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L-Glycine Alleviates Furfural-Induced Growth Inhibition during Isobutanol Production in *Escherichia coli*

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Lignocellulose is now a promising raw material for biofuel production. However, the lignin complex and crystalline cellulose require pretreatment steps for breakdown of the crystalline structure of cellulose for the generation of fermentable sugars. Moreover, several fermentation inhibitors are generated with sugar compounds, majorly furfural. The mitigation of these inhibitors is required for the further fermentation steps to proceed. Amino acids were investigated on furfural-induced growth inhibition in *E. coli* producing isobutanol. Glycine and serine were the most effective compounds against furfural. In minimal media, glycine conferred tolerance against furfural. From the IC_{50} value for inhibitors in the production media, only glycine could alleviate growth arrest for furfural, where 6 mM glycine addition led to a slight increase in growth rate and isobutanol production from 2.6 to 2.8 g/l under furfural stress. Overexpression of glycine pathway genes did not lead to alleviation. However, addition of glycine to engineered strains blocked the growth arrest and increased the isobutanol production about 2.3-fold.

Keywords: Lignocellulose, biomass, isobutanol, furfural, glycine

Introduction

Lignocellulose is the major building block of plant cell walls [1]. Lignocellulosic biomass comprises abundant raw material and has currently become a promising carbon source for the production of biofuel [2]. There are many types of raw material that are considered lignocellulosic biomass, such as corn stover, corn fiber, empty fruit bunch, pine wood, and wheat straw [3, 4]. Because lignocellulose cannot be directly used to make biofuel owing to its rigid structure, it needs to be converted to sugar monomers by pretreatment processes, such as dilute acid, concentrated acid, and enzymatic hydrolysis [1, 3, 5]. Such a pretreatment step with the hydrolysis process not only generates hexose sugars (glucose, galactose, and mannose) and pentose sugars (xylose and arabinose) but also makes toxic molecules that can inhibit cell growth, including weak acids, furan derivatives, and phenolic compounds [6, 7]. Among these, furfural, vanillin, 4-hydroxybenzaldehyde, and acetic acid are well-known inhibitors of biofuel production utilizing lignocellulosic biomass [8]. In particular, furfural is regarded as a major inhibitor in lignocellulosic biomass for its NAD(P)H scavenger action [9]. Although there have been many studies investigating their inhibitory mechanisms [10–12], the exact processes are still unknown. Until now, several possible inhibition mechanisms have been suggested, such as DNA damage, reactive oxygen species generation in *Saccharomyces cerevisiae*, and NADH or NADPH depletion in many bacterial species [13, 14]. For example, micro-organisms normally convert furfural to less toxic molecules,

such as furfuryl-alcohol and furoic acid [13, 15], which require additional NADPH consumption and consequently lead to decreased cell growth.

Many efforts have been conducted to increase the tolerance of production hosts to the inhibitory molecules. Adaptation methods have been conducted to isolate bacteria showing higher tolerance to furfural by serial transfer [16]. Additionally, the deletion of oxidoreductases *yqhD* (NADPH-dependent aldehyde reductase) and *dkgA* (beta-keto ester reductase) in ethanologenic *Escherichia coli* LY180 increased the tolerance to furfural [17]. Another oxidoreductase, FucO, which is NADH-dependent, has also been reported to increase the tolerance of *E. coli* [18, 19].

As a different way to evaluate the inhibitors, overliming using $Ca(OH)_2$ can successfully reduce the amount of furan aldehyde derivatives in the lignocellulosic biomass [20]. Cysteine supplementation in the medium can increase the tolerance of the organism [21, 22]. Yeast extract is known to promote bacterial growth in the presence of furfural [21]. Because furfural confers DNA damage to cells, the effects of serine, thymidine, and tetrahydrofolate, which are related to the de novo synthesis of pyrimidine, have also been studied to reduce the inhibitory effect on growth [22].

In this study, we report the effect of an additional medium component using amino acids in the presence of furfural on isobutanol-producing *E. coli* HM501 [13]. Additionally, the optimal concentration of the glycine against furfural has been studied. Finally, the glycine pathway genes were overexpressed to investigate the reduced furfural toxicity, and the IC_{50} for the inhibitory compounds acetic acid, vanillin, furfural, and 4-hydroxybenzaldehyde showed that glycine has a specific alleviation effect on furfural with the isobutanol-producing strain.

Materials and Methods

Bacterial Strains, Media, and Culture Conditions

The isobutanol-producing strain *E. coli* HM501 [13] was used in this study. For cell preparation, this strain was cultured in lysogeny broth (LB) agar and/or liquid broth. LB agar was prepared by dissolving 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 20 g of agar in 1 L of distilled water. For isobutanol production, the transformants were cultured in M9 minimal medium containing 2% glucose and 0.3% yeast extract. Appropriate antibiotics (100 μg/ml spectinomycin and 100 μg/ml ampicillin for recombinant *E. coli*) and 0.1 mM IPTG were also added when required. For the preculture, a single colony of the strain from an LB agar plate was used to inoculate 5 ml of LB medium. The culture was incubated overnight in a shaking incubator at 37°C. To conduct flask culture, the grown cells were inoculated into 30 ml of production medium in a 250 ml screw-cap flask at a 1:100 (v/v) dilution; the initial OD of this medium was 0.01. This flask was then sealed with the screw cap. Isopropyl β-D-1-thiogalactopyranoside was added initially to the culture medium to induce protein expression. The culture was continuously shaken in a shaking incubator. The temperature of this incubator was maintained at 30 °C. Aliquots were removed intermittently from the culture to carry out further steps. Test tube culture was carried out using 5 ml of the production medium containing the same concentration of glucose and yeast extract. This tube was sealed to create a microaerobic condition.

Reagents

Restriction enzymes and DNA polymerase were purchased from Enzynomics (Korea). The plasmid extraction kit and gel purification kit were purchased from GeneAll (Korea). Glucose, M9 minimal salts 5×, and yeast extract were purchased from Bacto or Difco (USA). Furfural, furfuryl alcohol, vanillin, sodium acetate, and 4-hydroxybenzaldehyde were purchased from Sigma-Aldrich (USA). Agarose and bacterial agar were supplied from the microbial carbohydrate resource bank at Konkuk University, Korea.

DNA Manipulations

Gene cloning for gene overexpression followed the general molecular biology method [23]. Genomic DNA of *E. coli* K-12 MG1655 was used as a template for *serA*, *glyA*, *purD*, and *gshB* for cloning. *serA* and *glyA* were amplified with serA-F (5′- CTCTGGAGCTCATGGCAAAGGTATCGCTGGAG-3′), serA-R (5′-CTCTAAGCTTTTAGTACAGCAGACGGGCGC-3′), glyA-F (5′-CTCTGGAGCTCATGTTAAAGCGTGAAATGAACATTG-3′), and glyA-R (5′-CTCTAAGCTTTTATGCGTAAACCGGGTAAC-3′) and digested with SacI and HindIII. Then, *serA* and *glyA* were ligated into pRSFDuet-1 in the same restriction site. *purD* was amplified with purD-F (5′-CTCTGGATCCGATGAAAGTATTAGTGATTGGTAAC-3′) and purD-R (5′-CTCTGTCGACTTAGTTCTGCTCGCGTTCGATAGCG-3′). BamHI and SalI were used for double restriction enzyme digestion for *purD,* which was cloned into pRSFDuet-1 in the same restriction site. *gshB* was amplified with gshB-F (5′-CTCTGACGTCATGATCAAGCTCGGCATCGTGATG-3′) and gshB-R (5′-AGTCCTCGAGTTACTGCTGCTGTAAACGTGC-3′) and digested with AatII and XhoI. Then, it was cloned into pRSFDuet-1 in the same restriction site. Once the intended plasmids were confirmed by sequencing, they were used for further study (Table 1).

Analysis Techniques

The concentration of isobutanol was determined by gas chromatography (Young Lin Tech, Korea); the chromatographic technique was performed using a HP-FFAP column (25 m × 0.20 mm × 0.3 μm) (Agilent Technologies, USA) and a flame ionization detector. The split ratio was 1:20. Two microliters of the sample was injected into the column. Helium was used as a carrier

Table 1. List of bacterial strains and plasmids used in this study.

Strain/primer/plasmid	Relevant information	Source/reference
Bacterial strains		
DH5α	F^- ϕ80*lacZ* M15 *endArecAhsdR*($r_k^- m_k^-$) *supEthigyrArelA* Δ(*lacZYA-argF*)U169	Laboratory stock
K12 MG1655	F^- *ompThsdS*$_B$($r_B^- m_B^-$) *gal dcm*	Novagen
DSM01	K12 MG1655 Δ*ldhA*::FRT, Δ*adhE*::FRT, Δ*frdA*::FRT, Δ*pta*::FRT	[26]
HM501	DSM01 harboring pET23a::*ilvC, ilvD, yqhD*, pCDF duet-1::*alsS, kivD*	[13]
HS44	HM501 harboring pRSF duet-1	[27]
HS40	HM501 harboring pHS4	This study
HS41	HM501 harboring pHS5	This study
HS42	HM501 harboring pHS6	This study
HS43	HM501 harboring pHS7	This study
Plasmids		
pRSF duet-1	RSF ori, Km^R	Novagen
pHS4	pRSF duet-1:: *serA*	This study
pHS5	pRSF duet-1:: *purD*	This study
pHS6	pRSF duet-1::*gshB*	This study
pHS7	pRSF duet-1::glyA	This study

gas; its flow rate was maintained at 3.0 ml/min. The oven was held at 40°C for 5 min and then heated to 230°C at a rate of 12°C/min, followed by maintenance of the temperature at 230°C for 5 min. The culture samples were centrifuged at 3,521 ×*g* for 10 min, and the isobutanol that was dissolved in the supernatant was extracted using chloroform. The same volume of chloroform was added, and the mixture was vortexed for 5 sec, followed by separation by centrifugation at 21,055 ×*g* for 1 min. The lower chloroform fraction was used for isobutanol determination. The concentrations of furfural and furfuryl alcohol were also determined under these conditions. The residual glucose concentration was calculated by high-performance liquid chromatography (PerkinElmer, Korea); the chromatographic technique was performed using an HPX-87H organic acid column (Bio-Rad, USA) at 60°C with 0.008 N sulfuric acid solution at a flow rate of 0.6 ml/min.

Amino Acid Screening

Amino acid screening was conducted using 5 ml of culture medium containing 2% glucose, 0.3% yeast extract for cell growth, 15 mM furfural, and a 1 mM concentration of the 20 L-amino acids. Cultivation was conducted under microaerobic conditions, and the culture media were sampled after 72 h to measure cell growth. The cell growth was measured in terms of cell density using a 96-well microplate reader (TECAN, Switzerland).

Measurement and Calculation of Parameters

Growth inhibition was determined using 0–15 mM furfural. Cell growth was determined by measuring the optical density at a wavelength of 595 nm. After 96 h, the inhibition of cell growth, the inhibitory effect on isobutanol production, and the residual glucose were investigated in the M9 minimal medium containing 2% glucose and 0.3% yeast extract.

Results and Discussion

Ability of Glycine to Alleviate Growth Inhibition by Furfural

Because yeast extract can increase the tolerance of microorganisms against furfural and produces robust cell growth due to the unnecessary additional biosynthesis of building blocks [17], we examined the effect of amino acids to overcome the toxicity of furfural in the isobutanol-producing strain. Among the 20 L-amino acids, glycine, aspartate, serine, and tyrosine mainly increased cell growth in the presence of 15 mM furfural in the medium (Fig. 1A). Leucine, valine, glutamate, isoleucine, lysine, histidine, phenylalanine, threonine, and tryptophan increased the cellular growth of HM501. However, the effect was minor compared with glycine, aspartate, serine, and tyrosine. In the case of isobutanol production, serine and glycine turned out to be effective (Fig. 1B).

To monitor the effect of glycine, its addition to the minimal medium was tested for its alleviation effect against furfural in M9 minimal medium in comparison with serine and thymidine, which are known to cause growth restoration [22]. The effect of different concentrations of glycine in the minimal media was investigated for optimal concentration. For the growth test, 5 ml of isobutanol-producing *E. coli* HM501 preculture was cultivated in LB medium at 37°C

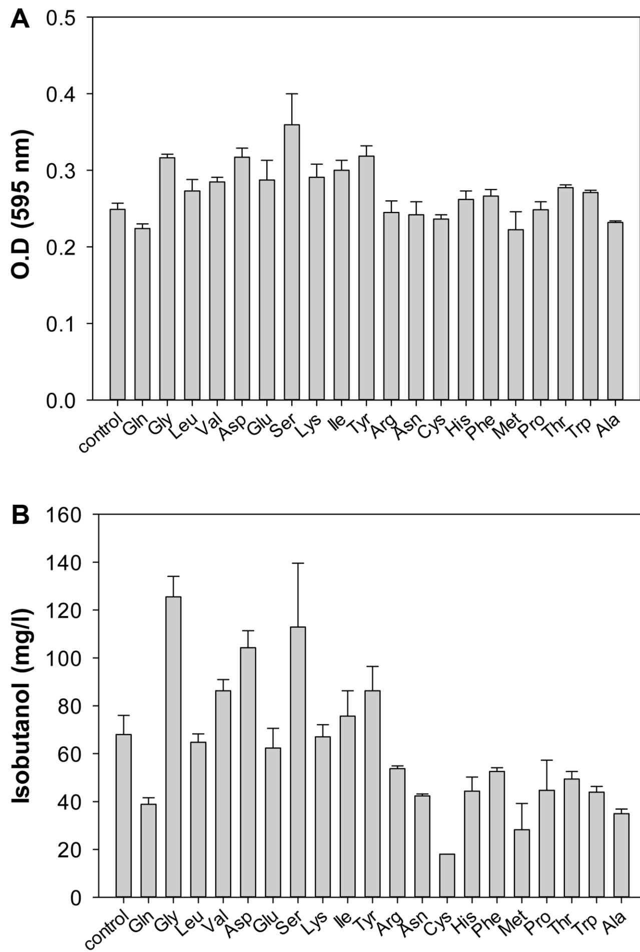

Fig. 1. Effects of L-amino acid addition on furfural tolerance and isobutanol production.

Possible anti-inhibitory molecules from 20 L-amino acids were screened. The growth restoration effect of additional L-amino acids was investigated. All the results were delivered after 72 h of culture cultivation. The error bars represent the standard deviation of two replicates. (**A**) Effect of L-amino acid supplementation on cell growth against furfural toxicity. (**B**) Effect of L-amino acid supplementation on the production of isobutanol against furfural toxicity.

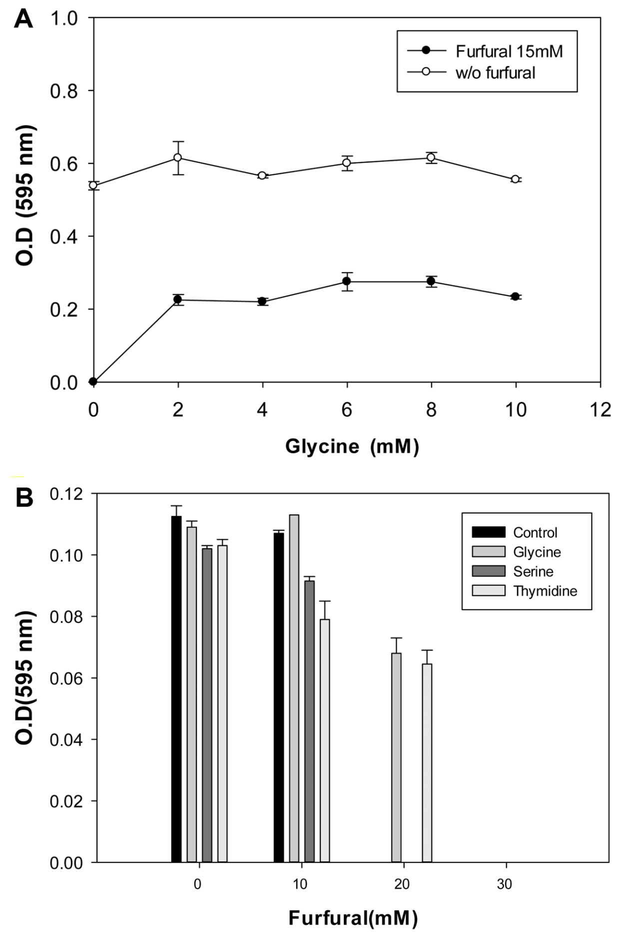

Fig. 2. Alleviation effect of medium supplements to furfural stress in M9 minimal medium.

The anti-inhibitory effect of glycine in the minimal medium was investigated. The optimal concentration of additional glycine was investigated for the cell growth restoration of *E. coli* HM501. In addition, the effects of other anti-inhibitory factors— serine and thymidine—were also investigated. The results were delivered after 48 h of culture cultivation. The error bars represent the standard deviation of two replicates. (**A**) Effect of glycine at different concentrations under furfural stress. (**B**) Effects of glycine, serine, and thymidine on the growth recovery under furfural stress.

overnight. The overnight culture was inoculated (1%) in M9 minimal medium with 0.4% glucose containing 15 mM furfural and different concentrations of glycine (from 0 to 10 mM) at 37°C for 48 h. The anti-inhibitory effect of glycine against furfural was maximized at 6 mM glycine in minimal medium (Fig. 2A). The addition of 6 mM glycine to the medium led to the tolerance of the cells to furfural. The positive effect of glycine, serine, and thymidine in the medium was also tested. For the cell growth test, 1% of overnight culture was inoculated into the M9 minimal medium mentioned above with 6 mM glycine, 0.2 mM thymidine, or 6 mM serine at 37°C for 48 h. At 20 mM furfural, cell growth was completely arrested in the minimal medium without additional supplements. The supplementation of thymidine also increased the furfural tolerance but the cell growth was completely inhibited at 30 mM furfural (Fig. 2B). However, additional serine in the medium was not effective when the furfural concentration was increased to 20 mM (Fig. 2B). Therefore, the anti-inhibitory effect of additional glycine in the M9 minimal medium was confirmed. Considering that glycine can be

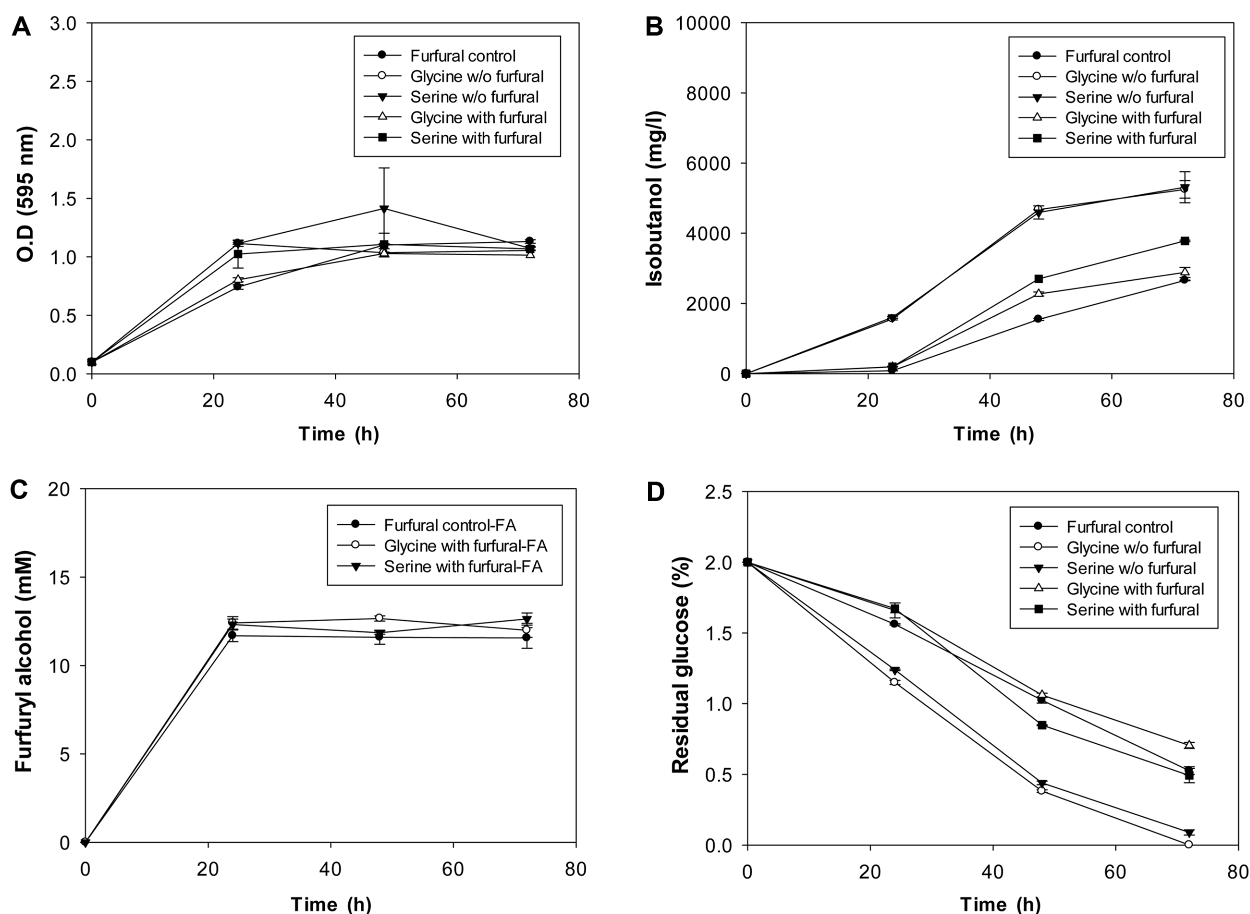

Fig. 3. Protective effect of glycine and serine with or without furfural during isobutanol production.

The effects of glycine and serine were monitored for 3 days for isobutanol production. The error bars represent the standard deviation of two replicates. (**A**) Effects of glycine and serine on cell growth with or without furfural. (**B**) Effects of glycine and serine on isobutanol production with or without furfural. (**C**) Effects of glycine and serine on furfural conversion into furfuryl alcohol. (**D**) Effects of glycine and serine on glucose consumption.

reversibly converted into serine, the functions of glycine and serine in the cell are expected to be similar [24].

Effects of Additional Glycine and Serine on the Production of Isobutanol

Cell growth, isobutanol production, furfural conversion, and residual glucose in the medium were monitored using production medium with additional 6 mM glycine and 6 mM serine and with or without 15 mM furfural. Cultures were grown in 30 ml of production medium for 72 h. The final cell concentration was not decreased by furfural in the medium, but the growth rate was inhibited. The growth rate was recovered with glycine and serine supplementation in the production medium (Fig. 3A). In the M9 minimal medium, glycine addition relieved furfural toxicity more effectively than serine addition. Contrary to minimal medium, in the production medium, which contained 0.3% yeast extract, the recoveries of cell growth and isobutanol production were better with serine supplementation (Figs. 3A and 3B). This is due to the yeast extract containing not only many amino acids but also other growth factors; the additive glycine effect seemed to have a threshold, and only a minimum amount of glycine was required to produce an alleviation effect.

During the fermentation of *E. coli* HM501 with glycine or serine, about 0.2 and 1.1 g/l more of isobutanol were produced, respectively, compared with control without addition of amino acid (Fig. 3B). When the conversion of furfural to furfuryl alcohol and glucose consumption were monitored, the addition of glycine did not show a significant difference (Figs. 3C and 3D). In contrast to glycine, serine seems to help glucose consumption in the production

Table 2. IC_{50} values with lignocellulosic-derived inhibitors with or without glycine.

Compound	Time (h)	IC_{50} (mM)	$IC_{50\ glycine}$ (mM)
Furfural	72	20 ± 1.2	25 ± 0.4
Acetic acid	72	33 ± 1.5	33 ± 2.2
Vanillin	72	5.2 ± 0.8	5.3 ± 1.2
4-Hydroxybenzaldehyde	72	7.7 ± 1.1	7.6 ± 1.5

medium (Fig. 3D). The additive effect of glycine seemed to be needed as the minimum amount, and it did not help to take up more glucose.

Investigation of Glycine for the Protective Effect against Lignocellulose-Derived Inhibitors and the Overexpression of Glycine Pathway Genes for Furfural Tolerance

The IC_{50} values for the other lignocellulose-derived inhibitors, vanillin, acetic acid, 4-hydroxy benzaldehyde, and furfural, were studied for further isobutanol production. The IC_{50} values for the inhibitors were measured in the isobutanol production medium. The concentrations were from 0 to 10 mM for vanillin and 4-hydroxybenzaldehyde, and the concentrations of acetic acid and furfural were from 0 to 30 mM. The IC_{50} of furfural without glycine was 20 mM. However, it was increased to 25 mM in the presence of glycine in the medium. However, the IC_{50} values of the other three lignocellulose-derived inhibitors did not differ; that is, vanillin (33 mM), 4-hydroxybenzaldehyde (5.2 mM), and acetic acid (33 mM) in the presence or absence of additional glycine in the medium as shown in Table 2, suggesting that glycine works on furfural only. Glycine can be generated via many other pathways and can be converted into various molecules, such as serine, IMP, and GSH (Fig. 4). Furthermore, glycine and serine were used for the one-carbon metabolism, generating NADPH and ATP. Thus, several genes were involved in the conversion of glycine to other compounds that have been investigated for the effectiveness against furfural. Furfural toxicity is related to DNA damage, osmotic stress, and NAD(P)H depletion. Thus, *purD* (phosphoribosylamine-glycine ligase), *serA* (D-3-phosphoglycerate dehydrogenase), *glyA* (serine hydroxymethyltranferase), and *gshB* (glutathione synthetase) from *E. coli* K-12 MG1655 were overexpressed in *E. coli* HM501 for the further evaluation of their effects on growth and isobutanol production. The preculture step was conducted as mentioned above, and the 1% overnight culture was then inoculated into 5 ml of production media containing 15 mM furfural with or without 6 mM glycine. After 72 h, among these genes, only *glyA* slightly alleviated cell growth under furfural stress, but the overexpression of the other genes had no effect on cell growth or decreased isobutanol production (Figs. 5A and 5B). This phenomenon is related to the overexpression caused only by the metabolic burden to cells without any advantages [21]. Glycine addition had a positive effect on all engineered strains and increased the isobutanol production. Although the volatile characteristic of furfural affected the effects, depending on the culture scale, glycine was again identified as an anti-inhibitory molecule for furfural, which is a major

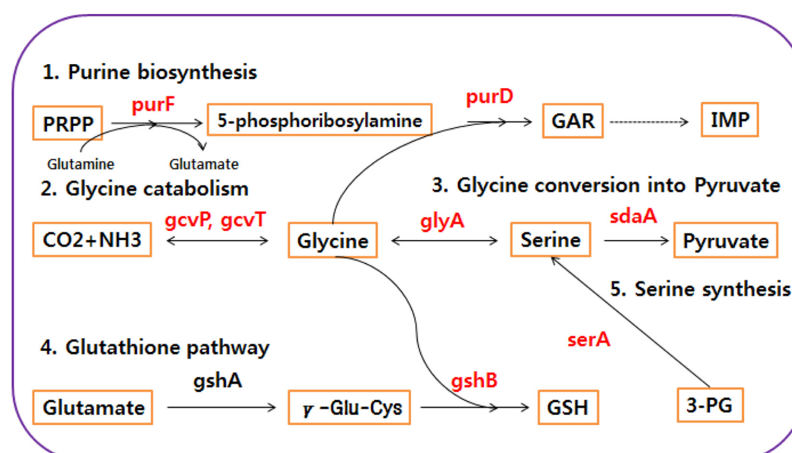

Fig. 4. Pathway map of glycine conversion into other compounds via several biosynthetic pathways in *E. coli.*

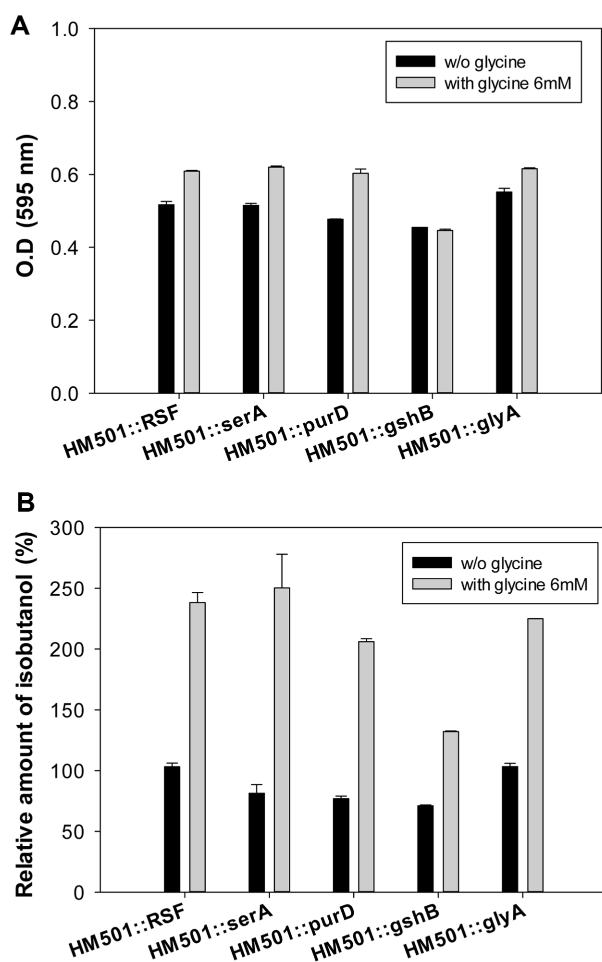

Fig. 5. Effects of glycine conversion pathway genes and serine synthesis genes on furfural toxicity.

Each gene was overexpressed to determine positive effects against furfural toxicity. The error bars represent the standard deviation of two replicates. (**A**) Overexpression effect of glycine-related pathway genes on cellular growth under furfural stress. (**B**) Overexpression effect of glycine-related pathway genes on isobutanol production under furfural stress.

inhibitor among lignocellulose-derived inhibitors. Still, the exact mechanism of such additions to the medium is unknown.

In summary, to overcome the toxicity of furfural, which is the major inhibitor molecule in lignocellulosic biomass, the L-amino acids were screened for a promising molecule to facilitate the better usage of the biomass. Among them, glycine and serine turned out to be effective. Furfural toxicity was better eliminated and produced more isobutanol in the production medium with glycine. Although the detailed mechanism of glycine was not revealed completely owing to its complex role, the versatile functions of glycine regarding the viability and productivity of serine, purine and pyrimidine synthesis, and NADPH generation [25] can help cells to be tolerant to furfural in various ways.

In conclusion, the presented results suggest that the additional medium component glycine would improve isobutanol production with lignocellulose containing furfural.

Acknowledgments

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