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BIOTECHNOLOGY

Overexpression of Oxidoreductase YghA Confers Tolerance of Furfural in Ethanologenic *Escherichia coli* Strain SSK42

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ABSTRACT Furfural is a common furan inhibitor formed due to dehydration of pentose sugars, like xylose, and acts as an inhibitor of microbial metabolism. Overexpression of NADH-specific FucO and deletion of NADPH-specific YqhD had been a successful strategy in the past in conferring tolerance against furfural in *Escherichia coli*, which highlights the importance of oxidoreductases in conferring tolerance against furfural. In a screen consisting of various oxidoreductases, dehydrogenases, and reductases, we identified the *yghA* gene as an overexpression target to confer tolerance against furfural. YghA preferably used NADH as a cofactor and had an apparent K_m value of 0.03 mM against furfural. In the presence of 1 g liter^{-1} furfural and 10% xylose (wt/vol), *yghA* overexpression in an ethanologenic *E. coli* strain SSK42 resulted in an ethanol efficiency of ~97%, with a 5.3-fold increase in ethanol titers compared to the control. YghA also exhibited activity against the less toxic inhibitor 5-hydroxymethyl furfural, which is formed due to dehydration of hexose sugars, and thus is a formidable target for overexpression in ethanologenic strain for fermentation of sugars in biomass hydrolysate.

IMPORTANCE Lignocellulosic biomass represents an inexhaustible source of carbon for second-generation biofuels. Thermo-acidic pretreatment of biomass is performed to loosen the lignocellulosic fibers and make the carbon bioavailable for microbial metabolism. The pretreatment process also results in the formation of inhibitors that inhibit microbial metabolism and increase production costs. Furfural is a potent furan inhibitor that increases the toxicity of other inhibitors present in the hydrolysate. Thus, it is desirable to engineer furfural tolerance in *E. coli* for efficient fermentation of hydrolysate sugars.

KEYWORDS *Escherichia coli*, ethanol, YghA, furfural, lignocellulose, tolerance

Lignocellulosic biomass presents an inexhaustible source of biocarbon that can be channeled toward the production of compounds with industrial relevance (1, 2). This has the potential to alleviate the ever-increasing need to burn fossil fuels for energy needs. However, biomass recalcitrance is a major impediment to cost-efficient conversion of lignocellulosic biomass into biofuels. Plants have evolved complex structural and physical mechanisms which resist the breakdown of complex oligosaccharides into simpler monosaccharides (3). Among sugars, the pentose content in lignocellulose can vary from 5 to 30% (2). On a dry basis, xylan can constitute up to 24 wt% of grasses (4). In order to maximize the yield of pentose sugars and make the biomass accessible for enzymatic saccharification and microbial metabolism, lignocellulosic biomass is pretreated to remove lignin and loosen the polysaccharides. Depending upon biomass composition and/or requirements, the pretreatment can be any from physical, physicochemical, chemical, and biological processes (5–8).

Dilute acid pretreatment at higher temperatures is frequently used to maximize yield of xylose from xylan polymers (9–11). However, pretreatment conditions also

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result in the formation of furan aldehydes, organic acids, and aromatic compounds, which act as inhibitors of cellular metabolism (5, 12, 13) and consequently lower productivity of the biofuel compound of interest. Furfural and 5-hydroxymethyl furfural (5-HMF) are the furan aldehydes that are generated due to dehydration of xylose and glucose, respectively, under acidic conditions at high temperatures. Furfural is a key inhibitor (12) which acts synergistically with other aldehyde inhibitors (14) to inhibit microbial growth. Furfural causes DNA damage (15–17), oxidative stress (18), and redox imbalance (19) in the microbial cells. Efforts targeted toward engineering furfural tolerance in *Escherichia coli* have resulted in improved fermentation of lignocellulosic sugars (20). To remove furfural toxicity from growth media, both biological (21) and chemical (22, 23) treatments have been applied. The drawbacks of using biological and chemical treatments are the reduction in the nitrogen content and an increase in processing costs, respectively, of the biomass. Genome analysis of a hydrolysate-resistant *E. coli* strain has identified the role of genes involved in primary and secondary metabolism, RNA metabolism, sugar transport, vitamin metabolism, and antioxidant activity (24) with potential to confer tolerance against lignocellulosic inhibitors. Published literature supports the idea that a multidimensional approach to engineer furfural tolerance has been successful. Furfural tolerance has been successfully engineered in *E. coli* via augmenting the cellular NADPH pools (25), silencing genes using NADPH pools to detoxify furfural (26), increasing expression of NADH-specific genes (27), multidrug-resistant (MDR) efflux pumps (28), enhancing DNA repair (29, 30), membrane biogenesis (30), and stress tolerance (30, 31). Tolerance against 5-HMF, a less toxic compound than furfural, has also been achieved using MDR efflux pumps (28) and enhancing NADPH pools (32).

Microbial cells respond to furfural challenge by converting it into furfuryl alcohol. Furfuryl alcohol with a 50% inhibitory concentration (IC_{50}) value of 4.0 g liter^{-1} (33) is relatively less toxic to *E. coli* than furfural with an IC_{50} value of 2.4 g liter^{-1} (14). Studies have used a strategy whereby deletion of aldehyde reductases (ALRs) of *E. coli* silenced the competing pathways with a consequent increase in titers of the biomolecule of interest (34, 35). This led us to ponder whether the activity of ALRs can be exploited to engineer tolerance in *E. coli* toward an aldehyde inhibitor, furfural. In the present study, we screened the collection of strains consisting of single-gene deletions against a series of reductases, oxidoreductases, and dehydrogenases to observe any significant alteration in tolerance of *E. coli* toward furfural. We used xylose as the primary carbon source in AM1 minimal growth medium, considering its predominance in the acid hydrolysate of biomass. Based on our screening results, we hypothesized that overexpression of *yghA* will confer tolerance against furfural. We report two important observations in engineered ethanologenic *E. coli* strain SSK42 (pTrcHisA-*yghA*), which has high expression levels of the *yghA* gene compared to the control SSK42 (pTrcHisA) strain. First, in the presence of furfural, SSK42 (pTrcHisA-*yghA*) has growth advantage compared to the control SSK42 (pTrcHisA) strain. Second, in contrast with SSK42 (pTrcHisA), the presence of inhibitor has no significant influence on maximum ethanol titers of SSK42 (pTrcHisA-*yghA*).

RESULTS

Screening of *E. coli* BW25113 mutants for target gene selection. In the first screen, a relatively low concentration of sugar at 0.2% (wt/vol) was used in AM1 medium. It has been reported that any concentration of xylose above 1.8% starts to exert osmotic stress under similar microaerobic conditions (36). Thus, the nonlimiting, as well as relatively lower, concentration of sugar would help to prevent any confounding variables from influencing the tolerance of microbe toward furfural. Bacterial growth (optical density at 600 nm [OD_{600}]) was monitored at 3 h and 6 h since the most impactful influence of gene mutation on productivity should be observed at the initial stages of growth (Fig. S2 in the supplemental material). A statistical approach was applied in order to select BW25113 mutant strains for the next screening step. Strains that displayed $\leq$5% variation in readings for both 3-h and 6-h time points (Table S2) were selected. The rationale was that the genetic makeup of such strain(s) is relatively better suited to withstand furfural stress and leads to a predictable growth response.

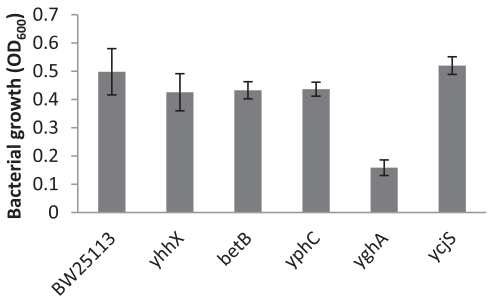

FIG 1 Screening of *E. coli* BW25113 strain deficient in respective genes indicated in the *x* axis. Cultures were grown in AM1 media containing 5% xylose (wt/vol) and 0.5 g liter^{-1} furfural. OD_{600} was recorded at 24 h. Values are averages of $n = 2$ independent experiments. Error bars represent SD of the mean.

Using the statistical approach, BW25113 derivatives, which had deletion in the *yhhX*, *betB*, *yphC*, *ycjS*, and *yghA* genes, respectively, were selected for the next screen.

In the second screen, both osmotic and furfural stresses were increased by increasing the concentration of xylose and furfural to 5% (wt/vol) and 1.0 g liter^{-1}, respectively. The high concentration of furfural did not result in significant growth in any of the selected strains at the end of 48 h (Fig. S3). Thus, in the third screening step, the concentration of furfural in AM1 medium was reduced to 0.5 g liter^{-1} while keeping the xylose concentration constant at 5% (wt/vol). At reduced furfural concentration, growth was observed in all strains at an earlier time point of 24 h (Fig. 1). Among the selected strains, the one with deletion of the *yghA* gene displayed a 3.15-fold decrease in biomass compared to the wild-type (WT) parent strain BW25113. Based upon this observation, it was hypothesized that overexpression of *yghA* shall confer tolerance against furfural in *E. coli*.

To validate if the deletion of *yghA* in BW25113Δ*yghA* was the only reason for reduced tolerance to furfural, pTrcHisA-*yghA* was transformed in strain BW25113Δ*yghA* and tested for its ability to tolerate furfural. The resulting strain was designated BW25113Δ*yghA* (pTrcHisA-*yghA*). As a control, an empty pTrcHisA was transformed into BW25113Δ*yghA* strain, and the resulting strain was labeled BW25113Δ*yghA* (pTrcHisA). The growth behavior of the transformed strains was monitored under similar sugar and furfural concentrations in the presence of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) as an inducer. A 1.85-fold increase in bacterial growth (OD_{600}) was observed in strain with cloned *yghA* compared to one carrying empty plasmid (Fig. S4) at 24 h. This observation suggested that the expression of *yghA* indeed is associated with an increase in biomass in the presence of furfural.

YghA predominantly uses NADH as a cofactor. His-tagged YghA was overexpressed in TOP10 cells and purified to homogeneity. YghA showed activity against furfural in the presence of NADH as well as NADPH. The apparent K_m values of YghA against furfural in the presence of NADH and NADPH were determined to be 0.03 and 0.05 mM, respectively, whereas the V_{max} values against furfural for NADH and NADPH were calculated to be 0.003 and 0.001 mM min^{-1}, respectively. The K_{cat}/K_m values in the presence of NADH and NADPH were determined to be 0.42 and 0.23 mM^{-1} min^{-1}, respectively. The enzyme kinetics data suggest that YghA has a preference to utilize NADH over NADPH as a cofactor in the presence of furfural as a substrate.

Furfural tolerance conferred by YghA is neutral to the tested carbon sources. Glucose is the preferred carbon source for *E. coli* due to enhanced energy and reducing power yield compared to xylose. It was thus tested whether glucose as the sole carbon source results in enhanced tolerance against furfural compared to the condition where xylose is the sole carbon source (Fig. 2). In the presence of 1.0 g liter^{-1} furfural, biomass increase in the control strain BW25113Δ*yghA* (pTrcHisA) was observed only in the presence of glucose (induced condition) and not that much in xylose (neither induced nor uninduced) (Fig. 2A). The biomass increase in BW25113Δ*yghA* (pTrcHisA-*yghA*), on the other hand, was similar in both of the carbon sources in the presence of the inhibitor. With xylose as carbon source, under induced conditions, the growth reduction for BW25113Δ*yghA*

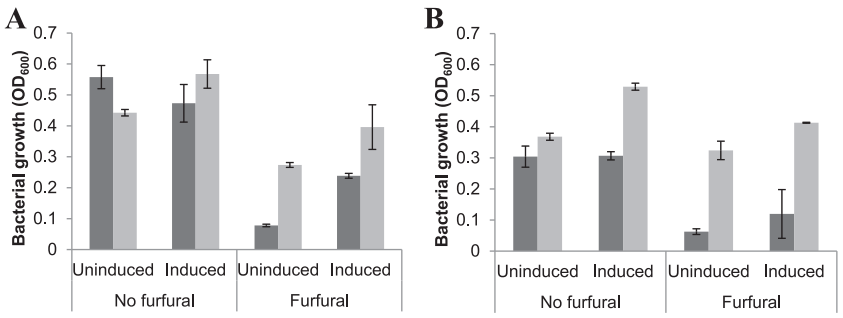

FIG 2 Influence of carbon source in promoting biomass formation in the *E. coli* BW25113Δ*yghA* host with either pTrcHisA (dark gray) or with pTrcHisA-*yghA* (light gray). Media consisted of 1 g liter^{-1} furfural, 0.1 mM IPTG, and either 5% (wt/vol) of glucose (A) or xylose (B). OD_{600} was recorded at 48 h. Values are averages of $n = 2$ independent experiments. Error bars represent SD of the mean.

(pTrcHisA-*yghA*) in the presence of furfural was 1.28-fold, while that for BW25113Δ*yghA* (pTrcHisA) was around 2.57-fold (Fig. 2B). BW25113Δ*yghA* (pTrcHisA-*yghA*) also showed higher growth under uninduced conditions in the presence of furfural for both xylose and glucose than BW25113Δ*yghA* (pTrcHisA). It suggested that leaky expression of *yghA* is sufficient to confer tolerance against furfural under tested conditions. Based on these results, it was concluded that *yghA* confers tolerance against furfural in a sugar-neutral manner.

It was observed that in the absence of furfural (Fig. 2A and B), *yghA* overexpression hardly resulted in any growth advantage when grown in glucose as a carbon source. However, in the presence of xylose, *yghA* overexpression resulted in 1.2- and 1.83-fold higher growth under uninduced and induced conditions, respectively (Fig. 2B), suggesting the supportive role of *yghA* during xylose metabolism beyond furfural tolerance.

The relevance of this idea was tested, under the same conditions, in the context of an *E. coli* strain, SSK42, whose genome has been optimized for ethanol production using xylose as the primary carbon source under microaerobic conditions (37, 38). Among other genetic changes, the promoter of PDH complex in SSK42 has been replaced by that of *gapA*, which allows production of an additional NADH under anaerobic conditions (37). However, this additional NADH was not able to rescue growth in the presence of furfural (Fig. S5A and B). It is difficult to ascertain a reason for cessation of growth for SSK42 in capped tube culture, as the strain has been evolutionarily adapted by passaging on AM1-xylose medium plates and then by alternating sole carbon source—either glucose or xylose—in planktonic culture (38), and the redox status of resultant SSK42 is not known. Despite the failure of YghA in improving the growth of the SSK42 strain in the presence of furfural, we observed that furfural could still be metabolized from the culture medium by SSK42 (pTrcHisA-*yghA*) (Fig. S5C).

In the previous studies, engineering furfural tolerance in *E. coli* has also been demonstrated to confer tolerance against the relatively less toxic furan aldehyde 5-HMF (28, 32). It was also found that overexpression of *yghA* leads to tolerance toward 5-HMF, and no 5-HMF could be detected in the media after 48 h in culture containing SSK42 (pTrcHisA-*yghA*) strain (Fig. S6).

Furfural clearance from medium is similar for both 0.01 and 0.1 mM IPTG concentrations. The reduction in furfural concentration in uninduced samples (Fig. S5C) made it prudent to investigate the role of IPTG in conferring competitive advantage in clearing furfural from the medium. It is a known fact that chemical properties of IPTG lead to induction of toxicity to *E. coli* BL21 cells (39). Any reduction in IPTG concentration will be beneficial in reducing toxicity and ultimately contribute toward increasing ethanol productivity by strain SSK42 (pTrcHisA-*yghA*). Since furfural metabolism is a multigenic trait, we added chloramphenicol to the culture medium to prevent the synthesis of any new cellular proteins and reduce any confounding effect of same on the results.

It was observed that in nongrowing whole cells of SSK42 (pTrcHisA-*yghA*), ~60% furfural was removed from the medium in the absence of IPTG at 2 h, representing a clearance rate of 0.60 g liter^{-1} h^{-1} (Fig. S7). While in the presence of IPTG, ~83 to 86%

furfural was removed from the medium. Interestingly, similar concentrations of furfural (0.27 to 0.34 g liter^{-1}) at 0.01- and 0.1-mM IPTG concentrations were observed after 2 h, which represents a furfural clearance rate of 0.86 and 0.83 g liter^{-1} h^{-1}, respectively. It suggests that increasing the concentration of IPTG from 0.01 to 0.1 mM does not confer any catalytic advantage via higher YghA protein levels to expedite the reduction of furfural from the media.

YghA overexpression leads to enhanced microbial and ethanol productivity in the bioreactor. The Hungate tube is a poorly buffered environment where mixing of nutrients and maintenance of microaerobic environment is also not optimum. The results (Fig. S5C) indicate that SSK42 (pTrcHisA-*yghA*) is effective in clearing furfural from the media even when an increase in biomass is severely compromised. The bioreactor is a controlled environment wherein pH and mixing of nutrients can be efficiently achieved. Thus, the increase in microbial biomass in a bioreactor was tested while keeping media components similar, as in Fig. S5C. Based upon results obtained from Fig. S7, the concentration of IPTG concentration was decreased from 0.1 to 0.01 mM in the bioreactor.

It was observed that in a controlled bioreactor environment and at 5% xylose (wt/vol) concentration, both SSK42 (pTrcHisA) and SSK42 (pTrcHisA-*yghA*) strains demonstrated their ability to increase the biomass in the presence of furfural (Table 1; Fig. 3A). The rate of increase in biomass for the *yghA*-overexpressing strain SSK42 (pTrcHisA-*yghA*) was 0.059 g liter^{-1} h^{-1} (1.6-fold higher than the control) and was achieved in the 24- to 48-h time period as against the value of 0.037 g liter^{-1} h^{-1} achieved in the 72- to 96-h time period for the control strain SSK42 (pTrcHisA). The corresponding difference in xylose consumption rate was also seen (Fig. 3B). The concentration of furfural at different time points is indicated in Fig. S8. When the ethanol production was evaluated in the presence of furfural and at 96 h, it was observed that the maximum ethanol titer of SSK42 (pTrcHisA-*yghA*) strain was 1.76-fold higher than that observed in SSK42 (pTrcHisA), and the value was comparable to the titer observed for the strains in the absence of furfural (Fig. 3C). In terms of volumetric ethanol productivity, the maximum value shifted to the 24-h earlier time point in *yghA* overexpressing strain and also resulted in an increase in productivity of almost 18% for SSK42 (pTrcHisA-*yghA*) compared to SSK42 (pTrcHisA) (Table 1). The maximum specific ethanol productivity value in the presence of 1 g liter^{-1} of furfural for SSK42 (pTrcHisA) was 0.35 g g^{-1} h^{-1} at 72 to 96 h, while it was 0.40 g g^{-1} h^{-1} for SSK42 (pTrcHisA-*yghA*) at 24 to 48 h (Table S3). The maximum ethanol yield in the case of SSK42 (pTrcHisA-*yghA*) was around 15% higher than that of SSK42 (pTrcHisA).

An increase in sugar concentration results in osmotic stress response and is characterized by expression of *soxS*, *sodA*, and *katE* genes, which are also involved in combating oxidative stress (40). This oxidative stress is in addition to the reactive oxygen species (ROS) accumulation induced in microbial cell in response to the furfural challenge (18). Consequently, it is desirable from an applied microbiology perspective that the furfural tolerance of ethanologenic SSK42 (pTrcHisA-*yghA*) strain can also withstand the stress imposed by high sugar concentration. Thus, the growth dynamics of strains SSK42 (pTrcHisA) and SSK42 (pTrcHisA-*yghA*) were tested at 10% sugar (wt/vol) loading.

It was observed that the maximum biomass titer at 10% sugar (wt/vol) load and in the presence of furfural for strain SSK42 (pTrcHisA-*yghA*) was 2.75 $\pm$ 0.12 g liter^{-1}, while for SSK42 (pTrcHisA), it was 1.36 $\pm$ 0.20 g liter^{-1}, which represented an $\sim$2-fold increase in biomass concentration and 1.5-fold increase in biomass productivity for YghA-overexpressing strain (Table 1; Fig. 4A). A similar difference in xylose consumption was also observed (Fig. 4B). The difference in maximum titers of ethanol was much more remarkable. The maximum ethanol titers for SSK42 (pTrcHisA-*yghA*) and SSK42 (pTrcHisA) were 41.58 $\pm$ 1.66 and 7.78 $\pm$ 2.69 g liter^{-1}, respectively, which represented a 5.3-fold higher ethanol titer. For SSK42 (pTrcHisA-*yghA*), a 1.4-fold increase in ethanol yield per gram of xylose consumed was also observed compared to SSK42 (pTrcHisA). The maximum theoretical yields for ethanol in the cases of SSK42 (pTrcHisA-*yghA*) and SSK42 (pTrcHisA) were 97.19 $\pm$ 12.64 and 68.92 $\pm$ 12.80%, respectively. It represents an $\sim$30% increase in the efficiency of ethanol production. The increase in ethanol productivity value was also, remarkably, 2.5-fold higher for SSK42

TABLE 1 Impact of furfural on biomass and ethanol formation by SSK42 (pTrcHisA) and SSK42 (pTrcHisA-*yghA*) strains under low (5%) and high (10%) xylose loading in a bioreactor[a]

Strain	Furfural concn. (g liter^{-1})	Data for: Cells: Final concn. (g liter^{-1})	Cells: Specific growth rate: Max value (h^{-1})	Cells: Specific growth rate: Time period (h)	Ethanol: Max titer (g liter^{-1})	Ethanol: Productivity: Max. value (g liter^{-1} h^{-1})	Ethanol: Productivity: Time period (h)	Ethanol: Yield: g g^{-1} sugar	Ethanol: Yield: % of max. theoretical
Strains under 5% xylose (wt/vol) loading									
SSK42 (pTrcHisA)	0	1.89 ± 0.02	0.113 ± 0.018	0–24	25.27 ± 0.02	0.41 ± 0.03	24–48	0.46 ± 0.03	89.84 ± 5.54
SSK42 (pTrcHisA-*yghA*)	0	1.74 ± 0.20	0.138 ± 0.004	0–24	20.97 ± 1.74	0.41 ± 0.05	24–48	0.42 ± 0.04	81.79 ± 8.23
SSK42 (pTrcHisA)	1	1.60 ± 0.06	0.056 ± 0.015	24–48	13.61 ± 0.53	0.41 ± 0.02	72–96	0.44 ± 0.02	85.53 ± 4.26
SSK42 (pTrcHisA-*yghA*)	1	1.77 ± 0.04	0.091 ± 0.007	24–48	23.90 ± 2.99	0.50 ± 0.04	48–72	0.52 ± 0.08	101.58 ± 14.99
Strains under 10% xylose (wt/vol) loading									
SSK42 (pTrcHisA)	0	2.31 ± 0.02	0.127 ± 0.008	0–24	31.40 ± 4.25	0.50 ± 0.07	48–72	0.46 ± 0.13	89.63 ± 24.56
SSK42 (pTrcHisA-*yghA*)	0	2.55 ± 0.08	0.139 ± 0.000	0–24	39.72 ± 4.38	0.62 ± 0.05	24–48	0.47 ± 0.07	92.13 ± 14.61
SSK42 (pTrcHisA)	1	1.36 ± 0.20	0.030 ± 0.013	48–72	7.78 ± 2.69	0.29 ± 0.11	96–120	0.35 ± 0.07	68.92 ± 12.80
SSK42 (pTrcHisA-*yghA*)	1	2.75 ± 0.12	0.104 ± 0.003	24–48	41.58 ± 1.66	0.72 ± 0.13	48–72	0.50 ± 0.06	97.19 ± 12.64

[a]Results are average of $n = 2$ independent experiments. Error is SD of the mean.

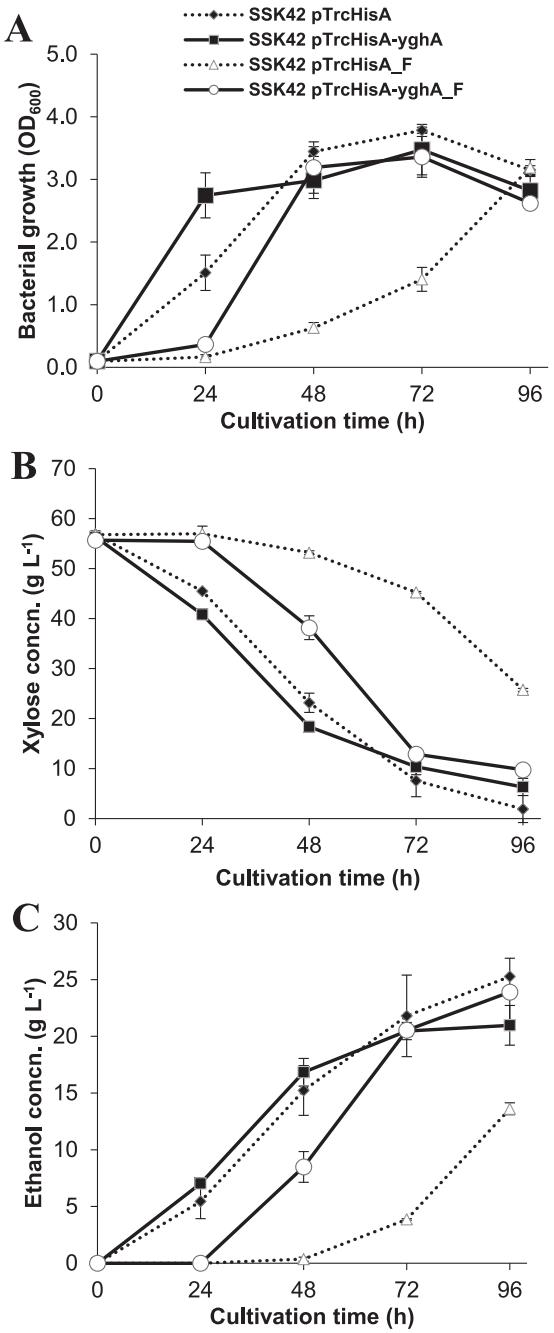

FIG 3 Fermentation profile of SSK42 strain with or without the overexpression of the *yghA* gene in a bioreactor at 5% xylose (wt/vol) load. "F" indicates furfural treatment at 1 g liter^{-1}. Bacterial growth (A) and xylose (B) and ethanol (C) concentrations are indicated in the respective panels. Values are averages of $n = 2$ independent experiments. Error bars represent SD of the mean.

(pTrcHisA-*yghA*) than SSK42 (pTrcHisA). At the end of 96 h, the average ethanol productivity was 0.39 g liter^{-1} h^{-1} (Fig. 4C), which was comparable with the 0.42 g liter^{-1} h^{-1} value obtained by overexpression of SDR pump *mdtJI* (28). The maximum specific ethanol productivity value in the presence of 1 g liter^{-1} of furfural for SSK42 (pTrcHisA) was 0.34 g g^{-1} h^{-1} at 96 to 120 h, while it was 0.39 g g^{-1} h^{-1} for SSK42 (pTrcHisA-*yghA*) at 48 to 72 h (Table S3). Our fermentation results prove that overexpression of YghA with relatively higher affinity toward NADH compared to NADPH is a useful strategy to confer tolerance against furfural at high sugar loadings.

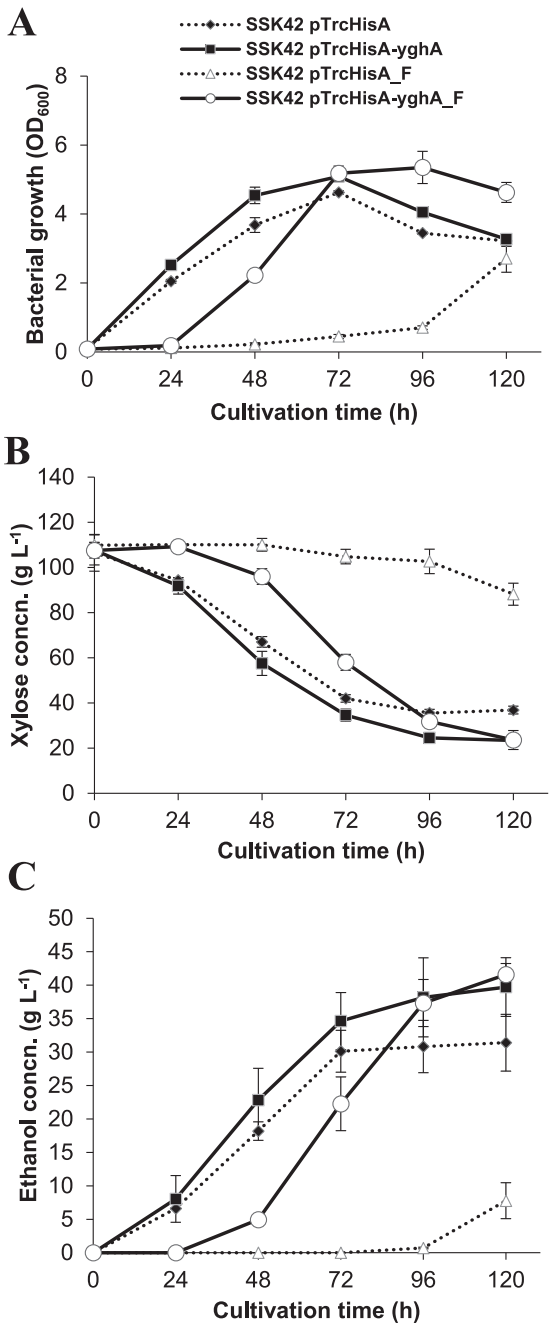

FIG 4 Fermentation profile of SSK42 strain with or without the overexpression of the *yghA* gene in a bioreactor at increased osmotic stress exerted by 10% xylose (wt/vol). Bacterial growth (A) and xylose (B) and ethanol (C) concentrations are indicated in the respective panels. "F" indicates furfural treatment at 1 g liter^{-1}. Values are averages of $n = 2$ independent experiments. Error bars represent SD of the mean.

DISCUSSION

In this study, we used a three-step screening strategy to investigate a set of *E. coli* oxidoreductases, dehydrogenases, and reductases that can confer tolerance against an aldehyde inhibitor, furfural, which is commonly generated during pretreatment of lignocellulose. Since, at the first screening step, either deletion or overexpression of the respective gene would be advantageous to the microbe to withstand furfural stress, our statistical approach was a nonbiased one to identify potential gene targets. Additionally, the stepwise increase in stresses (both osmotic and inhibitor) was helpful in keeping confounding

factors at bay in the first screening step and select strains for the next step of screening (Fig. S2 in the supplemental material). We found that the previously poorly characterized oxidoreductase YghA is able to confer tolerance against 1 g liter^{-1} furfural and ~10% xylose (wt/vol) as sugar source in an ethanologenic *E. coli* strain SSK42. Other screening studies have identified overexpression of *thyA* (29), *mdtJI* (28), and *groESL* and *lpcA* (30) to be effective in conferring tolerance against a concentration range of 0.75 to 1.25 g liter^{-1}of furfural.

Compared to glucose, xylose is a poor source of energy and reducing equivalents that are essential for microbial growth. The problem is further compounded under oxygen limitation where the cells are mainly dependent upon substrate-level phosphorylation and transhydrogenases for generation of energy and reducing equivalents, respectively. Under anaerobic conditions, the switch from glucose to xylose results in the reduction of cellular generation of NADH/$FADH_2$ and NADPH by 1.4- and 2.8-fold, respectively (41). This makes NADPH a scarce source for anabolic reactions under oxygen limitation, and its diversion for furfural detoxification further depletes cellular NADPH pools and compromises an increase in biomass. Compared to NADPH, YghA has relatively higher affinity for using NADH as a cofactor for furfural detoxification, which should be beneficial in increasing cellular NADPH pools. The low apparent K_m values of YghA for furfural (0.03 mM) in the presence of NADH also reflect in our data where expression at 0.01 mM IPTG concentration was sufficient to confer tolerance against furfural, and a further increase in YghA expression conferred no additional growth advantage in the presence of furfural. On the other hand, with an apparent K_m of 0.4 mM against furfural, overexpression of NADH-specific oxidoreductase FucO has also been reported to confer tolerance against furfural (27). Less is known about the function of YghA in the native *E. coli* host, and no structural studies have been carried out on it. In an earlier study, YghA has been defined to be an $NADP^+$-dependent aldehyde reductase with activity toward butyraldehyde and decanal (34). YghA has been reported to harbor a NAD(P) binding Rossman fold domain (42). Based on our results, we would further broaden the use of cofactor and aldehyde substrate by YghA.

Interestingly, the tolerance conferred by an oxidoreductase (YghA) against furfural and the maximum ethanol titers obtained are comparable to that offered by polyamines (43) and a small multidrug resistance (SDR) pump (28). This observation further lends credence to the fact that microbial metabolism harbors a multidimensional capability to efficiently counter the metabolic challenge of furfural, which exerts its toxicity by inhibiting different metabolic targets of cellular machinery.

MATERIALS AND METHODS

Strains and media. All strains used in this study are listed in Table S1 in the supplemental material. A set of mutants of strain BW25113 with a deletion of one additional gene was used in this study (44). The said strains were obtained from Coli Genetics Stock Center (CGSC), Yale University, New Haven, CT. The workhorse microbial strain of this study is SSK42 (genotype *E. coli* B P_{gapA} PDH Δ*ldh* Δ*frdA* Δ*pflB*), which has undergone extensive evolutionary adaptation in AM1 minimal media with either glucose or xylose as carbon sources (35, 44). The kanamycin resistance cassette was removed from SSK42 using temperature-sensitive pCP20, which harbors the FLP recombinase gene (45). The genome of *E. coli* B served as a template for PCR amplification of *yghA* gene using primer set YghA_pTrcHis_NheI, CCCGCTAGCATGTCTCATTTAAAAGACCCGACC, and YghA_pTrcHisA_BamHI, CCCGGATCCTTAACCTAAATGCTCGCCG. The open reading frame (ORF) of *yghA* was cloned into pTrcHisA at Nhe1 and BamH1 restriction sites to obtain the pTrcHisA-*yghA* construct. For physiological analysis, strains were grown in AM1 medium having composition $(NH_4)_2HPO_4$ (19.92 mM), $NH_4H_2PO_4$ (7.56 mM), KCl (2.00 mM), $MgSO_4{\cdot}7H_2O$ (1.50 mM), betaine HCl (1.00 mM), $FeCl_3{\cdot}6H_2O$ (8.88 μM), $CoCl_2{\cdot}6H_2O$ (1.26 μM), $CuCl_2{\cdot}2H_2O$ (0.88 μM), $ZnCl_2$ (2.20 μM), $Na_2MoO_4{\cdot}2H_2O$ (1.24 μM), H_3BO_3 (1.21 μM), $MnCl_2{\cdot}4H_2O$ (2.50 μM), and $CaCl_2{\cdot}2H_2O$ (1.36 μM) (46). Concentrations of xylose used have been mentioned at the respective experiments, and ampicillin was used at 12.5 mg liter^{-1}.

Culture conditions. Glycerol stocks of microbial strains were revived on petri plates containing AM1 media with 2% xylose (wt/vol) as carbon source. Two additional restreaks, each from a well-isolated colony, were performed, and a third streak was used to start primary overnight cultures, which were grown in Hungate tubes with capacity of 18 ml in a shaker incubator at 37°C and 150 rpm. In these tubes, 10 ml AM1 medium containing xylose, at the concentration mentioned against respective experiment, was used. All secondary cultures were seeded at OD_{600} of 0.1. Expression of *yghA* was induced using either 0.01 or 0.1 mM IPTG as indicated against the respective experiment.

Screening of *E. coli* BW25113 strains. Wild-type *E. coli* BW25113 was screened for sensitivity to the following concentrations of furfural: 0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, and 2.50 g liter^{-1}. One set consisted of 0.2% xylose (wt/vol) as the sole carbon source, while another set consisted of 0.2% (wt/vol) glucose. For both sugar sets, the 50% lethal dose (LD_{50}) concentration of furfural was determined to be 0.25 g liter^{-1} at 3 h of cultivation (Fig. S1 in the supplemental material). Furfural tolerance in the presence of xylose was of our primary interest; thus, it was included for further screening. The first screen of 54 *E. coli* BW25113 mutant and BW25113 parent strains was performed at 0.25 g liter^{-1} furfural and 0.2% xylose (wt/vol). Selected strains from the first screens were subjected to a second screen consisting of 1 g liter^{-1} furfural and 5% xylose (wt/vol). In the second screen, none of the selected strains displayed a significant increase in biomass at the end of 48 h. Thus, the same selected set of strains was subjected to the third screen consisting of 0.5 g liter^{-1} furfural and 5% xylose (wt/vol) in AM1 media. A significant change in biomass was observed in strains at 24 h at the reduced furfural concentration. All observations were recorded at least in duplicate.

Influence of IPTG concentration on furfural removal. Cultures of SSK42 (pTrcHisA) and SSK42 (pTrcHisA-*yghA*) strains were grown overnight in capped tubes in the presence of either 0, 0.01, or 0.1 mM IPTG concentration. After around 16 h, cultures were treated with 50 mg liter^{-1} chloramphenicol for 2 h. Furfural removal was then monitored in nongrowing whole-cell cultures at an OD_{600} of 2.0 in the medium consisting of 5% xylose (wt/vol), 12.5 mg liter^{-1} ampicillin, 50 mg liter^{-1} chloramphenicol, 2.0 g liter^{-1} furfural, and either 0, 0.01, or 0.1 mM IPTG. Tubes were incubated in 37°C at 150 rpm and sampled at indicated time points.

Purification of YghA. *E. coli* TOP10 harboring 6×His tagged pTrcHisA-*yghA* was cultured overnight in LB medium in the presence of ampicillin. The secondary culture was started using 1% of the overnight culture and induced using 0.1 mM IPTG at OD_{600} of ~0.4. Cells were harvested after 4 h and stored at −80°C overnight. Pellets were resuspended further in lysis buffer (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, and 5 mM imidazole). The cells were lysed using a microtip sonicator. After centrifugation of lysate at ~9,000 × *g* (1 h and 4°C), the supernatant was filtered (0.45 μm) and purified via Ni-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography. The purified protein was dialyzed in 100 mM sodium phosphate buffer, and the purity was checked on SDS-PAGE gel. The concentration of YghA was determined by bicinchoninic acid (BCA) protein assay kit (G-Biosciences, MO, USA).

Fermentation. Primary cultures were started in 250-ml shake flasks with 100 ml AM1 medium with 5% xylose (wt/vol), 12.5 mg liter^{-1} ampicillin, and 0.01 mM IPTG. After around 16 h growth, cells were harvested. Fermenters were seeded at OD_{600} of 0.1 in 300 ml AM1 media consisting of 1 g liter^{-1} furfural, 12.5 mg liter^{-1} ampicillin, 0.01 mM IPTG, and either 5% or 10% xylose (wt/vol) as sugar source. pH was maintained at 7.0 using 2N KOH. Air in headspace was pumped in at 0.03 liter per minute (LPM) for the first 24 h and then increased to 0.04 LPM for the remaining fermentation period.

Analysis. Xylose and ethanol concentration was determined using Shimadzu HPLC with Aminex HPX-87H (300 by 7.8 mm) column and refractive index (RI) detector. The column temperature was maintained at 60°C, and that of the detector was maintained at 50°C. We used 4 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 ml min^{-1}. A reference standard of 1 g liter^{-1} for each metabolite was obtained from Absolute Standards, USA. Biomass was estimated by recording optical density at 600 nm using a UV-visible (UV-Vis) spectrophotometer (Ultrospec 3100; Amersham Biosciences). The concentration of furfural was estimated using the UV-Vis method as reported before (47).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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