Increased Tolerance to Furfural by Introduction of Polyhydroxybutyrate Synthetic Genes to *Escherichia coli*

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Polyhydroxybutyrate (PHB), the most well-known polyhydroxyalkanoate, is a bio-based, biodegradable polymer that has the potential to replace petroleum-based plastics. Lignocellulose hydrolysate, a non-edible resource, is a promising substrate for the sustainable, fermentative production of PHB. However, its application is limited by the generation of inhibitors during the pretreatment processes. In this study, we investigated the feasibility of PHB production in *E. coli* in the presence of inhibitors found in lignocellulose hydrolysates. Our results show that the introduction of PHB synthetic genes (*bktB*, *phaB*, and *phaC* from *Ralstonia eutropha* H16) improved cell growth in the presence of the inhibitors such as furfural, 4-hydroxybenzaldehyde, and vanillin, suggesting that PHB synthetic genes confer resistance to these inhibitors. In addition, increased PHB production was observed in the presence of furfural as opposed to the absence of furfural, suggesting that this compound could be used to stimulate PHB production. Our findings indicate that PHB production using lignocellulose hydrolysates in recombinant *E. coli* could be an innovative strategy for cost-effective PHB production, and PHB could be a good target product from lignocellulose hydrolysates, especially glucose.

Keywords: Polyhydroxybutyrate, furfural, resistance, *Escherichia coli*, lignocellulose hydrolysate

Introduction

Lignocellulosic biomass is an abundant renewable resource for the production of biofuels, chemicals, and polymers [1–3]. Lignocellulose is mainly composed of three polymers, cellulose, hemicellulose, and lignin, together with other components, such as acetate, minerals, and phenolic substituents [4]. However, the main impediments in utilizing lignocellulose materials lie in the crystalline structure of cellulose sheathed by hemicellulose, degree of polymerization, biomass particle size, and recalcitrance of their bonding due to the protective covering of lignin [5]. To better utilize this biomass and efficiently extract carbohydrates, pretreatment processes are necessary [6–8]. Various pretreatment processes have been developed to purify sugar compounds, one of which is acid hydrolysis

[9, 10]. However, in pretreatment processes, several unwanted inhibitors (*e.g.*, furfural, 4-hydroxybenzaldehyde, vanillin, and acetate) are also produced [11, 12]. The quantity of these inhibitors depends on the type of biomass and the hydrolysis condition [13]. Among them, furfural is a major toxic byproduct from pretreated lignocellulose. Furfural is derived from pentose, and it damages DNA and inhibits glycolysis and sugar metabolism [13–15]. Furfural is converted to the less toxic furfuryl alcohol through reduction by aldehyde reductases [16]. To overcome the inhibitory effects of furfural, genes encoding NADH-dependent furfural reductase (FucO), NADPH-dependent oxidoreductase (YqhD), membrane-bound transhydrogenase interconverting NADH and NADPH (PntAB), and NAD salvage pathway (PncB, NadE) have been used to provide furfural resistance [17–20].

The polyhydroxyalkanoate (PHA) family of bio-based, biodegradable polymers is a promising next-generation product that can potentially substitute for petroleum-based plastics and is synthesized by many microorganisms as part of their natural metabolism [21, 22]. Previous studies on bacterial PHA accumulation have shown that it is largely affected by nutrient conditions, such as nitrogen and phosphate concentrations, and oxygen limitation [23–25]. Compared to petroleum-based plastics, PHA has many advantages including biodegradability and biocompatibility, while its thermal and mechanical properties are similar to those of petroleum-based plastics [26]. The most extensively studied member of the PHA family is poly(3-hydroxybutyrate)(PHB) [27]. The biosynthesis of PHB requires three reactions mediated by β -ketothiolase (BktB), acetoacetyl-CoA reductase (PhaB), and PHB polymerase (PhaC).

Bio-based resources are renewable and are expected to play a key role in the production of novel bio-based materials, contributing to a reduction in the negative environmental impact of petroleum-based products and thus addressing the bio-economy of the future [4]. The increasing worldwide need for bio-based plastic production will, therefore, be an important driver towards the use of renewable non-edible sources, such as lignocellulosic biomass [28]. To date, research has focused on the development of new bacterial strains and the discovery of cost-effective starting materials for PHB production [27, 29]. A number of bacteria that produce PHB from lignocellulose-derived monosaccharides have been identified [30–33].

However, their application is limited to using hydrolysates containing only small amounts of inhibitors or after eliminating toxic compounds [29, 31, 34]. Therefore, discovering an effective detoxification strategy remains important for efficient utilization of biomass hydrolysate for microbial growth and fermentation.

In this study, we investigated the feasibility of PHB production in *Escherichia coli* in the presence of inhibitors such as furfural. Interestingly, we found an association between furfural and PHB production in *E. coli*, in which PHB synthetic genes provided high resistance to furfural and the compound itself stimulated PHB production.

Materials and Methods

Bacterial Strains, Media, Reagents, and Culture Conditions

Strains and plasmids used in this study are listed in Table 1. *E. coli* DH5 α and KSYH(DE3) were used as host strains for gene cloning and PHB production, respectively [35–37]. For cell preparation and selection of transformants, the strains were 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 15 g of agar in 1 L of distilled water. For evaluation of furfural resistance and PHB production, transformants were cultured in M9 minimal medium containing 2% glucose and 0.1% yeast extract, which had an initial pH of 6.8. Appropriate antibiotics (100 μ g/ml spectinomycin and 25 μ g/ml chloramphenicol for *E. coli* transformants) were added when required, and 0.1 mM isopropyl- β -D-thiogalactoside (IPTG) was added at the beginning of culture. For preculture, a single colony from an LB agar plate was inoculated into 3 ml of LB broth. The

Table 1. Bacterial strains, plasmids, and primers used in this study.

Strain or plasmid	Description	Reference
<i>E. coli</i> strains		
DH5 α	General cloning strain	Invitrogen
KSYH(DE3)	BW25113 derivative containing DE3, <i>ΔaraBAD</i> , <i>ΔrhaBAD</i>	[35]
KSYH(DE3)/pCDF	KSYH(DE3) containing pCDFDuet-1	This study
KSYH(DE3):: <i>bktB</i>	KSYH(DE3) containing pCDF:: <i>bktB</i>	This study
KSYH(DE3):: <i>phaB</i>	KSYH(DE3) containing pCDF:: <i>phaB</i>	This study
KSYH(DE3):: <i>phaC</i>	KSYH(DE3) containing pCDF:: <i>phaC</i>	This study
YH090	KSYH(DE3) containing pLW487	[35]
Plasmids		
pCDFDuet-1	A compatible spectinomycin-selectable plasmid carrying T7/ <i>lac</i> promoter	Novagen
pCDF:: <i>bktB</i>	pCDFDuet-1 carrying <i>bktB</i> gene from <i>Ralstonia eutropha</i> H16	This study
pCDF:: <i>phaB</i>	pCDFDuet-1 carrying <i>phaB</i> gene from <i>Ralstonia eutropha</i> H16	This study
pCDF:: <i>phaC</i>	pCDFDuet-1 carrying <i>phaC</i> gene from <i>Ralstonia eutropha</i> H16	This study
pLW487	Spectinomycin-selectable pEP2-based plasmid carrying <i>bktB</i> , <i>phaB</i> and <i>phaC</i> genes from <i>Ralstonia eutropha</i> H16 under the control of <i>trc</i> promoter.	[48]

Table 2. Composition of lignocellulose hydrolysates from *Miscanthus*, barley straw, and pine tree used in this study.

Biomass		Components	Concentration (g/l)
<i>Miscanthus</i>	Monosugars	Glucose	102.19 ± 0.54
		Xylose	20.16 ± 0.25
		Galactose	1.42 ± 0.21
		Arabinose	2.97 ± 0.17
		Mannose	1.55 ± 0.21
	Byproducts	Formic acid	-
		Acetic acid	0.24 ± 0.01
		Levulinic acid	-
		5-Hydroxymethylfurfural	0.19 ± 0.01
		Furfural	0.48 ± 0.01
Barley straw	Monosugars	Glucose	135.80 ± 0.04
		Xylose	13.164 ± 0.67
		Galactose	-
		Arabinose	0.34 ± 0.01
		Mannose	0.70 ± 0.04
	Byproducts	Formic acid	0.17 ± 0.03
		Acetic acid	0.14 ± 0.02
		Levulinic acid	-
		5-Hydroxymethylfurfural	0.18 ± 0.01
		Furfural	0.09 ± 0.01
Pine tree	Monosugars	Glucose	127.44 ± 0.22
		Xylose	14.02 ± 0.33
		Galactose	-
		Arabinose	-
		Mannose	2.95 ± 0.23
	Byproducts	Formic acid	-
		Acetic acid	0.88 ± 0.02
		Levulinic acid	-
		5-Hydroxymethylfurfural	0.15 ± 0.01
		Furfural	0.17 ± 0.01

culture was incubated overnight in a shaking incubator at 37°C and 200 rpm. For flask culture, grown cells were inoculated into 100 ml of production medium in 250-ml screw-cap flasks at a dilution of 1:100 (v/v). The culture was continuously shaken in an incubator at 200 rpm and 30°C. Restriction enzymes and DNA polymerase were purchased from Enzymomics (Korea), plasmid extraction and gel purification kits from GeneAll (Korea), medium components from Bacto or Difco (USA), and furfural, vanillin, and 4-hydroxybenzaldehyde from Sigma-Aldrich (USA). Lignocellulose hydrolysates (*Miscanthus*, barley straw, and pine tree) were purchased from Sugaren (Korea) and their composition is described in Table 2 [8].

DNA Manipulation

Gene cloning was conducted according to standard protocols [38]. PHB synthetic genes (*bktB*, *phaB*, and *phaC*) were from

Ralstonia eutropha H16. In brief, each target gene was amplified by PCR, and the amplification product was purified and digested with restriction enzymes. The digested fragment was ligated into a plasmid, which was digested with the same restriction enzymes. Ligated plasmids were transformed into *E. coli* DH5α using the heat-shock method. Constructed plasmids were used for further study only after they were confirmed by sequencing. Detailed information is shown in Table 1.

Analytical Methods

PHB production was determined by gas chromatography and a slightly modified version of a previously described method [39, 40]. For analysis, culture samples were centrifuged at 10,000 ×g for 10 min, washed with deionized water twice, and suspended in 1 ml of water. The suspended samples were lyophilized, weighed, and placed in Teflon-stoppered glass vials. For methanolysis of

PHB samples, 1 ml of chloroform and 1 ml of a methanol/sulfuric acid (85:15, v/v) mixture were added to the vials, which were then incubated at 100°C for 2 h. Samples were cooled to room temperature and incubated on ice for approximately 10 min. After adding 1 ml of ice-cold water, samples were thoroughly vortex-mixed for 1 min and centrifuged at 2,000 ×g. The (lower) organic phase was collected using a pipette and transferred to a clean borosilicate glass tube containing anhydrous sodium sulfate (Na₂SO₄). Then, the samples were injected into a gas chromatograph (Young Lin Tech, Korea) equipped with a DB-Wax column (30 m × 0.32 mm × 0.5 μm) (Agilent Technologies, USA). The split ratio was 1:10. Helium was used as a carrier gas at a flow rate of 3.0 ml/min. Two microliters of the organic phase was injected using an autosampler. The inlet was maintained at 210°C. The column oven was held at 80°C for 5 min, heated to 220°C at 20°C/min, and then held at 220°C for 5 min. Peak detection was conducted using a flame ionization detector, which was maintained at 230°C. Furfural concentrations were determined by

gas chromatography as mentioned above, except that the column oven was held at 50°C for 5 min, heated to 230°C at 20°C/min, and then held at 230°C for 5 min.

Results

Effects of PHB on Furfural Resistance

It is well known that pretreatment of lignocellulose generates several potentially toxic compounds, such as organic acids and aldehydes (*e.g.*, acetic acid, levulinic acid, 4-hydroxybenzaldehyde, vanillin, and furfural) [33]. Furfural is considered the most potent inhibitor of *E. coli* growth [41] and has various cell-inhibitory effects [17, 18, 42]. It damages DNA, affects the hydrophobicity of intracellular membranes and depletes NAD(P)H in the cell [18]. In addition to general toxicity, because both PHB synthesis and furfural detoxification require NADH and

Fig. 1. Enhancement of polyhydroxybutyrate (PHB) production by furfural.

Cell growth and PHB production in *E. coli* YH090 (pLW487) were investigated in the presence of furfural. Furfural concentration ranged from 0 to 20 mM. (A) DCW (dry cell weight, g/l), (B) PHB content (w/w %), (C) Residual biomass (g/l), and (D) PHB concentration (g/l). Cells were grown for 72 h. Error bars represent the standard deviation of two replicates.

NADPH as cofactors, cofactor depletion affects cell growth and productivity in *E. coli* and *R. eutropha* H16 [17, 18, 30, 33, 42]. Therefore, when cell growth and PHB content were monitored in *E. coli* YH090 containing PHB synthetic genes in the presence of different concentrations of furfural, we expected to observe a decrease in cell growth. However, interestingly, in the presence of 15 mM furfural, *E. coli* YH090 cell growth and PHB content were respectively 1.2-fold and 1.3-fold higher than in the absence of furfural, and the highest PHB concentration of 0.85 g/l was noted in this condition (Figs. 1A, 1B, and 1D). When more than 20 mM furfural was used, introducing PHB synthetic genes had no effect on furfural resistance. The residual biomass, defined as dry cell weight excluding that of PHB, was constant at different concentrations of furfural (Fig. 1C). These results are consistent with previous reports that *E. coli* does not contain furfural oxidative degradation pathways and is unable to catabolize furfural as a carbon source [43]. Although it is well known that furfural inhibits sugar metabolism, such as glycolysis, carbon assimilation is accelerated along with PHB accumulation in the presence of furfural (Figs. 1B and 1D) [13].

To confirm that PHB production is increased in the presence of furfural, we monitored PHB production with or without 15 mM furfural in *E. coli* YH090 for 72 h (Fig. 2). During the initial 24 h of cultivation, cell growth and PHB production were lower in the presence of 15 mM furfural than in the absence of furfural. However, after 24 h of cultivation in the presence of 15 mM furfural, cell growth and PHB content sharply increased and, at 72 h, cell growth was 1.55-fold higher and the PHB concentration

was 4-fold higher than in the absence of furfural (Fig. 2). Therefore, addition of 15 mM furfural strongly stimulated cell growth and PHB production. The mechanisms underlying these effects, however, remain to be elucidated.

Investigation of Furfural Resistance Induced by Individual Genes and the Protective Effect against Lignocellulose-Derived Inhibitors

It is widely known that many reductases are effective for inducing furfural resistance [19, 20, 44]. Similarly, we expected that PhaB, an NADPH-dependent acetoacetyl-CoA reductase, would be effective in restoring cell growth and improving PHB production in the presence of furfural. Thus, to determine which gene was responsible for increased cell growth and PHB production in the presence of furfural, individual gene (*phaB*) of the PHB synthesis pathway was overexpressed separately in *E. coli* KSYH(DE3), and cell growth and the furfural conversion rate were evaluated for 24 h (Fig. 3). Contrary to our expectations, KSYH(DE3) strains containing individual gene (*phaB*) separately showed no clear difference in cell growth and furfural conversion rate (Fig. 3). In contrast, *E. coli* YH090 strain containing the three PHB synthetic genes showed notably improved cell growth and the highest furfural conversion rate during 24 h cultivation. During the initial 6 h of cultivation, this YH090 strain showed significantly lower cell growth and furfural conversion rate than the other strains; however, after 12 h cultivation, cell growth was completely restored, and the furfural conversion rate was the highest observed. These results were consistent with the findings that *E. coli* YH090

Fig. 2. Time-course profiles of polyhydroxybutyrate (PHB) production in *E. coli* YH090 in the presence or absence of furfural. (A) DCW (dry cell weight, g/l) and (B) PHB concentration (g/l). Error bars represent the standard deviation of two replicates.

Fig. 3. Effects of overexpression of PHB synthetic genes on cell growth and furfural consumption.

(A) Optical density (OD) at 600 nm and (B) Furfural concentration (mM). Cells were grown for 24 h. Error bars represent the standard deviation of two replicates.

showed a longer lag phase in cell growth and PHB production in the presence of furfural as discussed above (Fig. 2). Based on these findings, we conclude that furfural resistance depends on the overexpression of the three PHB synthetic genes, not any individual gene of the PHB synthesis pathway.

To test whether introducing PHB synthetic genes might affect resistance to other lignocellulose-derived inhibitors, such as vanillin and 4-hydroxybenzaldehyde, cell growth of *E. coli* KSYH(DE3) and YH090 was evaluated in medium

containing 4-hydroxybenzaldehyde (5 mM) or vanillin (10 mM) [42]. Similar to our findings for furfural, when the three PHB synthetic genes were introduced, cell growth was increased by 1.58-fold and 2-fold in the presence of 4-hydroxybenzaldehyde and vanillin, respectively (Fig. 4). We conclude that the introduction of PHB synthetic genes confers resistance not only to furfural, but also to vanillin and 4-hydroxybenzaldehyde.

Effects of Lignocellulose Hydrolysates on PHB Production

To evaluate the effects of lignocellulose hydrolysates on PHB production, *E. coli* YH090 was grown in M9 minimal medium containing lignocellulose hydrolysates. The composition of hydrolysates from *Miscanthus*, barley straw, and pine tree, which contain several inhibitors, are shown in Table 2. As the initial glucose concentration was adjusted to 2%, each lignocellulose hydrolysate was diluted appropriately. Because only small amounts of pentose sugars (xylose and arabinose) were not detected by HPLC, their concentrations were ignored. To evaluate the effects of lignocellulose hydrolysates, cell growth and PHB production in synthetic medium containing M9 minimal medium and 2% glucose as a sole carbon source, without inhibitors, were also investigated. PHB production was significantly increased with increasing fermentation time, especially in the presence of hydrolysates of *Miscanthus*, barley straw, or pine tree. Especially, in the presence of *Miscanthus* hydrolysate, the PHB concentration reached 0.67 g/l at 24 h, 1.06 g/l at 48 h, and 1.29 g/l at 72 h, corresponding to an approximately 2-fold increase compared

Fig. 4. Increased resistance to lignocellulose-derived inhibitors. Inhibitory effects of lignocellulose-derived inhibitors and protective effects of PHB synthesis were investigated. 4-Hydroxybenzaldehyde (4-Hb, 5 mM) and vanillin (10 mM) were used. DCW, dry cell weight, g/l. Cells were grown for 48 h.

Fig. 5. Polyhydroxybutyrate (PHB) production using lignocellulose hydrolysates.

Cell growth and PHB production in *E. coli* YH090 (pLW487) using lignocellulose hydrolysates were compared. Lignocellulose hydrolysates from *Miscanthus*, barley straw, and pine tree were used. (A) DCW (dry cell weight, g/L) and (B) PHB concentration (g/L). Cells were grown for 72 h. Error bars represent the standard deviation of two replicates.

to synthetic medium without any inhibitor. When lignocellulose hydrolysates containing well-known fermentation inhibitors (formic acid, 5-hydroxymethylfurfural, and furfural) were used, PHB production was increased compared to that in synthetic medium. Although it cannot be definitively concluded that the inhibitors increase PHB production, the results indicate that PHB production is possible in lignocellulose hydrolysates in the presence of inhibitors such as furfural, vanillin, and 4-hydroxybenzaldehyde.

Discussion

For efficient utilization of lignocellulose as a sugar source, a pretreatment process, such as acid hydrolysis, is required [6, 7]. However, in the pretreatment process, several fermentation inhibitors are formed in addition to monosaccharides [11, 12]. In particular, furfural is considered one of the major inhibitors of *E. coli* growth [41].

Here, we report the protective effect of PHB on growth inhibition by furfural and the stimulating effect of furfural on PHB production. Although the mechanisms underlying these effects still need to be investigated, we demonstrated the synergetic effects of furfural and PHB production and the feasibility of PHB production in the presence of lignocellulose hydrolysates. We showed that these effects were not related to a supplemental carbon source (Fig. 1C) or to only one of the enzymes in the PHB synthesis pathway (Fig. 3). In other words, the cell growth inhibition

effect of furfural was restored by overexpression of three PHB synthetic genes in *E. coli* KSYH(DE3). In particular, PHB production was increased in the presence of furfural compared to in its absence.

Multiple reports have described that PHA formation and mobilization enhance stress tolerance [45-47]. A previous study indicated that a complete PHB mobilization system (expression of PHB synthetic genes and PHB depolymerase) serves as an intracellular energy and carbon storage system in *E. coli*, and it increases tolerance to stress conditions, such as carbon starvation, heat shock, and osmotic pressure [46]. In addition, PHA mobilization influences chaperone protein levels [37, 45]. It is well known that PHB accumulation in recombinant *E. coli* can cause stress in cells and induces the expression of various protective proteins related to stress resistance (e.g., heat shock proteins, such as GroEL, GroES, and DnaK) [45]. Therefore, stress responses might be responsible for the increased cell growth and PHB production in the presence of furfural. In conclusion, PHB synthetic genes can improve tolerance to toxic inhibitors, such as furfural, vanillin, and 4-hydroxybenzaldehyde. In addition, although these are well-known fermentation inhibitors, they could be used to stimulate PHB production. PHB production by *E. coli* YH090 in the presence of lignocellulose hydrolysates was approximately 2-fold higher than that in the synthetic medium. Consequently, PHB production in recombinant *E. coli* using lignocellulose hydrolysates could be useful for cost-effective bio-based plastic production.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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