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Original article

Enhanced production of ginsenoside Rh2(S) from PPD-type major ginsenosides using BglSk cloned from *Saccharibacillus kuerlensis* together with two glycosidase in seriesMuhammad Zubair Siddiqi^{a,b,1}, Hipolito Amaral Ximenes^{a,c,1}, Bong-Kyu Song^c, Hye Yoon Park^d, Woong Hee Lee^e, Hyosang Han^f, Wan-Taek Im^{a,c,*}^a Department of Biotechnology, Hankyong National University, 327 Jungang-ro, Anseong-si, Gyeonggi-do 17579, Republic of Korea^b AceMzyme Co., Ltd, Room 403, Academic Industry Cooperation, 327 Jungang-ro, Anseong-si, Gyeonggi-do 17579, Republic of Korea^c Major in Integrated Chemical and Environmental Technology, Graduate School of Chemical Engineering, Hankyong National University, 327 Jungang-ro, Anseong-si, Gyeonggi-do 17579, Republic of Korea^d National Institute of Biological Resources (NIBR), Incheon, Seo-gu, Gyeongseo-dong, Hankyong-ro 42, Republic of Korea^e Institute of Biotechnology, Chungnam National University, 99 Daehak-ro, Eoeun-dong, Yuseong-gu, Daejeon, Republic of Korea^f Department of Health Administration, Joongbu University, Chungcheongnam-do, Geumsan-gun, Chubu-myeon, Daehak-ro, 201, Republic of Korea

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ABSTRACT

Background: Ginsenoside Rh2(S) is a promising compound for the prevention of various kinds of cancers, inflammation, and diabetes. However, due to its low concentration (<0.02%), researchers are still trying to find an efficient glycoside hydrolase for the scaled-up production of Rh2(S).

Method: Three glycoside hydrolases (BglBX10, Abf22-3, and BglSk) were cloned in *Escherichia coli* BL21 (DE3) and the expressed recombinant enzyme was used for the scaled-up production of Rh2(S) through the conversion of PPD-type (protopanaxadiol) major ginsenosides (Rb1, Rc, and Rd, except Rb2) extracted from Korean red ginseng. Specific and specialized bioconversion pathways were designed that evolved the initial bioconversion of PPD-mix → Rg3(S) → Rh2(S). The reaction was started with 50 mg/mL of PPD-mix, 20 mg/mL of BglBX10, Abf22-3, and BglSk in series, respectively. The process was completed in a 10 L jar fermenter with a 5 L working volume at 37 °C for 48 hrs.

Results: The designed bioconversion pathways show that Abf22-3 and BglBX10 were responsible for the conversion of Rb1, Rc and Rd → Rg3(S), and then Rg3(S) was completely transformed to Rh2(S) by BglSk. As a result, 15.1 g of ginsenoside Rh2(S) with 98.0 ± 0.2% purity was obtained after strict purification using the Prep-HPLC system with a 100 φ diameter column. Additionally, BglSk was also investigated for its production activity with seven different kinds of PPD-mix type ginsenosides.

Conclusion: Our pilot data demonstrate that BglSk is a suitable enzyme for the gram unit production of ginsenoside Rh2(S) at the industrial level.

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1. Introduction

Ginseng is used as a powerful tonic for health in Korea, China, and Japan (Attele et al., 1999). The major compounds of ginseng are commonly known as ginsenosides, which are biologically active, and thus based on aglycone structure. The major ginsenosides are classified as oleanane type, protopanaxadiol (PPD) and protopanaxatriol (PPT) types. The PPD type ginsenosides are Rb1, Rc, Rb2, Rb3, and Rd, while PPT type ginsenosides are Rg1 and Re; more than 90% of ginseng extract is composed of these PPD and PPT type major ginsenosides (Attele et al., 1999; Christensen,

2009). Previously, research studies showed that these major ginsenosides could be used for the treatment of various kinds of human diseases such as cancers, neuroprotective agents, inflammation, fatigue, memory enhancement, and general health benefits or energy (Nag et al., 2012; Zheng et al., 2017; Kimura et al., 2012; Shen, 2005). To date, the researchers have described high biological activity associated with the transformation of major ginsenosides into minor ginsenosides, as well as the chemical reactivity of minor ginsenosides not seen with major ginsenosides (Choi and Hong, 2011; Jia and Zhao, 2009; Siddiqi et al., 2014).

Ginseng extract contains $\leq 0.2\%$ minor ginsenosides [such as; Rg3, Rh1, Rg5, Rk1, Rh3, C-Mc, C-Mx, Rh2, F2, C-K, F1, and so on] which cannot satisfy clinical requirements (An et al., 2019; Cui et al., 2014), as well as industrial demand. Among minor ginsenosides, C-K, F1, Rg3, Rh2(S), and F2 are well studied for their anti-tumor, anti-diabetes, anti-osteoporosis, anti-inflammatory, anti-cancer, and anti-obesity effects (Song et al., 2017; Baatar et al., 2018; Choi and Hong, 2011; Jia and Zhao, 2009; Siddiqi et al., 2014). Additionally, the ginsenoside, Rh2(S), which is one of the most notable cancer-preventing components of red ginseng, has not yet been produced in scaled-up production as shown in Table S1 (Song et al., 2017; Baatar et al., 2018; Kim, 2017; Lv et al., 2016; Li et al., 2015; Wang et al., 2012; Oh et al., 1999; Chung et al., 2013; Kang et al., 2017; Zhang et al., 2011; Lo et al., 2017).

Therefore, based on the strong efficacy of Rh2(S), the production is very limited and it can only be produced at the laboratory level. Previously, Rh2(S) was obtained through acid or base hydrolysis, but these methods generated a lot of waste and by-products (Siddiqi et al., 2015; Cao et al., 2012). Thus, the use of recombinant enzymes for gram unit production of minor ginsenosides is recognized as one of the preferred methods, as opposed to heat-acid treatment, due to its low cost, high specificity, high purity (85–95% chromatography purity), and environmentally friendliness (Zheng et al., 2017). Previously, researchers used the recombinant enzyme or strain itself to produce Rh2 by the conversion of Rb1 or Rg3 ginsenosides (Rb1 \rightarrow Rd \rightarrow Rg3 \rightarrow Rh2) (Palaniyandi et al., 2016; Su et al., 2006; Hou et al., 2012; Kim et al., 2013). However, the conversion efficiency of these enzymes was very low, and not specific to gram unit production of the ginsenoside Rh2(S). Moreover, these methods have been reported in quite some time and were not investigated for scale-up production of the ginsenoside Rh2(S). Similarly, (Siddiqi et al., 2015) produced the gram unit of Rh2mix from PPD-mix (Rb1, Rc, and Rd) using the combined method of both heat-acid treatment and enzymatic transformation, but pure Rh2(S) was not produced and instead many byproducts such as Rh2(R), Rh3, and Rk2 were produced.

In this study, we designed experimental techniques for the enhanced production of Rh2(S) [(15.1 g with high purity $\geq 98\%$) using three recombinant enzymes (BglBX10, Abf22-3, and BglSk) in series. Furthermore, we aimed to show that the enhanced production of Rh2(S) using these three enzymes will fulfill the needs of both the pharmaceutical and cosmetic industries.

2. Materials and methods

2.1. Ginsenoside standards and other chemicals and reagents

Standard ginsenosides, including Rb1, Rg3(S), Rd, Rb2, Rg3(R), Rh2(S), C-K, Rh2(R), and F2, were purchased from AceEmzyme Co., Ltd., Anseong (Korea). Three recombinant enzymes were used in this enzymatic hydrolysis; BglBX10 (β -glucosidase from *Flavobacterium johnsoniae* UW101^T) (Kim et al., 2013), Abf22-3 (α -L-arabinofuranosidase from *Leuconostoc* sp. 22–3), and BglSk (β -glucosidase from *Saccharibacillus kuerlensis*).

2.2. Substrate [Rg3(S)] preparation

Recombinant glycoside hydrolases (BglBX10 and Abf22-3) for the production of Rg3(S) ginsenoside were prepared as described previously by (Kim et al., 2013). Briefly, the PPD-mix substrate from Korean ginseng (Rc, Rd, Rb1, and Rb2) at final concentration of 50 mg/mL (150 g in total) was dissolved in 0.1 M phosphate buffer (pH 7.0) with the crude recombinant enzyme BglBX10 (10 mg/mL) and Abf22-3 (10 mg/mL) up to the final work volume of 5 L. The reaction mixture was performed in 10 L jar fermenter with the pH 7.0 ± 0.5 at a temperature of 37°C for 24 h. After 5 h time intervals the samples were collected and the biotransformation of PPD-mix to Rg3(S) was confirmed by TLC analysis. After the complete conversion of PPD-mix (except Rb2) to Rg3(S), the solution was heated at temperature 80°C for 2 h to inactivate the recombinant BglBX10 and Abf22-3.

2.3. Molecular cloning, expression, and purification of recombinant BglSk

The genomic DNA from *S. kuerlensis* was extracted using a genomic DNA extraction kit (Solgent, Daejeon, Korea). The DNA was amplified using PCR to obtain the *Saccharibacillus kuerlensis* DNA segment. The oligonucleotide primers based on the BglSk DNA sequence were designed as 5'-GGT TCC GCG TGG ATC CAT GGA ATT CAA TTT TCC G-3'_F [forward] and 5'-GAT GCG GCC GCT CGA GTT ACC CGC TGA CAA GTG A-3'_R [reverse] by Macrogen Co. Ltd. Korea. The amplified PCR purified product was used to introduce the BamHI and XhoI restriction sites (underline), respectively. Subsequently, the PCR product was purified and injected into the GST fusion vector (pGEX-4 T-1) using the Ez-Cloning Kit (Enzygnomics Co. Ltd., Korea). The β -glucosidase DNA sequence (GenBank accession number: WP 018975927.1) was used as a base for primer identification. The resulting recombinant pGEX-BglSk was transformed into *Escherichia coli* BL21 (DE3) and grown in Luria-Bertani (LB) broth supplemented with ampicillin (100 mg/mL) at 37°C . The recombinant enzyme was collected as previously described (Cui et al., 2014; Song et al., 2017). Furthermore, GST-bind agarose resin (Elpisbiotech-, Korea) was used for the GST tag purification. After purification, the recombinant protein homogeneity was evaluated by 10% SDS-PAGE analysis. The SDS-PAGE result showed the precise molecular weight as was identified by amino acid sequence analysis.

2.4. Substrate specificity and effect of temperature, pH, and metal ions on the activity of BglSk

Substrate preference was tested using 2 mM of 13 different kinds of p-nitrophenyl (pNP) and 4 different kinds of o-nitrophenyl (oNP) substrates (as shown in Table S2) at 37°C for 10 min. One unit activity is defined as the release of $1\ \mu\text{mol}$ o-nitrophenol or p-nitrophenol per min.

The purified BglSk was tested for the substrate-specific activity, effect of various temperatures [$5\text{--}60^\circ\text{C}$ (with a 5°C interval)], pH values [$2\text{--}10$ (with 1.0 pH interval)] and different metal ions [NaCl, CoCl_2 , CaCl_2 , KCl, MgCl_2 , MgSO_4 , MnSO_4 , EDTA, and β -Mercaptoethanol (with a final concentration of 1 mM and 10 mM)] were identified as previously described (An et al., 2019; Cui et al., 2014). Furthermore, the Kinetic parameter analysis was done with freshly purified enzymes using $1\text{--}20$ mM of pNPG (4-nitrophenyl β -D-glucopyranoside) concentrations. One unit activity is defined as the amount of enzyme required to produce $1\ \mu\text{mol}$ of p-nitrophenol per minute. All the assays were performed in triplicate. The parameters, K_m and V_{max} , were determined using the

enzyme kinetics program described by Cleland (Cleland, 1979). All the analyses were carried out in triplicates.

2.5. Biotransformation activity of BglSk using seven PPD type ginsenosides

The enzyme solutions at a concentration of 1 mg/mL in 100 mM of sodium phosphate buffer (pH 7.0) were reacted with an equal volume of ginsenosides Rb1, Rb2, Rc Rd, Rg3, F2, and Gyp-XVII, solution at a concentration of 1 mg/mL (wt/vol) in 100 mM of sodium phosphate buffer (pH 7.0) at 37 °C. The samples were taken at regular intervals for 5 h and analyzed via TLC after pretreatment (see analytic methods).

2.6. Optimization of substrate and enzyme concentration for biotransformation

To effectively optimize the substrate [PPD-mix (Rb1, Rb2, Rc, and Rd)] and enzyme (BglBx10, Abf22-3) concentrations for the production Rg3(S) as reported previously by Kim et al. (Kim et al., 2013), 10 mg/mL of each enzymes (BglBx10, Abf22-3) were mixed with 50 mg/mL of PPD-mix substrate. The results show that the ginsenosides Rb1, Rc and Rd were completely converted ginsenoside Rg3(S) within 24 h. After the complete transformation of PPD-mix to Rg3(S), the samples were heated at 80 °C for 2 hrs to inactivate the recombinant BglBX10 and Abf22-3. Then produced Rg3(S) was diluted as one half (25 mg/mL as calculated as PPD mix conc.) and was reacted with different concentrations 5, 10, 20, 50 mg/mL (as final concentration) of BglSk. All the initial analyses were performed in a 15 mL tube with a 3 mL working volume at 37 °C for 24 h in a shaking incubator at 150 rpm. After every 4 h, the samples were collected and the transformation of Rg3(S) to Rg2(S) was confirmed by TLC analysis.

2.7. Preparation of three recombinant enzymes using high cell density culture

A 5L working volume of LB (Luria-Bertani) broth supplemented with 80 mg/mL ampicillin in a 10 L stirred-tank reactor was used (FMT10SA, Fermentech, Korea) for the bulk production of recombinant BglBx10, Abf22-3, and BglSk. All three recombinant *Escherichia coli* were cultured in 3 different stirred-tank reactors (FMT10SA, Fermentech, Korea). The growth of the recombinant *Escherichia coli* cultures in the fermenters was maintained at 37 °C and 400 rpm. When the OD₆₀₀ nm cultures reached to 3.0–3.5, the temperatures were reduced to 25 °C (BglBX10), 22 °C (Abf22-3), and 18 °C (BglSk), respectively. After the temperatures reduction, the BglBX10 Abf22-3 and BglSk clones were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 18–24 h, the cells were broken using a digital sonicator (Digital sonifier S-450D, Branson, USA), and the crude recombinant enzymes (BglBX10, Abf22-3, and BglSk) were collected in the soluble form after centrifugation (Component R, Hanil science Co Ltd., Korea) at 5000 rpm for 20 min at 4 °C. The soluble crude enzyme (recombinant BglSk) was used for further experiments.

2.8. Scaled up production of Rh2(S) from the fermentation of PPD-mix

The scaled-up production of Rh2(S) was performed in a 10 L stirred-tank reactor [300 rpm (Biotron GX, Hanil science Co., Korea) with 6.0 L working volume at 37 °C for 24 h. Briefly, the 100 g unit of crude Rg3(S) was produced from 50 mg/mL of PPD-mix (150 g total) using the combination of BglBX10 (10 mg/mL final concentration) and Abf22-3 (10 mg/mL final concentration) recombinant enzymes. The bioconversion reaction of PPD-mix to Rg3(S) was completed after 24 h at 37 °C. After complete conversion of PPD-

mix (except Rb2) to Rg3(S) the recombinant enzyme BglBX10 and Abf22-3 was inactivated at 80 °C for 2 h, and the Rg3(S) solution was diluted as half concentration and cooled down to 37 °C. In order to produce the scaled-up production of Rh2(S), the crude recombinant BglSk (20 mg/mL final concentration) was added to the diluted Rg3(S) solution, and the reaction mixture was further continued for 24 h. The samples were collected every 4 h and analyzed by TLC to determine the exact time for the transformation of Rg3(S) to Rh2(S). Furthermore, the transformation was confirmed by HPLC analysis.

2.9. Purification of Rh2(S) with high purity 98%

After the scaled-up production of Rh2(S), the 6 L reaction mixture (containing Rh2(S), a small quantity of C-O and C-Y with recombinant BglBX10, Abf22-3, and BglSk) was centrifuged at 2898g for 15 min (2236R, Labogene, Denmark). In order to purify the Rh2(S), both the supernatant and pellet were processed separately. The Rh2(S) precipitates were dissolved twice in 6 L of 80% ethanol and filtered through a filter paper (Advantec, Japan). After filtration, the filtrate was combined with the supernatants and adjusted to a 40% ethanol solution. The glass column chromatography [400(L) × 115(D) mm] packed with HP20 resin (Mitsubishi, Japan) was used to remove the impurities, except the ginsenoside Rh2(S). The 40% ethanol solution was loaded onto the column and the free sugar molecules and unwanted hydrophilic compounds from HP-20, which were adsorbed by the beads, and were washed with 6-bed volumes (BV) of water. After washing, the Rh2(S) was eluted with 95% ethanol (extra pure grade; SK Chemicals, Korea) and the eluent was evaporated in *vacuo* evaporator. The partially dried purified Rh2(S) was further purified by a preparative HPLC system (Hanbon, China) equipped with DAC column [500(L) × 100 (D) mm] packed with ODS resin (Daiso, Japan). The dried crude Rh2(S) was dissolved in 54% ACN and loaded onto the DAC column and eluted with 54% acetonitrile (ACN). Ginsenoside Rh2(S) was eluted in retention time 60 to 90 min (data not shown). The ACN eluent was evaporated in *vacuo* and its purity was determined by HPLC.

2.10. Analytical methods

2.10.1. TLC (Thin layer chromatography) analysis

The minor ginsenosides combined with ginsenosides standards were spotted on TLC plates (60F₂₅₄ silica gel Merck, Germany) using CHCl₃:CH₃OH:H₂O (65:35:10, v/v/v) as the mobile phase. The ginsenosides spots were visualized by spraying the plates with 10% (v/v) H₂SO₄, followed by heating at 110 °C for 5 min and the results were compared with the ginsenosides standards.

2.10.2. HPLC (or High-performance liquid chromatography) analysis

The HPLC analysis of the ginsenosides was performed using an HPLC machine (Younglin Co. Ltd., Korea) with automatic injector, quaternary pump, and single wavelength UV detector (model 730D). For ginsenosides peaks were identified by the Younglin's AutoChro 3000 software. After 30 μl sample injection, the flow rate was kept constant at 1.0 mL/min and the ginsenosides peaks detection were measured by 203 nm UV absorbance.

3. Results

3.1. Molecular Cloning, expression and purification of recombinant BglSk

The DNA of β-glucosidase *Saccharibacillus kuerlensis* comprises of 1263 bp, with a theoretically molecular weight of 47.7 kDa. The amino acids sequence analysis show that the recombinant

BglSk belong to glycoside hydrolase family 1. The BglSk gene was amplified by PCR and then hosted into the pGEX 4 T-1 vector. Additionally, the expression levels of the recombinant BglSk and predictive molecular masses were also confirmed by 10% SDS-PAGE analysis. The molecular mass of the expressed BglSk (calculated via an amino acid sequence) and fusion tag protein found to 73.7 kDa (Fig. 1). The GST-BglSk was purified using the GST-tag resin column (Elpis Biotech). After protein purification, non-induced, induced soluble fraction, induced precipitated fraction, and purified protein soluble fractions were examined by 10% SDS-PAGE. The SDS-PAGE analysis shows 47.7 kDa molecular weight of the purified recombinant protein, as shown in Fig. 1.

3.2. Effect of pH, temperature, and metal ions on BglSk activity

Enzyme characterization is necessary to confirm the identity and functional activity of an enzyme product. Therefore, the effect of various parameters such as temperature, pH, metal ions, substrate preference, and specific activity was determined for the recombinant BglSk.

The optimum temperature and pH for bioconversion of ginsenosides were tested using the crude BglSk. The BglSk had optimal activity at pH 7.5 in phosphate buffer, and it was stable at pH 6.5–10.0 (Fig. 2A). The stability of BglSk reduced very quickly when the pH values goes below 6.0 or over 10.0. Thus, at pH 6.0 and below 6.0, the enzyme activity reduced to 40.0% and 15.0% of the optimum activity (Fig. 2A).

The recombinant BglSk show its optimum (96.5%) enzyme activity at 45 °C. The thermo-stability of BglSk reduced considerably less than 37 °C, and loses about 90% of its activity when incubated for 1 h above 50 °C (Fig. 2B). As a result, the BglSk was stable below 40 °C and lose the activity above 50 °C. Thus, the BglSk is little mesophilic and stable at a neutral pH range. Moreover, the optimum temperature and near neutral optimum pH (7.0) of BglSk was similar to the previously describe ginsenoside-hydrolyzing from *Arachidococcus ginsenosidimitans*, *Niabella ginsenosidivorans* and *Lactobacillus ginsenosidimitans*

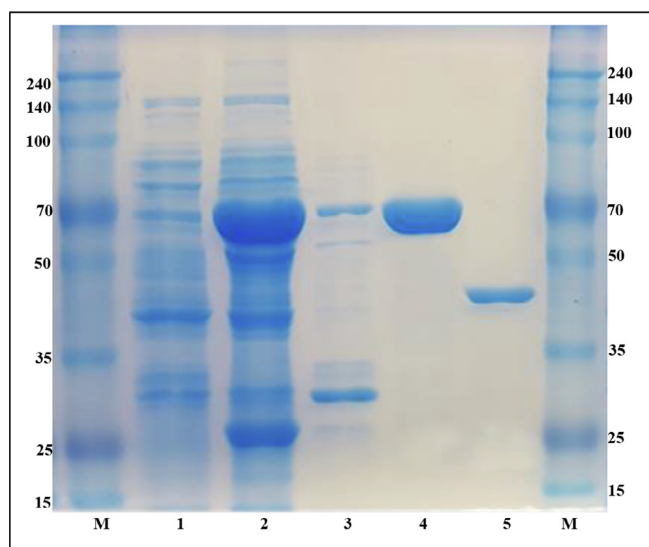


Fig. 1. SDS PAGE analysis of recombinant BglSk after purification using GST-bind agarose resin. 1, un-induced crude enzyme of BglSk; 2, soluble fraction of the induce BglSk; 3, precipitated fraction of the induce BglSk; 4, GST-bind of BglSk after purification with GST-bind agarose resin; 5, purified BglSk after GST cleavage by thrombin.

(Siddiqi et al., 2019; Siddiqi et al., 2020; Wang et al., 2019). Although, the optimal temperature of BglSk for hydrolysis of pNPG was 45 °C, but during the extended and stable bioconversion of ginsenoside, the reaction was maintained at 37 °C with an optimum pH of 7.0 ± 0.5 .

The kinetic parameters (V_{max} and K_m) values of BglSk were identified by putting the substrate concentration vs. the initial velocity of each reaction. After each reaction, the values were subjected to a linear regression analysis. Thus, the V_{max} and K_m values of BglSk for pNPG were $29.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and 8.7 mM, respectively.

As stated in previous sections, the presence of metal ion can either activate or inhibit the enzymatic activity. So, the effects of metal ions, β -mercaptoethanol, and EDTA on the activity of BglSk were examined. The results were stated as a percentage of the activity in the absence of metal ions (as shown in Table S3). The BglSk activity was not disturbed by 1 mM of metal ions [K^+ , Na^+ , Mg^{2+} , Ca^{2+} , Co^{2+} , and Mn^{2+}] and other chemicals [EDTA, and β -mercaptoethanol]; however, its activity was decreased by 10 mM of Mg^{2+} , Mn^{2+} , Co^{2+} , Ca^{2+} , and β -mercaptoethanol. Further, the 10 mM EDTA inhibit the BglSk activity up to 60%, which show that the divalent cations might be required for its activity. The result proved that BglSk does not require any catalytic activity and only β -mercaptoethanol which had an ability to stimulate the enzyme BglSk.

The substrate preference of BglSk was confirmed by means of 2.0 mM of o-nitrophenyl (oNP)-glycosides and p-nitrophenol (pNP) with α and β configurations. The study revealed that BglSk was more active for the hydrolysis of pNP- β -D-glucopyranoside ($100.0\% \pm 1.2\%$), pNP- β -D-galactopyranoside ($60.3\% \pm 3.2\%$), and pNP- β -D-fucopyranoside ($53.7\% \pm 1.9\%$). While, weakly active for oNP- β -D-glucopyranoside (33.4 ± 3.5), pNP- α -L-arabinopyranoside ($30.2\% \pm 5.2\%$), and oNP- β -D-fucopyranoside ($23.4\% \pm 2.7\%$). As a result, seeing the relatively high value, pNP- β -D-glucopyranoside (100%) was used as the control for the determination of the relative activity of other substrates (Table S4).

3.3. Bioconversion characteristics of BglSk

The specific activity of the recombinant BglSk was determined to find out the pathway of the bioconversion of ginsenosides. The specific activity of enzyme BglSk was performed through various substrates of PPD-mix type ginsenosides (Rc, Rd, Rb1, Rg3, Rb2, F2, and Gyp-XVII). The conversion of each ginsenoside (at 37 °C for 24 h) was confirmed by TLC (Fig. 3). The BglSk exhibit a very specific transforming activity for the following ginsenosides: Rb1 to Rd \rightarrow F2 to C-K, Rb2 \rightarrow C-O to C-Y, Rc to C-Mc1 \rightarrow C-Mc, Rg3 \rightarrow Rh2, and Gyp-XVII to F2 \rightarrow C-K (Fig. 4A, B). This distinctive biotransformation ability by BglSk makes it possible to produce gram-scale of minor ginsenoside Rh₂(S). As a result, we recommend that BglSk can be used for the future production of other minor ginsenosides as well.

3.4. Optimization of substrate and enzyme concentration

BglSk concentration was optimized to determine the appropriate enzyme concentration to minimize the excessive use of enzyme. Four different concentrations of BglSk enzyme (5, 10, 20, 50 mg/mL) were used in this study. The result illustrates that 20 mg/mL of BglSk was very good for the conversion of 25 mg/mL of ginsenoside Rg3(S) to Rh2(S) within 10–24 h. However, high substrate concentration (25 mg/mL) with low concentration of enzyme (5, 10 mg/mL) did not complete within 24 h (Fig. 5). Therefore, 25 mg/mL concentration of Rg3(S) and 20 mg/mL concentration enzyme BglSk are the ideal concentration for mass

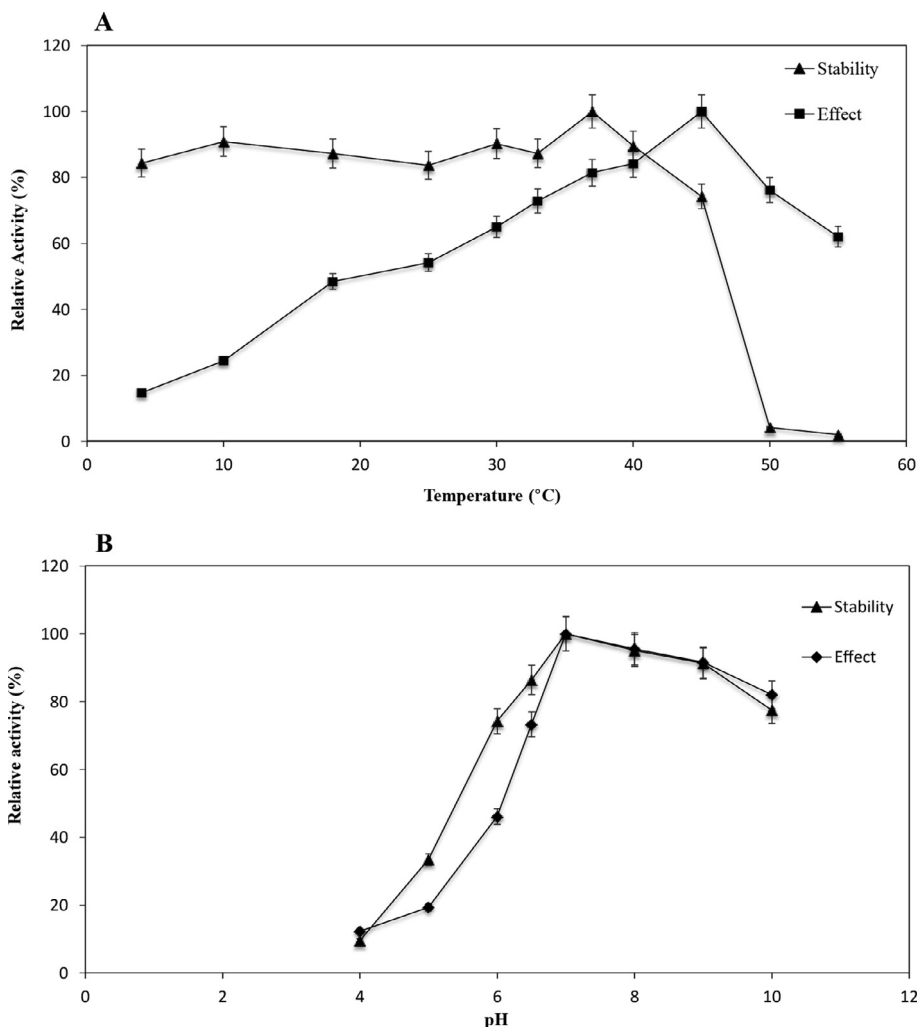


Fig. 2. A, B. Show the effect of various temperature and pH on enzyme activity of recombinant BglSk.

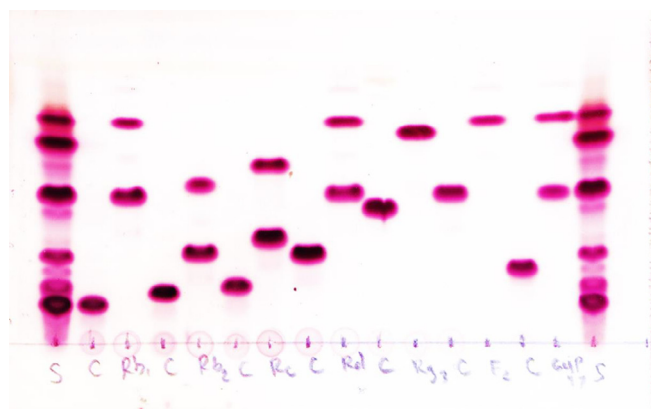


Fig. 3. Thin Layer chromatography (TLC) analysis show the conversion of PPD type ginsenosides (Rb1, Rb2, Rc, Rd, Rg3, F2, Gyp-XVII) to minor ginsenosides after 24 h by BglSk. S: ginsenoside standard; 1 and 2, Rb1 control and bioconversion; 3 and 4, Rb2 control and bioconversion; 5 and 6, Rc control and bioconversion; 7 and 8, Rd control and bioconversion; 9 and 10 Rg3 control and bioconversion; 11 and 12, F2 control and bioconversion; 13 and 14, Gyp-XVII control and bioconversion.

production of ginsenoside Rh2(S) from Rg3(S). Furthermore, the bioconversion pathway of Rg3(S) to Rh2(S) is shown in Fig. 4B.

3.5. Preparation of high cell density culture of BglSk, BglBX10, and Abf22-3

The *Escherichia coli* BL21 (DE3) cells that harbored pGEX-BglSk, pGEX-BglBX10, and pGEX-Abf22-3 were cultured separately in a 10 L stirred-tank reactor with a 5 L working volume for each recombinant cell at 400 rpm. The temperatures of the cultures were maintained at 37 °C, and the pH values of the media were adjusted to 7.0 using 0.1 mM phosphate buffer. After induction at different temperatures [18 °C for BglSk, (25 °C for BglBX10 and 22 °C for Abf22-3)], the cultures were further incubated for bulk production of recombinant of protein. When all cultures reached an OD_{600 nm} of 40 ± 2.1, the recombinant cells (BglSk, BglBX10, and Abf22-3) were collected by centrifugation. 150 g of wet cells (for each BglSk, BglBX10, and Abf22-3, respectively) were harvested and 50 g/500 mL[w/v] were resuspended in 0.1 M of sodium-phosphate buffer (pH 7.0). The suspended cells were dis-

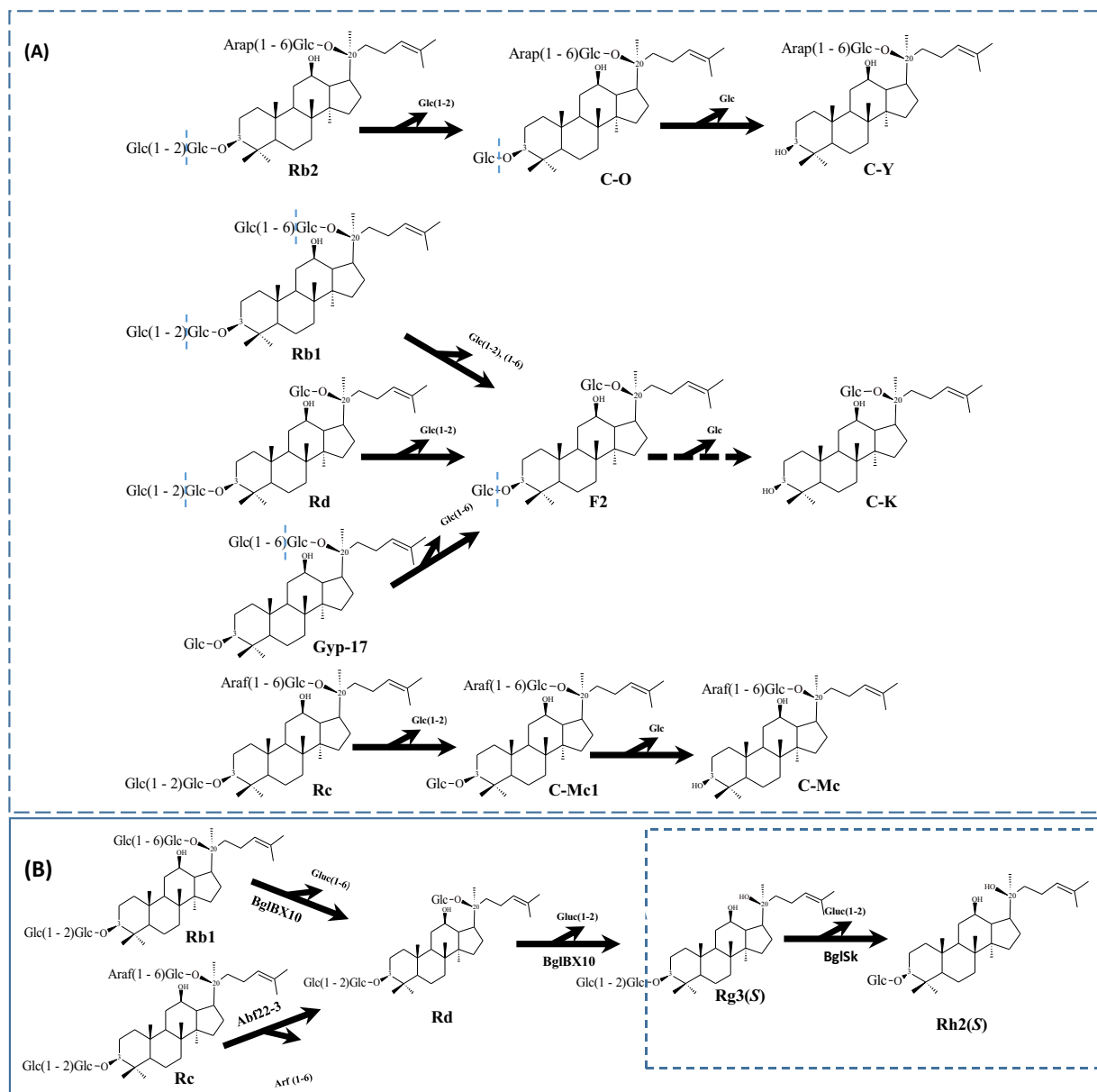


Fig. 4. A, B. Show the conversion pathway of recombinant BglSk using the PPD type ginsenosides Rb1, Rb2, Rc, Rd, Rg3, F2, and Gyp-XVII (4A, 4B), the mass production ginsenoside Rh2(S) from Rb1, Rc and Rd using the recombinant BglBX10, Abf22-3 and BglSk.

rupted via ultrasonication and the crude soluble enzymes were used for the bioconversion of PPD type ginsenosides.

3.6. Scaled-up production of Rh2(S)

After cell (BglBX10, Abf-22-3, and BglSk) sonication, the crude recombinant enzyme was used for the scaled-up production of Rh2(S) from the initial substrate of PPD-mix. Briefly, ginsenoside Rg3(S) was produced from PPD-mix [20 mg/mL (as final concentration in 100 mM sod-phosphate buffer)] using the crude soluble form of BglBX10 (20 mg/mL final concentration) and Abf22-3 (10 mg/mL final concentration). The enzymatic reaction was started at 37 °C for 24 h in a 10 L reactor tank with 3 L working volume, in order to produce the Rg3(S). After TLC analysis, the ginsenosides Rb1 and Rc dissipated from the reaction mixture within 5–10 h of incubation, whereas there was a more gradual

disappearance of Rd to Rg3(S) (Data not show). After 24 h incubation, the HPLC analysis revealed that ginsenoside Rc, Rb1, and Rd were completely transformed to Rg3(S) as shown in Fig. 6C and Fig. S1. While the biotransformation of Rg3(S) to Rh2(S) was carried out after the deactivation of BglBX10 and Abf22-3 at 80 °C, 20 mg/mL (as final concentration) of BglSk was added to diluted Rg3(S) solution as a half concentration in order to transform the ginsenoside Rg3(S) to Rh2(S). After time intervals (each 6 h), the samples from the reaction mixture were collected in order to determine the conversion of Rg3(S) to Rh2(S) using TLC analysis. Thus, after TLC analysis the conversion of Rg3(S) to Rh2(S) was confirmed by HPLC, and the results show that the biotransformation of Rg3(S) to Rh2(S) was almost completed within 24 h, which was no longer detected by the HPLC analysis (except C-O and C-Y) (Fig. 6D). The ginsenosides C-O and C-Y were detected in the last reaction due to the presence of Rb2 that was transformed by BglSk

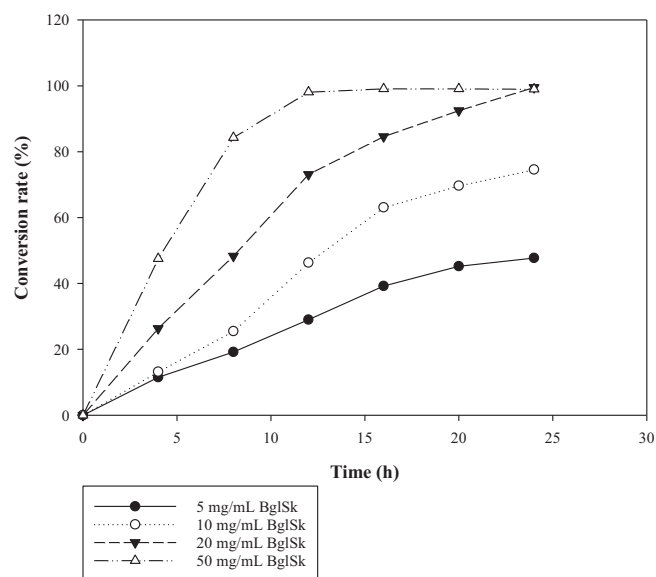


Fig. 5. Effect of various concentrations [5 (●), 10 (○), 20 (▼), 50 (Δ) mg/mL (as final concentration)] of BglSk on the production of ginsenoside Rh₂(S) from Rg₃(S) by BglSk.

during the transformation of Rg₃(S) to Rh₂(S). The biotransformation of PPD mix to Rh₂(S) using three recombinant enzymes (BglBX10, Abf22-3, and BglSk) was completed in two phases in order to minimize the competitiveness of substrate binding to the three enzymes.

3.7. Purification of Rh₂(S)

To get the high purity of Rh₂(S), the Rh₂(S) solution was centrifuged at 5000 rpm for 15 min to remove the recombinant enzymes. In addition, the salts and free sugar molecules from Rh₂(S) were removed using glass column chromatography with 5 L of the 95% ethanol eluent. Then, the Rh₂(S) was vaporized *in vacuo* in order to create 86.4 g Rh₂(S) combined with C-O and C-Y. Moreover, to get the highly pure Rh₂(S), the Rh₂(S) containing the C-O and C-Y was loaded into a self-packed silica cartridge using the chloroform–methanol–water (90:10:1) as mobile phase solvent. In order to get the pure Rh₂(S) [free from C-O and C-Y], the eluents (250 mL/flask) were collected in different flasks and analyzed by TLC. After TLC analysis, those fractions which contained only Rh₂(S) were collected and evaporated. Finally, the 32.5 g powder was obtained from a self-packed silica cartridge and was subjected to recycling preparative HPLC for standard production of Rh₂(S). As a result,

15.1 g of Rh₂(S) with a purity of 98.0% was obtained from the initial bioconversion of 150 g of PPD-mix as shown in (Fig. S2). The chromatographic purity of the Rh₂(S) was more than 98%, as determined by HPLC.

4. Discussion

Even though ginsenoside Rh₂(S) has a high biological and pharmacological activity for curing human diseases such as cancer, inflammation, diabetes, tumor, and obesity (Table S1), the lack of technology for the scaled-up production of Rh₂(S) has prevented the pharmaceutical industries from fulfilling their requirement. Korean ginseng contains approximately less than 0.01% of Rh₂(S) on dry weight basis (Siddiqi et al., 2020), and different methods (such as physical, chemical, and biological methods) have been developed for the transformation of PPD-mix type major ginsenoside to Rg₃(S) and Rh₂(S); however, these methods still have some constraints and are limited to laboratory level (Shen, 2005; Palaniyandi et al., 2016; Siddiqi et al., 2019). Thus, for gram unit production of Rh₂(S), only two researchers have been able to produce the Rh₂(S) from the initial substrate of PPD-mix (Rb1, Rc, and Rd) using the combined methods of heat-acid, and enzymatic conversion; however, this process produced byproducts such as Rh₂(R), Rh₃, and Rk₂ and the concentration of Rh₂(S) was very low (Song et al., 2017; Palaniyandi et al., 2016; Su et al., 2006). In general, the selectivity was poor and causing side reactions (like epimerization, hydroxylation, and hydration) and also produces some byproduct that cause environmental pollution [12, 26, 27, 32 (Table S5)].

In this study, we briefly describe and found the possibility of scaled-up production of Rh₂(S) from the initial substrate of PPD-mix type ginsenosides using the combination of three recombinant enzymes (BglBX10, Abf22-3, and BglSk) expressed in *Escherichia coli* BL21 (DE3) in series. Moreover, we found that BglSk was very efficient for the bioconversion of Rg₃(S) and mass production of Rh₂(S). The BglSk displays a very specific ginsenoside conversion pathways for: Rb1 → Rd → Rg₃ → Rh₂, Rb2 → C-O → C-Y, Rc → CM-c1 → CM-c, and Gyp-XVII → F2 (Fig. 4A,B). This matchless biotransformation capability of BglSk with its optimum reaction condition (pH 7 and 37 °C), makes it possible to yield 15.1 g-scale Rh₂(S) with 98% of chromatographic purity. Here, we describe for the first time that the BglSk expressed in *Escherichia coli* can be used to transform 50 mg/mL of PPD-Mix to Rg₃(S) and then Rg₃(S) to 86.4 g-scale Rh₂(S) within 24 h. The combination of BglBX10, Abf22-3 and BglSk (expressed in *E. coli*) enables the usage of ginsenoside Rh₂(S) derived from PPD-Mix (Korean red ginseng) in the pharmaceutical industries and cosmetics industries. Additionally, we demonstrated that scaling up of recombinant BglSk can be used at commercial level.

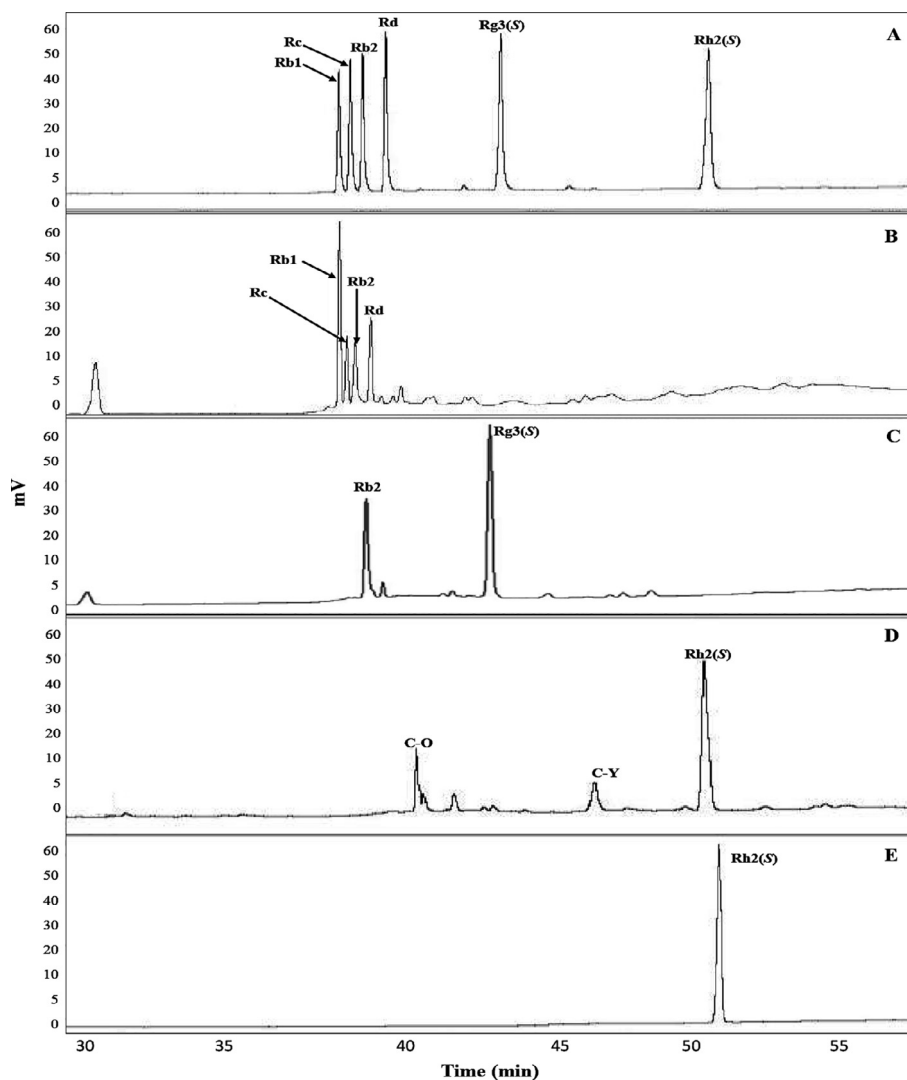


Fig. 6. (A–E). HPLC profiles for the biotransformation of ginsenosides PPD mix (Rb1, Rc, and Rd) to Rg3(S) and Rh2(S) by three recombinant enzymes BglBX10, Abf22-3, and BglSk. 6A), ginsenosides standard; 6B), Control of PPD-mix; 6C), Biotransformation PPD-mix to Rg3(S); 6D), Biotransformation Rg3(S) to Rh2(S); 6E), Purified Rh2(S) after strict purification by Prep-HPLC.

5. Conclusion

Choosing a suitable organism for glycoside hydrolase (β -glucosidase) gene expression, we have seek out a very promising recombinant enzyme expressed in *Escherichia coli* for enhanced production of Rh₂(S) from Rg₃(S). By means of BglSk, 15.1 g of Rh₂(S) was obtained via conversion of 150 g of major ginsenosides Rb₂, Rc, Rb₁, and Rd. The reaction mixture (recombinant enzymes + PPD-mix) was started in a 10 L jar fermenter at neutral pH (7.0) and 37 °C for 48 h, with an initial substrate (PPD-mix) concentration of 50 mg/mL. Thus, the combination of these three recombinant enzymes (BglBX10, Abf22-3, and BglSk) for conversion of PPD-Mix to Rg₃(S) and gram-scale production of Rh₂(S) offers an efficient method for the preparation of minor ginsenoside Rh₂(S) on a large scale to meet industrial needs.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2021.04.079>.

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