Sustainable Production of Dihydroxybenzene Glucosides Using Immobilized Amylosucrase from *Deinococcus geothermalis*^S

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Received: May 23, 2018

Revised: June 15, 2018

Accepted: July 4, 2018

First published online
July 3, 2018

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Supplementary data for this
paper are available on-line only at
<http://jmb.or.kr>.

pISSN 1017-7825, eISSN 1738-8872

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The amylosucrase encoding gene from *Deinococcus geothermalis* DSM 11300 (DgAS) was codon-optimized and expressed in *Escherichia coli*. The enzyme was employed for biosynthesis of three different dihydroxybenzene glucosides using sucrose as the source of glucose moiety. The reaction parameters, including temperature, pH, and donor (sucrose) and acceptor substrate concentrations, were optimized to increase the production yield. This study demonstrates the highest ever reported molar yield of hydroquinone glucosides 325.6 mM (88.6 g/l), resorcinol glucosides 130.2 mM (35.4 g/l) and catechol glucosides 284.4 mM (77.4 g/l) when 400 mM hydroquinone, 200 mM resorcinol and 300 mM catechol, respectively, were used as an acceptor substrate. Furthermore, the use of commercially available amyloglucosidase at the end of the transglycosylation reaction minimized the gluco-oligosaccharides, thereby enhancing the target productivity of mono-glucosides. Moreover, the immobilized DgAS on Amicogen LKZ118 beads led to a 278.4 mM (75.8 g/l), 108.8 mM (29.6 g/l) and 211.2 mM (57.5 g/l) final concentration of mono-glycosylated product of hydroquinone, catechol and resorcinol at 35 cycles, respectively, when the same substrate concentration was used as mentioned above. The percent yield of the total glycosides of hydroquinone and catechol varied from 85% to 90% during 35 cycles of reactions in an immobilized system, however, in case of resorcinol the yield was in between 65% to 70%. The immobilized DgAS enhanced the efficiency of the glycosylation reaction and is therefore considered effective for industrial application.

Keywords: Dihydroxybenzene glucosides, amylosucrase, amyloglucosidase, *Deinococcus geothermalis*, enzyme immobilization

Introduction

Amylosucrase (AS) is a glycosyltransferase (GT) structurally belonging to the glycoside hydrolase [1, 2]. Transglycosylation reactions of AS are usually conjugations of single and/or multiple (gluco-oligosaccharides) sugar(s) with a great advantage compared with Leloir GTs. For production of bioactive phenolic glycosides [2, 3], AS has been used in the synthesis of a variety of attractive biomaterials including amylose-like polymers, starch, dendritic nanoparticles, and microparticle encapsulation [4–6]. Although AS has potential application in glycodiversification, it is rarely reported for

transglycosylation of phenolic compounds.

The biologically active phenolic compounds usually contain single or multiple functional groups such as hydroxyl and methyl moieties bonded to aromatic hydrocarbons. These hydroxyl groups are prone to subsequent modifications, which alters their physiochemical functions compared with the parent compounds. Glycosides of hydroquinone (*para*-dihydroxybenzene), popularly known as skin-whitening agents and widely used in the cosmetic industry, inhibit melanin formation in the skin and lighten dark skin, freckles and age spots, as well as repair sun damage [7]. Resorcinol and catechol are derivatives of *meta*- and *ortho*-

dihydroxybenzenes, respectively. They also exhibit potent biological activities and therefore have pharmaceutical as well as cosmeceutical applications. Resorcinol is used as a skin-whitening agent and as an ointment to relieve pain and itching [8–10], while catechol is used as an anti-cancer compound and as a cosmeceutical agent [4, 11]. Although these phenolic compounds are highly useful, their applications have been limited due to issues related to stability, poor solubility, and short life in a photosensitive environment. Bulky sugar attachment to these compounds has been shown to resolve these issues of water solubility, stability, and biological properties [12].

In this study, three dihydroxybenzenes (hydroquinone, catechol and resorcinol) were glycosylated using the amylosucrase enzyme prepared using a codon optimized gene of *Deinococcus geothermalis* DSM 11300 (DgAS). The oligosaccharide-conjugated phenolic glycosides were also generated during transglycosylation. The productions of these phenolic mono-glucosides were enhanced by optimization of various reaction parameters, enzyme immobilization and treatment with commercial amyloglucosidase from *Aspergillus niger*. Previous studies reported the synthesis of α -arbutin and its potential use in the cosmeceutical industry [13, 14].

Materials and Methods

Chemicals and Reagents

Hydroquinone, resorcinol, catechol, ascorbic acid, sucrose, and amyloglucosidase (from *Aspergillus niger*) were purchased from Sigma-Aldrich (USA). High performance liquid chromatography (HPLC) grade acetonitrile and water were purchased from Mallinckrodt Baker (USA). Restriction enzymes were purchased from Takara Bio (Japan) and Promega (USA). Amicogen LKZ118 beads were purchased from Amicogen (Korea).

Bacterial Strains, Cloning and Culture Conditions

Escherichia coli XL1 blue was used as a host for all DNA manipulation while *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) was used for the expression and production of DgAS enzyme using pHCE IIB (NdeI) vector. Recombinant strains were grown in Luria-Bertani (LB) agar plate and broth medium supplemented with ampicillin (100 μ g/ml) at 37°C. The nucleotide sequence of amylosucrase (GenBank Accession No. ABF44874.1) sourced from *Deinococcus geothermalis* DSM 11300 was codon-optimized and synthesized by General Biosystems (USA). The 1,984-bp gene fragment was flanked by NdeI and HindIII restriction sites.

Enzyme Expression and Purification

Recombinant *E. coli* BL21 (DE3) harboring DgAS was cultured

in 50 ml LB broth at 37°C for 24 h. The cells were harvested by centrifugation at 840 $\times$ g (for 10 min at 4°C and washed using a buffer containing 50 mM Tris-HCl and 10% glycerol (pH 7.5). These cells were re-suspended in the same buffer and disrupted with a sonicator in an ice bath. The supernatant and cell debris were separated by centrifugation at 13,475 $\times$ g for 15 min at 4°C. Expressed protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% (w/v) acrylamide gel. The supernatant was purified using nickel nitrilotriacetic acid (Ni-NTA) affinity column chromatography (Qiagen Inc., USA) and eluted at various concentrations (10, 100, 200, and 500 mM) of imidazole, respectively, as previously reported [15, 16].

Enzymatic Reaction Conditions for DgAS and Amyloglucosidase

The transglycosylation reaction was performed using dihydroxybenzenes (hydroquinone, resorcinol and catechol) (Sigma-Aldrich, USA) as acceptor molecules in the presence of sucrose as glucose donor catalyzed by amylosucrase (DgAS). The 200 μ l of the total reaction mixture contained 20 mM dihydroxybenzene, 2 mM ascorbic acid, 200 mM sucrose, 0.4 mg/ml amylosucrase enzyme and 200 mM Tris-HCl buffer at pH 7. The reactions were incubated at 37°C for 30 min and stopped by keeping the reaction mixture in a boiling water bath for 10 min. The reaction mixtures were treated with 13 U/ml of commercial amyloglucosidase (Sigma-Aldrich, USA) at 40°C for 3 h. The samples were treated with chilled methanol to stop further reaction and centrifuged at 13,475 $\times$ g for 15 min to separate them from protein precipitates. The reaction mixtures were analyzed by high performance liquid chromatography-photo diode array (HPLC-PDA) and confirmed by electrospray ionization mass spectrometry (ESI/MS) and nuclear magnetic resonance (NMR) analyses. The production yield was enhanced by the addition of 10% of various antioxidants (ascorbic acid) to the reaction mixtures.

Analytical Methods

Reverse-phase HPLC-PDA analyses was performed at 280 nm UV absorbance using a C₁₈ column packed with YMC-Pack ODS-AQ; 4.6 mm internal diameter, 150 mm long and 5 μ m particle size long, connected to photo diode array (PDA) on binary condition consisting of solvent A (0.05% trifluoroacetic acid buffer-TFA), water, and 100% acetonitrile maintained at a flow rate of 1 ml/min for a 25-min program. The acetonitrile was set throughout as 0–40% (0–15 min), 40–75% (15–20 min), 75–0% (20–25 min). The purification of compounds was carried out by preparative HPLC with a C₁₈ column (YMC-PACK ODS-AQ; 150–250 mm long, 20 mm internal diameter, 10 μ m particle size) connected to a UV detector using a 36-min binary program. The flow was 10 ml/min throughout the program as acetonitrile flowed for 0–20 min (40%), 20–30 min (75%), 30–36 min (0%).

The high resolution quadrupole-time of flight electrospray ionization-mass spectrometry (HRQTOF-ESI/MS) was performed in positive-ion mode using Acquity mass spectrometer (with

UPLC; Waters, USA) coupled with a Synapt G2-S system (Waters). The purified compounds were structurally determined by Avance II 300 Bruker (Germany) BioSpin NMR spectrometer equipped with a TCI CryoProbe (5 mm). All the samples were exchanged with D₂O and dissolved in methanol-*d*₄ for ¹H NMR (proton), ¹³C NMR (carbon).

Bioconversion of Phenolic Compounds and Purification of Reaction Products

An identical DgAS transglycosylation was carried out in the presence of three dihydroxybenzenes according to the optimized parameters listed in the supplementary section. To enhance the production yield and substrate stability, the selected antioxidant (10% ascorbic acid) was added to the reaction mixture followed by 13 U/ml of glucosidase enzyme resulting in the highest yield. The reaction was maintained at optimized donor and acceptor substrate concentrations, temperature, and pH for 30 min. The large preparative scale reaction was carried out and purified using preparative HPLC in binary condition as mentioned in the analytical section.

Optimization of Transglycosylation Reaction Conditions

The transglycosylation reaction was optimized by studying the required concentration of donor and acceptor substrates in different ratio, temperature and pH. The identical reactions were carried out in the presence of reaction components as mentioned above varying the ratio of phenolic compounds and sucrose from 2.5:1, 5:1, 10:1, 15:1, and 20:1 followed by incubation at 37°C for 30 min. For temperature optimization, reaction mixtures were incubated in ranges from 35°C to 60°C while to compare reactivity according to pH, the reaction mixtures were made in varying buffer conditions of pH 5 to pH 9. Each time, the reaction mixtures were analyzed by HPLC-PDA to determine the optimized parameters based percentage production yields.

Immobilization of Amylosucrase

The enzyme immobilization experiment was performed using AMICOGEN LKZ118 beads. The beads were washed three times with distilled water followed by dehydration for 40 sec using a vacuum pump. Around 5 g of beads in an Erlenmeyer flask was mixed in 10.2 ml of distilled water followed by addition of 1.1 M Na₂SO₄. This solution was dissolved and incubated at 25°C at 100 rpm. Around 70 mg of enzyme per gram of beads were mixed and immobilized at 25°C at 100 rpm for 24 h. After immobilization, it was washed using distilled water three times and dehydrated again for 40 sec through a vacuum pump before being stored at 4°C.

The immobilized enzyme reactions were carried out at 40°C for 30 min. The reaction mixture was vigorously shaken (200 rpm) throughout the reaction incubation time. The reactions were carried out in 5 ml volume containing 200 mM Tris-HCl (pH 7.0), 2 M sucrose, 400 mM hydroquinone, 40 mM ascorbic acid and 300 mg of DgAS-immobilized beads. Identical reactions were carried out with resorcinol and catechol, but the concentration of

sucrose, acceptor substrate, and ascorbic acid was different. For resorcinol, 1 M sucrose, 200 mM resorcinol, and 20 mM ascorbic acid were used which was 1.5 M sucrose, 300 mM catechol, and 20 mM ascorbic acid in the reaction mixture of catechol. The immobilization rate was calculated from the initial and final concentrations of immobilized enzymes. These enzymes were washed and reacted repeatedly 50 times, which also suggests the efficiency of the technique and phenolic glycoside synthesis in this experiment.

Results

DgAS Expression and Purification

The codon-optimized and synthesized DgAS gene (supplementary S1) was cloned into pHCE IIB (NdeI) with 6× histidine tagged at its carboxyl terminus. The cloned gene of 1.984 Kb was confirmed by restriction digestion with NdeI/HindIII enzymes. The gene was expressed in *E. coli* BL21(DE3). The expressed soluble fraction of the protein was purified as described in methods and analyzed by SDS-PAGE analysis which showed protein of 72.49 kDa resembling the 6x-his tagged fused DgAS (Fig. S1).

Transglycosylation of Phenolic Compounds

Amylosucrase (DgAS) enzyme was used for enzymatic catalysis in the presence of sucrose as a sugar donor. Hydroquinone, resorcinol and catechol were used as substrates. The aim of this work was to synthesize a mono-glucoside derivative of each substrate using an inexpensive glucose donor source catalyzed by the glucosyltransferase activity of DgAS. Characteristically, this enzyme hydrolyzes sucrose and transfers glucose moiety to the hydroxyl group of phenolic substrates during glycosylation [13, 17].

The HPLC-PDA analysis of the reaction mixture of all three dihydroxybenzenes showed glycosylation of each substrate to generate multiple products. The standard hydroquinone that appeared at a retention time (*t_R*) of 8.2 min was converted to mono and multiple products appeared below *t_R* of 6.6 min (Figs. 1A and 1B). Similar results were observed with resorcinol *t_R* = 10.4 min (Figs. 1C and 1D) and catechol *t_R* 11 min as a mono-glucoside (Figs. 1E and 1F). Multi-glucoside products of resorcinol and catechol appeared at *t_R* = 7.2 to *t_R* = 8.4 min and *t_R* = 8.2 to *t_R* = 10.8 min, respectively. Minor products conjugated with gluco-oligosaccharides (glucotriose, glucotetraose, glucopentaose, etc) attached to hydroquinone, resorcinol and catechol were detected along with mono-glucosides of each substrate (Fig. 1). The conjugation of multiple sugar units into aglycons was confirmed by high resolution HRQTOF-ESI/MS (Fig. S2).

Fig. 1. HPLC-PDA chromatogram of transglycosylation reactions of hydroquinone, resorcinol and catechol compared with standards.

(A) Hydroquinone standard (B) Hydroquinone reaction (C) Resorcinol standard (D) Resorcinol reaction (E) Catechol standard (F) Catechol reaction.

Optimization of Sucrose Concentration, Temperature and pH

Different concentrations of sucrose ranging from 50 to 400 mM were added in the reaction mixtures of hydroquinone, resorcinol and catechol, keeping 20 mM of each substrate and other components constant. Enhanced production occurred with optimization of the maximum donor (sucrose) substrate concentration yielded the highest target products. By using 20 mM hydroquinone as the substrate for transglycosylation, the hydroquinone mono-glucoside (arbutin) increased with the rise in sucrose concentration up to 300 mM, and the production was constant upon further increase of the sucrose to 400 mM (Fig. 2A). The conversion rate of hydroquinone was 95.1% when 300–400 mM of sucrose was used. Similarly, in the case of resorcinol, the synthesis of resorcinol mono-glucoside and oligosaccharide-conjugated resorcinol (multi-glucosides) occurred. The synthesis of mono-glucoside increased continuously up to 200 mM of sucrose concentration and it remained stable up to 400 mM whereas the production of oligosaccharide-conjugated resorcinol derivatives continued to increase until the donor substrate concentration increased to 400 mM (Fig. 2B). When catechol was used as the substrate, the formation of catechol mono-glucoside (mono) increased up to 100 mM of sucrose concentration followed by eventual decline in the conversion percentage (Fig. 2C). Concurrently, the oligosaccharide-conjugated catechol gradually increased up to a sucrose concentration of 400 mM (Fig. 2C). These results suggested that minimization of the synthesis of the oligosaccharide-conjugated products of resorcinol and catechol by sugar hydrolyzing enzymes increased the production of mono-glucosides.

The reactions were carried out using 200 mM Tris-HCl buffer (pH 7.0), 2 mM ascorbic acid, 100 mM sucrose, and 20 mM of phenolic compounds at varying incubation temperatures ranging from 35°C to 60°C. The optimal temperature for transglycosylation reaction was found to be 40°C for hydroquinone (Fig. 2D). The highest conversion level was observed at 35°C with resorcinol and catechol. However, for pH optimization, different ranges of pH buffer were used along with other reaction components. The reactions were incubated at 40°C. Reactions with hydroquinone (98% conversion) and resorcinol (43.6% conversion) yielded the highest products at pH ~ 7 while the yield was the highest at pH 8.0 in case of catechol (60.6% conversion) (Figs. 2G–2I).

Improved Transglycosylation by Amyloglucosidase

Amyloglucosidase hydrolyzes the glucosidic bonds from

Fig. 2. Optimization of transglycosylation parameters in the reaction mixtures.

Sucrose concentration optimization in: (A) Hydroquinone reaction, (B) Resorcinol reaction and (C) Catechol reaction. Incubation temperature optimization in: (D) Hydroquinone reaction, (E) Resorcinol reaction and (F) Catechol reaction. pH optimization for (G) Hydroquinone reaction, (H) Resorcinol reaction and (I) Catechol reaction. Each reaction was performed multiple times as shown by error bars.

oligosaccharides, with extensive industrial applications for the commercial production of D-glucose from starch [18]. The same amyloglucosidase was applied for the treatment of DgAS-catalyzed reaction products to enhance the production of phenolic mono-glucosides. The enzyme from *A. niger* was used after the transglycosylation reaction was terminated. Approximately 13 U/ml amyloglucosidase (optimized concentration data not shown) was used to treat each reaction mixture of hydroquinone, resorcinol and catechol. The effect of glucosidase activity was directly observed by HPLC-PDA chromatographic profile during the product formation from the reaction mixtures (Figs. S3C–S3F). The treatment of amyloglucosidase decreased the oligosaccharide-conjugated products while increasing the

single sugar-conjugated products. However, this phenomenon was not observed in the hydroquinone reaction mixture. Most of the products were mono-glucosides (Figs. S3A and S3B). This data supported the possibility of converting multi-sugar-conjugated products to a mono-glucose-conjugated product, which results in target product enhancement.

Optimization of Dihydroxybenzene Concentrations

Finally, the production of each hydroquinone glucoside, resorcinol glucoside, and catechol glucoside, was observed under optimized conditions of incubation temperature, pH and sucrose concentration. In addition, the data were obtained after treatment with 13 U/ml of amyloglucosidase.

Fig. 3. Substrate concentration optimization resulted in the highest yield of hydroquinone, resorcinol and catechol glucosides.

Substrates increased in concentration from 20 mM to 500 mM and the reaction mixtures were analyzed by HPLC-PDA. (A) Hydroquinone concentration optimization. (B) Resorcinol concentration optimization and (C) Catechol concentration optimization.

Total reaction volume was carried out in 500 μ l for the transglycosylation reaction of hydroquinone. The substrate

concentrations were increased from 20 to 500 mM while maintaining other reaction components constant. We have achieved an efficient conversion of substrates into products while optimizing the sucrose concentration from 50 to 100 mM (Figs. 2A–2C). The reactions were analyzed by HPLC-PDA at different time intervals, *i.e.*, 0.5, 1, 3, 6, and 12 h, respectively. The results showed that 81% of 400 mM hydroquinone was converted to glucosides in 30 min of incubation time. More than 65% of 200 mM resorcinol was converted to glucoside in 30 min (Figs. 3A and 3B). Similarly, at the same incubation period 94% of 300 mM catechol was converted to products (Fig. 3C). Approximately 325.6 mM (88.6 g/l) of hydroquinone glucosides, 130.2 mM (35.4 g/l) of resorcinol glucosides and 284.4 mM (77.4 g/l) of catechol glucosides were obtained within 60 min of reaction. This is the highest yield of hydroquinone glucoside (α -arbutin), resorcinol glucoside, and catechol glucoside ever reported in such a short reaction time.

Structural Confirmation

Each reaction product was purified by prep-HPLC and subjected to NMR analyses to determine whether it was a mono-glucoside or a multiple sugar-conjugated product. The HPLC-PDA chromatogram showed distinct single peaks after purification of compounds (Fig. S4). The ^1H NMR signals of each glucoside product were compared with the substrates. Hydroquinone glucoside shows the anomeric signals at 5.28 ppm with a J value of 3.7 Hz including the presence of other sugar protons at 3.2 to 4 ppm and confirms the product as single sugar attached at alpha configuration (Fig. 4). Resorcinol glucoside and catechol glucoside also show the anomeric signals at 5.43 ppm with a J value of 3.7 Hz and at 5.28 ppm with a J value of 3.5 Hz as alpha configuration.

Immobilization of AS

The enzyme immobilization experiment was performed using AMICOGEN LKZ118 beads. The immobilized enzyme was washed and reacted repeatedly 50 times for transglycosylation. In each cycle the conversion of supplied dihydroxybenzene conversion to glucosides was measured. In the case of hydroquinone, the reactivity remained constant up to 35 times on average, and the yield was maintained between 85% and 90%. However, the reactivity decreased sharply after the 35th round. On the other hand, resorcinol and catechol exhibited close to 40-fold reactivity. The percent yield of resorcinol varied between 65% and 70% and that of catechol ranged between 85% and 90% (Fig. 5). The immobilized DgAS led to a 95, 36.9, and

Fig. 4. ^1H NMR signal comparison of each substrate and glucoside

72.2 g/l final concentration of total glycosylated product of hydroquinone, catechol and resorcinol in a single cycle, respectively. Further, the efficiency of the experiments was

increased using enzyme immobilization with Amicogen LKZ118 beads, suggesting that the same immobilized enzyme can be used to synthesize phenolic glucosides inexpensively and efficiently (Fig. 5).

Fig. 5. Efficient use of immobilized enzymes repeatedly to generate phenolic glucosides up to 50 cycles.

Discussion

Even though phenolic compounds are highly valuable, their applications in industry have been restricted due to their short life in a photosensitive environment and poor water solubility [19]. Generally, the toxicity of glycosylated product was found to be drastically lowered when compared to their aglycones [20]. Thus, transglycosylation reactions are widely employed to modify phenolic compounds such as hydroquinone, resveratrol, genistein and catechin to produce their respective glucosides [21–24]. There are several reports of transglycosylation reactions and comparative study of their biological activities with aglycones [25–28]. However, in some instances, the trace amount of substrate remained in the sample could be harmful. Thus, approaches to remove such selective toxic compounds from the reaction

mixtures could be useful to purify the glucosylated derivatives [29, 30].

In this study, we generated glucosylated derivatives of three dihydroxybenzenes (hydroquinone, resorcinol and catechol) using a sustainable approach of employing immobilized amylosucrase (DgAS) and they are expected to exhibit better industrial applicability than parent compounds. In the transglycosylation reactions of biomolecules using sucrose as the sugar donor, DgAS is the most thermostable AS among various microbial sources [17]. However, all enzymes are not equally expressed in soluble fraction when they are heterologously overexpressed in *E. coli* systems. Thus, to express the recombinant enzyme efficiently in soluble fraction in *E. coli*, the codon-optimized gene is generally used to overcome the issues of protein translation and processing in soluble fractions. We have codon-optimized the nucleotide sequence of DgAS from *Deinococcus geothermalis* DSM 11300 and cloned it into an expression vector for protein synthesis. The transglycosylation reaction was performed using three dihydroxybenzenes. Many biocatalysts used for production of α -arbutin have been reported and discussed recently in the presence of various sugar donors such as sucrose, maltopentose, and maltose, using the AS derived from *Cellulomonas carbaniz* T26 [31]. Indeed α -arbutin has widespread applications including industrial use, as an effective skin-lightening cosmeceutical agent. However, resorcinol and catechol were not used in such catalysis and synthesis of glycoside derivatives, which explains the comparatively limited attention focused on their biological significance.

All the three derivatives of dihydroxybenzenes (hydroquinone, resorcinol and catechol) were used as substrates for transglycosylation. We targeted the synthesis of mono-glucosides of each phenolic compound using sucrose as a sugar donor and AS as a biocatalyst. Based on the preliminary transglycosylation reaction, the substrate was efficiently converted to glucosides in all three reactions. Mass analysis revealed multiple sugar (gluco-oligosaccharides)-conjugated products. Hydroquinone was found to be conjugated with up to three sugars while resorcinol and catechol were linked to nearly nine sugar chains (Fig. 2). However, the target was to obtain single sugar-conjugated products of all the three phenolic compounds. Amyloglucosidase is an enzyme commercially used to obtain glucose from starch. Since this enzyme catalyzes the breakage of the α -(1,4) glycoside bond between the two sugars, the transglycosylation reaction mixtures were subsequently treated with commercially available amyloglucosidase to obtain mono-glucosides. In this experiment, the production of sugar

chain-attached glycosides was reduced while the levels of mono-glucosides increased during the HPLC-PDA chromatogram analyses (Fig. S3).

We optimized the reaction parameters to enhance productivity. Firstly, the sucrose concentration was optimized where the glucoside production increased until 400 mM of sucrose concentration in each reaction. As shown in Fig. 2, the optimal sucrose concentration of hydroquinone, resorcinol, catechol was 300, 200, and 100 mM (mono-glucoside), respectively.

Temperature and pH were also optimized to 40°C and at pH 7, respectively, for reaction products resulting in a higher yield. The concentration of dihydroxybenzenes was increased under the optimum conditions of sucrose concentration, temperature, and pH. More than 95% conversion was obtained from the hydroquinone reaction mixture while >70% of resorcinol was converted to its glycosides. However, the catechol glycosides biosynthesis was elevated up to 100 mM sucrose concentration with above 90% yield. This conversion percentage is substantially high for production of glycosylated product. After optimization of each reaction parameter in the transglycosylation reaction, the substrate concentration was increased and the products were analyzed at different incubation times. In each reaction mixture, the substrates were increased from 20 to 500 mM while that of sucrose increased 5-fold resulting in the highest production of hydroquinone glucoside (α -arbutin), resorcinol glucoside and catechol glucoside ever reported in a such short incubation time. The products were finally purified and analyzed using NMR to confirm their identity as mono-glucosides of phenolic compounds.

The stability of enzymes often hindered their practical application in biotechnological processes. An approach to stabilize an enzyme's activity is to prevent enzyme aggregation and reduce irreversible unfolding by immobilization onto a suitable carrier [32, 33]. Immobilization of enzymes is usually performed to use them as an industrial biocatalyst. Epoxy supports are proven to be very easy to use in immobilizing enzymes [34–38]. In this article, we report a high-performing epoxy support (Amicogen LKZ118) for the immobilization of DgAS.

The transglycosylation reaction using immobilized enzyme revealed that the reactivity remained constant up to 35 cycles with hydroquinone. But, the conversion of resorcinol and catechol remained constant up to 40 cycles. Hydroquinone's earlier (5 cycles earlier than resorcinol and catechol) conversion in immobilized reaction system could be attributed to DgAS's different catalysis towards

hydroquinone. Moreover, the hydroquinone reaction was performed with a higher dose of donor (2 M) and acceptor (400 mM) concentrations than used with resorcinol and catechol. This could be another reason for the early decrease in conversion percentage of hydroquinone. The immobilized DgAS led to a 278.4 mM (75.8 g/l), 108.8 mM (29.6 g/l), and 211.2 mM (57.5 g/l) final concentration of mono-glycosylated hydroquinone, catechol and resorcinol at 35 cycles, respectively. This result revealed that DgAS is a stable enzyme and can be employed for conversion of large quantities of dihydroxybenzenes to glucosides by immobilizing the enzyme without decrease in any significant activity for glucoside production. This approach provides a cost-effective and easy to process immobilization (use of epoxy activated) of DgAS to generate immobilized codon optimized enzyme biocatalysts, which have great potential for industrial production of glucosylated derivatives of dihydroxybenzenes.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) and a grant funded by the Korea government (MEST) (NRF-2017R1A2A2A05000939).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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