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Sustainable production of dihydroxybenzene glucosides using immobilized amyllosucrase from *Deinococcus geothermalis*

Hun Sang Lee¹, Tae-Su Kim¹, Prakash Parajuli¹, Ramesh Prasad Pandey¹,², and Jae Kyung Sohng¹,²*

¹Institute of Biomolecule Reconstruction (iBR), Department of Life Science and Biochemical Engineering, Sun Moon University, 70 SunMoon-ro 221, Tangjeong-myeon, Asan-si, Chungnam 31460, Korea.

²Department of Pharmaceutical Engineering and Biotechnology, Sun Moon University, 70 SunMoon-ro 221, Tangjeong-myeon, Asan-si, Chungnam 31460, Korea.

†These authors equally contributed.

*Corresponding author.

Prof. Jae Kyung Sohng

Department of BT-Convergent Pharmaceutical Engineering, SunMoon University, 70 SunMoon-ro 221, Tangjeong-myeon, Asan-si, Chungnam 31460, Korea

Tel: +82-41-530-2246

Fax: +82-41-530-8229

E-mail address: sohng@sunmoon.ac.kr (J. K. Sohng)

Running title: Production of dihydroxybenzene glucosides
Abstract

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, donor (sucrose) and acceptor substrates 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 glucooligosaccharides, 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% until 35 cycles of reactions in immobilized system, however, in case of resorcinol the yield was in between 65% to 70%. The immobilized DgAS amylosucrase enhanced the efficiency of glycosylation reaction and to be 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 reaction of AS are usually conjugation 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 (paradihydroxybenzene), 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-dihydroxybenzens, respectively. They also exhibit potent biological activities, thus applicable in pharmaceutical as well as cosmeceutical. 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 anticancer compound and as a cosmeceutical agent [4, 11]. Although these phenolic compounds are highly useful, their application has 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 dihydroxybenzens (hydroquinone, catechol and resorcinol) were glycosylated using the amylosucrase enzyme prepared using 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 it studies reported the synthesis of α-arbutin and s cosmeceutical property [13, 14].

**Materials and Methods**

**Chemicals and reagents**

Hydroquinone, resorcinol, catechol, ascorbic acid, sucrose, amyloglucosidase (from *Aspergillus niger*) were purchased from Sigma-Aldrich (St. Louis, MO, USA). High performance liquid chromatography (HPLC) grade acetonitrile and water purchased from Mallinckrodt Baker (Phillipsburg, USA). Restriction enzymes were purchased from Takara Bio (Shiga, Japan) and Promega (Madison, WI, USA). Amicogen LKZ118 beads were purchased from Amicogen (Gyonggi-Do, Republic of 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 μg/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 from General Biosystems (USA). The 1984-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×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 was separated by centrifugation at 13,475×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, Valencia, CA, USA and eluted at various concentrations (10 mM, 100 mM, 200 mM 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 µL 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 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 xg for 15 min to separate 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 C_{18} column packed with YMC-Pack ODS-AQ; 4.6 mm internal diameter, 150 mm long and 5 μm particle size along connected to photo diode array (PDA) on binary condition consists of solvent A (0.05% trifluoroacetic acid buffer-TFA) water and 100% acetonitrile maintained at a flow rate of 1 mL/min for 25 min program. The acetonitrile was set throughout as 0-40% (0-15 min), 40-75% (15-20 min), 75-0% (20-25min). The purification of compounds were carried out by preparative HPLC with a C_{18} column (YMC-PACK ODS-AQ; 150 250 mm long, 20 mm internal diameter, 10 μm particle size) connected to a UV detector using 36-min binary program. The flow was 10 mL/min throughout the program as acetonitrile flow for 0-20 min (40%), 20-30 min (75%), 30-36 min (0%).

The high resolution quadruple-time of flight electrospray ionization-mass spectrometry (HRQTOF-ESI/MS) was performed in positive ion mode using Acquity mass spectrometer
(with UPLC; Waters, Milford, MA USA) coupled with 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 D$_2$O and dissolved in methanol-d$_4$ for $^1$H NMR (proton), $^{13}$C NMR (carbon).

**Bioconversion of phenolic compounds and purification of reaction products**

An identical DgAS transglycosylation was carried out in the presence of three dihydroxybenzens 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 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, reactions 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 condition 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. Beads were washed three times with distilled water followed by dehydrated for 40 seconds using a vacuum pump. Around 5 g of beads in Erlenmeyer flask was mixed on 10.2 mL of distilled water followed by addition of 1.1 M Na$_2$SO$_4$ to dissolve and incubated at 25 °C at 100 rpm. Around 70 mg of enzyme per gram of beads were mixed and immobilized at 25 °C in 100 rpm for 24 h. After immobilization, it was washed using distilled water three times and dehydrated again for 40 second through vacuum pump and stored at 4 °C.

The immobilized enzyme reactions were carried at 40 °C for 30 min. The reaction mixture was vigorously shaked (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 was used which was 1.5 M sucrose, 300 mM catechol, and 20 mM ascorbic acid in the reaction mixture of catechol. Immobilization rate was calculated from the initial and final concentration of immobilized enzymes. The immobilized enzyme was washed and reacted repeatedly for 50 times which also suggests the efficiency of the technique and phenolic glycosides 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 6x 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 with 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. Aim of this work was to synthesize a mono-glucoside derivative of each substrate using an inexpensive glucose donor source catalyzed by glucosyltransferase activity of DgAS. Characteristically, this enzyme hydrolyzes sucrose and transfers glucose moiety to the hydroxyl group of phenolic substrates during the glycosylation [13, 17].

The HPLC-PDA analysis of the reaction mixture of all three dihydroxybenzenes showed glucosylation of each substrate to generate multiple products. The standard hydroquinone appeared at retention time ($t_R$) of 8.2 min was converted to mono and multiple products appeared below $t_R$ of 6.6 min (Fig. 1 A and 1B). Similar results were observed with resorcinol $t_R$ 10.4 min (Fig. 1 C and 1D) and catechol $t_R$ 11 min as a mono-glucoside (Fig. 1 E and 1F).

Multi glucoside product 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
Optimization of sucrose concentration, temperature and pH

Different concentration of sucrose ranging from 50 mM to 400 mM was added in the reaction mixture of hydroquinone, resorcinol and catechol keeping 20 mM of each substrate and other components constant. Enhanced production occurred via optimization of the maximum donor (sucrose) substrate concentration yielding the highest target products. 20 mM hydroquinone as the substrate for transglycosylation, the hydroquinone mono-glucoside (arbutin) was increased with the rise in sucrose concentration up to 300 mM, and the production was constant upon further increase of sucrose to 400 mM (Fig. 2A). 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) was produced. The synthesis of mono-glucoside was increased continuously until 200 mM of sucrose concentration and it was stable until 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 until 100 mM sucrose concentration followed by eventual decline in the conversion percentage (Fig. 2C). Concurrently, the oligosaccharide-conjugated catechol was gradually increased until sucrose concentration of 400 mM (Fig. 2C). These results suggested that minimization of the synthesis of oligosaccharide-conjugated products of resorcinol and catechol by the 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 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) (Fig. 2G, 2H and 2I).

**Improved transglycosylation by amyloglucosidase**

Amyloglucosidase hydrolyzes the glucosidic bonds from 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 (Fig. S3 C, 3D, 3E and 3F). 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 (Fig. S3 A and 3B). 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 dihydroxybenzens concentration**

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. Total reaction volume was carried out in 500 μL for transglycosylation reaction of hydroquinone. The substrate concentrations were increased from 20 mM 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 mM to 100 mM (Fig. 2A, 2B and 2C). The reactions were analyzed by HPLC-PDA at different time intervals, i.e., 0.5 h, 1 h, 3 h, 6 h 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 (Fig. 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 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 $^1$H NMR signals of each glucoside products were compared with the substrates. Hydroquinone glucoside shows the anomic signals at 5.28 ppm with a $J$ value 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 anomic signals at 5.43 ppm with a $J$ value 3.7 Hz and at 5.28 ppm
with a $J$ value 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 for 50 times for transglycosylation. In each cycle the conversion of supplied dihydroxybenzen 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 g/L, 36.9 g/L and 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 glycosides inexpensively and efficiently (Fig. 5).

**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, 22, 23, 24]. There are several reports of transglycosylation reactions and comparative study of their biological activities with
aglycones [25, 26, 27, 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) which are anticipated 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 genes 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 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
330 glucosides in all the three reactions. Mass analysis revealed multiple sugar (gluco-
331 oligosaccharides)-conjugated products. Hydroquinone was found to be conjugated with up to
333 three sugars while resorcinol and catechol were linked to nearly nine sugar chains (Fig. 2).
334 However, the target was to obtain single sugar-conjugated products of all the three phenolic
335 compounds. Amyloglucosidase is an enzyme commercially used to obtain glucose from
336 starch. Since this enzyme catalyzes the breakage of the α-(1,4) glycoside bond between the
337 two sugars, the transglycosylation reaction mixtures were subsequently treated with
338 commercially available amyloglucosidase to obtain mono-glucosides. In this experiment, the
339 production of sugar chain-attached glycosides was reduced while the levels of mono-
340 glucosides increased during the HPLC-PDA chromatogram analyses (Fig. S3).
341 We optimized the reaction parameters to enhance productivity. Firstly, the sucrose
342 concentration was optimized where the glucoside production increased until 400 mM of
343 sucrose concentration in each reaction. As shown in Fig. 2, the optimal sucrose concentration
344 of hydroquinone, resorcinol, catechol was 300 mM, 200 mM and 100 mM (mono-glucoside),
345 respectively.
346 Temperature and pH were also optimized to 40 °C and at pH 7, respectively, for reaction
347 products resulting in a higher yield. The concentration of dihydroxybenzens was increased
348 under the optimum conditions of sucrose concentration, temperature, and pH. More than 95%
349 conversion was obtained from the hydroquinone reaction mixture while >70% of resorcinol
350 was converted to its glycosides. However, the catechol glycosides biosynthesis were elevated
351 until 100 mM sucrose concentration with above 90% yield. This conversion percentage is
352 substantially high for production of glycosylated product. After optimization of each reaction
353 parameter in the transglycosylation reaction, the substrate concentration was increased and
354 the products were analyzed at different incubation times. In each reaction mixture, the
substrates were increased from 20 mM to 500 mM while that of sucrose increased 5-fold resulting in the maximum production of hydroquinone glucoside (α-arbutin), resorcinol glucoside and catechol glucoside ever reported in a 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 an enzyme often hindered practical application of enzyme in biotechnological processes. An approach to stabilize enzyme’s activity is to prevent enzyme aggregation and reducing irreversible unfolding by immobilization onto a suitable carrier [32, 33]. Immobilization of enzymes is usually performed to use enzymes as an industrial biocatalyst. Epoxy supports are proven to be very easy to immobilize enzymes [34, 35, 36, 37, 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 remain constant up to 40 cycles. The early (5 cycles earlier than resorcinol and catechol) decline of hydroquinone conversion in immobilized reaction system could be attributed to DgAS’s different catalysis towards hydroquinone. Moreover, the hydroquinone reaction was performed with high dose of donor (2 M) and acceptor (400 mM) concentration than resorcinol and catechol. This could be another reason to 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 of 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 quantity of dihydroxybenzens 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 has a great potential for industrial production of
glucosylated derivatives of dihydroxybenzenes.

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Conflict of interest
We declare that we have no conflicts of interest
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Figure legends

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

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 shown by errors bars.

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.

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

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