salts and sugars (103).

Here I investigated the combined effects of genetically increasing trehalose biosynthesis and supplementing the medium with betaine on osmotic tolerance to stress agents encountered during fermentation in hydrolysate by measuring growth in mineral salts medium containing 20 g liter^{-1} (w/v) glucose as the primary carbon source.

Materials and Methods

Two *E. coli* strains were used in these studies: W3110 (wild type) and JP20 (103). Strain JP20 is an isogenic derivative in which an IPTG-inducible operon for trehalose biosynthesis (Φ*ampH:: lacI*P_{tac}-*otsBA*) has been chromosomally integrated into ampH, and the genes for trehalose degradation (*treA, treC,* and *treF*) have been deleted. Levels of trehalose in JP20 were previously shown to be elevated without induction due to leaky expression, and further increased upon induction by IPTG (103).

Osmotolerance was examined as described by Purvis *et al.* (103). Tolerance was investigated using M9 mineral salts medium containing 20 g liter^{-1} (111 mM) glucose, unless indicated otherwise. Inocula were prepared by resuspending cells from fresh colonies on solid medium in broth. Cultures were inoculated to provide an initial $OD_{550\ nm}$ of 0.03 (10 mg dcw l^{-1}). Cell density was determined after incubation for 24 h at 37ºC. The minimum inhibitory concentrations (MIC) for each stress agent was estimated by extrapolating plots of cell mass versus solute concentration to zero growth.

Results and Discussion

Although the beneficial effects of intracellular trehalose and betaine during growth under osmotic stress are well-established for sodium chloride and glucose (23, 58, 104), little work has been reported concerning the combined effect of two intracellular osmolytes. Prior experiments

have established that 1 mM betaine is near the optimal concentration for osmotic tolerance in *E. coli* for several stress agents (123). Previous studies in our laboratory have examined the effects of different levels of trehalose on osmotolerance by comparing the wild type parent to an isogenic strain (JP20) containing a chromosomally integrated, IPTG-inducible operon for trehalose biosynthesis (103). Strain JP20 exhibits elevated levels of trehalose synthesis in the absence of induction, and higher levels with added IPTG. Estimates of intracellular trehalose levels for these strains were as follows: less than1 mM for W3110 (parent), 28 mM for JP20 without induction, and 360 mM for JP20 after induction (103). Based on these results, experiments were designed to examine the combined effects of betaine (1 mM) and trehalose. A qualitatively similar effect on trehalose biosynthesis (JP20 induced>JP20>W3110) was assumed independent of stress agent.

Tolerance to Sugars

The addition of betaine to the media prior to growth led to a substainial increase in tolerance to high concentrations of glucose, increasing the minimal inhibitory concentration (MIC) from 0.7 to 1.1 M (Fig. 2-1A). As expected, JP20 also had increased tolerance to glucose even in the absence of induction by IPTG (due to leaky expression of the otsAB operon), and induction further enunciated this trend. Most interestingly, the combination of betaine addition and increased trehalose production yielded a synergistic benefit above which either osmoprotectant could provide alone.

A similar effect was observed in the presence of another sugar commonly found in hydrolysate, xylose (Fig. 2-1B). Again, W3110 without betaine exhibited the least tolerance to the osmolyte, with either betaine addition or increased trehalose expression seperately providing a benefit to tolerance. JP20 grown in the presence of betaine resulted in the highest tolerance to xylose. Taken together the data serves as an indication that a synergistic osmoprotective effect is

observed with relation to betaine and trehalose in the presence of otherwise inhibitory sugar concentrations.

Tolerance to Salt

The greatest benefit observed from the tested osmoprotectants was in the presence of sodium chloride (Fig. 2-2). As shown with sugar tolerance assays, tolerance to NaCl increased when cells were grown in the presence of 1 mM betaine. JP20, the strain developed for increased trehalose production, also had improved tolerance to NaCl compared to W3110 in an uninduced state, with induction by IPTG providing an added benefit. JP20 in the presence of betaine exhibited the greatest tolerance to NaCl, although induction by IPTG in this instance did provide an additional effect. This indicates that although betaine and trehalose can act synergistingly, saturating osmoprotectant concentrations still exist, above which no further benefit is observed.

Tolerance to Organic Acids

In addition to osmotic stress agents present in media prior to growth, *E. coli* releases fermentation products that can have an osmotic effect, two of which being lactate and succinate. By far the best osmoprotectant in the presence of lactate appears to be betaine, allowing growth in up to 0.6 M lactate (Fig. 2-3A). Increased production of trehalose appeared to exhibit a minor benefit. Unfortunately, no synergistic benefit was observed during growth in lactate.

Addition of betaine was most beneficial to succinate tolerance, increasing the MIC from 0.3 M to 0.6 M (Fig. 2-3B). Trehalose over-expression provided a noticeable advantage, but again, no synergistic effect was observed between the two osmoprotectants. Interestingly, either betaine addition or trehalose overproduction led to an increase in final cell density when grown in low succinate concentrations compared with growth in the absense of succinate, indicating that once osmotic hinderance has been overcome succinate can serve as a carbon source.

Tolerance to Alcohol

Unlike xylose, glucose, sodium chloride, lactate, or succinate, no beneficial effect was observed by either osmoprotectant in the presence of ethanol (Fig. 2-4). This result is consistent with the small size and previous reports of high cellular permeability (28, 29, 80). Other actions such as metabolic stress or chemical stress are presumed to be important actions leading to growth inhibition by ethanol. Oddly, osmprotectant addition led to a slight antogonistic effect on growth, with this trend enuciated when the trehalose over-producing strain, JP20, was grown in the presence of betaine.

Conclusions

A combination of supplementing fermentations with 1 mM betaine and enhancing biosynthesis of trehalose by the biocatalyst may be more useful than either alone for increased tolerance to sugars (glucose and xylose) and products such as sodium lactate. Although this combination did not improve tolerance to ethanol, strain productivity may be improved in high sugar environments. The molar toxicity of xylose was over 3-fold higher than glucose and NaCl.

Figures and Tables

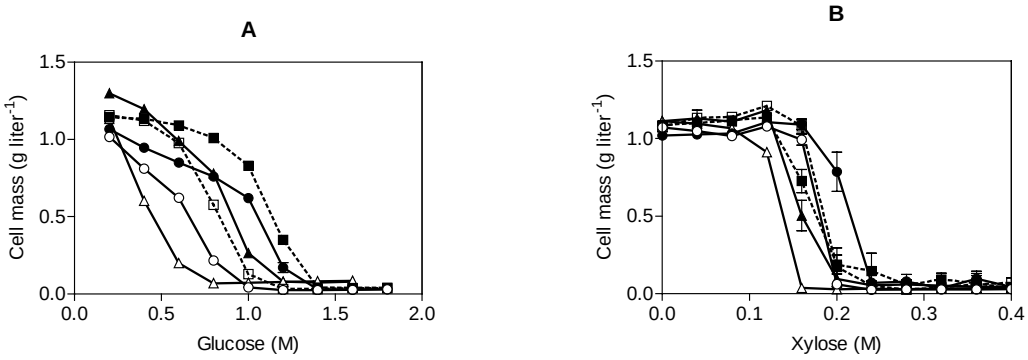

Figure 2-1. Effect of betaine and trehalose on sugar tolerance. Tolerance to (A) glucose, and (B) xylose. Δ, W3110 without betaine; ▲, W3110 with 1 mM betaine; ○, JP20 without betaine; ●, JP20 with betaine; □, JP20 induced without betaine; ■, JP20 induced with 1 mM betaine.

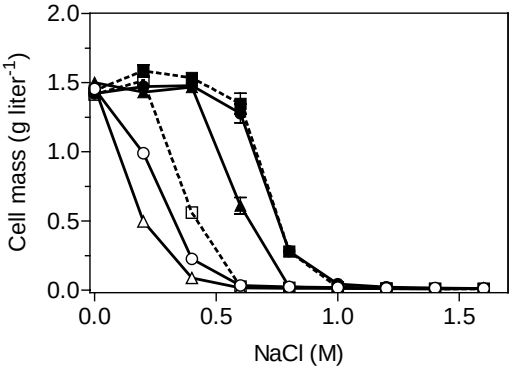

Figure 2-2. Effect of betaine and trehalose on salt tolerance. Tolerance to NaCl. Δ, W3110 without betaine; ▲, W3110 with 1 mM betaine; ○, JP20 without betaine; ●, JP20 with betaine; □, JP20 induced without betaine; ■, JP20 induced with 1 mM betaine.

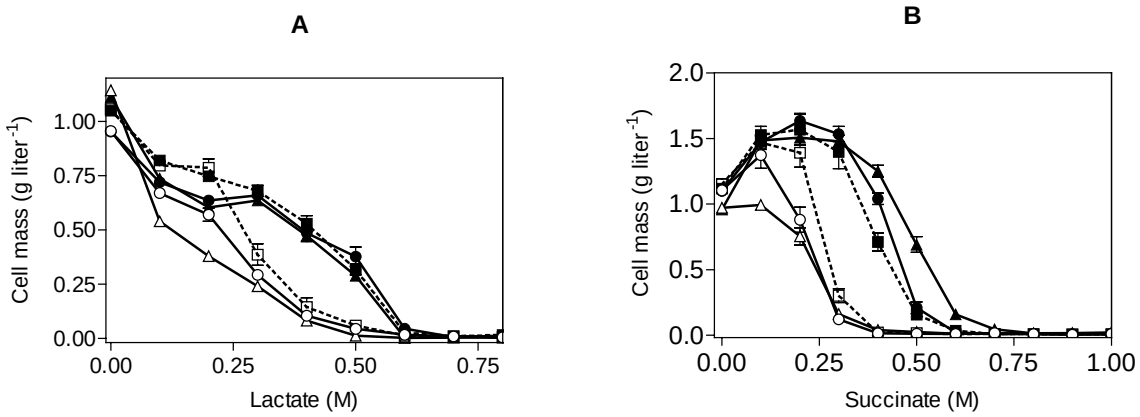

Figure 2-3. Effect of betaine and trehalose on organic acid tolerance . Tolerance to (A) lactate, and (B) succinate. Δ, W3110 without betaine; ▲, W3110 with 1 mM betaine; ○, JP20 without betaine; ●, JP20 with betaine; □, JP20 induced without betaine; ■, JP20 induced with 1 mM betaine.

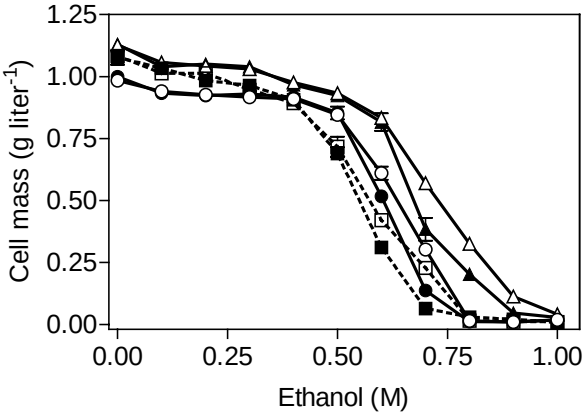

Figure 2-4. Effect of betaine and trehalose on alcohol tolerance. Tolerance to ethanol. Δ, W3110 without betaine; ▲, W3110 with 1 mM betaine; ○, JP20 without betaine; ●, JP20 with betaine; □, JP20 induced without betaine; ■, JP20 induced with 1 mM betaine.

CHAPTER 3
INCREASING TOLERANCE TO DESICCATION

Introduction

Desiccation tolerance has been a field of much interest to researchers, with extensive studies conducted on both bacterial and mammalian cells. This research has delineated mechanisms allowing certain organisms to survive in an anhydrobiotic state and come away unscathed. *S. cerevisae*, for instance, produces high levels of the disaccharide trehalose as well as the osmoprotectant betaine in response to osmotic stress. *E. coli* can implement a similar action of defense, producing trehalose and taking in betaine through a specific transport system (20, 100). While betaine has been shown to act as an invaluable tool against high osmolyte concentrations, its effects are not evident during desiccation (129). As such, trehalose, as well as other disaccharides such as sucrose, serve as the prevailing protectors under these circumstances, acting to take the place of water in order to prevent membrane fusion as well as protein inactivation (65, 101). Efforts have been made in the past to further enhance the ability of these organisms to survive the desiccation process, with *E. coli* being at the forefront of these ventures. *E. coli* cells have been dried in trehalose solutions to increase extra-cellular trehalose while at the same time intra-cellular levels were increased through osmotic induction, allowing for survival levels up to approximately 80% after a week of desiccation (25). In addition, recombinant expression of sucrose-6-phosphate synthase in *E. coli* using the *spsA* gene from *Synechocystis* has been implemented as a means by which to allow the cells to produce sucrose as a protection mechanism, increasing survival when desiccated over phosphorus pentoxide from approximately $2*10^{-4}$ % to 2.3% (11). This study looks to further expound upon the benefits provided by these and other sugars by the recombinant over-expression of trehalose production in concert with

separate addition of xylose, glucose or sucrose, as well as gain insight into the mechanism by which this effect occurs.

Materials and Methods

Growth of Organisms

LY163, an ethanologenic *E. coli* having all native fermentative routes for NADH reoxidation replaced with the *Zymomonas mobilis* ethanol producing pathway (118), and its derivatives were grown for desiccation in NBS minimal medium with the addition of 5 % w/v xylose, sucrose or glucose prior to growth. Cultures were transferred on NBS agar plates containing 2 percent glucose and were re-streaked using an applicator stick on a daily basis. KO11, a recombinant strain of *E. coli* containing the *pdc* and *adh* genes from *Z. mobilis* (90), and its derivatives were grown in Corn Steep Liquor medium, also with the addition of 50 g liter^{-1} xylose, glucose or sucrose prior to growth. Cultures were transferred daily on CSL plates containing 20 g liter^{-1} xylose by restreaking via applicator stick. TG106, an *E. coli* B derivative (*pfl*$^{-}$, *frd*$^{-}$, *adhE*$^{-}$, *ackA*$^{-}$, Δ*ldhA*::*ldhL mgsA*$^{-}$) made to produce L-lactate and EM2T, a further derivative of TG106 made to over-express the genes for trehalose production were transferred on NBS 20 g liter^{-1} glucose plates with 0.1 M MOPS, pH 7.0, on a daily basis. Organisms were frozen for long-term storage by growing the cells to approximately 0.5 optical density (550 nm), adding 0.75 mL of this culture to 0.75 mL 80% w/v glycerol stock and then placing the tube into a Nalgene Cyro freezing container to be frozen at negative 75 degrees Celsius

Over-Expression of Trehalose Production

LY163, KO11, and TG106 were transformed with pLOI3650, a plasmid containing the otsBA operon under control of a tac promoter that inserts the operon into the genome as a transposon (103). Each strain was grown in 50 mL of LB medium in a 250 mL Erlenmeyer flask after inoculation with a single colony and was grown to approximately 0.5 OD. The culture was

then centrifuged at 5,000 g for 5 minutes and the pellet was resuspended in 10 mL of 50 mM ice-cold calcium chloride solution. 3 uL of plasmid was added to 200 uL of the resuspended cells and this tube was held on ice for 30 minutes. The suspension was then placed in a 37-degree water bath for 2 minutes and was then removed and held at room temperature for 5 minutes. Subsequently, 1 mL of LB with 50 g liter^{-1} glucose was added to the suspension and this was transferred to a 37 shaker for 1 hour. These cells were then plated and selected for kanamycin resistance and ampicillin sensitivity to make sure that only the otsBA operon had been inserted. Preliminary tests were then conducted on the resulting colonies with regard to desiccation resistance when grown for 24 hours in either CSL (KO11) or NBS (LY163 and TG106) with 50 g liter^{-1} glucose. The optimal strains were selected and designated EM2L for the LY163 derivative, EM2P for the KO11 derivative, and EM2T for the TG106 derivative.

Testing for Desiccation Survival

Cultures were grown in either NBS (for LY163) or CSL (for KO11)--neither medium contained betaine--with 50 g liter^{-1} xylose, glucose, or sucrose for either 24 hours or to approximately 7 OD. A sample of 10 uL of these cultures were then pipeted into 1.5 mL microfuge tubes that were thereafter transferred to a Pyrex desiccation chamber containing phosphorous pentoxide. Argon was added to the desiccator, via a tube which connected the chamber to a gas tank, to purge the majority of the oxygen from the atmosphere in the chamber. After 3 days of desiccation the cultures were resuspended in 1 mL of 50 g liter^{-1} xylose solution, and appropriate dilutions were made in 50 g liter^{-1} xylose so that the colony forming units could then be counted and compared to those of the non-desiccated cultures in order to determine survival. To test for desiccation survival in TG106 and EM2T pH had to be maintained since the organism produces high quantities of lactate. To do this, the cultures were first grown in fleakers

containing NBS with 1 mM betaine and 50 g liter^{-1} of the appropriate sugar for 24 hours with pH maintained at 7.0 by 6 N KOH addition prior to desiccation over phosphorus pentoxide.

Results and Discussion

Effect of Sugar Substrate on Survival

As a preliminary study KO11 was grown in a variety of sugars and was then desiccated for 3 days, at which point the cells were rehydrated and plated to obtain colony-forming unit counts (Fig. 3-1). Growth in xylose led to the lowest levels of survival, with growth on glucose, lactose, arabinose, fructose or mannose giving intermediate survival levels, and sucrose yielding the highest percentage survival by far. From this data it was decided that xylose, glucose, and sucrose be selected as the growth substrates for further investigation with regard to survival to allow for a comprehensive comparison. Addition of 1 mM betaine to KO11 cultures grown for 24 hours with 50 g liter^{-1} sucrose was also tested to see if it would improve survival, but survival levels of cultures grown in betaine were actually slightly lower than if no betaine was added (data not shown). This was not surprising since similar studies had shown this to be the case under separate conditions (129), and serves as an indication that the principles cells use to cope with osmotic stress as opposed to desiccation differ significantly.

Effect of Strain on Survival

When grown for 24 hours in NBS with 50 g liter^{-1} xylose and subsequently rehydrated after 3 days desiccation, extremely low survival was observed for the ethanol producing strains KO11 and LY163 (Table 3-1). Growth in glucose for 24 hours prior to desiccation, compared to xylose, led to slightly higher survival for these strains. Growth in 50 g liter^{-1} sucrose prior to desiccation led to the highest survival by far of all sugars tested for both strains, although survival of KO11 grown in sucrose was higher than that of LY163. One possible reason for this

discontinuity is that KO11 was grown in corn steep liquor, which is undefined and contains various osmoprotectants, whereas the NBS medium used to grow LY163 does not.

In addition to testing the ability of the ethanol-producing *E. coli* to survive the desiccation process, it was also of interest to determine whether or not *E. coli* producing other fermentation products could achieve similar levels of recovery. To do this the lactate producing strain TG106 was desiccated after 24 hours growth in fleakers containing NBS with 1 mM betaine and 50 g liter^{-1} of either, xylose, glucose, or sucrose, and was then rehydrated after 3 days and plated to measure cell counts. As expected, desiccation in xylose led to the lowest levels of survival of the three substrates, although survival was still considerably higher then with either LY163 or KO11. Interestingly, while addition of sucrose again allowed a higher degree of survival than xylose, it was glucose in this instance that led to the highest levels of survival.

Cell mass, product levels, remaining sugar, and pH were examined for clues to these differences in survival. Higher survival after desiccation was generally associated with sucrose, lactate instead of ethanol as a fermentation product, lower cell mass, and higher pH. Xylose as the fermentable sugar and accumulated ethanol (above 6 g liter^{-1}) were associated with a decrease in survival.

Effect of Trehalose Overproduction on Survival

Since it was evident that the sugar the cells were grown on had a profound impact on the organism's survival, the question of whether or not increased production of a similar sugar would also confer such tolerance to desiccation arose, and if this effect in conjunction with sugar substrate would lead to further increases in survival. To test this KO11, EM2P, and EM2P induced were grown for 24 hours in CSL with 5 g liter^{-1} of either xylose, glucose, or sucrose, desiccated for 3 days, and then rehydrated. When grown on xylose, both KO11 and EM2P

exhibited very low levels of survival, but survival of EM2P was relatively higher, and trehalose induction increased this trend (Fig. 3-2A,B,C). Survival was higher overall when the cultures were grown in glucose, and even higher when the cultures were grown in sucrose, with a combined benefit resulting from the overexpression of trehalose, as shown by higher survival levels in EM2P in all instances compared to KO11.

Over-expression of trehalose production in concert with growth on sucrose led to survival levels higher than that which either could provide alone, so it seemed prudent to ask if this phenomenon could be applied to other strains as well. To answer this question the same transposon was inserted in LY163, producing EM2L. Although survival was slightly lower overall in LY163 and its derivatives than with KOll and EM2P, possibly due to faster growth and hence greater consumption of sucrose, inserting the genes and inducing them led to significantly higher levels of survival than in the parent strain (Fig. 3-2E). Finally, this technique was applied to the lactate producing organism, TG106, making EM2T. EM2T grown in sucrose had higher levels of survival than TG106, with survival rising upon induction (Fig. 3-2F).

Effect of Growth Stage on Survival

From this data it became clear that growth in sucrose in conjunction with over-expression of trehalose production led to levels of survival higher than that which either could provide alone. The next pertinent question regarded the optimal growth point that *E. coli* cells should be desiccated at in order to achieve optimal survival. To test this, EM2L was grown uninduced to a range of optical densities. From this experiment it was determined that the optimal desiccation point was late log phase or approximately 7 OD (Fig. 3-3).

Using this information LY163, EM2L, and EM2L induced were grown in NBS with 50 g liter^{-1} sucrose to approximately 7 OD and were then desiccated for three days before rehydration

and plating (Fig. 3-2D). This led to a significant increase in survival for both strains. Possible reasons accounting for this outcome include higher levels of sucrose left in the medium due to lower biomass and fermentation product production (which was shown to be the case by HPLC measurements), as well as a decrease in waste product concentration that might hinder survival.

Mechanism of Survival

In order to begin to touch upon the mode by which survival is selectively conferred by varying sugar substrates, LY163 was grown for 24hrs in NBS with 50 g liter^{-1} xylose, glucose, or sucrose, and then directly prior to desiccation was centrifuged to allow for replacement of the spent medium with fresh NBS with 30 g liter^{-1} xylose, glucose, or sucrose. Interestingly, it was found that if the cultures were grown in xylose and then resuspended in sucrose, high levels of survival were achieved (Fig. 3-4). Conversely, cultures grown in sucrose and then resuspended in xylose had relatively poor levels of survival. This indicates that the mechanism by which the sugar serves to protect the cell involves acting in a direct physiological fashion on the outer cell membrane. Strangely, glucose added to xylose grown cultures also allowed for high survival levels, which may be attributed to the replacement of the supernatant containing various waste products that could have had a separate impact on survival.

To test to see if addition of supplemental sucrose directly prior to desiccation would contribute to increased survival, EM2L uninduced was grown for 24 hours and either 0, 1, 2 or 5 percent additional sucrose was added to the culture before transfer to the desiccation chamber (Fig. 3-5). The additional sucrose led to higher survival at all sucrose concentrations, although this trend appeared to offer diminishing returns after the one percent sucrose addition. Since percentage survival was higher than without additional sucrose, but lower than if desiccation occurred at an early growth phase, it indicates that sugar levels are not the only factor involved. Separate experiments were conducted in which KO11 was grown in CSL with 5% sucrose and

was then washed and resuspended in CSL with varying concentrations of sucrose prior to desiccation (Fig. 3-6). This experiment indicates that the optimal concentration of sucrose prior to desiccation to achieve a high level of survival is approximately 5 percent, and that over-addition of sucrose (sucrose concentrations higher than 70 g liter^{-1}) leads to a decrease in survival.

Effect of Desiccation on Fermentation

Desiccation had no measurable effect on growth and ethanol production by strain EM2L. Fresh and desiccated cells grew to the same density in the seed fermentor and produced ethanol at the same rate in the test fermentor with 100 g L^{-1} xylose (Fig. 3-7). Yields of over 90% theoretical (0.51 g ethanol per g xylose) were achieved with both.

Conclusions

The choice of sugar substrate dramatically affected desiccation tolerance of *E. coli* strains engineered for ethanol production and for lactate production. Survival was highest with sucrose, particularly for cells tested during log phase. Further improvements in desiccation tolerance were obtained by increasing the expression of genes for trehalose biosynthesis (*ostBA*) or resuspending cells in fresh medium to remove accumulated products of metabolism prior to desiccation. The benefit of trehalose and sucrose for desiccation tolerance may result in part from their unreactive nature. The presence of xylose, a reactive sugar, was detrimental for tolerance during desiccation. For EM2L containing a second copy of *otsBA*, ethanol production using desiccated inocula for seed fermentation were equivalent to that of undessicated inocula.

Figures and Tables

Table 3-1. Desiccation of ethanol versus lactate producing *E. coli*

Strain	OD 550 nm	Ethanol or lactate (g liter^{-1})	Remaining sugar (g liter^{-1})	pH	% Survival
KO11					
Xylose	10.1	12	14	4.1	<0.001
Glucose	9.5	12	14	3.7	<0.002
Sucrose	8.6	5	29	4.7	20 +/- 2
LY163					
Xylose	11.9	16	2	5.1	<0.02
Glucose	11.2	16	4	5.0	<0.20
Sucrose	10.9	6	24	5.6	5 +/- 1
TG106					
Xylose	3.0	13	29	7.0	2 +/- 1
Glucose	5.5	26	7	7.0	9 +/- 5
Sucrose	3.6	15	22	7.0	8 +/- 4

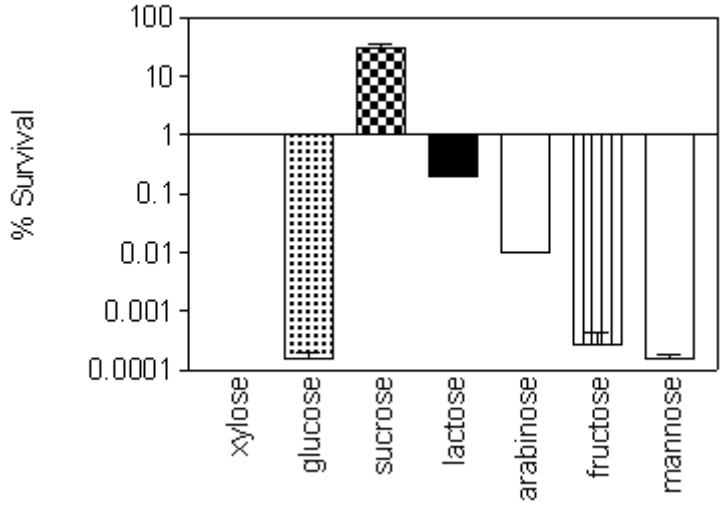

Figure 3-1. Growth substrate versus desiccation tolerance. Survival of KO11 grown in CSL with 50 g liter^{-1} xylose, glucose, sucrose, lactose, arabinose, fructose, or mannose prior to desiccation for 3 days and subsequent dehydration in 50 g liter^{-1} xylose and plating to obtain colony-forming unit counts.

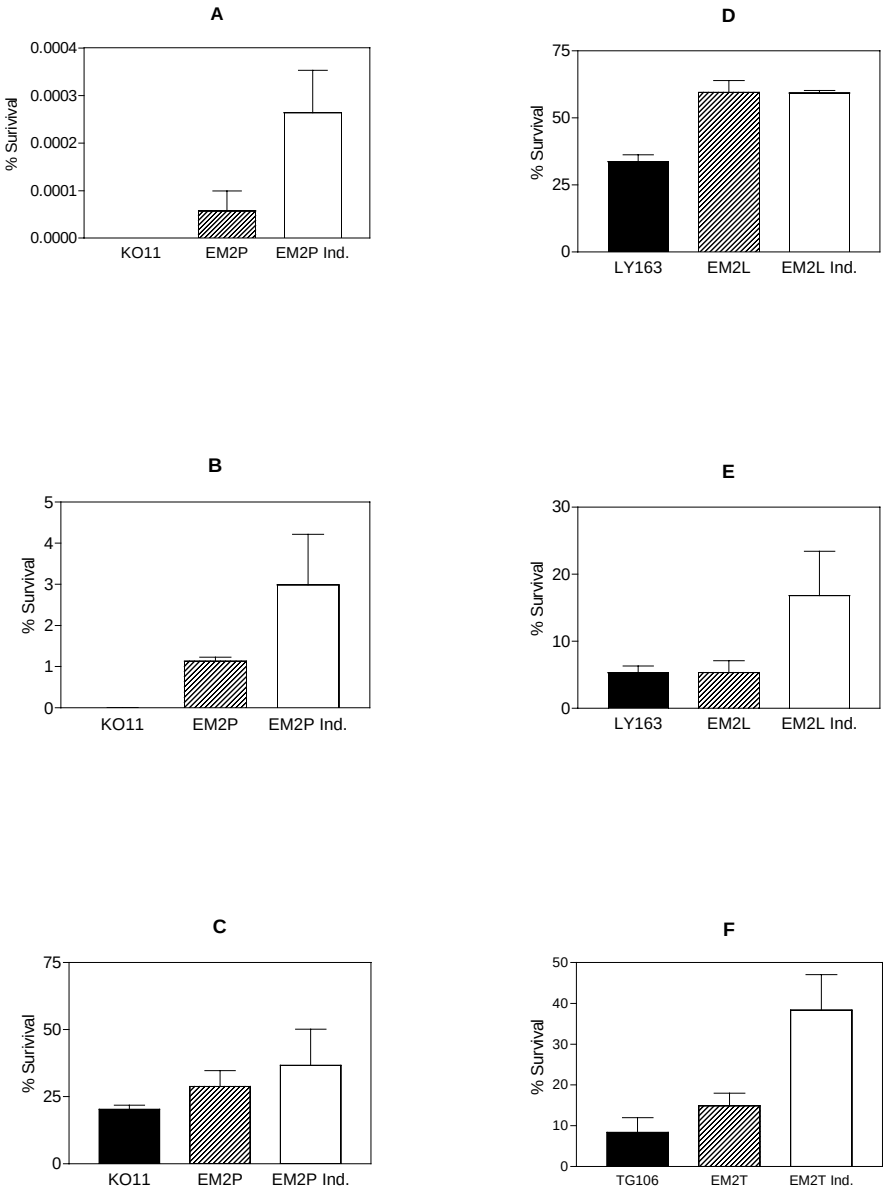

Figure 3-2. Trehalose production and sugar addition's combined effect on desiccation tolerance. Survival of KO11, the ethanol producing parent strain, and EM2P, the derivative over-expressing the genes for trehalose production, after being grown in CSL with 50 g liter^{-1} xylose (A), glucose (B), or sucrose (C) for 24 hrs prior to chemical desiccation and subsequent rehydration and plating. Survival of LY163, the parent ethanol producing strain, and EM2L, a derivative of LY163 over-expressing trehalose, after being grown to 7OD (D) or for 24 hrs (E) in NBS with 50 g liter^{-1} sucrose prior to desiccation. (F) Survival of TG106, the parent lactate producing strain, as well as EM2T, the derivative made to over-express the genes for trehalose production, after growth in fleakers containing NBS with 1 mM betaine and 50 g liter^{-1} sucrose prior to subsequent desiccation.

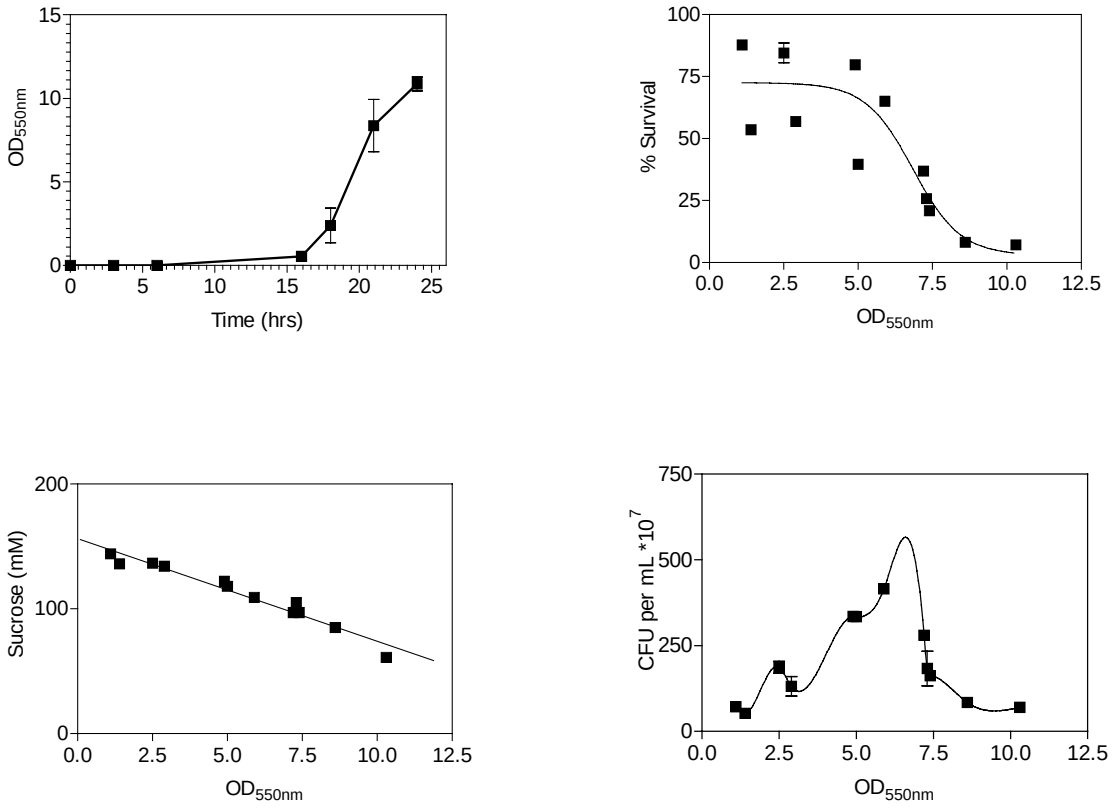

Figure 3-3. Cell density's effect on desiccation tolerance. Survival of EM2L grown to a range of optical densities in NBS with 50 g liter^{-1} sucrose prior to chemical desiccation for 3 days and subsequent rehydration and plating to measure colony-forming units. Survival shown as either CFU per mL or percentage survival. Corresponding sucrose levels as well as a growth curve of EM2L in 50 g liter^{-1} sucrose are also depicted.

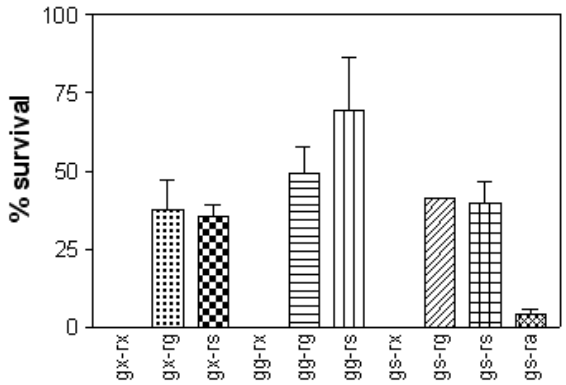

Figure 3-4. Growth sugar versus added sugar's affect on desiccation tolerance. Survival of LY163 after growth for 24 hours in NBS with 50 g liter^{-1} xylose, glucose, or sucrose, with subsequent resuspension of cells--directly prior to desiccation—in 30 g liter^{-1} of the indicated sugar (xylose, glucose, sucrose, or arabinose). For instance, gx-rx means grown in xylose (gx) and resuspended in xylose (rx).

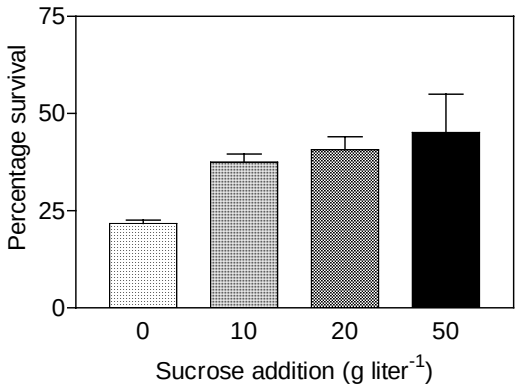

Figure 3-5. Effect of sugar addition directly prior to desiccation. Survival of EM2L grown in NBS with 50 g liter^{-1} sucrose and subsequent addition of a range of sucrose concentrations directly prior to chemical desiccation for 3 days followed by rehydration and plating.

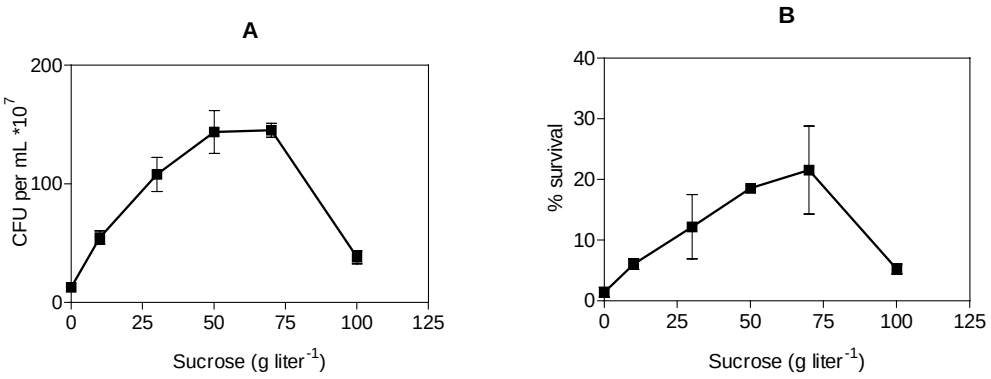

Figure 3-6. Sugar concentration versus desiccation tolerance. Survival of KO11 grown in CSL with 50 g liter^{-1} sucrose and then washed and resuspended in a range of sucrose concentrations prior to chemical desiccation, rehydration, and plating, expressed as either cfu per ml (A) or percentage survival (B).

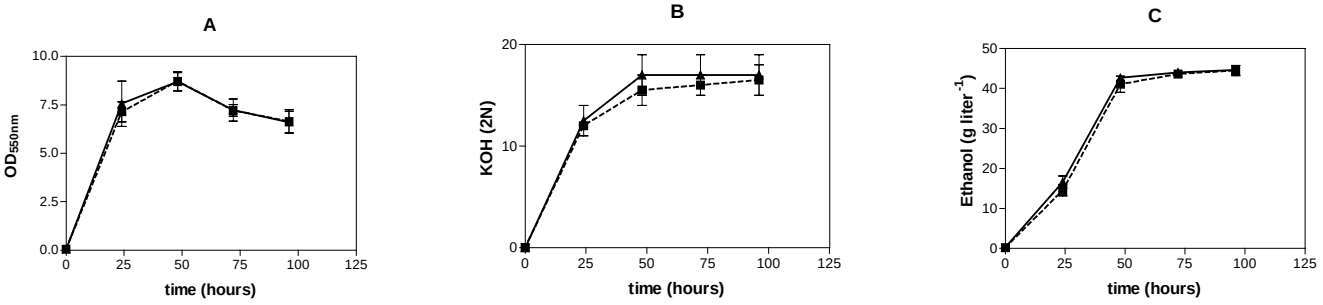

Figure 3-7. Ability of previously desiccated cells to ferment. Strain EM2L was grown overnight as described for desiccation and used either directly to inoculate a seed fermentation (3.5 ml, 1% by volume; NBS medium, 50 g liter^{-1} xylose) or dessicated (35 droplets of 0.1 ml each on parafilm; stored for 3 days) prior to use in a second seed fermentation. After 24 h incubation (37_C, 150 rpm, controlled at pH 6.5 with 2 M KOH), each seed fermentation was used to inoculate a test fermentation containing NBS media under the same conditions. Cell mass, base addition, and ethanol were measured during a 96 h incubation. Symbols for all: previously dessicated EM2L (■) versus undessicated EM2L (▲).

CHAPTER 4
INCREASING TOLERANCE TO FURFURAL

Introduction

A wide variety of fermentation products can be made using sugars from lignocellulosic biomass as a substrate (40, 51, 55, 135). Prior to fermentation, however, the carbohydrate polymers cellulose and hemicellulose must be converted to soluble sugars using a combination of chemical and enzymatic processes (122, 131). Chemical processes are accompanied by side reactions that produce a mixture of minor products such as alcohols, acids, and aldehydes which have a negative effect on the metabolism of microbial biocatalysts. Alcohols (catechol, syringol, etc.) have been shown to act by permeablizing the cell membrane and toxicity correlates well with the hydrophobicity of the molecule (137). Organic acids (acetate, formate, etc.) are thought to cross the membrane in neutral form and ionize within the cytoplasm, inhibiting growth by collapsing the proton motive force (93, 136). The inhibitory mechanisms of aldehydes are more complex. Aldehydes can react to form products with many cellular constituents in addition to direct physical and metabolic effects (82, 113). In aggregate, these minor products from chemical pretreatments can retard cell growth and slow the fermentation of biomass-derived sugars (46, 92).

Furfural (a dehydration product of pentose sugars) is of particular importance (3). Furfural content in dilute acid hydrolysates of hemicellulose has been correlated with toxicity (74). Removal of furfural by lime addition (pH 10) rendered hydrolysates readily fermentable while re-addition of furfural restored toxicity (73). Furfural has also been shown to potentiate the toxicity of other compounds known to be present in acid hydrolysates of hemicellulose (136-138). Furfural has been reported to alter DNA structure and sequence (9, 59), inhibit glycolytic enzymes (34), and slow sugar metabolism (48).

The ability of fermenting organisms to function in the presence of these inhibitors has been researched extensively. Encapsulation of *S. cerevisiae* in alginate has been shown to be protective and improve fermentation in acid hydrolysates of hemicellulose (119). Strains of *S. cerevisiae* have been previously described with improved resistance to hydrolysate inhibitors (4, 71, 89). *E. coli* (36), *S. cerevisiae* (5) and other microorganisms (13) have been shown to contain enzymes that catalyze the reduction of furfural to the less toxic product, furfuryl alcohol (137). In *E. coli*, furfural reductase activity appears to be NADPH-dependent (36). An NADPH-dependent furfural reductase was purified from *E. coli* although others may also be present. An NADPH-dependent enzyme capable of reducing 5-hydroxymethyl furfural (a dehydration product of hexose sugars) has been characterized in *S. cerevisiae* and identified as the ADH6 gene (99).

Isolation of a furfural-resistant *E. coli* mutant (EMFR9) in which furfural reductase activity is lower than that of the parent (LY180) due to decreased expression of *yqhD* and *dkgA* is described in this section. The reduction of furfural by these two NADPH-dependent oxidoreductases is proposed to inhibit growth by depleting NADPH needed for biosynthesis. Thereafter, we use global transcript analysis to expand our investigations in order to include the broader cellular response to added furfural using the parent organism, strain LY180.

Materials and Methods

Strains, Media, and Growth Conditions

Strains and plasmids used in this study are listed in Table 4-1. Plasmid and strain constructions were made using Luria broth (78). Antibiotics were included as appropriate. Temperature-conditional plasmids were grown at 30^{o}C; all others were grown at 37^{o}C. Ethanologenic strains were maintained in AM1 mineral salts medium (72) supplemented with 20 g liter^{-1} xylose for solid medium and 50 g liter^{-1} xylose or higher for liquid medium used in

fermentation experiments. *E. coli* strain LY168 (51) is a derivative of KO11 and served as the starting point for this investigation. Note that *E. coli* W (ATCC 9637) is the parent for strain KO11, initially reported to be a derivative of *E. coli* B (90).

Construction of Strain LY180

Strain LY168 has been previously described for the fermentation of sugars in hemicellulose hydrolysates (51). Several modifications were made to improve substrate range (restoration of lactose utilization, integration of an endoglucanase, and integration of cellobiose utilization) resulting in LY180. Linear DNA fragments used for integration are shown in Figure 4-1 and have been deposited in GenBank. The FRT region in *lacY* was replaced with the native *E. coli* ATCC 9637 sequence by double homologous recombination using Fragment A containing *lacZ lacY lacA cynX'* (24, 53). Integrated strains were selected directly for lactose fermentation. The *frdBC* region downstream from *frdA::Zm frg celY* $_{Ec}$ (*Erwinia chrysanthemi*) was deleted by double homologous recombination using a two step process (53). Fragment B (*frdB'*, a *cat, sacB* cassette, and *frdC'*) was integrated first with selection for chloramphenicol resistance. The *cat-sacB* cassette was then replaced with Fragment C consisting of *frdA'*, *Z. mobilis* promoter fragment, *E. chrysanthemi celY*, and *frdC'* by selecting for resistance to sucrose. This replacement also deleted an FRT site. The *K. oxytoca* genes encoding cellobiose utilization (*casAB*) were inserted into *ldhA* by double homologous recombination also using a two step process (53). Fragment D (*ldhA'*, a *cat-sacB* cassette, *casAB*, and *'ldhA*) was used to replace the FRT site in *ldhA* with selection for resistance to chloramphenicol. The *cat-sacB* cassette was then replaced with Fragment E consisting of *ldhA'*, a promoter fragment from *Z. mobilis,* and *K. oxytoca casA'*. Integrated strains were isolated by selecting directly for cellobiose fermentation. All constructs were verified by analyses of phenotypes and PCR products.

Growth-Based Selection for a Furfural Resistant Strain

LY180 was inoculated into a 500-mL vessel (initial inoculum of 50 mg dcw liter^{-1}) containing 350 ml of AM1 supplemented with 100 g liter^{-1} xylose and 0.5 g liter^{-1} furfural (37°C, 150 rpm, pH 6.5). Cultures were serially diluted into new fermentors at 24-h intervals, or when cell mass exceeded 330 mg dcw liter^{-1}. Furfural was gradually increased to 1.3 g liter^{-1} as growth permitted. After 54 serial transfers, a resistant strain was isolated and designated EMFR9.

Furfural Resistance and Metabolism During Fermentation

Furfural resistance was compared in small fermentors (37°C, 150 rpm, pH 6.5, 350-ml working volume) using AM1 medium (72) containing 100 g liter^{-1} xylose. Seed cultures were inoculated to approximately 33 mg dcw liter^{-1}. Samples were removed periodically to measure cell mass, ethanol, and furfural.

Furfural toxicity (MIC) was also examined using tube cultures (13x100 mm) containing 4 ml of AM1 broth with 50 g liter^{-1} (wt/vol) filtered-sterilized sugar, furfural, and other supplements. Cultures were inoculated to an initial density of 17 mg dcw liter^{-1}. Cell mass was measured after incubation at 37°C for 24 h and 48 h.

Comparison of Hydrolysate Toxicity

A hemicellulose hydrolysate of sugar cane bagasse was produced using dilute sulfuric acid at elevated temperature and pressure and supplied by Verenium Corporation (Boston, MA). This hydrolysate contained 82 g liter^{-1} total sugar (primarily xylose), 1.4 g liter^{-1} furfural, and other constituents. Hydrolysate was supplemented with the mineral components of AM1 medium, adjusted to pH 6.5 using 45% KOH, and diluted with complete AM1 (80 g liter^{-1} xylose). Diluted samples of hydrolysate were distributed into 13 mm X 100 mm culture tubes (4 mL each), inoculated to an initial cell density of 17 mg dcw liter^{-1}, and incubated at 37°C. Cell

mass (after centrifugation and resuspending in broth) and ethanol concentration were measured after 48 h.

Microarray Analysis

Cultures were grown in small fermentors to a density of 670 mg dcw/L. An initial sample was removed that served as a control. Furfural was immediately added from a 50 g L^{-1}aqueous stock (0.5 g $liter^{-1}$ final concentration) and incubation continued for 15 minutes prior to a second sampling. Samples were rapidly cooled in an ethanol-dry ice bath, harvested by centrifugation at 4^0C, resuspended in Qiagen RNA Later and stored at -80^0C until RNA extraction. RNA was purified using a Qiagen RNeasy Mini Kit, treated with DNase I and purified by phenol/chloroform extraction and ethanol precipitation. RNA was sent to NimbleGen (Madison, WI) for microarray comparisons using templates designed for *E. coli* K12. Each sample consisted of pooled material from four fermentors. The complete experiment was performed twice. Data was analyzed with ArrayStar software (DNA Star, Madison, WI), and by SimPheny (Genomatica Inc., San Diego, CA). Expression ratios are presented as the average of the two-pooled datasets, although it should be noted that the oxidoreductase experiments were based on the initial microarray dataset only. A control experiment was performed during which water was added. 54 genes (>2% of chromosome) changed more than 2-fold after water addition. Only 8 of these were also affected by furfural addition indicating that the effect of disturbing the culture by liquid addition was negligible for the furfural response.

Network Component Analysis

Network Component Analysis (NCA) calculates transcription factor activity ratios from expression ratios and known regulatory connections and was performed as previously described (49). The connectivity file was updated according to Regulon DB and Ecocyc (33, 54). The regulon of the "stringent factor" was defined as previously described, via analysis of the 5-

minute response to serine starvation via serine hydroxamate treatment during mid-log growth of BW25113 in MOPS glucose (49). Regulators with significantly altered regulatory activity were identified by comparison to a null distribution and using a P-value cutoff of 0.05.

Cloning and Deletion of Genes

Oxidoreductase genes for expression studies (ribosomal-binding sites, coding regions, and 200 base pair terminator regions) were amplified from strain LY180 genomic DNA using a BioRad iCycler (Hercules, CA), ligated into pCR 2.1 TOPO vector, and cloned into *E. coli* TOP10F' using an Invitrogen TOPO TA Cloning Kit (Carlsbad, CA). Plasmids were purified using a QiaPrep Spin Mini Prep Kit. Gene orientation was established by PCR.

E. coli transhydrogenase genes were amplified (ribosomal-binding sites, coding regions, and a 200 bp terminator region) from strain LY180 genomic DNA using a BioRad iCycler (Hercules, CA) with primers that provided flanking HindIII sites. After digestion with HindIII, the product was ligated into HindIII digested pTrc99a (vector) and transformed into *E. coli* TOP10F' (Carlsbad, CA). Plasmids were purified using a QiaPrep Spin Mini Prep Kit (Valencia, CA). Gene orientation was established by digestion with restriction enzymes and by polymerase chain reaction.

A *yqhD* deletion was constructed in LY180 as described by Datsenko and Wanner (24) using the plasmids pKD4 and pKD46. A *dkgA* deletion in LY180 was constructed as described by Jantama et al. (53). A double mutant with deletions in both *yqhD* and *dkgA* was also constructed. Repeated attempts to delete the *yqfA* gene were not successful.

Purification and Kinetic Analysis of YqhD and DkgA

Both the *yqhD* and *dkgA* genes were cloned into a Novagen pET-15b vector and expressed as a His-tagged protein in *E. coli* BL21 (DE3). Cells were grown with IPTG to approximately 1.3 g dcw liter^{-1}, washed with 100 mM phosphate buffer, and lysed using MP Fast

Prep-24 (MP Biomedical, Solon, OH) and Lysing Matrix B. Crude extracts were passed through a 0.22 μm PVDF filter and further purified using a 1 mL HiTrap nickel column. Purified enzymes were dialyzed in 100 mM phosphate buffer using a Thermo Slide-A-Lyser and quantified using a Thermo BCA Protein Assay Kit. Purity of YqhD and DkgA were estimated to be greater than 90% by SDS-PAGE. A single band was observed for each in an SDS-PAGE gel. Estimated sizes of the purified proteins were in agreement with predicted values of 43 kD and 31 kD, respectively. Apparent Kcat and apparent Km values were determined for both purified enzymes using NADPH and furfural.

Whole-cell Assays of Furfural Metabolism in Vivo during Fermentation

Whole-cell furfural metabolism was measured using fermentors in which cultures were grown to a density of 670 mg dcw liter^{-1} (mid log phase). Furfural was added to an initial concentration of 0.5 g liter^{-1}. Samples were removed at zero time and after 15, 30, and 60 min of incubation for the measurement of furfural and cell mass. The specific rate of furfural metabolism was calculated using the average cell mass during each assay interval. Results are expressed as μmoles min^{-1} mg dcw^{-1}.

In Vitro Assay of Furfural Reduction

Anaerobic tube cultures were grown in AM1 medium containing 50 g liter^{-1} xylose and harvested in mid log phase (0.7-1.0 g dcw liter^{-1}). Cells were washed once with 20 mL 100 mM potassium phosphate buffer (pH 7.0), resuspended in phosphate buffer to approximately 6.5 g dcw liter^{-1}, chilled on ice, and lysed for 20 sec using a FastPrep-24 cell disruptor and Lysing Matrix B. Debris was removed by centrifugation (13,000 x g; 10 min) and the supernatant used to measure furfural-dependent oxidation of NADH and NADPH. Assays contained 100 mM phosphate buffer (pH 7.0), 20 mM furfural, and 0.2 mM reductant (NADPH or NADH).

Furfural-dependent activity (μmoles min^{-1} mg $protein^{-1}$) was measured as the change in absorbance at 340nm. Greater than 80% of activity was NADPH-dependent.

Analyses

Ethanol was measured using an Agilent 6890N gas chromatograph (Palo Alto, CA) equipped with flame ionization detectors and a 15-meter HP-PlotQ megabore column. Dry cell weight was estimated by measuring optical density at 550nm using a Bausch & Lomb Spectronic 70 spectrophotometer. Each OD_{550nm} is equivalent to approximately 333.3 mg dcw $liter^{-1}$.

Furfural levels in AM1 medium were measured by absorbance at OD_{284nm} and OD_{320nm} (20). The accuracy of this method was confirmed by HPLC analysis. Furfural content of bagasse hemicellulose hydrolysate was measured using an Agilent LC1100 liquid chromatograph (refractive index monitor and UV detector) and an Aminex HPX-87P ion exclusion column (BioRad, Hercules, CA) with water as the mobile phase

Furfural tolerance for growth was measured in standing tubes with 4 mL total volume of AM1 and 50 g $liter^{-1}$ filter-sterilized xylose. Tubes were incubated at 37^0C and measured after 24 and 48 h. Values reported are an average of at least 3 measurements.

Results and Discussion

Isolation and Initial Characterization of a Furfural-Resistant Mutant

A furfural-resistant derivative of LY180 was isolated after 53 serial transfers in pH-controlled fermentors (Fig. 4-2) containing AM1 mineral salts medium with 100 g $liter^{-1}$ xylose and increasing concentrations of furfural (0.5 g $liter^{-1}$ initially to final concentration of 1.3 g $liter^{-1}$). Attempts to directly isolate mutants resistant to 1.0 g $liter^{-1}$ furfural in a single step (solid medium and broth) were not successful. Step-wise improvement in furfural tolerance was observed during serial transfers, consistent with multiple changes. The resulting strain, EMFR9, grew and fermented xylose in the presence of 1.0 g $liter^{-1}$ furfural at a rate equivalent to the

parent LY180 in the absence of furfural (Fig. 4-3). Growth and ethanol production by EMFR9 also exceeded that of the parent LY180 in the absence of furfural.

Addition of a low furfural concentration (0.4 g liter^{-1}) to the parent LY180 caused an initial lag in growth and ethanol production (Fig. 4-3A and 4-3B). During this lag, furfural was chemically reduced to the less toxic furfuryl alcohol (137, 138) (Fig. 4-3C). Growth and fermentation increased by more than 3-fold immediately following the complete removal of furfural. Growth and ethanol production by LY180 were strongly inhibited by 1.0 g liter^{-1} furfural throughout the 72-h incubation (Fig. 4-3D and 4-3E). During this time, approximately 20% of the furfural was reduced indicating that LY180 remained metabolically active (Fig. 4-3F). In contrast to LY180, EMFR9 was virtually unaffected by the presence of furfural (0.4 g liter^{-1} or 1.0 g liter^{-1}) (Fig. 4). The volumetric rate of furfural reduction was higher for EMFR9 than LY180 at both furfural concentrations (Figure 4-3C and 4-3F), primarily due to the larger amount of cell mass (Fig. 4-3A). This was confirmed by further experiments in which the *in vivo* rate of NADPH-dependent furfural reduction by EMFR9 (per mg dcw) was found to be about half that of the parent LY180. In contrast to LY180, growth and fermentation of EMFR9 did not require prior reductive removal of furfural. With EMFR9, both 0.4 g liter^{-1} and 1.0 g liter^{-1} furfural were reduced to furfuryl alcohol concurrently with growth. Reduction by EMFR9 was complete after 12 h and 18 h, respectively (Fig. 4-3C and 4-3F).

Together, these results suggest that the process of reducing furfural rather effects of the compound itself may be the primary site of growth inhibition at low concentrations. The loss of function, i.e. a decrease in furfural reducing activity, correlated with an increase in furfural tolerance in the mutant. Based on these results, we propose that the inhibition of growth by

furfural results from competition between biosynthetic needs and furfural reduction for a limited pool of NADPH.

Effect of Media Composition on Furfural Resistance (MIC)

Unlike glucose, the production of NADPH is problematic during xylose fermentation (130) and offers an approach to test the NADPH-competition hypothesis by measuring the MIC for furfural in different media. In mineral salts media with 50 g liter^{-1} xylose (Fig. 4-4A), the minimal inhibitory concentration (MIC) of furfural was approximately 1.0 g liter^{-1} for LY180 (parent) and 2.0 g liter^{-1} for the mutant EMFR9. Replacement of xylose with glucose would be expected to increase the pool of NADPH. This change (Fig. 4-4B) increased the furfural MIC by 50% for LY180 (1.5 g liter^{-1}) and by 25% for EMFR9 (2.5 g liter^{-1}). Addition of a small amount of yeast extract (1.0 g liter^{-1}) to xylose-mineral salts medium would be expected to decrease biosynthetic demands for NADPH. This supplement (Fig. 4-4C) doubled the furfural MIC for the parent LY180 (2.0 g liter^{-1}) and increased the MIC for EMFR9 (2.5 g liter^{-1}) by 25%. With all media, EMFR9 was more resistant to furfural than the parent LY180. Both glucose (increased NADPH production) and yeast extract (decreased need for biosynthesis) increased furfural tolerance. However, this benefit was more pronounced for the parent, strain LY180, than for the mutant EMFR9, consistent with the lower level of furfural reductase activity in EMFR9.

The MIC for three other compounds known to be present in hemicellulose hydrolysates were also examined: 2-hydroxymethyl furfural (analogue, dehydration product of hexose sugars), furfuryl alcohol (reduced product of furfural), and syringaldehyde (degradation product of lignin). EMFR9 was slightly more tolerant to 2-hydroxymethyl furfural (MIC of 3.0 g liter^{-1}) than LY180 (MIC of 2.5 g liter^{-1}). Both strains were equally sensitive to syringaldehyde (MIC 2.0 g liter^{-1}) and furfuryl alcohol (15 g liter^{-1}) (data not shown). The absence of an increase in

tolerance to other compounds in EMFR9 is consistent with a specific site or target for furfural toxicity.

Comparison of Oxidoreductase Expression by mRNA Microarray Analysis

Previous studies have demonstrated that *E. coli* contains NADPH-dependent enzyme(s) capable of reducing furfural to a less toxic compound (furfuryl alcohol) but no gene was identified (36). The dependence of the parent LY180 on the complete reduction of furfural prior to growth and the loss of this dependence by EMFR9 further implicates oxidoreductases as being of primary importance for furfural sensitivity.

Microarray analysis of mRNA was used to identify candidate oxidoreductase genes for furfural reduction. Cultures of LY180 and EMFR9 were grown to mid-log phase in pH-controlled fermentations with 100 g liter^{-1} (wt/vol) xylose. For this comparison, RNA was isolated 15 min after the addition of 0.5 g liter^{-1} furfural. A total of 12 known and putative oxidoreductases were found that differed by approximately 2-fold or higher (Table 4-2).

Four oxidoreductases were identified that were expressed at lower levels in EMFR9 (Table 4-2). Each of these four genes was cloned into plasmids and transformed into EMFR9. When expressed from plasmids, three of these genes (*dkgA*, *yqhD*, and *yqfA*) were found to decrease furfural tolerance (Fig. 4-5). Expression of *yqhD* and *dkgA* were most detrimental and both were shown to increase furfural reductase activity in EMFR9 (Fig. 4-6). Expression of *yqfA* did not restore furfural reductase activity of EMFR9 and its effect on growth inhibition may be related to other functions. No detrimental effect on growth was observed for *yjjN*. Thus the decrease in expression of *yqhD, dkgA*, and *yqfA* in EMFR9 can be inferred to be beneficial for furfural tolerance. Silencing of *yqhD* and *dkgA* in EMFR9 would decrease the competition with biosynthesis for NADPH during furfural reduction. It should be noted that effects seen under

uninduced conditions can be attributed to a leaky promoter that allows expression of each cloned gene, in conjunction with the high copy number of the pCR2.1 TOPO vector.

The other eight genes were cloned from LY180 into pCR2.1 TOPO for expression. These oxidoreductases had increased expression in EMFR9 (1.8-fold to 4.5 fold) relative to the parent LY180. Plasmids containing each of these genes were transformed into LY180. However, none of these 8 caused an increase or decrease furfural tolerance (data not shown).

To further examine the potential importance of *yqhD, dkgA*, and *yqfA* silencing, attempts were made to delete each of these genes from LY180. Although deletions of both *yqhD* and *dkgA* were readily recovered, similar methods were not successful with *yqfA*. In LY180, deletion of *yqhD* alone or in combination with *dkgA* caused an increase in furfural tolerance (Fig. 4-7) and a decrease in furfural reductase activity *in vivo* similar to that of EMFR9 (Fig. 4-6). Since deletion of *dkgA* alone in LY180 did not lower the *in vivo* reductase activity or increase furfural tolerance, YqhD is presumed to be the more important activity for growth inhibition by low concentrations of furfural. The lowest furfural reductase activity was found after deletion of both genes.

Characterization of YqhD and DkgA

The largest changes in gene expression among oxidoreductases were the silencing of *yqhD* and *dkgA*. Both YqhD and DkgA were expressed as his-tagged proteins in BL21 (λDE3) and purified to discernable homogeneity. Both enzymes catalyzed the NADPH-dependent reduction of furfural to furfuryl alcohol. The apparent *K*m values for furfural were relatively high for YqhD (9.0 mM) and DkgA (>130 mM). With such values, it is unlikely that furfural is the native substrate of either enzyme. Reasonably assuming that cells are permeable to furfural, the intracellular activities of YqhD and DkgA would be expected to vary over the range of furfural concentrations used for selection (5-14 mM; 0.5-1.3 g liter^{-1}). The apparent Km values for

NADPH were quite low for both YqhD (8 μM) and DkgA (23 μM). In the presence of furfural, the high affinity of both enzymes for NADPH would compete with biosynthetic reactions for NADPH. Partitioning of NADPH among pathways would be determined by the *K*m for NADPH, steady state pool size of NADPH, and the relative abundance of competing oxidoreductase activities. Several key metabolic enzymes have a Km for NADPH higher than that of YqhD (8 uM), including CysJ (80 uM), required for sulfate assimilation to form cysteine and methionine, ThrA (90uM), required for the formation of threonine, and DapB (17 uM), for lysine formation (109).

Tolerance to Acid Hydrolysate of Hemicellulose

Hemicellulose hydrolysates contain a mixture of compounds that act in combination to inhibit microbial growth and fermentation (73, 74, 136-138). Growth and fermentation were examined in dilutions of a neutralized hydrolysate that contained 1.4 g liter^{-1} furfural (Fig. 4-8). Although the MIC values for growth and ethanol production were similar (30% hydrolysate), EMFR9 grew to a 3-fold higher density and produced over 10-fold more ethanol in 20% hydrolysate than the parent LY180. Selection of EMFR9 for increased resistance to furfural was accompanied by an increase in resistance to hemicellulose hydrolysate, confirming the importance of furfural as a component of hydrolysate toxicity.

Global Effect of Furfural on the Transcriptome

Message levels were compared in actively growing cells before and 15 min after the addition of 0.5 g L^{-1} fufural. Expression levels for 412 genes (10% of the transcriptome) were altered (2-fold or greater) by the addition of furfural. The distribution of these altered genes varied widely among functional groups, providing useful insight into the mechanism of furfural's action (Table 4-3). In most functional groups, expression levels of less than 10% of the gene members were altered by 2-fold or greater. Groups with this low frequency of change included

cofactors, carbon compounds, regulatory, macromolecular synthesis (Cell structure, DNA, Lipids, Transcription, and Translation), and others (Phage, Putative/IS, Regulatory, and Unclassified/Unknown). Expression levels for 10% to 20% of the member genes were altered in four groups (Cell processes, Central metabolism, Energy, and Transport). Most of the affected genes associated with central metabolism, energy, and transport increased in expression upon furfural addition. These changes could provide an opportunity to scavenge and metabolize additional compounds that may be available and to increase carbon flow for energy production. Although strain LY180 is non-motile, many of the altered genes concerned with cell processes are involved in motility and chemotaxis and were not investigated further.

Expression levels for over 20% of the member genes in two functional groups were altered by the addition of furfural, Amino acids and Nucleotides. In these groups, over 2/3 of the altered genes were reduced by 2-fold or greater upon the addition of furfural. Expression levels for individual genes affecting the biosynthesis of purines, pyrimidines, and every family of amino acids were reduced by 2-fold or greater upon the addition of furfural. A single gene in nucleotide metabolism and only a minority of genes involved in amino acid metabolism exhibited a furfural-dependent increase in expression. Together, these changes agree well with the generalized decrease in biosynthesis and growth observed upon the addition of furfural.

Effect of Furfural on Regulatory Activity

Network component analysis (NCA) was used to provide a global view of the cellular response to furfural. This analysis uses known regulatory network structure to identify regulators with perturbed activity from transcriptome data (15, 66). Of the 60 regulators included in this analysis, 22 were identified as being altered in expression by furfural relative to a random network (Table 4-4, Fig. 4-9). Perturbation of RpoS, a sigma factor that acts as a signal for general stress response, indicates that the cell recognizes the presence of a stress-

inducing agent. Since up to 10% of *E. coli's* genome is regulated in some fashion by RpoS (95, 128), it is difficult to determine a specific inhibitory response. RpoS-regulated genes with increased expression upon furfural addition include *poxB*, involved in conversion of pyruvate into acetate and CO_2 (139), and *otsA*, required for trehalose protection during osmotic stress response (115).

Regulators of cysteine and methionine biosynthesis (CysB and MetJ) as well as repressors of amino acid (ArgR) and nucleotide biosynthesis (PurR) were also significantly affected by furfural addition. The stringent factor, a collective indicator of the stringent response (a diversion of resources away from growth to amino acid biosynthesis during amino acid and carbon starvation) also shows activation consistent with stalled biosynthesis and an excess of many intermediates. Together, these results indicate that the pools of many amino acids and biosynthetic intermediates have been altered by furfural addition. The fact that expression of genes concerned with cysteine and methionine biosynthesis increased while expression of other biosynthesis pathways declined is consistent with a depletion of cysteine and methionine pools as an early event resulting from a furfural challenge. Histidine may also be limited by the addition of furfural. Genes (*hisA*, *hisB*, *hisC*, *hisD*, *hisF*, *hisH*, and *hisI*) under control of the His regulator (histidinyl-tRNA) were generally increased after the addition of furfural, although less than 2-fold (Fig. 4-10). The two terminal steps in histidine biosynthesis involve the reduction of NAD^+ to NADH, a reaction that may be slowed by the high NADH/NAD^+ ratio associated with fermentation.

The up-regulation of several glycerol metabolism genes (*glpT* and *glpD* in Figure 4-9, and *glpF* and *glpK* in Table 4-3) led us to investigate the effect of glycerol supplementation on furfural tolerance. However, the addition of glycerol (1.0 to 20 g $liter^{-1}$) had no effect on furfural

tolerance (data not included). Several other regulators, including *fis* and *crp,* were found to be significantly altered by NCA.

Effect of Furfural on Amino Acid Sulfur Assimilation Gene Expression

Genes concerned with sulfur assimilation into cysteine, and methionine are scattered within several Functional groups (Amino acid, Central metabolism, Regulation, and Transport). All that were perturbed by 2-fold or greater (Table 4-3) were increased by the addition of furfural (*cysC, cysH, cysI, cysM, cysN, cysQ, metA, metB*, *metC*, *metL, sbp, tauA, tauB, tauC* and *tauD*). Many additional genes involved in sulfur assimilation were also up-regulated less than 2-fold and have been included to demonstrate the furfural response (Fig. 4-11). Sulfur is supplied as sulfate in AM1 medium and must be reduced to the level of hydrogen sulfide for incorporation, an energy intensive reaction requiring 4 molecules of NADPH. The furfural-induced increase in expression of these genes is in sharp contrast to the decreases observed for many other genes concerned with the biosynthesis of amino acids, purines, and pyrimidines (Table 4-3). Expression of the taurine transport genes (*tauABCD*; alternative source of sulfur), the sulfate-binding transport protein (*sbp*), and the transcriptional activator of many cysteine biosynthetic genes were increased by more than 5-fold in response to added furfural. Together, these results suggest that the addition of furfural results in an intracellular deficit in sulfur-containing amino acids (cysteine and methionine) which may be associated with the high NADPH requirement in this pathway.

Effect of Amino Acid Supplements on Furfural Tolerance

All 20 amino acids were individually tested for their ability to improve the growth of LY180 in AM1 mineral salts medium (Fig. 4-12A, 4-12B). A concentration of 0.1 mM was selected roughly based on the cellular content of individual amino acids (87). Only four amino acids improved furfural resistance when supplied at this low concentration: cysteine >

methionine > serine, arginine > histidine. The two sulfur amino acids were clearly the most beneficial for furfural resistance. When supplied at a 5-fold higher concentration (0.5 mM), all amino acids were beneficial to some degree (Fig. 4-12B). However, cysteine remained the most effective followed by serine, methionine, and arginine. A cysteine concentration of 0.05 mM allowed LY180 to grow to a density of 1 g liter^{-1} in the presence of 1.0 g liter^{-1} furfural, approximately equal to the total cellular sulfur (Fig. 4-12C). No measurable improvement in furfural resistance was observed with 0.01 mM cysteine.

I considered the possibility that the protective effect of L-cysteine could result from a chemical reaction with furfural in AM1 medium. However, the protective concentration of cysteine (0.05 mM) was 200-fold lower than that of 1.0 g liter^{-1} furfural (10 mM) making this unlikely. Furfural in mineral salts medium can be readily quantified by its characteristic spectrum (75) and remained unchanged during a 48 hr incubation at 37ºC, consistent with minimal chemical reactivity.

The beneficial effects of histidine, serine, and arginine for furfural tolerance are not immediately apparent. Most genes concerned with histidine biosynthesis increased in response to furfural addition, although less than 2-fold (Fig. 4-10). De novo biosynthesis of histidine during fermentation may be constrained by the high NADH/NAD^+ ratio during anaerobic growth and the requirement for further reduction of NAD^+ in the two terminal steps of biosynthesis. Similarly, the first committed step in serine biosynthesis also involves the reduction of NAD^+ and may be hindered during fermentation. Increasing serine may also increase the efficiency of incorporating reduced sulfur from H_2S into cysteine. Genes concerned with arginine biosynthesis (*argA, argB,, argC, argD, carA, carB, and argG*) were generally lowered by the addition of furfural. However, the expression level of *speA* encoding arginine decarboxylase was increased

by the addition of furfural. The degradation of arginine may provide useful intermediates and co-factors for biosynthesis.

Effect of Alternative Sulfur Sources on Furfural Tolerance

The addition of furfural inhibited growth and increased the transcription of genes concerned with sulfur assimilation. Genes involved in the uptake and incorporation of the alternative sulfur compound, taurine (*tauABC* and *tauD*), were among the 10 genes with the largest increases in expression. The *tau* genes are typically expressed only during sulfur starvation (125). Since cysteine was effective in relieving furfural inhibition, the increased expression of these genes can be presumed to result from a reduction in the pool of sulfur amino acids by furfural. Furfural could inhibit sulfur amino acid biosynthesis either by limiting the availability of reduced sulfur (H_2S) from sulfate or by inhibiting the incorporation of reduced sulfur into cysteine.

These possibilities were examined during growth in AM1 medium containing 1.0 g liter^{-1} furfural by comparing the effects of alternative sulfur sources (L-cysteine, D-cysteine, taurine, sulfite, and sodium thiosulfate) that enter metabolism at different levels of reduction. Note that 4 NADPH molecules and two reductase enzymes (CysH and CysIJ) are required to fully reduce sulfate prior to assimilation into cysteine. L-cysteine, D-cysteine and thiosulfate bypass both reductase enzymes and all were effective at relieving furfural inhibition (Fig. 12D). D-cysteine cannot be incorporated directly and is first catabolized to H_2S. Thiosulfate also serves as a source of reduced sulfur for incorporation by CysM (69). Taurine is catabolized to sulfite in the cytoplasm and must be reduced by sulfite reductase (CysIJ and 3 NADPH molecules) prior to assimilation into cysteine (111). Unlike cysteine and thiosulfate, taurine was not effective in preventing the inhibition of growth by 1.0 g liter^{-1} furfural.

These results with alternative sulfur sources indicate that furfural acts to inhibit growth

by limiting the production of reduced H_2S from sulfate rather than by inhibiting the incorporation of reduced sulfur into cysteine. With taurine as a sulfur source, furfural must act at the level of sulfite reductase (CysIJ). With sulfate as a sulfur source, further effects of furfural at earlier steps in anabolism cannot be excluded.

Effect of Increasing Transhydrogenase Expression on Furfural Tolerance

Growth of LY180 is inhibited only while furfural is being metabolized and resumes after complete reduction to furfuryl alcohol by NADPH-dependent enzymes. Silencing two genes (*yqhD* and *dkgA*) encoding low Km, NADPH-dependent furfural reductases provided resistance to over 1.0 g liter^{-1} furfural. Although furfural may inhibit sulfur amino acid production by directly affecting enzymes concerned with the conversion of sulfate and sulfite to H_2S, it is also possible that the inhibition of this process results from an indirect effect of furfural on the availability of NADPH. To test this hypothesis, the two *E. coli* transhydrogenases (SthA and PntAB) were cloned into pTrc99a and confirmed by sequencing. The growth of LY180 was reduced on plates and in broth by the presence pTrc99a plasmids and antibiotics (antibiotics may have forced the cells to maintain pTrc99a, depleting the cells of energy for other processes).

SthA is a cytoplasmic transhydrogenase with kinetic characteristics that promote function primarily in the direction of NADPH oxidation (108). Expression of the *sthA* gene from a plasmid did not alter furfural tolerance with or without IPTG induction (Fig. 4-13). Functionality of the cloned gene was confirmed in vitro. Upon induction with 0.1 mM IPTG, activity was found to increase from approximately 1.0 nmol min^{-1} mg protein^{-1} to 18 nmol min^{-1} mg protein^{-1}. PntAB is a proton translocating transhydrogenase that is not known to function during fermentative growth but is potentially capable of increasing the pool of NADPH (108). Leaky expression of *pntAB* from an uninduced plasmid partially restored growth in the presence of 1.0 g liter^{-1} furfural (Fig. 4-13). Adding IPTG to express higher levels of this enzyme

eliminated resistance to furfural and also inhibited the growth of cells in the absence of furfural. Based on these results, furfural appears to inhibit growth by depleting the supply of NADPH needed for biosynthesis. The large requirement of NADPH for sulfate assimilation, 4 per cysteine equivalent, and the limited routes for NADPH production from xylose during fermentation appear to have made the production of sulfur amino acids most vulnerable to competition by furfural reductases for NADPH.

Conclusions

Furfural is a natural product of lignocellulosic decomposition. Furfural is also formed by the dehydration of pentose sugars during the depolymerization of cellulosic biomass under acidic conditions (3, 73). This compound is an important contributor to toxicity of hemicellulose syrups, and increases the toxicity of other compounds (138). Selection for a furfural-resistant mutant of *E. coli* during growth in xylose-mineral salts medium resulted in a strain (EMFR9) with improved resistance to hemicellulose hydrolysate, confirming the practical importance of this compound.

The ability to reduce furfural into the less toxic furfuryl alcohol is widely distributed in nature (13). An enzyme has been purified from *E. coli* that catalyzes this reaction (36) and a gene that reduces the analogue 5-hydroxymethyl furfural has been identified in *S. cerevisiae* (5). The mRNA levels of oxidoreductases were compared in the furfural-resistant mutant EMFR9 and the parent LY180. Twelve differed by 2-fold or more. Of these, 8 were higher in EMFR9 and 4 were lower in EMFR9. All were cloned and tested either for their ability to confer furfural tolerance in LY180 or decrease furfural tolerance in EMFR9. None of these gene products increased furfural tolerance when over-expressed from plasmids. Expression of three genes (*yqhD, dkgA*, and *yqfA*) decreased the furfural tolerance of EMFR9. Contrary to initial expectations that furfural tolerance would be improved by increased expression of reductase

activity, these results demonstrated that the increased tolerance in EMFR9 results in large part from gene silencing (*yqhD, dkgA*) that decreased the level of NADPH-dependent furfural reductase activities. Deletion of *yqhD* encoding the lower *K*m oxidoreductase (NADPH) increased furfural tolerance in LY180 while deletion of *dkgA* had no effect. No change in furfural reductase activity was detected from the over-expression of *yqfA* and the role of this gene in furfural tolerance remains unknown. No mutation was found in these genes in EMFR9 and the mechanism of this gene silencing is under investigation.

The *yqhD* gene has been previously shown to encode an NADPH-dependent aldehyde oxidoreductase (116) that can be used for the production of propanediol (84, 140). This gene has also been shown to confer resistance to damage by reactive species of oxygen (97). The *dkgA* gene has been shown to catalyze the reduction of 2,5-diketo-D-gluconic acid, a key step in the production of ascorbic acid (38, 134). This enzyme is also thought to function in the reduction of methylglyoxal (52, 60). The function of the *yqfA* gene is unknown but is proposed to be a membrane subunit of an oxidoreductase that may be involved in fatty acid metabolism (77).

Enzymes encoded by *yqhD* and *dkgA* were purified and demonstrated to have NADPH-dependent furfural reductase activities. Both YqhD and DkgA have low *K*ms for NADPH that would allow competition with biosynthetic reactions. This competition for NADPH appears to be the primary basis for growth inhibition by furfural. Growth of the parent resumed upon complete reduction of added furfural. Replacing xylose with glucose and adding yeast extract to xylose medium would be expected to increase the availability of NADPH and both changes increased furfural tolerance. Deleting *yqhD* and *dkgA* in the parent LY180 increased furfural tolerance, but not to the full extent present in the mutant EMFR9, indicating additional mutations may also contribute to increased furfural tolerance in EMFR9.

These results show that the low concentration of furfural (up to about 1.5 g liter^{-1}) found in hemicellulose hydrolysates of sugar cane bagasse is not inhibitory to the growth or fermentation of ethanologenic strain EMFR9. The observed growth inhibition of the parent LY180 appears to be due to the diversion of NADPH away from biosynthesis by enzymes such as YqhD and DkgA.

Based on the transcriptional and regulatory changes that were observed in response to the addition of furfural, a partial response map was assembled (Fig. 4-14). This map combined with a summary of highly perturbed genes (Table 4-3) allowed the identification of sulfur assimilation into amino acids as an early site of furfural action. Furfural increased the expression of many genes and regulators concerned with sulfur assimilation into cysteine and methionine (Figure 4-9), consistent with deficiency in these sulfur amino acids. In contrast, furfural lowered the expression of many other biosynthetic genes for building block molecules, consistent with their excess. Further, the addition of low concentrations (0.1 mM) of cysteine and methionine relieved growth inhibition by 1.0 g liter^{-1} furfural (Fig. 4-12). The minimum effective level of cysteine (0.05 mM) was similar to the estimated sulfur amino acid content of the cells that grew in the presence of 1.0 g liter^{1} furfural.

Previous studies investigated the transcriptional response of *E. coli* strain K12 to sulfur limitation during growth in minimal medium. Sulfur limitation was induced by replacing sulfate with either 0.25 mM taurine or 0.25 mM glutathione (37). In their study, a sulfur limitation reduced the rate of synthesis of cysteine and methionine and induced oxidative stress. Interestingly, the sulfur limitation also increased the transcription of *cbl* and the taurine transport genes (*tauABC*), two effects also observed in our furfural response data (Table 4-3).

At low concentrations, serine (a precursor of cysteine), histidine, and arginine were also

effective at reducing furfural inhibition of growth. All amino acids were effective to some extent when added at a 5-fold higher concentration (0.5 mM). Intracellular pools of histidine and serine may be limited to some extend during anaerobic growth since both biosynthetic pathways include reactions that reduced NAD^+ to NADH. These reactions may be hindered by the high $NADH/NAD^+$ ratios typical of fermentation, reducing pool sizes. The beneficial action of arginine was surprising because expression levels for genes concerned with arginine biosynthesis (*argA, argB,, argC, argD, carA, carB, and argG*) were lowered by the addition of furfural, consistent with an excess of this amino acid. Furfural also increased the expression of arginine decarboxylase (*speA*), an enzyme concerned with arginine increasing intracellular pH and degradation. It is possible that degradation products of arginine increase furfural tolerance.

The inhibition of sulfur amino acid synthesis by furfural was localized to the steps prior to sulfur assimilation into cysteine (Fig. 4-12). Alternative sulfur sources at differing degrees of reduction were tested as supplements during growth with furfural (1.0 g $liter^{-1}$) (Fig. 4-12D). The cytoplasmic degradation of D-cysteine and thiosulfate both provide a source of reduced sulfur for direct incorporation into cysteine and both relieved the inhibition of growth by furfural. Taurine is degraded intracellularly to sulfite, a partially reduced sulfur source that needs further reduction by CysIJ prior to incorporation into cysteine. Unlike thiosulfate, the addition of taurine had no effect on furfural tolerance despite the high expression levels of genes encoding taurine transport (*tauABC*) and degradation (*tauD*). Together, these results indicate that furfural inhibits sulfate assimilation by interfering with the reduction of sulfite by CysIJ. Additional inhibitory effects may also be present at earlier steps and cannot be excluded.

The inhibition of sulfate reduction is unlikely to represent the initial action of furfural that inhibits growth. Resistance appears to result from the silencing of two NADPH-dependent

enzymes (YqhD and DkgA) that reduce furfural to furfuryl alcohol, a less toxic compound (137, 138). Based on these results, furfural is proposed to inhibit growth by limiting the available NADPH for biosynthesis. Results from our gene array studies provide further support for this hypothesis. Sulfate assimilation, the most NADPH-intensive pathway in metabolism, was found to the most vulnerable site for furfural action. Growth inhibition by 1.0 g liter^{-1} furfural was relieved by supplying reduced sulfur for amino acid biosynthesis. Many other NADPH-dependent biosynthetic reactions would also be adversely affected by the NADPH-dependent reduction of furfural. Supplying reduced sulfur for biosynthesis as well as other building block metabolites would have a general sparing effect on the NADPH pool, consistent with the general growth benefit provided by individual amino acids. A direct linkage between furfural inhibition of growth and NADPH was further demonstrated by expression of the proton-translocating transhydrogenase, *pntAB*. Low-level expression of these genes without inducer was shown to increase furfural tolerance.

Depletion of cysteine and methionine levels by furfural would be expected to initiate a cascade of cellular events (Fig. 4-14) including stalled ribosomes that trigger a stringent response (35). The accumulation of other amino acids and nucleotides would activate repressors of biosynthesis such as ArgR and PurR, and decrease expression of many biosynthetic pathways. NADPH depletion can also explain the altered activity of ArcA and the resulting expression increase of TCA-cycle related genes, as ArcAB activity is known to be sensitive to the cellular redox ratio (127).

Figures and Tables

Table 4-1. Bacterial strains, plasmids, and primers.

Strain, plasmid, or primer	Relevant characteristics	Reference of source
Strains		
LY168	*frdA::(Zm frg celY$_{Ec}$* FRT*)* Δ*ldhA::*FRT Δ*adhE::(Zm* frg *estZ$_{Pp}$* FRT) Δ*ackA*::FRT *rrlE::(pdc adhA adhB* FRT) *lacY::*FRT Δ*mgsA*::FRT,	(51, 133)
LY180	Δ*frdBC::(Zm frg celY$_{Ec}$*) Δ*ldhA::(Zm* frg *casAB$_{Ko}$) adhE::(Zm* frg *estZ$_{Pp}$* FRT) Δ*ackA*::FRT *rrlE::(pdc adhA adhB* FRT) Δ*mgsA*::FRT	This study
EMFR9	LY180 improved for furfural tolerance	This study
EMFR9 Δ*yqhD*	EMFR9 Δ*yqhD:kan*	This study
EMFR9 Δ*dkgA*	EMFR9 Δ*dkgA:cat sacB*	This study
EMFR9 Δ*yqhD* Δ*dkgA*	EMFR9 Δ*yqhD::kan*, Δ*dkgA::cat sacB*	This study
BL21 (λDE3)	F$^-$ *ompT gal dcm lon hsdS$_B$(r$_B^-$ m$_B^-$)* λ(DE3 [*lacI lacUV5*-T7 gene 1 *ind1 sam7 nin5*])	Promega (Madison, WI)
E. coli TOP10F'	F´ethanol *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen (Carlsbad, CA)
Plasmids[1]		
PCR 2.1 TOPO	*bla kan lacZ P$_{lac}$*	Invitrogen (Carlsbad, CA)
pLOI4301	*yqhD* gene in pCR 2.1 TOPO	This study
pLOI4302	*yjjN* gene in pCR 2.1 TOPO	This study
pLOI4303	*dkgA* gene in pCR 2.1 TOPO	This study
pLOI4304	*yqfA* gene in pCR 2.1 TOPO	This study
pLOI4305	*yajO* gene in pCR 2.1 TOPO	This study
pLOI4306	*ydhU* gene in pCR 2.1 TOPO	This study
pLOI4307	*ydhV* gene in pCR 2.1 TOPO	This study
pLOI4308	*ygcW* gene in pCR 2.1 TOPO	This study
pLOI4309	*nemA* gene in pCR 2.1 TOPO	This study
pLOI4310	*yjgB* gene in pCR 2.1 TOPO	This study
pLOI4311	*ydhS* gene in pCR 2.1 TOPO	This study
pLOI4312	*ydhY* gene in pCR 2.1 TOPO	This study
pLOI4313	His-tagged *yqhD* in pET15b	This study
PLOI4314	His-tagged *dkgA* in pET15b	This study
pET15b	T7 promoter, *bla*, His-tag vector	Novagen (Madison, WI)
pKD4	FRT *kan* FRT	(24)
PKD46	*P$_{ara}$ bla,* red recombinase (γ,β,exo)	(24)
pTrc99a	Ptrc bla oriR rrnB lacIq	(6)
pLOI4315	sthA gene in pTrc99a	This study
pLOI4316	pntAB in pTrc99a	This study

Table 4-1. Continued

Strain, plasmid, or primer	Relevant characteristics	Reference of source
Primers[2] (5' to 3')		
yqhD cloning	For-ACATCAGGCAGATCGTTCTC Rev-CCACAGCTTAGTGGTGATGA	This study
yjjN cloning	For-GGAGAGCCGAATCATGTCTA Rev-CCGGAACCTGTCTCAACCAA	This study
dkgA cloning	For-GCCTGCTCCGGTGAGTTCAT Rev-CCGGCTCTGCATGATGATGT	This study
yqfA cloning	For-GCTGGAGAGGTATACATGTG Rev-GCCGTATTCGCTCGAAGAGT	This study
yajO cloning	For-CCGCAGCACATGCAACTTGA Rev-ATGGCGCTGCCGACCAATGA	This study
ydhU cloning	For-CCGCATCTGTATCGCCGGTT Rev-GCCGATGCGAGCATGATTCGT	This study
ydhV cloning	For-ATTATCGAGTGGAAAGATAT Rev-CGTAGTCTCCGTTCTGCTTA	This study
ygcW cloning	For-ACCTTTCTTTTTTTTTGCCT Rev-TTACGACCGCTGCCGGAATC	This study
nemA cloning	For-TTATTGCGACGCCTGCCGTT Rev-GTTCAATCACCGCTTCTTCG	This study
yjgB cloning	For-CCTGCCATGCTCTACACTTC Rev-CTGGTTAGATGGCGACTATG	This study
ydhS cloning	For-AACTTATCTGATAACACTAA Rev-CCAACAGCGGCGACAATGTA	This study
ydhY cloning	For-TCAGGCTGCTGAATTGTCAG Rev-GGCACCAGATCCAGTTAATG	This study
Deletion of *yqhD*	For-GTTCTCTGCCCTCATATTGGCCC AGCAAAGGGAGCAAGTAGTGTAGG CTGGAGCTGCTTC Rev-GACGAAATGCCCGAAAACGAA AGTTTGAGGCGTAAAAAGCCATAT GAATATCCTCCTTA	This study
Deletion of *dkgA*	Outward 1-ACGGTTGGATTAGCCATACG Outward 2-GACCAGTTCGGCGGCTAACA For-GCCTGCTCCGGTGAGTTCAT Rev-CCGGCTCTGCATGATGATGT	This study
yqhD cloning into pET15b	For-TGACTCTCGAGATGAACAACTT TAATCTGCA Rev-AGTCAGGATCCTTAGCGGGCG GCTTCGTATA	This study
dkgA cloning into pET15b	For-ATATGCCTCGAGATGGCT AATCCAACCGTTAT Rev- CCGATAGGATCCTTAGCCGC CGAACTGGTCAGG	This study
Sequencing *yqhD*	*yqhD*_for1 CGGCGAGGTACTGGTGAC *yqhD*_rev1 CATGTTAGCCGCCGAACT *yqhD*_seq1 TCATGTTGGCTTCTGCCG *yqhD*_seq2 GCGCAATCGCTGGTTTAC *yqhD*_seq3 GTTCCGATGATGAGCGTATTG *yqhD*_seq4 AGGCGTTTTCGATCAGAAAG	This study

Table 4-1. Continued

Strain, plasmid, or primer	Relevant characteristics	Reference of source
Sequencing *dkgA*	*dkgA*_for1 CCAGCAACCGGTTCAGAAT *dkgA*_rev1 AACGCGTGAAAATAGCGACT *dkgA*_seq1 GCGGTAAAGAGATTAAAAGCGC *dkgA*_seq2 TATGGCTAATCCAACCGTTATTAAG *dkgA*_seq3 CCCGCCCGTTGTTACTCT	This study
Sequencing of *yqfA*	pcr_for: CCATCCGCGACGAGTCTGAA pcr_rev: GGTGAAGCGGAACTGAACAA seq1: CCATCCGCGACGAGTCTGAA seq2 : CGACGCTCTATCACGCCATT	This study
Sequencing of *yjjN*	pcr_for: TGCGCTGTTTAAGATCGCT pcr_rev CATGATTGCCTTCTCGGG seq1 ACTGAGATGATCTCAAGCGATTG seq2 GGAAACAACGCGAGATACCT seq3 CCACGCTGGCAGAAACCTA	This study
pntAB cloning	For- CTCTCTAAGCTTGCTTGTGTGGCTCCTGACAC Rev- CTCTCTAAGCTTGTTCAGTCCTCGCGGCAATC	This study
sthA cloning	For- CTCTCTAAGCTTATGTTACCATTCTGTTGCTT Rev- CTCTCTAAGCTTGATGCTGGAAGATGGTCACT	This study

[1] The genes inserted into pCR 2.1 TOPO include a native ribosomal binding site and transcriptional terminator. Expression is from the plasmid promoter (P_{lac}).

[2] Orientation of genes cloned into pCR 2.1 TOPO was verified by PCR analysis

Table 4-2. Expression of oxidoreductase genes perturbed by furfural addition.

Effect of over-expression of cloned genes on MIC for furfural			
Transcripts that were approximately 2-fold or greater in EMFR9 relative to LY180			
Gene	Ascension number	Fold increase	Expression in LY180
yajO	b0419	1.9	No increase in MIC
ydhU	b1670	1.8	No increase in MIC
ydhV	b1673	2.0	No increase in MIC
ygcW	b2774	2.1	No increase in MIC
nemA	b1650	4.5	No increase in MIC
yjgB	b4269	2.0	No increase in MIC
ydhS	b1668	1. 9	No increase in MIC
ydhY	b1674	1.9	No increase in MIC
Transcripts that were approximately 2-fold or more lower in EMFR9 relative to LY180			
Gene	Ascension number	Fold decrease	Expression in EMFR9
yqhD	b3011	-48	Reduced MIC
dkgA	b3012	-12	Reduced MIC
yjjN	b4358	-4.4	No effect on MIC
yqfA	b2899	-2.5	Reduced MIC

Table 4-3. Gobal comparison of genes perturbed by furfural addition.

Functional group	Total	Differentially regulated (no, %)	Downregulated	Upregulated
Amino acid biosynthesis and metabolism	123	28, 22.7	*argC, aroL, argA, tyrB, asnA, argD, thrA, ilvD, trpD, argB, trpE, thrC, ilvA, argG, aroH, thrB, sdaB, dapB, ilvM, ilvC*	*cysM, metC, IdcC, dadX, metA, metB, metL, dadA*
Biosynthesis of cofactors, prosthetic groups and carriers	120	8, 6.7	*pdxA, ubiX, folC, bioD*	*idi, trxC, pabC, ybdK*
Carbon compound metabolism	133	12, 9		*xdhB, acnB, gcd, xdhA, amyA, dhaK, aldB, dhaL, ygjG, fucO, treF, tauD*
Cell processes (incl. adaptation, protection)	198	29, 14.6	*fliQ, fliJ, fliE, fliP, fliL, fliK, fliF, fliG, fliH, cspA, fliN, flgJ, ymcE, lpxP, flgK, fliO, flgH, flgL*	*ibpA, yfiA, otsA, osmC, sodC, hchA, fic, b4411, osmY, yqhD, nemA*
Cell structure	114	8, 7.0	*mreC, mreD, rfaC, fliS, etk, yeiU, lpxB*	*ybhO*
Central intermediary metabolism	162	27, 16.7	*pykF, fumB, tktA, pyrH, ppa*	*poxB, cysQ, gabD, cysN, mqo, acnA, cysC, aceB, aceA, cysI, cysH, dcyD,aldA, gloA, glpK, fumA, glpD, sdhB, sdhC, sdhA, sdhD, dkgA*
DNA replication, recombination, modification and repair	105	5, 4.8	*rnhB, fliA, recG*	*yjiD, aidB*
Energy metabolism	139	14, 10.1	*hypC, ackA, hypB, atpC*	*cyoE, frmA, aceE, aceF, fdoH, fdoG, cyoD, cyoB, cyoA, cyoC*
Fatty acid and phospholipid metabolism	42	3, 7.1	*accC, accB*	*fadI*
Nucleotide biosynthesis and metabolism	62	13, 21	*pyrB, purE, carA, pyrD, purH, guaB, purF, purN, purK, purD, carB, pyrE*	*xdhC*
Phage/IS	295	6, 2	*ydfK, ynaE, cspB, cvpA, cspI, ynfN*	
Putative	1167	102, 8.7	*mltD, ydjH, fliR, yqeI, yijP, ydjI, bioC, ynjE, yibK, ydjJ, ydjZ, ynjC, ykgK, flhA, dctR, yhgF, yejM, ybjE, yfcC, ydgR, flgI, sdaC, yhiD, ybhA, ecfG, yibQ, ynjI, yliF, yliE, ydjK, yjjB, yibA,yedV*	*ycbB, paaY, yjgH, ybiC, yeaQ, yecC, yqgD, ydjN, yhbW, ybaT, yagT, yehZ, yfcG, ymgE, yhbO, ygcE, yedY, sfsA, ydgD, nanK, yjiA, yohJ, yhiP, ydcO, yiaG, yigM, yhjG, ydcN, yqfA, dhaM, ygeV, ybaS, ydcT, ynfM, ygiV, nanE, yqaE, yqeF, yfdY, yniA, ydcS, yncG, maeB, ybhP, ygaW, ybdH, yohF, yhcO, ydcK, yddV, yciW, yeiA, sufB, ybeM, yohC, ychH, yeiT, yeeE, yhdW, yjfF, uspB, ytfT, glgS, yqhC, b4485, cstA, ytfQ, ydhM, yjiX*
Regulatory function	253	18, 7.1	*adiY, evgS, cspG, suhB, cadC, flhC, fis*	*rssB, sbmC, sdiA, pdhR, crl, bolA, hcaR, metR, phoU, galS, cbl*
Transcription, RNA processing and degradation	61	2, 3.3	*trmH, xseB*	
Translation, post-translational modification	184	8, 4.3	*truC, rpsT, etp, rpsA*	*msrB, msrA, clpA, pphA*
Transport	353	69, 19.5	*artM, nikB, nikA, lysP, proV, artP, proW, nikD, nikC, thiP, tyrP, proX, hisP, aroP, artI, hisJ, nikE, artQ, cusB, btuF, artJ, ampG, cusA, mtr, dcuC, narK, pitA, hisM, emrA, thiQ*	*xylE, gabP, manX, nagE, araF, sufD, sufC, mntH, livH, kgtP, cysA, ssuC, blc, gltJ, cycA, yahN, pstA, pstC, glpT, glpF, argT, ssuA, pstB, gltK, gltI b4460, mtlA, pstS, narU, mdtM, sufA, dctA, mglC mglA, sbp, mglB, tauA, tauB, tauC*
Unclassified	14	3, 21.4		*ssuD, ssuE, ybdL*
Unknown	681	57, 8.5	*yiiQ, intG, flhE, ymdA, ynjB, ydjY, yeeN, ymcA, yibL, yghG, b1172, yjaH*	*yccT, ybiJ, yjdI, yahO, yjdN, yhcN, ybaA, yedK, yqjD, eutQ, ybgS, yhhA, ompW, yhjY, yghX, yqeB, rtcB, ygaU, erfK, yegS, yeeD, yhcH, ydhS, yegP, yebV, yjfN, yehE, ydcJ, ygaM, yqeC, ybiL, psiF, yhfG, yjfO, nlpA, ybeH, ynhG, ycfR, yjiY, yodD, csiD, yeaH,yedP, yeaG, ycgB*
Total	4206	412, 9.8		

Table 4-4. Regulators perturbed by furfural addition.

Regulator	Perturbation direction	Description	Activation mechanism
ArcA	down	aerobic respiration control	phosphorylation by ArcB
ArgR	up	repressor of arginine biosynthesis	binding of L-arginine
BirA	up	repressor of biotin biosynthesis	binding of bio-5'-AMP
CRP	up	global regulator of catabolite-sensitive operons	binding of cAMP
CysB	up	regulator of cysteine biosynthesis	binding of O-acetyl-L-serine
DcuR	unclear	activator of genes involved in C-4 dicarboxylate metabolism	phosphorylation by DcuS
FIS	down	global regulator associated with nutritional upshift	inherently active
FlhDC	down	master motility regulator	[FlhD]4[FlhC]2
FliA	down	minor sigma factor, regulates motility-associated genes	inherently active
his	up	histidine, regulates histidine biosynthesis via transcriptional attenuation	inherently active
MetJ	down	repressor of methionine biosynthesis	binding of S-adenosyl-methi
NagC	down	coordinates biosynthesis and catabolism of amino sugars	binding of GlcNAc-6-P
PdhR	down	repressor of pyruvate dehydrogenase complex	absence of binding by pyruva
PhoB	up	regulator of inorganic phosphate uptake	phosphorylation by PhoR
PhoP	up	regulator of divalent cation starvation response	phosphorylation by PhoQ
PurR	up	repressor of purine nucleotide biosynthesis	binding of hypoxanthine
RpoH	up	heat shock sigma factor	inherently active
RpoN	up	nitrogen-related sigma factor	inherently active
RpoS	up	general stress response sigma factor	multiple mechanisms
RutR	up	proposed repressor of pyrimidine degradation	unknown
SF	up	lumped "stringent factor"	amino acid starvation

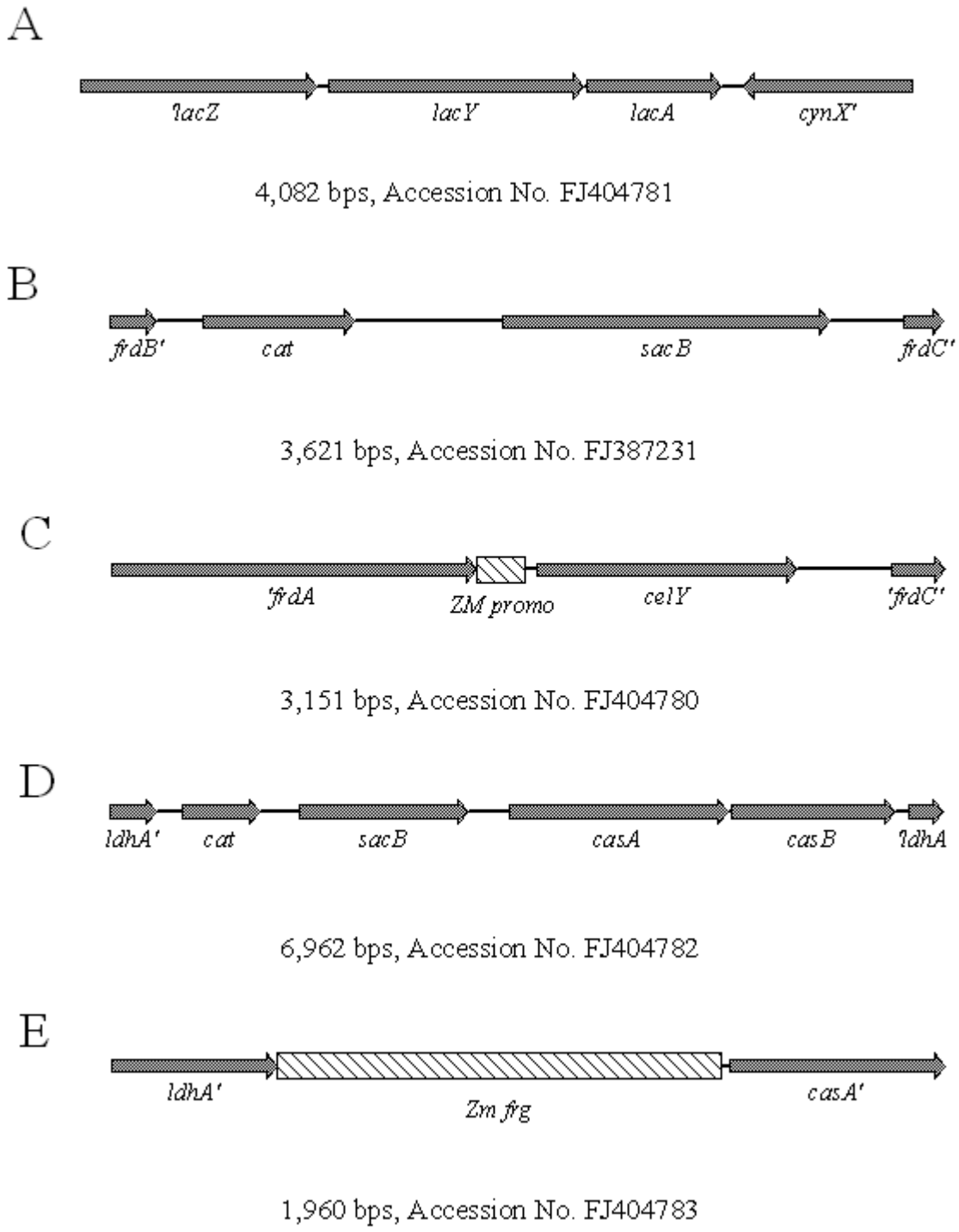

Figure 4-1. Linear DNA fragments used in construction of LY180.

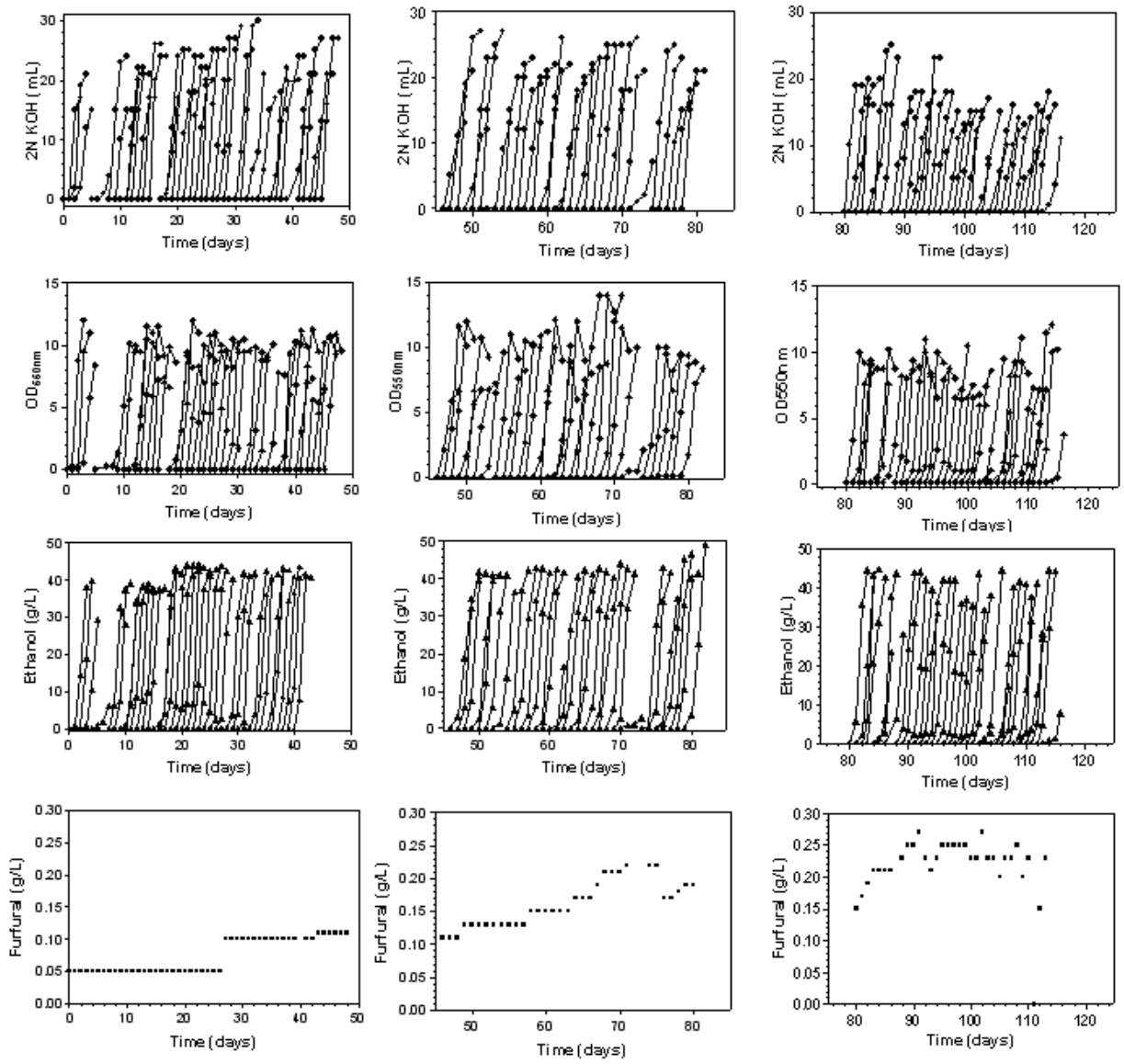

Figure 4-2. Directed evolution of *E. coli* for furfural tolerance. Cultures were grown anaerobically in 100 g liter^{-1} xylose and AM1 minimal media to 1-4 OD and transferred to fresh media with increasing initial furfural concentrations as tolerance to furfural increased. pH was maintined at 6.5 by automatic addition of 2N KOH. Ethanol production, cell density, and KOH addition were measured at 0, 24, 48, and 72 hrs for each transfer.

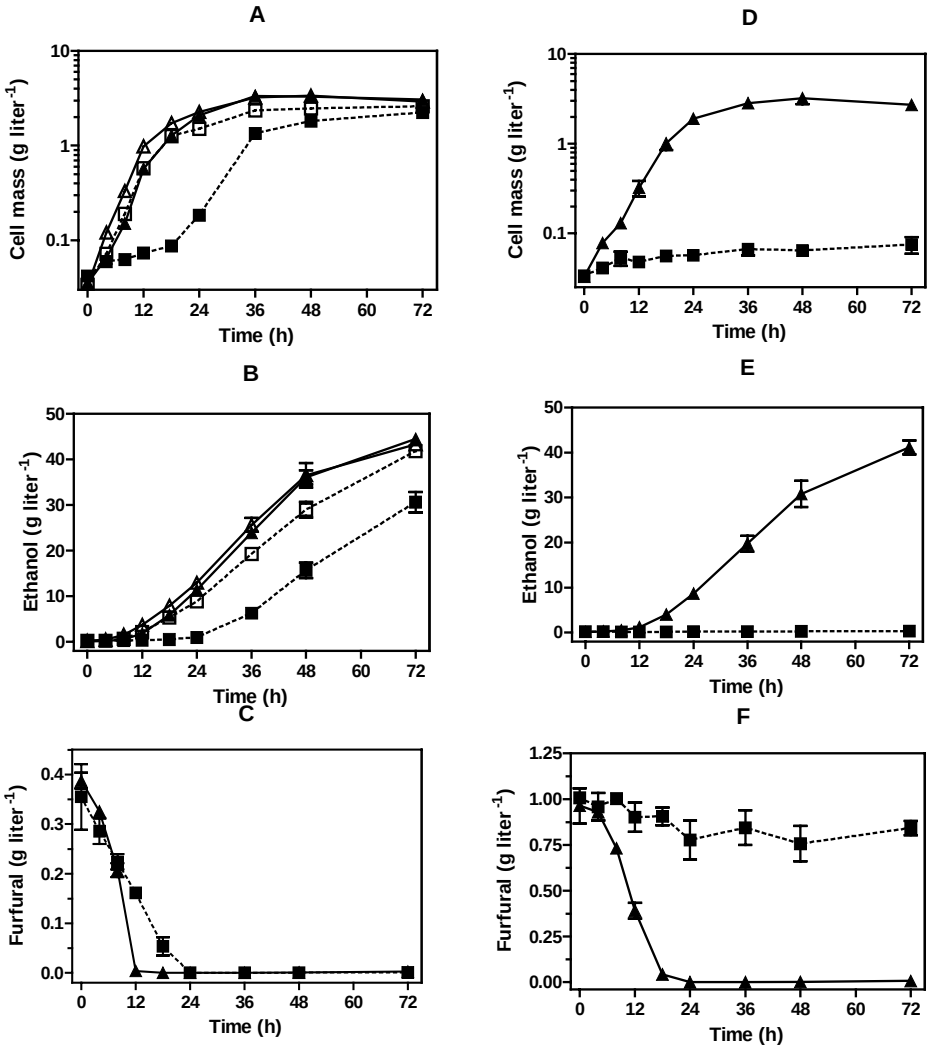

Figure 4-3. Tolerance of furfural resistant strain EMFR9 versus LY180. Effect of furfural on pH-controlled fermentation of 100 g liter^{-1} xylose. Fermentation with 0.4 g liter^{-1} furfural (A, B, and C). Fermentations with 1.0 g liter^{-1} furfural (D, E, and F). For clarity, data for EMFR9 and LY180 are connected by solid and broken lines, respectively.
Symbols for all: ■, LY180 with furfural; ▲, EMFR9 with furfural; □, LY180 without furfural; and Δ, EMFR9 without furfural.

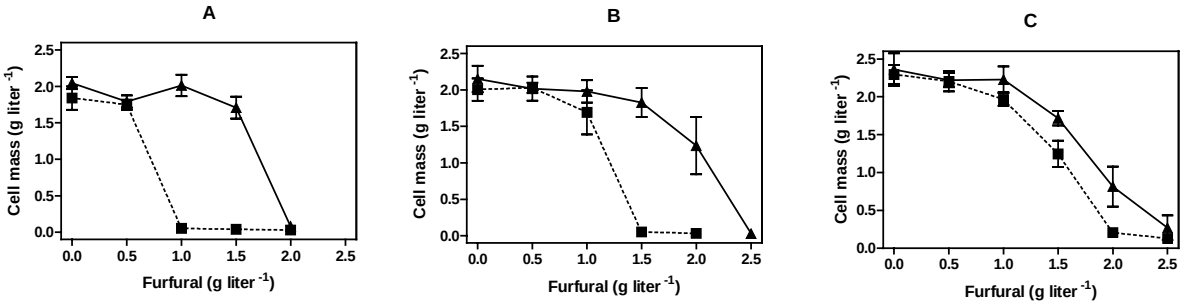

Figure 4-4. Effect of media composition on furfural tolerance. A. AM1 medium containing xylose (50 g liter^{-1}); B. AM1 medium containing glucose; C. AM1 medium containing xylose and yeast extract (1.0 g liter^{-1}); Symbols for all: ■, LY180 (dashed line); and ▲, EMFR9 (solid line) after incubation for 48 hours.

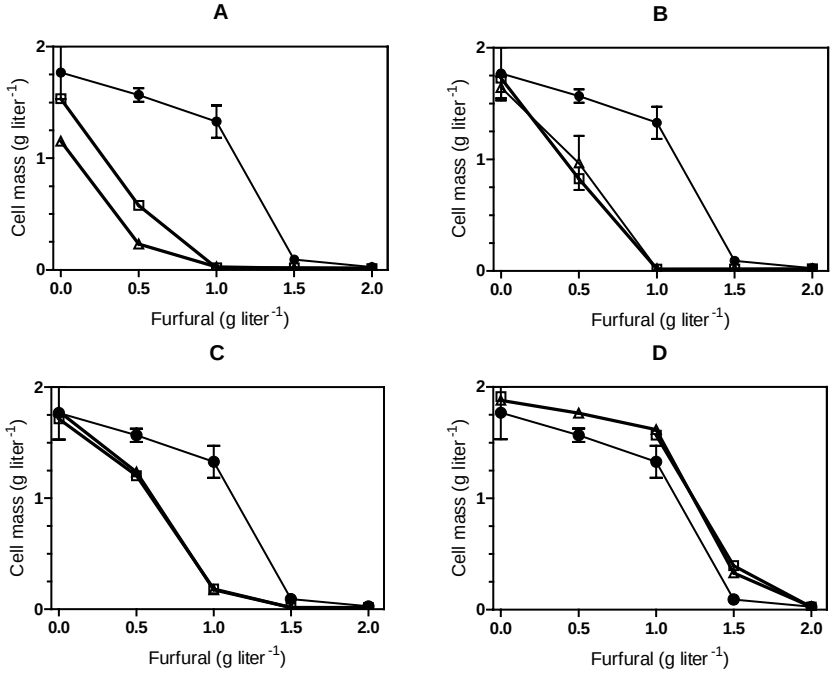

Figure 4-5. Effect of oxidoreductase expression on furfural tolerance. In EMFR9, A. Expression of *dkgA*; B. Expression of *yqhD*; C. Expression of *yqfA*; D. Expression of *yjjN*. Symbols for all: ●, pCR2.1 control without insert; □, uninduced expression; Δ, expression induced with 0.1 mM IPTG.

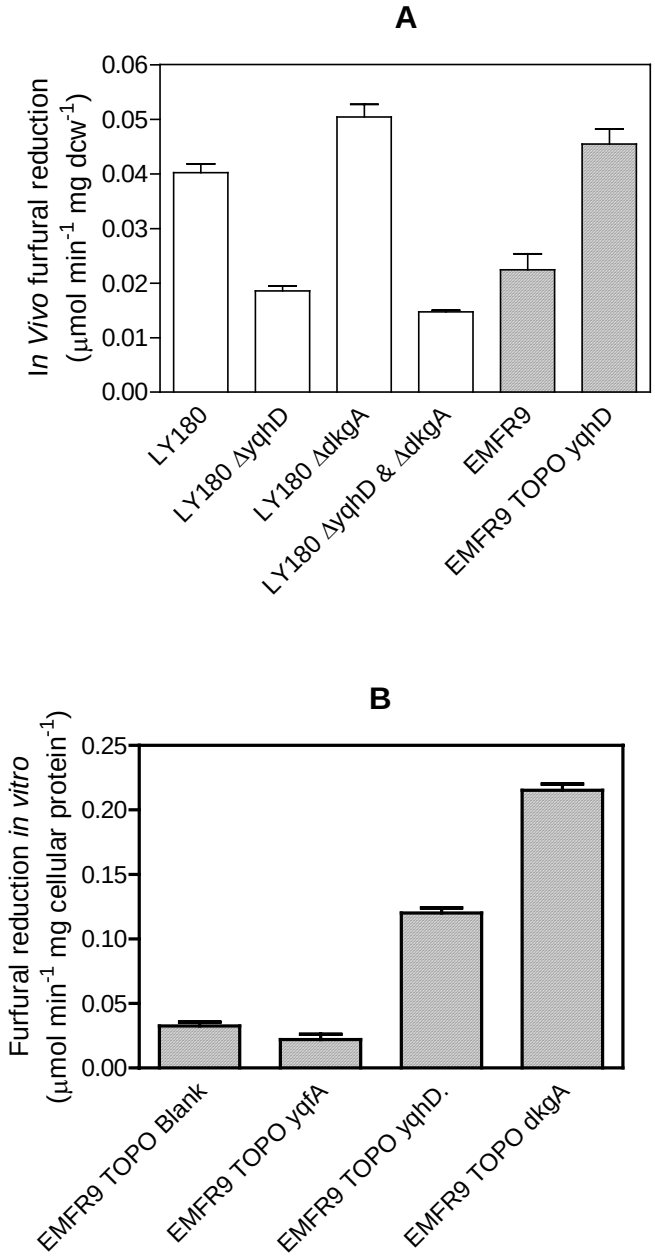

Figure 4-6. In vivo and in vitro furfural reduction comparison. A. *In vivo* activity of whole cells during fermentation. LY180 and deleted derivatives are shown as open bars. The furfural-resistant mutant, EMFR9 and EMFR9 (pLOI4301) expressing *yqhD* are shown as shaded bars. B. Comparison of *in vitro* furfural-reducing activities in cell-free extracts of EMFR9 harboring plasmids expressing cloned genes (forward direction, induced with 0.1 mM IPTG).

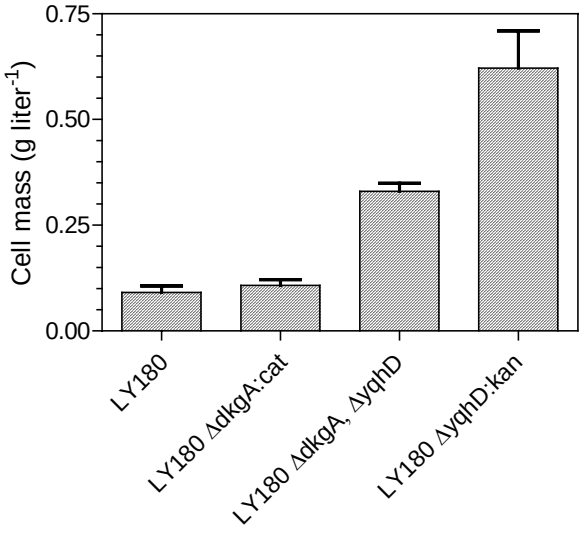

Figure 4-7. Effect of *yqhD* and/or *dkgA* deletion on furfural tolerance. Growth after 48 hr incubation in the presence of 1.0 g liter^{-1} furfural.

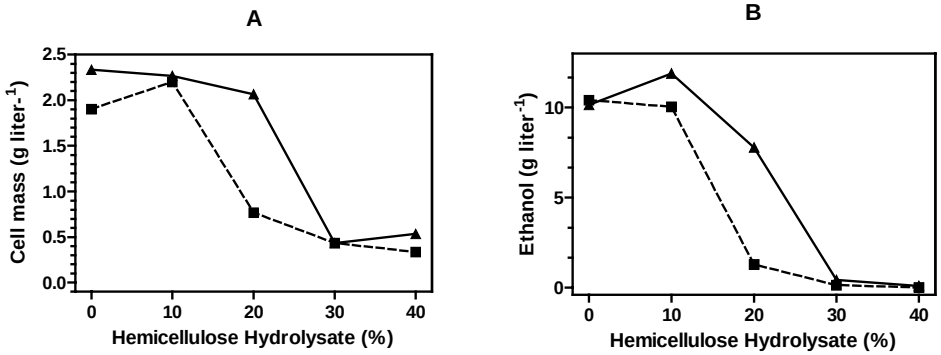

Figure 4-8. Growth in hemicellulose hydrolysate. Growth (A) and ethanol production (B) of LY180 (■) and EMFR9 (▲).

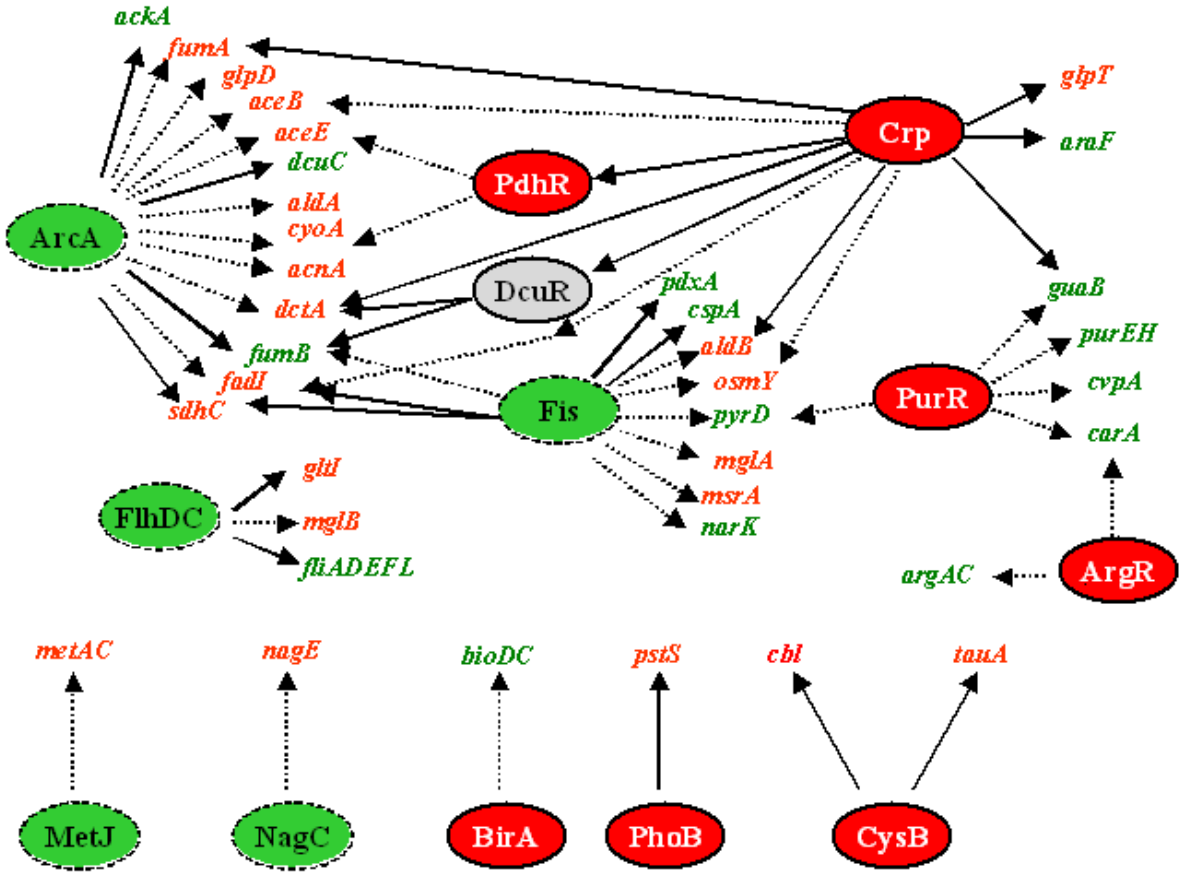

Figure 4-9. Partial regulatory response of LY180 to furfural. Response map following challenge with 0.5 g liter^{-1} furfural. Regulatory genes that were significantly perturbed were identified by NCA with a P-value cutoff of 0.05 relative to a null distribution. Regulators with increased activity are shown in red with a solid border, regulators with decreased activity are shown in green with a dashed border. Regulators that showed a mixed activity are shown in grey. Representative genes that were perturbed greater than 2-fold are shown, with red (green) indicating genes with increased (decreased) expression. Solid lines indicate activation by the connected regulator, dashed lines indicate repression. Because the direction of perturbation for DcuR is unclear, this regulator is shown in gray.

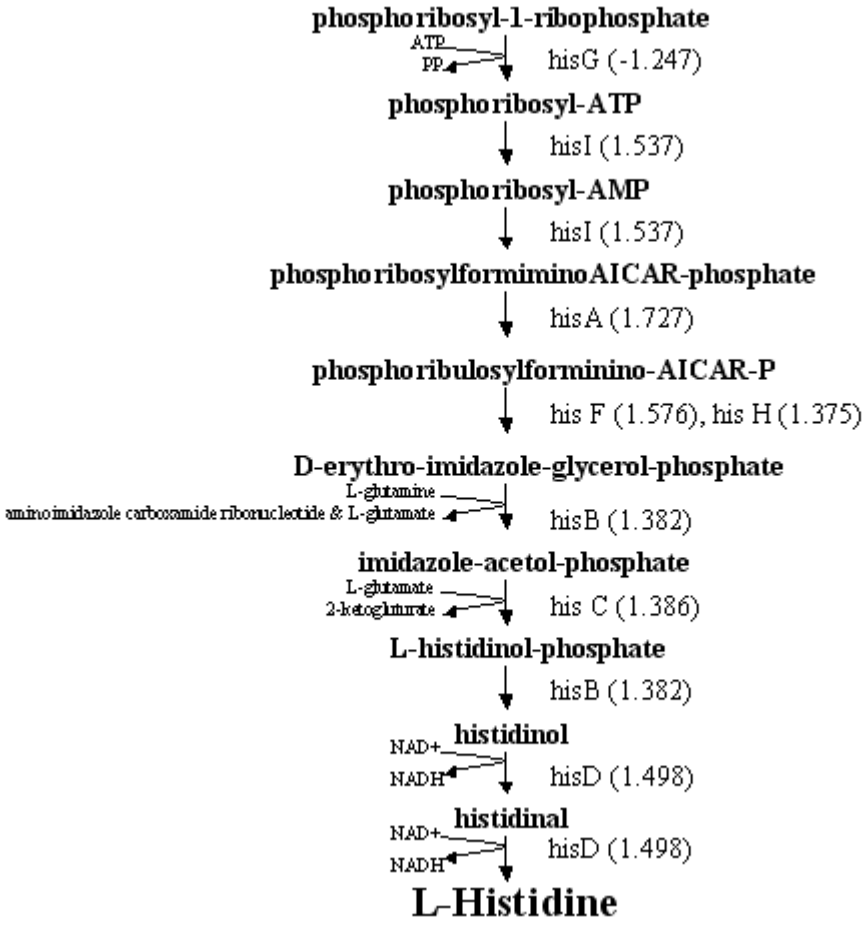

Figure 4-10. Histidine pathway genes perturbations upon furfural addition. Changes in gene transcipt levels in LY180 upon addition of 0.5 g liter^{-1} furfural as determined by microarray analysis are listed quantitatively beside the corresponding gene.

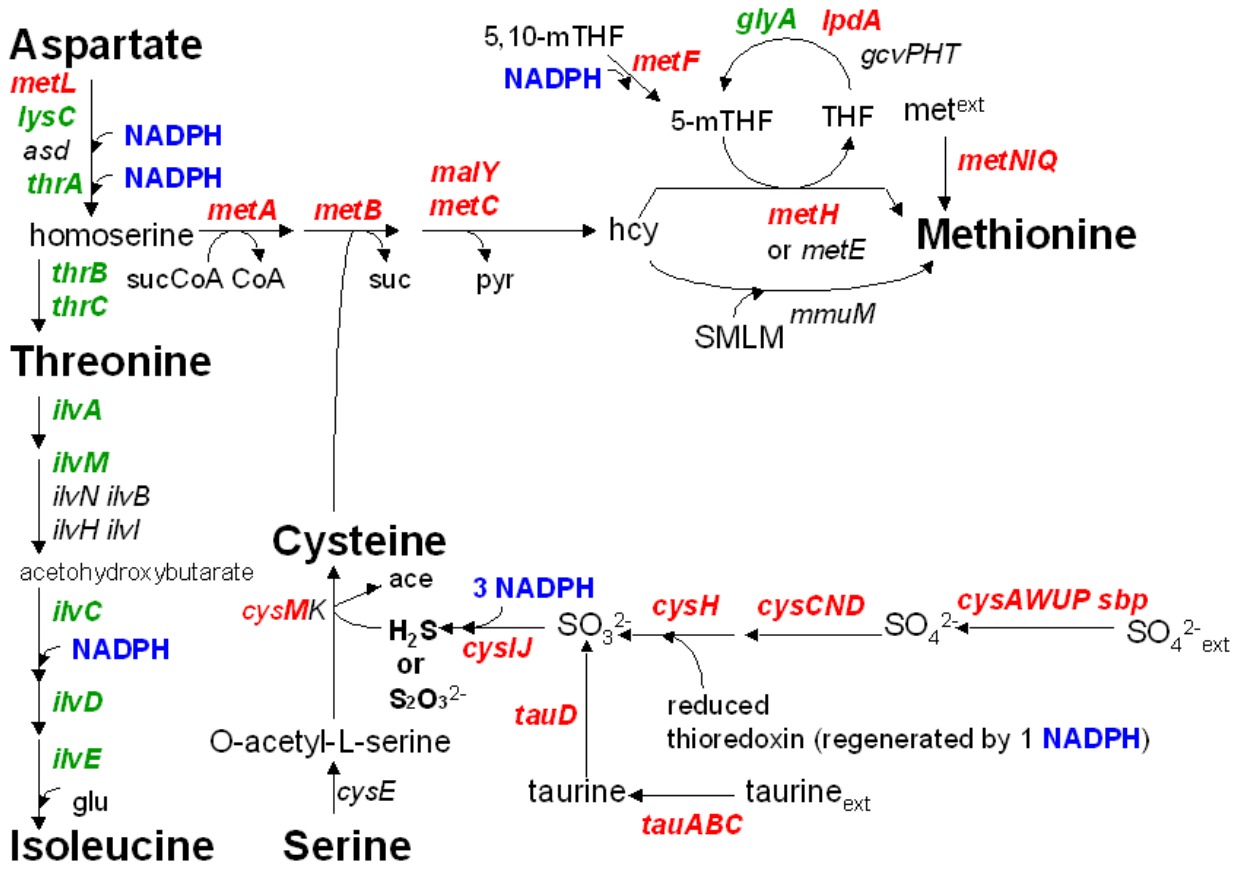

Figure 4-11. Cysteine and methionine pathway gene perturbations upon furfural addition. Furfural increased expression of most genes concerned with sulfur assimilation into cysteine and methionine. Pathways for the synthesis of threonine and isoleucine from aspartate are included for comparison. Genes up-regulated by 1.5-fold or greater are shown in red. Genes down-regulated by 1.5-fold or greater are shown in green. All others are shown in black.

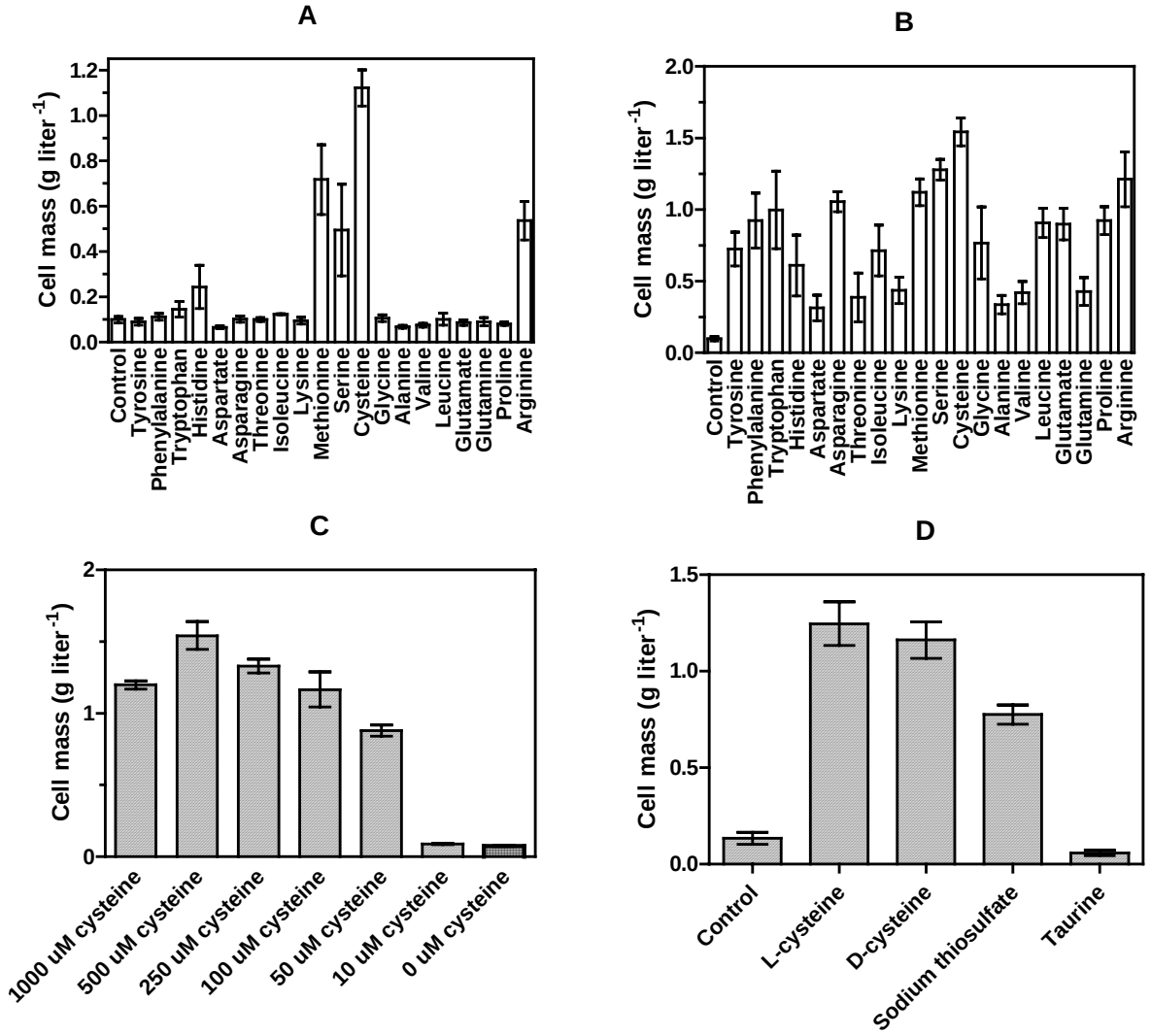

Figure 4-12. Supplementation with specific metabolites increases furfural tolerance. Cultures were compared after incubation for 48 hrs (AM1 medium, 50 g liter^{-1} xylose, 1.0 g liter^{-1} furfural, 37°C). A. Addition of individual amino acids (0.1 mM each). B. Addition of individual amino acids (0.5 mM each). C. Addition of cysteine. D. Addition of alternative sulfur sources.

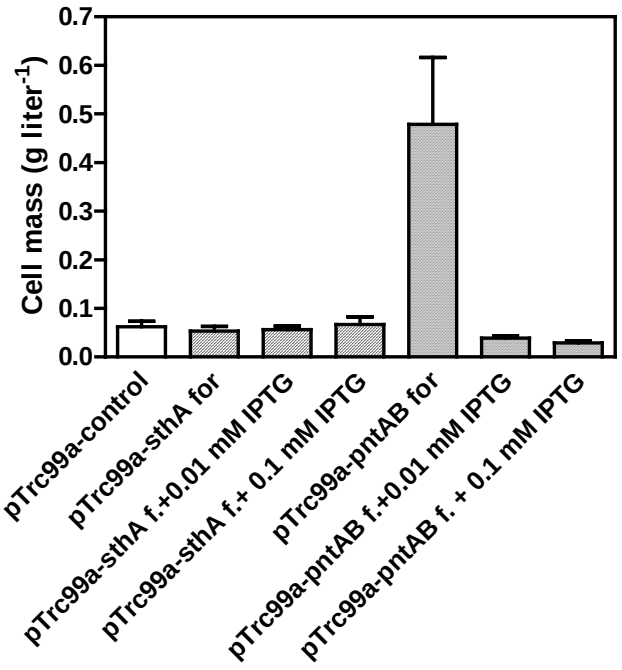

Figure 4-13. Effect of increased transhydrogenase expression on furfural tolerance. Cultures were grown for 48 hrs in AM1 minimal media containing 50 g liter^{-1} xylose and 1.0 g liter^{-1} furfural. The empty vector served as a control. Inducer was added prior to inoculation.

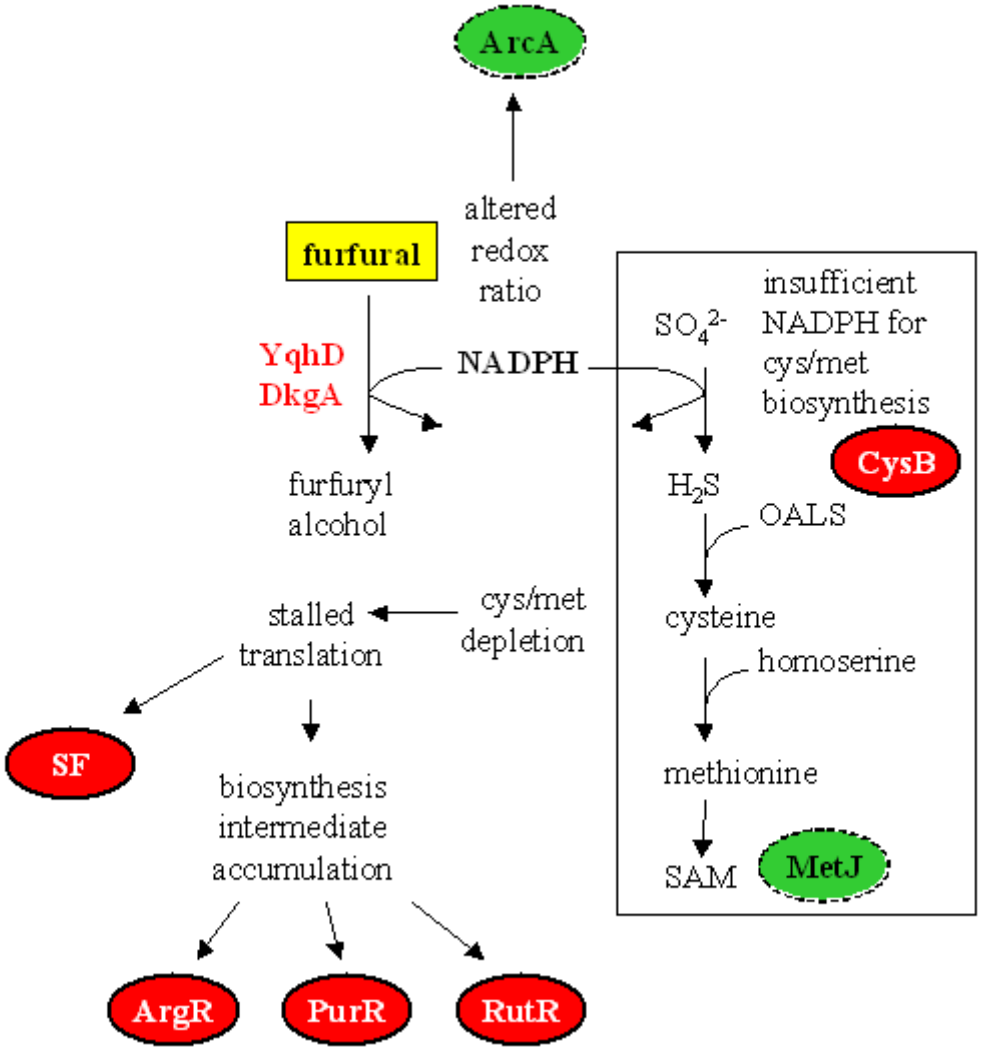

Figure 4-14. Model of furfural challenge. Regulators with increased activity are shown in red with a solid border, regulators with decreased activity are shown in green with a dashed border. The addition of furfural induces two NADPH-dependent reductases (*yqhD* and *dkgA*) that inhibit growth by out-competing essential biosynthetic reactions. Assimilation of sulfur into amino acids requires 4 NADPH per cysteine and appears to be the most vulnerable of these biosynthetic reactions. Secondary consequences from furfural addition include depletion of sulfur amino acids and a cascade of events from stalled translation and accumulation of many non-sulfur building block intermediates to a more general stress response.

CHAPTER 5
GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

General Accomplishments

Engineering tolerance of *E. coli* to hemicellulose hydrolysate inhibitors was conducted by focusing seperately on osmotic stress and chemical stress. *E. coli* was previously made to produce an increased level of trehalose by transposon insertion of Ptac-*otsBA* and deletion of *treA*, *treC*, and *treF* (strain JP20) (103). JP20 exhibited increased tolerance to sugars (glucose, xylose), salt (sodium chloride), and organic acids (lactate, succinate) compare with parent strain W3110. W3110 grown with 1mM betaine also displayed increased tolerance to glucose, xylose, sodium chloride, lactate, and succinate compared to W3110 without betaine. Overexpressing trehalose during growth with betaine led to a greater benefit than either osmoprotectant could provide alone in the cases of glucose, xylose, and sodium chloride.

In order to determine if the benefit of increased trehalose production on osmotolerance extended to desiccation survival, three strains with transposon inserted Ptac-*otsBA* were tested, EM2P (an ethanol producing KO11 derivative), EM2L (an ethanol producing LY163 derivative), and EM2T (a lactate producing TG106 derivative). In all cases, increased expression of the trehalose producing genes *otsBA* promoted survival during desiccation. Growth sugar was also found to impact survival, with survival during growth in xylose < glucose < mannose < fructose < arabinose < lactose <sucrose. Log phase was found to be the optimal period of growth for harvesting prior to desiccation, and harvesting cells during mid-log phase when over-producing trehalose and growing on sucrose together resulted in a combined benefit, leading to survival levels as high as 80%. Desiccation did not impact the growth or fermentation of rehydrated cells.

Furfural is one of the most significant chemical inhibitors present in hemicellulose hydrolysate (39, 48, 73, 82, 138). A furfural tolerant *E. coli* was developed by growing and transferring ethanologenic strain LY180 in fermentation vessels containing furfural. The furfural tolerant strain (designated EMFR9) was shown to have a MIC towards furfural of 2.0 g liter^{-1}, twice that of LY180. EMFR9 grew and reduced furfural simultaneously, whearas LY180 would only grow after all furfural had been removed from the media. On a dcw basis, EMFR9 reduced furfural at a lower rate than the parent. Growing cultures in glucose improved furfural tolerance for LY180, and to a lesser extent EMFR9, compared to growth on xylose. Addition of yeast extract to xylose grown cultures increased furfural tolerance of LY180, making it nearly as tolerant as EMFR9. EMFR9 displayed an increased ability to grow in sulfuric acid treated hemicellulose hydrolysate compared to LY180.

An NADPH dependent furfural reductase had previously been isolated in *E. coli* (36), although a corresponding gene could not be determined. Messanger RNA microarray with EMFR9 and LY180 was conducted, comparing genes transcript levels of cultures grown to 2 OD before and 15 minutes after furfural addition. 8 known or putative oxidoreductases had at least 2 fold higher transcript levels in EMFR9 than LY180, while 4 displayed at least 2 fold lower transcript levels in EMFR9 than LY180. Cloning the 8 genes into LY180 did not increase furfural tolerance, but cloning the 4 genes into EMFR9 reduced furfural tolerance in the cases of *yqfA*, *yqhD*, and *dkgA*. Deletion of *yqhD* from LY180 increased furfural tolerance, but deletion of *dkgA* yielded no effect. By testing purified histidine tagged protein, YqhD and DkgA were both shown to have NADPH dependent furfural reductase activity with a low Km for NADPH.

Microarray analysis revealed an increase in transcript abundance of genes related to cysteine and methionine biosynthesis. 4 NADPH are required to reduce sulfate so that it can be

incorporated into cysteine, but in the presence of furfural this pool is redirected to forming furfuryl alcohol. Addition of 500 uM alternative sulfur sources that require less NADPH to be incorpated than sulfate (L-cysteine, D-cysteine, sodium thiosulfate) increased furfural tolerance of LY180 but did not affect EMFR9. Histidine biosynthesis gene transcript levels also increased in LY180 upon furfural addition. Growth of LY180 seperately in 100 uM of each of 20 amino acids revealed a benefit from cysteine, methionine, serine, histidine, and arginine. Increasing amino acid concentrations to 500 um led to a more generalized benefit. Finally, over-expression of the transhydrogenase *pntAB* in LY180 led to an increase in furfural tolerance. Taken together it appears that NADPH dependent furfural reduction by YqhD and DkgA competes for NADPH pools required for biosynthesis of cysteine, resulting in a stringent response which prevents cell growth.

Future Works

In addition to furfural, other significant inhibitors are present in hemicellulose hydrolysate that interfere with the growth of fermenting organisms. Acetate is one such compound, an organic acid that is released from the cleavage of hemiacetyl groups (136). It has the ability to collapse the proton motive force of the cell, preventing growth and fermentation (136).

Acetate has been shown to impact cellular transcipt levels in *E. coli* including *rpoS* (7), a sigma factor important in general stress response. *E. coli* tolerant to acid (112) and acetate (41) have been evaluated, but the specific mechanisms that convey this tolerance remain unknown. A segment of *cbpA* from *E. coli* that encodes a 24 amino acid proton buffering peptide was cloned into *Z. mobilis*, increasing tolerance to both hydrochloric acid and acetic acid (10). In addition, the ABC acetate transporter AatA from *Acetobacter aceti* when cloned into *E. coli* conveyed

acetate resistance (86). Furthermore, increased expression of aconitase from *A. aceti* led to increased acetate tolerance (85). This illustrates that bacteria already have strategies for coping with acetate, which may be used as a platform for further increasing tolerance.

As with the engineering of furfural tolerance, acetic acid tolerance might be developed by selectively evolving through continual transfers in the presence of acetate. Microarray analysis can then be preformed in the presence and absence of acetate, and perturbed genes can be expressed or deleted. Single Nucleotide Polymorphism analysis can also be implemented in order to directly determine the location of mutations within the chromosome. These mutations can be transferred into a clean genetic background using the parent organism, and resistance to acetate can be determined.

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BIOGRAPHICAL SKETCH

Elliot Norman Miller was born in San Diego, CA, in the year 1982. He traveled the countryside with his parents and brother, settling sporadically as the nature of his father's miltitary career necessitated. At age nine Mr. Miller became a resident of Florida and has remained since. His education includes a high school diploma from Seminole High School in Sanford, FL, as well as a bachelor's degree in microbiology from the University of Florida, Gainesville, FL.