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Improvement of isoflavone aglycones production using β-glucosidase secretory produced in recombinant *Aspergillus oryzae*

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ABSTRACT

β-Glucosidase (BGL1) from *Aspergillus oryzae* was efficiently produced in recombinant *A. oryzae* using *sodM* promoter-mediated expression system. The yield of BGL1 was 960 mg/l in liquid culture, which is 20-fold higher than the yield of BGL1 produced using the yeast *Saccharomyces cerevisiae*. Recombinant BGL1 converted isoflavone glycosides into isoflavone aglycones more efficiently than β-glucosidase from almond. In addition, BGL1 produced isoflavone aglycones even in the presence of the insoluble form of isoflavone glycosides.

1. Introduction

Isoflavones have a structural and functional similarity to human estrogen, and may provide protection from cardiovascular disease, osteoporosis, and reduce the risk of cancers and post-menopausal symptoms [1–5]. Isoflavone glycosides such as daidzin and genistin are abundant in soybeans; however, for effective absorption of isoflavones into the human body, glycosidic forms of isoflavones need to be converted to isoflavone aglycones such as daidzein and genistein [6].

β-Glucosidases (β-D-glucoside glucohydrolase; EC 3.2.1.21; BGL) play an important role in the hydrolysis of glucosides of isoflavones, and they have been utilized in isoflavone aglycone production. According to previous reports, β-glucosidases from *Saccharopolyspora erythraea* can hydrolyze genistin during fermentation of soy-based media [7], and β-glucosidases from *Bifidobacteria* in soy milk are capable of converting glucosides to their aglycones [8]. Pandjaitan et al. [9] treated soy protein isolate with almond β-glucosidases to convert most of its isoflavone glucosides to their respective aglycones.

Recently, we identified several kinds of genes encoding β-glucosidase (*BGL1*, *BGL3*, and *BGL5*) in *Aspergillus oryzae*. These *BGLs* were cloned and expressed using *Saccharomyces cerevisiae* in cell surface displayed and secreted forms [10,11]. These displayed or secreted BGLs are able to hydrolyze isoflavone glycosides and produce isoflavone aglycones efficiently. Compared to BGL3 or BGL5, BGL1 hydrolyzes isoflavone glycosides rapidly and achieves high-yield aglycone production. Although the displayed BGL1 is advantageous, the secreted BGL1 is regard in industrial application. However, the amount of secreted BGLs produced by using yeast as a host is too small for industrial production of isoflavone aglycones.

Here, we demonstrate that a large amount of BGL can be produced by recombinant *A. oryzae*, and that the enzyme is highly effective in producing isoflavone aglycones. *A. oryzae* has two advantages as a host of protein production. One is that it is capable of secreting large amounts of proteins with post-translational modifications [12,13]. The other is that it has a "generally regarded as safe" (GRAS) status and has been used for a long time in the food industry. To produce BGL1 from *A. oryzae* efficiently, we utilized the *sodM* promoter-mediated expression system [14]. The enzymatic

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activity and stability of recombinant BGL were investigated and the reaction conditions were optimized. In addition, we demonstrate that isoflavone aglycones production of BGL1 is superior to that of β-glucosidase from almond. We succeeded in efficient production of isoflavone aglycones by using recombinant BGL1.

2. Experimental

2.1. Bacterial strains and media

Escherichia coli, NovaBlue (Novagen, Inc., Madison, WI, USA), was used as the cloning host for recombinant DNA manipulations, and was grown in Luria-Bertani (LB) medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl] containing 0.1 mg/ml ampicillin. The *A. oryzae niaD* mutant (strain IF4), derived from wild-type *A. oryzae* OSI1031, isolated in Research Institute, Gekkeikan Sake Co., was used as the expression host [15].

Czapek-Dox (CD) medium plates [2% (w/v) glucose, 0.3% (w/v) $NaNO_2$ (CD-NO_2) or $NaNO_3$ (CD-NO_3), 0.2% (w/v) KCl, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4{\cdot}7H_2O$, 0.002% (w/v) $FeSO_4{\cdot}7H_2O$, and 0.8 M NaCl, pH 6.0] containing 1.5% (w/v) agar were used as the minimal medium. CD-NO_2 medium plates were used to preserve the *A. oryzae niaD* mutants. CD-NO_3 medium plates were used to select the fungal transformants. GPY medium [3% (w/v) glucose, 0.2% (w/v) KCl, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4{\cdot}7H_2O$, 1% (w/v) peptone, and 0.5% (w/v) yeast extract, pH 6.0] was used for cultivation of *A. oryzae niaD* mutant and transformants.

2.2. Plasmid construction and A. oryzae transformation

PCR was carried out using KOD plus DNA polymerase (TOYOBO, Osaka, Japan) according to the manufacture's procedure. The gene fragment encoding BGL was amplified from the *A. oryzae* genome with the following primers: 5′-GGCGCGCCATGAAGCTGTC-AGCGGCACTTTC-3′ and 5′-ATTTAAATCTATAAGCTCAATGATCCGGTC-3′. The amplified fragments were digested with *Asc*I/*Swa*I and subcloned into pISI [15], which contained the *sodM* promoter and *glaB* terminator from *A. oryzae* [14]. The resulting plasmid was abbreviated as pISI-BGL1 (Fig. 1). The DNA sequences of amplified fragments were confirmed by DNA sequencing (ABI PRISM 3100, Applied Biosystems, Tokyo, Japan).

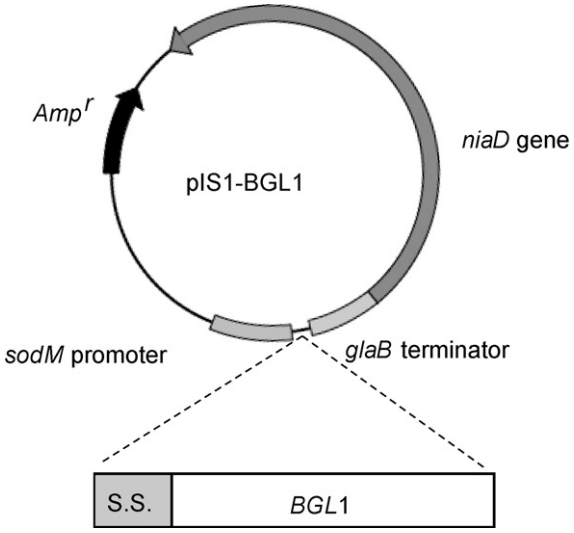

Fig. 1. Expression plasmid for secretion of BGL1.
The *niaD* gene encoding nitrate reductase of *A. oryzae* was used as a selectable marker gene. The *BGL1* gene was inserted into the *Asc*I–*Swa*I site between the *sodM* promoter and the termination region of the *A. oryzae* glucoamylase gene, *glaB*. S.S.: secretion signal sequence of *A. oryzae BGL1* gene.

The plasmid pISI-BGL1 was introduced into *A. oryzae niaD* mutants using the protoplast-polyethyleneglycol method [16]. The obtained transformants were subcultured on CD-NO_3 medium plate three times. According to our previous experiments, the copy number of the expression vectors integrated into genome was assumed to be one [15,17].

2.3. Production of BGL1

The transformants harboring plasmid pISI-BGL1 were cultivated in 100 ml of GPY medium at 30 °C for 14 d and the mycelia were removed by filtration through a Myracloth (Calbiochem, La Jolla, CA, USA). After cultivation for 4 d, the supernatant was analyzed by 12% SDS-PAGE stained with Coomassie brilliant blue R-250. The protein concentration in the supernatant of the cultured media was determined with the Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using bovine serum albumin (BSA) as the standard. The enzymatic deglycosylation of the recombinant protein was carried out using a peptide N-glycosidase F (PNGase F, New England BioLabs, Inc., Ipswich, MA, USA) and Endo Hf (New England BioLabs) according to the manufacture's procedure. After enzymatic treatment, the samples were analyzed by SDS-PAGE and stained with Coomassie brilliant blue R-250.

2.4. Characterization of enzymatic activity and stability

About 80 ml of culture media after 4 d cultivation was filtered through a 0.45 μm polyvinylidene difluoride (PVDF) filter unit (Millipore, Bedford, MA, USA) to remove the insoluble materials, and the filtered media was concentrated using a centrifugal filter device (Millipore) with 50,000 nominal molecular weight limit. To remove the medium component, 10 ml water was added to 1 ml of concentrated culture media including recombinant BGL1 and then concentrated as described above, with the procedure repeated twice. The optimal pH for purified BGL1 was assayed by measuring BGL activity with 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPβG, Nacalai Tesque, Inc., Kyoto, Japan) as the substrate in a pH range from 2.0–8.0. To adjust pH, the following buffers were used: 50 mM glycine–HCl for pH 2.0–3.0, 50 mM acetate for 4.0–5.0, and 50 mM potassium phosphate for pH 6.0–8.0. The stability of BGL1 was determined by pre-incubating the BGL1 at various pH at 30 °C for 17 h. Then remaining BGL1 activity was assayed using *p*NPβG as a substrate. The optimal temperature of BGL1 was also determined using *p*NPβG as a substrate at pH 5.0. The thermostability of the enzyme was determined by incubating the enzyme at various temperatures for 4 h. Then remaining activity was measured using *p*NPβG as a substrate. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the production of 1 μl of *p*-nitrophenol/min.

2.5. Production of isoflavone aglycones

Production of isoflavone aglycones was performed in 50 mM sodium acetate buffer (pH 5.0) containing β-glucosidase (5 U/l or 50 U/l) and 0.045% (w/v) or 0.5% (w/v) of Fujiflavone P40 (Fujicco, Kobe, Japan), which contains 35% (w/w) isoflavones, at 40 °C for 1–5 h. After incubation, the mixture was treated at 90 °C for 10 min to deactivate BGL1. To extract isoflavone aglycones, 99.5% (v/v) ethanol was added to the 1 ml mixture up to 10 ml. In the case of 0.5% (w/v) of Fujiflavone P40 use, 70% (v/v) ethanol was added to the 1 ml mixture up to 50 ml. In a similar way, β-glucosidase from almond (Sigma, St. Louis, MO, USA) was examined. A portion of the sample was filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filter unit (Millipore) and analyzed by HPLC.

2.6. HPLC analysis

For HPLC analysis of isoflavones, a UV detector (CDD10A; Shimadzu, Kyoto, Japan) at 254 nm, and an YMC-pack ODS-AM-304 column (5 μm, 30 cm × 4.6 mm; YMC, Kyoto, Japan) were used. The HPLC conditions were as follows: the column oven temperature was 40 °C; the flow rate was 0.5 ml/min; and the concentration of the mobile phase increased from 0% (0 min) to 100% (70 min) with a linear gradient of solvent A acetonitrile:water:acetic acid mixture (15:85:0.1 (v/v/v)) and solvent B acetonitrile:water:acetic acid mixture (35:65:0.1 (v/v/v)).

3. Results and discussion

3.1. Expression of BGL1

To enhance the production of BGL1 in *A. oryzae*, we constructed a *sodM*-mediated BGL1 overexpression system. The *sodM* promoter is significantly strong and suitable for large amounts of protein expression [14]. The nucleotide sequence of secretion signal was the original sequence of *A. oryzae*. After 4 d of cultivation, the supernatant was directly analyzed by SDS-PAGE, and there was only one major band corresponded to BGL1. It was suggested that the target protein in the culture media of recombinant *A. oryzae* was of high purity. The molecular mass was estimated to be 120 kDa (Fig. 2, lane 1), which is higher than that of the predicted molecular mass of BGL1 (87 kDa). We assumed that the higher molecular mass was caused by glycosylation, so therefore the supernatant was treated with PNGaseF or Endo Hf to deglycosylate the BGL1. PNGase F and Endo Hf are two of the amidases that cleave between the innermost GlcNAc and the asparagine residue of complex N-linked oligosaccharides from glycoproteins. After treatment with PNGaseF and Endo Hf, the band shifted to 87 kDa (Fig. 2, lanes 2 and 3), which corresponds to the molecular mass estimated from the BGL1 amino acid sequence. BGL1 produced by recombinant *A. oryzae* were smaller than that produced by recombinant *S. cerevisiae* (approximately 160 kDa) (data not shown). The discrepancies between the molecular masses of BGL1 produced by *A. oryzae* and *S. cerevisiae* were caused by the difference of the mode of glycosylation. This result is consistent with the previous report, showing that recombinant *Trichoderma reesei* cellulases, including β-glucosidase, produced by *A. oryzae* were smaller than those of recombinant cellulases produced by *S. cerevisiae* [18].

Then we investigated the time-course of BGL1 production in the supernatant. As shown in Fig. 3, when BGLl was produced using the *sodM*-mediated expression system, the amount of secreted BGL1 significantly increased with culture time and that the highest yield was 960 mg/l at 4 d of cultivation. The enzymatic activity of the culture supernatant was 240 U/ml after 4 d cultivation. It was

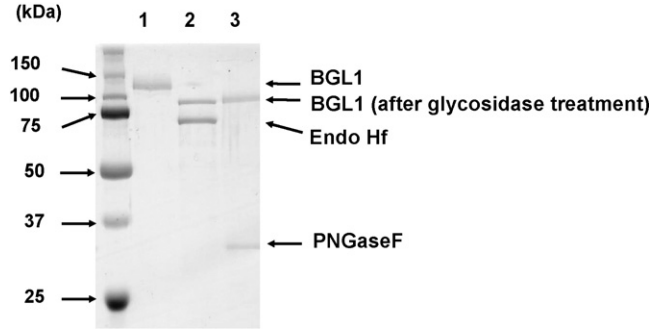

Fig. 2. SDS-PAGE analysis of BGL1 production by *A. oryzae*. Lane 1, culture media (after 4 d); lane 2, culture media treated with Endo Hf; lane 3, culture media treated with PNGase F.

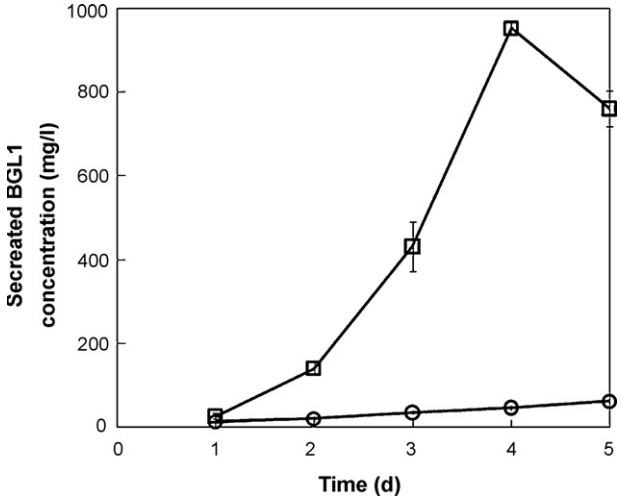

Fig. 3. Time-course of BGL1 concentration in culture media, secreted by *A. oryzae niaD*⁻ transformed with pISI-BGL1 (open squares) and intact *A. oryzae* (open circles). Each value is the mean of three different experiments, and error bars indicate standard deviation.

approximately 27,500-fold higher than BGL activity produced by intact *A. oryzae* (0.009 U/ml) and was also 180-fold higher than BGLl activity produced by *S. cerevisiae* (1.3 U/ml) [11]. These results show that *A. oryzae* can produce large amounts of BGL while maintaining its activity, suggesting that *A. oryzae* is an appropriate host for large-scale production of BGL1.

3.2. Characterization of BGL1

The specific activity of BGL1 was estimated to be 250 U/mg, and it was comparable to that of other β-glucosidase (β-glucosidase from *E. coli*, 300 U/mg; β-glucosidase from almond, 3542 U/mg [19]). It was assumed that BGL1 produced by *A. oryzae* was well functioned as well as native β-glucosidase. Next, to characterize the properties of BGL1, the effects of pH (2.0–8.0) on BGL1 activity were investigated. Using *p*NPG as a substrate, the enzymatic reaction was carried out in various pH range buffers at 30 °C. Fig. 4A shows that the optimal pH for BGL1 was 4.0–5.0. In higher or lower pH conditions, the enzymatic activity significantly decreased. We also investigated the stability of BGL1 in various pH buffers. BGL1 was pre-incubated at various pH (2.0–8.0) at 30 °C for 17 h, then the remaining activity was measured using *p*NPG as a substrate. Fig. 4B shows that under pH 5–7, more than 90% of BGL1 activity remained. However, under pH 4, only about 70% of BGL1 activity remained.

Next, the effect of temperature on BGL1 activity was investigated. The enzymatic reaction was carried out at various temperatures at pH 5.0 using *p*NPG as a substrate. Fig. 4C shows that the optimal temperature for BGL1 was 50 °C, while at higher temperatures the enzymatic activity significantly decreased. We also investigated the stability of BGL1 at various temperatures. BGL1 was pre-incubated at pH 5.0 at several different temperatures for 4 h. Then the residual activity of BGL1 was measured using *p*NPG as a substrate at 30 °C. Fig. 4D shows that at 30–40 °C, the BGL1 activity was almost completely retained, and at higher temperatures, enzymatic stability significantly decreased. These results correspond to a previous report [11] suggesting that *A. oryzae* can produce active BGL1 as well as yeast.

3.3. Isoflavone aglycones production

The time-course of isoflavone aglycones production from 0.045% (w/v) Fujiflavone P40 was measured with 5 U/l of BGL1 at pH 5.0 at

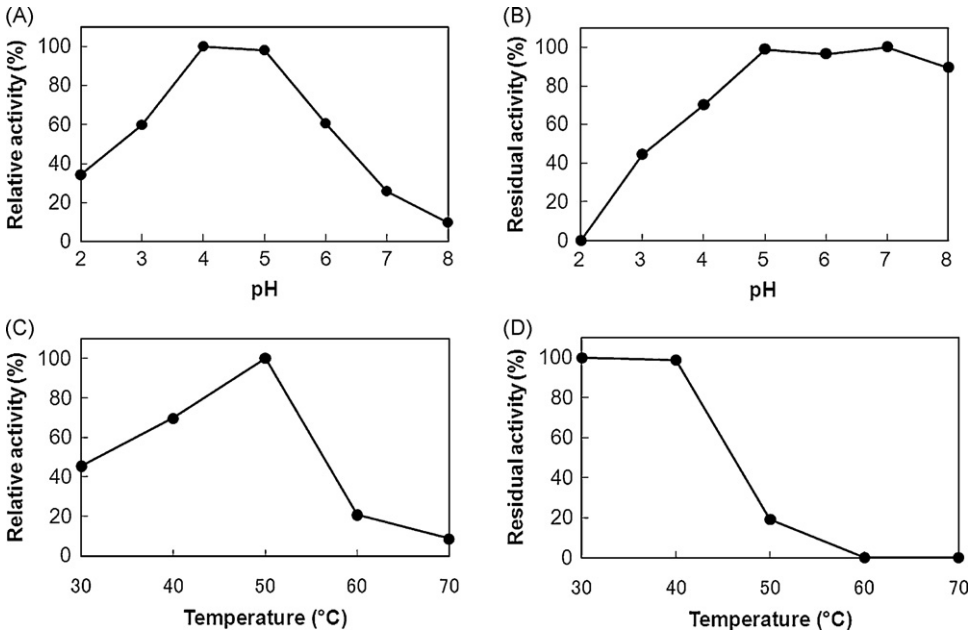

Fig. 4. Enzyme activity and stability of BGL1. Effect of pH on the activity (A) and stability (B) of BGL1. Effect of temperature on the activity (C) and stability (D) of BGL1. Each value is the mean of three different experiments, and the standard deviation was less than 5%.

40 °C for 5 h. β-Glucosidase from almond, which is widely utilized to convert isoflavone glucosides into isoflavone aglycones [9], was examined in a similar way. Isoflavone glucosides were digested by BGL1 and isoflavone aglycones (daidzein, genistein, and glycitein) were successfully produced. The yield of daidzein after 3 h and 5 h reactions was 90% and 94%, respectively. The residual ratio of daidzin, genistin, and glycitin after the 5 h reaction was 1.0%, 1.8%, and 2.5%, respectively (Fig. 5A). In the case of β-glucosidase from almond, the yield of daidzein after 3 h and 5 h reactions was 67% and 78%, respectively. The residual ratio of daidzin, genistin, and glycitin after the 5 h reaction was 17%, 62%, and 32%, respectively (Fig. 5B). These results show that the yield of isoflavone aglycones by BGL1

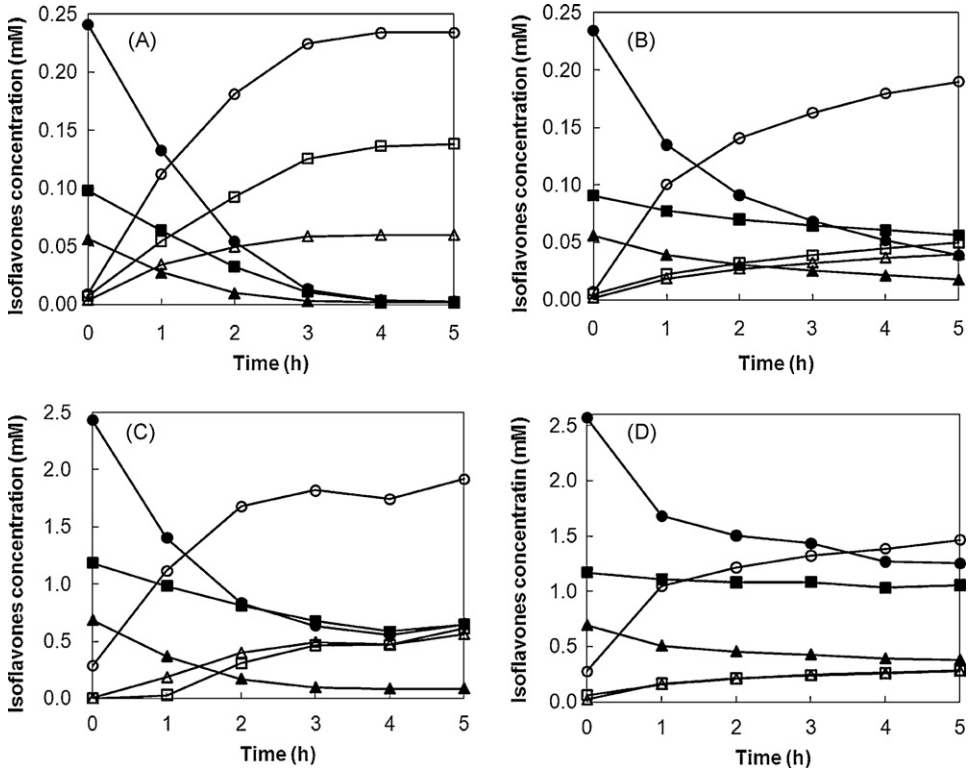

Fig. 5. Time-course of isoflavone aglycones production. Isoflavone aglycones production of (A) BGL1 with 0.045% (w/v) Fujiflavone P40, (B) β-glucosidase from almond with 0.045% (w/v) Fujiflavone P40, (C) BGL1 with 0.5% (w/v) Fujiflavone P40 and (D) β-glucosidase from almond with 0.5% (w/v) Fujiflavone P40. Symbols for isoflavones: closed circles, daizin; open circles, daidzein; closed squares, genistin; open squares, genistein; closed triangles, glycitin; open triangles, glycitein. Each value is the mean of three different experiments, and the standard deviation was less than 5%.

in this system was superior to that of β-glucosidase from almond. BGL1 rapidly hydrolyzed isoflavone glucosides and attained high yields of isoflavone aglycones.

In industrial production of isoflavones, low solubility of isoflavones reduces the productivity of isoflavone aglycones. To test the reactivity of BGL1 with high concentrations of isoflavone, the time-course of isoflavone aglycones production from 0.5% (w/v) Fujiflavone P40 was measured with 50 U/l of BGL1. In this condition, almost all isoflavone was insoluble. After the reaction, approximately 70% of isoflavone glucosides were converted into isoflavone aglycones, and the residual ratio of daidzin, genistin, and glycitin was 27%, 55%, and 12%, respectively (Fig. 5C). After 5 h, the hydrolysis reaction proceeded only slightly, which may have been caused by glucose inhibition [20,21]. In the case of β-glucosidase from almond, only 50% of isoflavone glucosides were converted into isoflavone aglycones, and the residual ratio of daidzin, genistin, and glycitin was 49%, 90%, and 55%, respectively (Fig. 5D). From β-glucosidase from almond, the amount of residual isoflavone was higher than that from isoflavone using BGL1 from *A. oryzae*, which indicates that the effect of glucose inhibition on β-glucosidase from almond was significantly higher than that of BGL1 from *A. oryzae*. These results clearly show that BGL1 from *A. oryzae* can efficiently convert isoflavone glucosides into isoflavone aglycones under insoluble conditions of isoflavone glucosides.

4. Conclusion

In this work, we succeeded in efficiently producing *A. oryzae* BGL1 in *A. oryzae niaD* mutant using the *sodM* promoter. The highest secretion level of recombinant BGL1 was 960 mg/l, which is a quantity sufficient for commercial application. Recombinant BGL1 hydrolyzed high concentrations of soy isoflavone glucosides efficiently and is thus suitable for isoflavone aglycones production.

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