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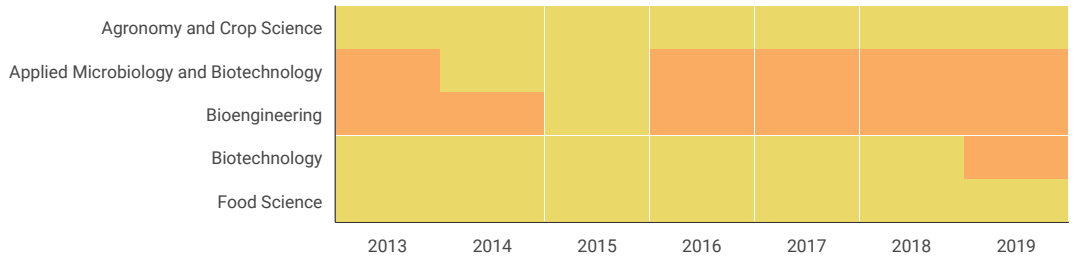
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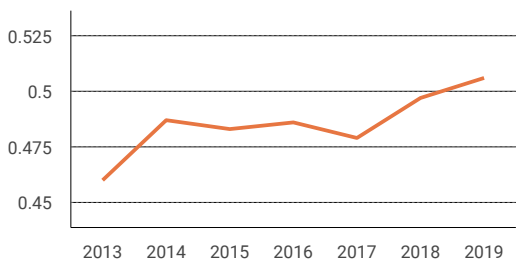
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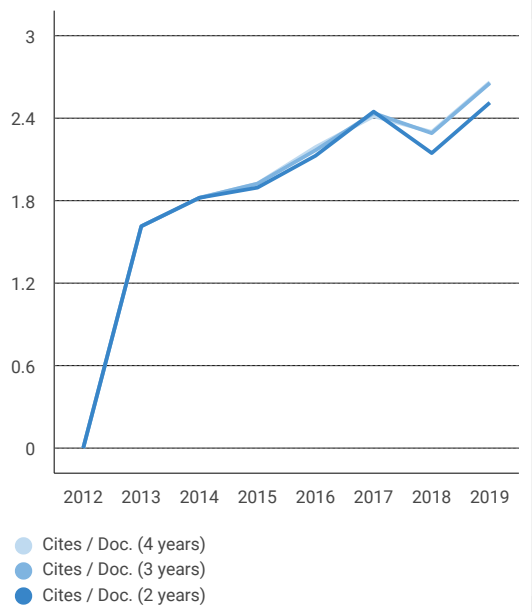
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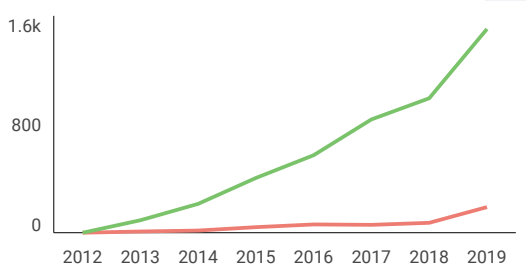
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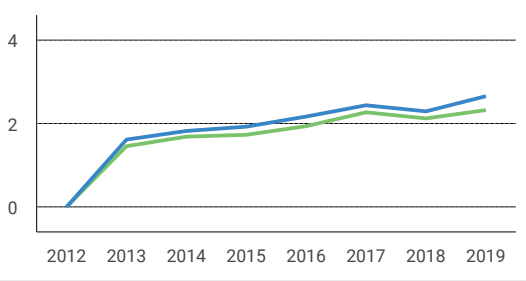
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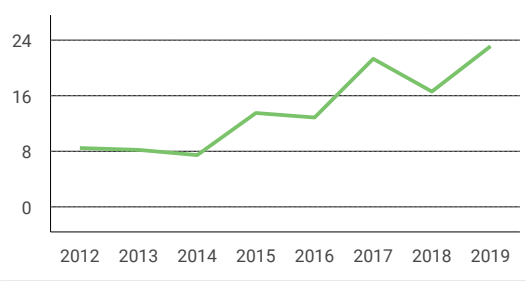
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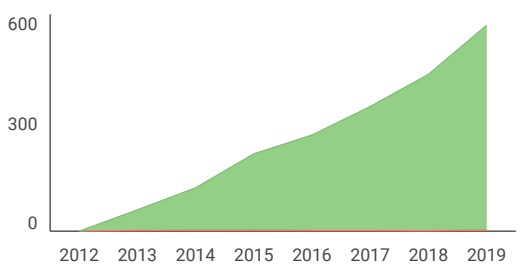
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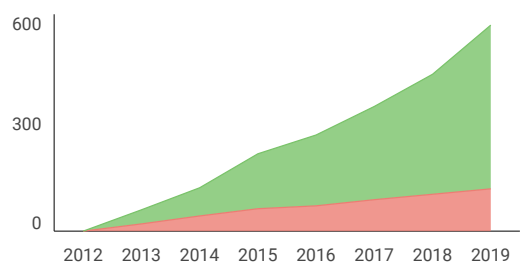
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Enzymatic biotransformation of ginsenoside Rb1 by recombinant β -glucosidase of bacterial isolates from Indonesia

Almando Geraldi, Ni'matuzahroh, Fatimah, Chang-Hao Cui, Thi Thuy Nguyen, Sun Chang Kim



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5
6 Enzymatic biotransformation of ginsenoside Rb1 by recombinant β -
7 glucosidase of bacterial isolates from Indonesia

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15 **Abstract**

16 β -Glucosidase (EC. 3.2.1.21) is of industrial interest due to its critical role in the utilization of cellulosic biomass to produce high-value chemical
17 compounds and biofuels. Moreover, β -glucosidases can be utilized in the biotransformation of high value plant active materials such as ginsenosides.
18 In this report, we confirmed the biotransformation activity of ginsenosides by β -glucosidases from *Bacillus* sp. 3KP and *Serratia marcescens* LH61
19 strains isolated from Indonesia. β -Glucosidases from both bacterial strains were cloned and overexpressed in *Escherichia coli* BL21(DE3). Crude cell
20 extract of *E. coli* BL21(DE3) overexpressing the β -glucosidase were used for the biotransformation of ginsenosides Rb1. Results showed that the Rb1
21 was biotransformed to the more pharmacologically active rare ginsenosides, gypenoside XVII and F2. This work is the first effort to use β -
22 glucosidases from Indonesian bacterial strains for ginsenosides biotransformation and is expected to encourage further exploration of β -glucosidase-
23 producing bacterial strains from Indonesia.

24
25 **Keywords:** β -glucosidase; biotransformation; ginsenosides; *Bacillus* sp.; *Serratia marcescens*

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1 1. Introduction

2 β -Glucosidases (β -d-glucopyranoside glucohydrolase) [E.C.3.2.1.21] are diverse group of enzymes which hydrolyse
3 the glycosidic bond of a carbohydrate moiety (Singh et al., 2016; Zang et al., 2018). The enzymes are best-known for
4 their critical role in the degradation of lignocellulosic materials into glucose in chemical and biofuel industries
5 (Srivastava et al., 2019). Recently, the enzymes are also highly sought after due to their ability to transform plant active
6 materials with high economic value such as ginsenosides.

7 Ginsenosides, a group of triterpenoid saponins, are the major active components of ginseng, roots of *Panax* species
8 plants (Park et al., 2019). For thousands of years ginseng has been used as traditional medicine in East Asian countries,
9 and recently ginseng has been increasingly used in foods and dietary supplements worldwide (Cui et al., 2019). More
10 than 90% of total ginsenosides in ginseng are less pharmacologically active major ginsenosides group such as
11 ginsenoside (G)-Rb1, G-Rb2, G-Rc, and G-Rd (Du et al., 2014). Those major ginsenosides are poorly absorbed by
12 human intestinal track (Upadhyaya et al., 2016). Hydrolysis of sugar moieties of major ginsenosides is needed to obtain
13 more easily absorbed, thus more pharmacologically active minor/rare ginsenosides (Kim et al., 2012; Zhong et al.,
14 2016). Among various hydrolysis methods (heating, acid treatment, enzymatic transformation), the use of enzymes
15 especially β -glucosidase is preferred due to their advantages of higher stereo-specificity, conversion yield, as well as
16 lower negative effects to the environment (Kim et al., 2019).

17 β -Glucosidases for ginsenoside biotransformation are commonly cloned from microorganisms isolated from the soils
18 of ginseng plantation in East Asia (An et al., 2010; Cui et al., 2017; Yan et al., 2008). A considerable number of those
19 ginsenoside-transforming β -glucosidases belong to glycosyl hydrolase (GH) family 1, which hydrolyse the sugar
20 moieties at both the C-3 and C-20 positions in PPD-type ginsenosides (Noh et al., 2009; Wang et al., 2011; Yuan et al.,
21 2015). In this study, we report the first effort to clone ginsenoside-transforming β -glucosidase from GH family 1-
22 producing bacterial isolates from Indonesia, *Bacillus* sp. 3KP (Ni'matuzahroh et al., 2017; Triawan et al., 2017) and
23 *Serratia marcescens* LII61 (Fatimah et al., 2019). The efficacy of β -glucosidases from those isolates to catalyse the
24 biotransformation of PPD-type major ginsenoside Rb1 to the more pharmacologically active minor ginsenosides
25 Gypenoside XVII (Gyp-XVII) and F2 was also investigated.

26 2. Materials and Methods

27 2.1. Chemicals

28 Ginsenoside Rb1, Rd, and F2 standards (98% purity), were purchased from Zelang Medical Technology Co., Ltd.
29 (China). *p*-Nitrophenyl- β -D-glucopyranoside (*p*NP β Glc) was purchased from Sigma Aldrich (USA), while 5-Bromo-4-
30 chloro-3-indolyl β -D-glucopyranoside (*X*-Glc) was purchased from Wako Co. Ltd. (Japan). All the chemicals used in
31 this study were at least analytical or reagent grade, and the sources are noted individually.

32 2.2. Molecular cloning of the β -glucosidase genes

33 Genomic DNA of *Bacillus* 3KP and *S. marcescens* was extracted by using a genomic DNA extraction kit (Qiagen,
34 USA). The extracted genomic DNA was used as template for the amplification of β -glucosidase encoding genes
35 designated as *bgl3KP* and *bglSM* via polymerase chain reaction (PCR) using Pfu DNA polymerase (Solgent, Korea).
36 The primers used in the PCR reaction were designed based on the sequence glycosyl hydrolase family 1 genes of
37 *Bacillus* sp. (GenBank accession no. CP042874.1) and of *Serratia marcescens* (GenBank accession no. CP027300.1),
38 respectively. Primers (*bgl3KP* forward: 5'- GTCTAACCATGGAATAAGTTTCCACATGATTTTTTATTCGGA-3';
39 *bgl3KP* reverse: 5'- CCGTTAGGATCCTTATCATTATAAACTCTTCCCTCTCGTTTCG-3'; *bglSM* forward; 5'-
40 GTCTAACATATGGAATATCAATTTGCCGACGG-3' *bglSM* reverse: 5'-
41 CCGTTACTCGAGTTATCATTAGTCAAATCCGTTGCGTCTGG-3') which include NcoI and BamHI (*bgl3KP*) and
42 NdeI and XhoI (*bglSM*) restriction sites (underlined) were synthesized by Genotech (Korea). The amplified DNA
43 fragments of *bgl3KP* and *bglSM* were purified and inserted into the pRSFDuet-1 vector (Novagen, USA) digested with
44 appropriate enzymes (NEB, USA).

45 2.3. Expression of recombinant β -glucosidase

46 The resulting recombinant pRSF-*bgl3KP* and pRSF-*bglSM* was each transformed into *E. coli* BL21(DE3). The *E.*
47 *coli* BL21(DE3) harbouring the recombinant plasmid was grown in LB kanamycin medium at 37°C (200 rpm) until the
48 culture reached OD₆₀₀ of 0.6, at which point induction with 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was
49 conducted. The bacterial cells were grown at 25°C (200 rpm) for a further 8 h and were then harvested by centrifugation
50 at 13,000 rpm for 10 min at 4°C. The cells were resuspended in Phosphate Buffer Saline (pH 7.0), and then disrupted by
51 ultrasonication (Bioruptor; Cosmobio, Korea). Soluble and precipitate (cell debris and inclusion bodies) fractions were

1 separated by centrifugation at 21,600× g for 15 min at 4 °C. The insoluble pellet was washed twice in 1% Triton X-100
2 and resuspended in 10 mM TE buffer (pH 7.6) (precipitate fraction). The expression of *bgl3KP* and *bglSM* genes was
3 confirmed by SDS-PAGE (Elpisbio, Korea) and EZ-Gel staining solution (Daeillab, Korea).

4 2.4. Activity confirmation of the recombinant β -glucosidase

5 The activity of the recombinant β -glucosidase Bgl3KP and BglSM was tested using X-Glc assay (An et al., 2012). *E.*
6 *coli* BL21(DE3) harbouring pRSF-bgl3KP (Bgl3KP strain) and pRSF-bglSM (BglSM strain) were inoculated on LB agar
7 plate supplemented with 50 mg/ml kanamycin, 0.1 M IPTG, and 50 mg/ml X-Glc and incubated at 37°C for 18 h. The
8 β -glucosidase activity was indicated by the presence of blue coloured colonies. While, the specific activity of the
9 recombinant β -glucosidase was confirmed using *p*-nitrophenyl- β -D-glucopyranoside (*p*NP β Glc) as surrogate substrate
10 (Cui et al., 2019; Gouripur and Kaliwal, 2017). Twenty-five mg of *E. coli* bgl3KP and bglSM strains were harvested
11 after 8 h induction at 25°C using 0.1 mM IPTG, resuspended in 500 μ l Phosphate Buffer Saline (pH 7.0) and disrupted
12 by ultrasonication (Bioruptor; Cosmobio, Korea). The cell lysate was then mixed with *p*NP β Glc and incubated at 37°C
13 for 1 hour until yellow colour appeared, indicating the release of *p*-nitrophenol.

14 2.5. Ginsenosides biotransformation assay

15 The biotransformation ability of recombinant bgl3KP and bglSM was evaluated using ginsenoside Rb1 as a
16 substrate. Twenty-five mg of *E. coli* Bgl3KP and BglSM strains were harvested after 8 h induction at 25°C using 0.1 mM
17 IPTG, resuspended in 500 μ l Phosphate Buffer Saline (pH 7.0) and disrupted by ultrasonication (Bioruptor; Cosmobio,
18 Korea). One hundred μ l of the cell lysate were reacted with an equal volume of 0.1% (wt/vol) in 50 mM of sodium
19 phosphate buffer (pH 6.0) at 37°C for 48 hours. An equal volume of water-saturated *n*-butanol was added to stop the
20 reaction, and the reactant present in the *n*-butanol fraction was analysed by thin-layer chromatography (TLC) (Park et
21 al., 2014).

22 TLC was performed using 60 F254 silica gel plates (Merck, Germany) with CHCl₃-CH₃OH-H₂O (65:35:10,
23 vol/vol/vol, lower phase) in the solvent system. Spots detection on the TLC plate was conducted by spraying with 10%
24 (vol/vol) H₂SO₄, followed by heating at 110°C for 5 min.
25

26 3. Results and Discussion

27 3.1. Cloning, expression, and purification of recombinant Bgl3KP and BglSM

28 The β -glucosidase genes *bgl3KP* and *bglSM* both were 1,410 and 1,386 bp in lengths, encoding proteins of 469 and
29 461 amino acids with molecular masses of 54.7 and 52.8 kDa (Figure 2) and theoretical pI values of 5.67 and 5.95
30 (http://web.expasy.org/compute_pi/), respectively. Bgl3KP and BglSM both belong to the glycoside hydrolase family 1
31 (GH1). Analysis of the amino acid sequences of Bgl3KP indicated that it was most similar (99.6%) to the glycoside
32 hydrolase family 1 protein of *Bacillus* sp. (GenBank accession no. WP_000671629.1), while BglSM showed high
33 similarity (99.6%) to the glycoside hydrolase family 1 protein of *Serratia marcescens* (GenBank accession no.
34 WP_110146706.1).

35 To determine the evolutionary position of Bgl3KP and BglSM within the enzymes in GH family 1 with reported
36 ginsenoside biotransformation activity (Cui et al., 2013; Park et al., 2014), neighbor joining phylogenetic tree was
37 constructed using the MEGA X program (Kumar et al., 2018). Bootstrap value of 1000 was used, and the resulting tree
38 is presented in Figure 1. Bgl3KP and BglSM are clustered with β -glucosidases from *Sphingomonas* sp. 2F2 and
39 *Pyrococcus furiosus* DSM 3638 (Oh et al., 2014; Wang et al., 2011). BglSM especially formed a separate, well-
40 supported
41 clade (bootstrap of 100) with β -glucosidases of *Sphingomonas* sp. 2F2, indicating that both enzymes showed high
42 amino acid sequences similarities.
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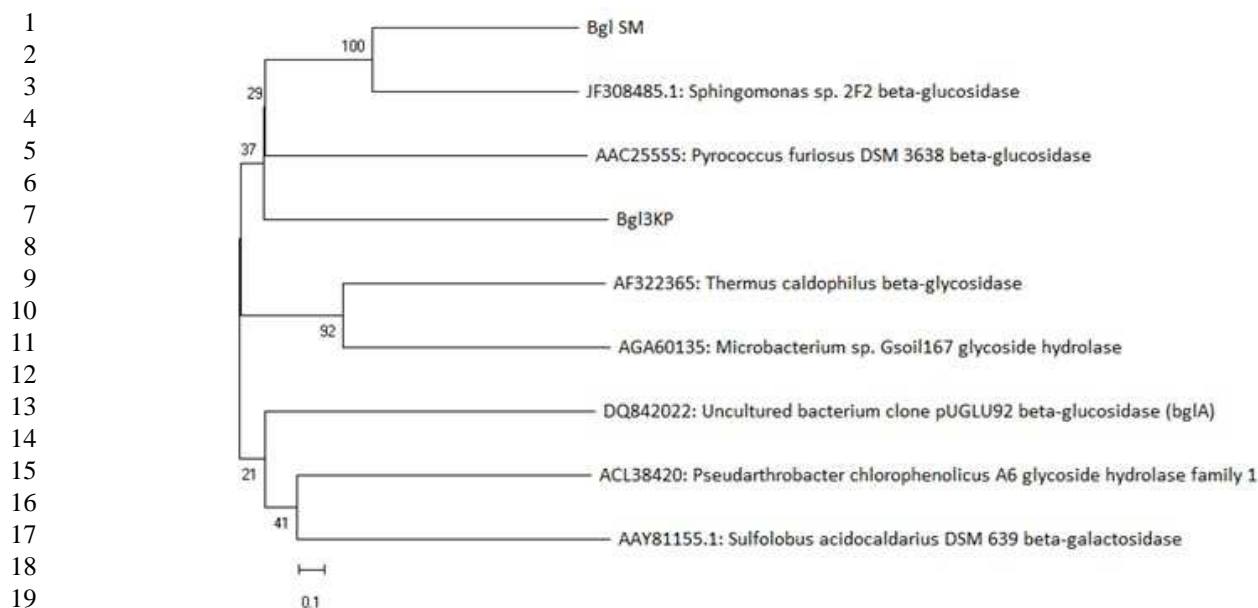


Fig. 1. Phylogenetic analysis of Bgl3KP and BglSM and other ginsenoside-hydrolysing glycoside hydrolase family 1 (GH1) enzymes with reported ginsenoside-biotransforming activity. Amino acid sequences were obtained from the NCBI database with accession numbers as indicated in the tree. This tree was made using the neighbour-joining method with a Poisson model and pairwise deletion (Du et al., 2014). Bootstrap values are expressed as percentages of 1000 replications where greater than 65% values are shown at the branch points. The bar represents 10 amino acid residue substitutions per 100 amino acid residues.

Bgl3KP and BglSM were successfully expressed in *E. coli* BL21(DE3) after 8 hours at 25°C induction using 0.1 M IPTG. Based on the SDS PAGE result, most of the β -glucosidase were in soluble fraction (Figure 2), thus the crude cell extracts were expected to exhibit enzymatic activities.

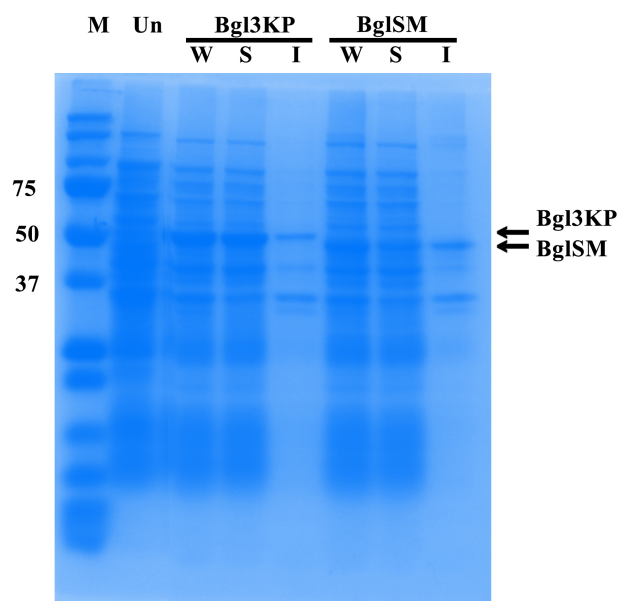
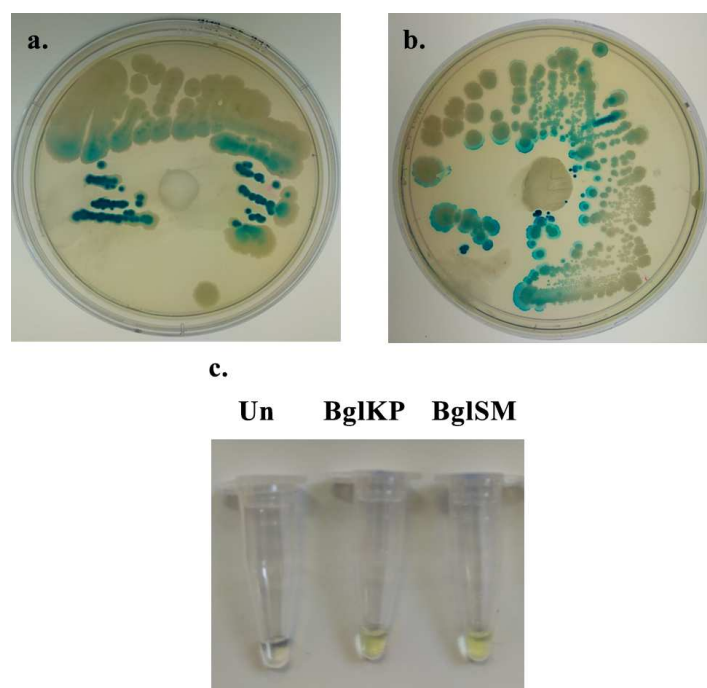


Fig. 2. SDS-PAGE analysis of Bgl3KP and BglSM expression: lane M, protein marker with bands corresponding to proteins with size of 75, 50, and 37 kDa marked (Biorad, USA); lane Un, cell lysate of Bgl3KP strain growth for 8 h at 25°C without IPTG induction; lane Bgl3KP/BglSM W, cell lysate of Bgl3KP / BglSM strain induced with IPTG for 8h at 25°C; S, soluble fraction of cell lysate of Bgl3KP / BglSM strain induced with IPTG for 8h at 25°C; I, precipitated fraction of cell lysate of Bgl3KP / BglSM strain induced with IPTG for 8h at 25°C.

3.2. Activity of the recombinant β -glucosidases in crude cell extract

The activity of expressed β -glucosidases was confirmed using X-Glc. The presence of blue colonies indicated that the expressed Bgl3KP and BglSM were active (Figure 3a and 3b). The activity was further confirmed using pNP β Glc assay. Hydrolysis of pNP β Glc by crude cells extract of *E. coli* strain Bgl3KP and BglSM induced with IPTG for 8 h at

1 25°C was observed after 1-hour incubation (Figure 3c). The results suggest that both enzymes show potential to catalyse
 2 the biotransformation of major ginsenoside.

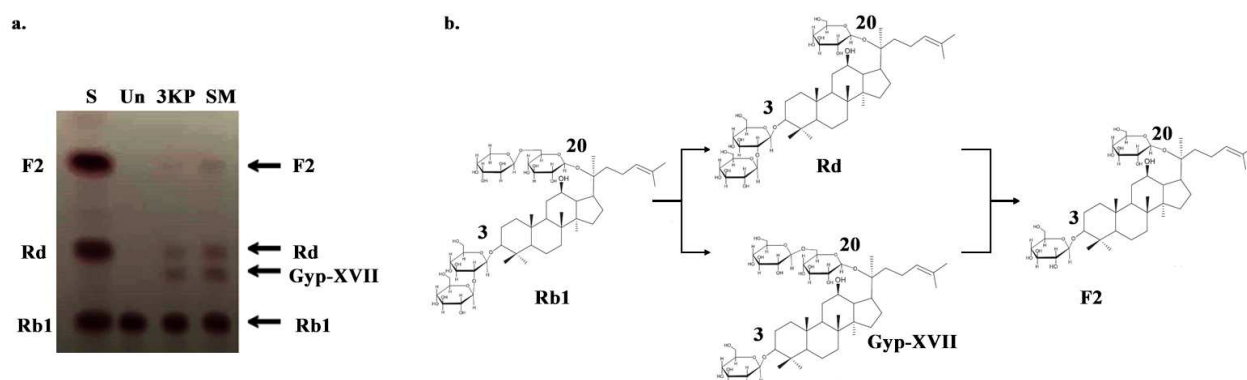


5 Fig. 3. Blue colonies of *E. coli* strain Bgl3KP (a) and BglSM (b) on LB + IPTG + X-Glc plate after 18 hours incubation at 37°C.

6 *pNPβGlc* hydrolysis was observed in the presence of crude cell extract of Bgl3KP and BglSM strains induced with 0.1 M IPTG for 8 hours (c). The
 7 cell lysate of uninduced Bgl3KP strain was used as control (Un).
 8

9 3.3. Biotransformation of ginsenosides

10 Rb1 biotransformation activity of Bgl3KP and BglSM from crude cell extract were confirmed using TLC analysis.
 11 After 48 hours incubation, spots corresponding to ginsenosides Rd and another spot below the spot corresponding to
 12 ginsenoside Rd, which based on previous reports was predicted to be Gyp-XVII (An et al., 2010; Wang et al., 2011)
 13 were observed, indicating that the two β -glucosidases were able to catalyze the hydrolysis of outer glucose moieties at
 14 the 3 and 20 position on Rb1 (Figure 4a and 4b). These results are amenable with previous reports where GH family 1
 15 β -glucosidases cloned from *Sulfolobus solfataricus*, *Sulfolobus acidocaldarius*, *Pyrococcus furiosus*, and *Arthrobacter*
 16 *chlorophenolicus* (Noh et al., 2009; Noh and Oh, 2009; Park et al., 2014; Yoo et al., 2011). However, only BglSM
 17 showed significant biotransformation of Rd and Gyp-XVII to F2 (Figure 4b). Interestingly, BglSM showed similar
 18 ginsenoside transformation activity with as β -glucosidases from *Sphingomonas* sp. 2F2 which belong to the same clade
 19 in the constructed neighbour joining tree (Wang et al., 2011) (Figure 1).



22 Fig. 4. (a) Thin layer chromatography (TLC) analyses of biotransformation of ginsenosides Rb1. Lane S, Ginsenoside standards (Zelang Medical Technology Co., Ltd., China); Lane Un, biotransformation of Rb1 by cell lysate of Bgl3KP strain growth for 8 h at 25°C without IPTG induction;

1 Lane 3KP / SM, biotransformation of Rb1 by soluble fraction of cell lysate of IPTG induced Bgl3KP/ BglSM strain.

2 (b) The proposed conversion pathway of ginsenoside Rb1 using crude cell extract of induced Bgl3KP and BglSM strains.

3 Ginsenoside Gyp-XVII and F2 have been reported to show beneficial health effects. Gyp-XVII has been reported
4 for its cardiovascular protective effect by alleviating atherosclerosis via the ER α -mediated PI3K/Akt pathway and
5 neuroprotective protective effect against Alzheimer's disease by activating transcription factor EB (Meng et al., 2016;
6 Yang et al., 2017), while F2 has been reported for its anti-cancer activity which mediated through inhibition of
7 proliferation judged by Ki67 and apoptosis induced by activation of caspase-3 and -8 in glioblastoma multiforme, as
8 well as anti-inflammatory effects (Mai et al., 2012; Park et al., 2016; Shin et al., 2012). Therefore, Bgl3KP and BglSM
9 has potential application in the pharmaceutical industry.

10 This report is one of the first endeavors on exploring the potency of bacterial isolates from outside East Asia
11 especially from Indonesia as sources for ginsenoside-biotransforming β -glucosidase. As one of the mega-biodiversity
12 countries with various unique ecosystems such as hot springs, acidic crater lakes, and peatlands, Indonesia could
13 provide the sources for β -glucosidase enzymes with unique characteristic which are useful for efficient ginsenosides
14 biotransformation at industrial scale (i.e. thermostability and tolerance to low pH) (Geraldi et al, 2019). Thus, further
15 exploration of β -glucosidases producing microbes in Indonesia would to be conducted.

16 4. Conclusion

17 Genes encoding recombinant ginsenoside-hydrolysing β -glycosidases, Bgl3KP and BglSM, belonging to the GH1
18 family, were cloned from *Bacillus* sp. 3KP and *Serratia marcescens* LII61, respectively. These enzymes were expressed
19 in *E. coli* BL21(DE3) in a soluble form and could convert major ginsenoside Rb1 into pharmacologically active minor
20 ginsenoside F2 via Rd and Gyp-XVII. To our knowledge this is the first report on using β -glucosidases of bacterial
21 isolates from Indonesia for ginsenoside biotransformation. This report is expected to encourage further exploration of
22 ginsenoside transforming- β -glucosidases, especially those with industrial potential, in Indonesia.

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