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Effects of intron retention on properties of β-glucosidase in *Aspergillus niger*

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ABSTRACT

Intron retention, one of the major types of alternative splicing in plants and animals, has also been reported existing in filamentous fungi's glycoside hydrolases. In this study, an intron-retained β-glucosidase gene transcript (bgl1B) from *A. niger* B2 strain was obtained. Compared with the normally spliced transcript bgl1A, bgl1B had an extra 51bp insertion, which was confirmed to be the sixth (the last) intron of this β-glucosidase gene. The bgl1A and bgl1B were expressed in *Pichia pastoris* and the purified enzymes were used to compare their catalytic properties. The results showed that the intron retention didn't impair the catalytic function. Instead, the intron-retained enzyme BGL1B had a better thermostability with a higher optimal temperature and a longer half-life under 50 °C. Also it exhibited a little higher k_{cat} for 4-nitrophenyl-β-D-glucopyranoside (PNPG) and a noticeable higher hydrolysis efficiency towards geniposide. This work suggested that the β-glucosidase gene in *A. niger* most likely underwent an alternative splicing presented as intron retention type, and intron retention might be a source of enzyme diversity in fungi.

1. Introduction

Alternative splicing is a post-transcriptional regulatory mechanism for gene expression commonly existing in eukaryotic organisms, by which one gene can produce multiple proteins. Of the different classes of alternative splicing, intron retention (IR), the process in which specific introns remains unspliced in precursor RNAs, is one of the major types of alternative splicing (Nilsen and Graveley, 2010; Wang et al., 2008).

Although IR is frequently proved to participate in the modulation of important development events in plants and animals (Marquez et al., 2012; Braunschweig et al., 2014), in lower eukaryotes filamentous fungi, the functional involvement of IR is not well investigated. It was reported that IR existed in two exocellobiohydrolase I-like genes in *Phanerochaete chrysosporium*, both resulting in a changed carbohydrate binding domain, and that these IR events occurred with Avicel as a substrate, while with carboxymethyl cellulose or cellobiose, only fully spliced mRNA transcripts were formed, hinting that alternative splicing by IR might be a way for this fungus to regulate the enzyme's substrate-binding specificities (Birch et al., 1995). In the genus *Phytophthora* family 5 endoglucanase genes, zero to three introns were observed to retain in the processed RNA transcripts, yet changing the growing conditions didn't change the relative proportions of partially processed transcripts observed with *P. sojae* (Costanzo et al., 2007).

Recently, RNA-Seq technique allows for a comprehensive analysis of alternative splicing on a genome-wide level. Based on RNA-Seq data of seven human-pathogenic fungi under response-to-host conditions or stress conditions, Sieber et al. (2018) proved that IR was the most occurring pattern among the 6 known alternative splicing patterns in fungi, and proposed that the higher rate of IR took place as a response to host conditions. Also, specific genes with IR, such as superoxide dismutase *SOD3* in *Candida albicans*, were identified to be related with response-to-host and/or stress conditions. Using the same method, Xu et al. (2016) revealed that the genes encoding fumarase and malate dehydrogenase, the key enzymes for fumaric acid production in *Rhizopus oryzae*, underwent IR events with glucose medium, whereas no IR events with these genes were detected with xylose medium, which was expected to account for, to some extent, the higher fumaric acid yield on glucose medium. Despite these valuable investigations on fungal IR events, few of intron-containing gene products were characterized in comparison with their fully spliced counterparts.

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β-Glucosidase (E.C. 3.2.1.21) catalyzes the hydrolysis of the β-1,4-linked glycosidic bonds in beta-D-glucosides and oligosaccharides, with release of glucose. As an indispensable component of cellulase complex, β-glucosidase has been widely used in lignocellulose and cellodextrin hydrolysis for purpose of biofuel productions (Fan et al., 2012; Galazka et al., 2010; Singhania et al., 2013). Also, it is of great importance in transformation of beta-D-glucoside compounds to their corresponding aglycones, such as genipin (Gong et al., 2014), resveratrol (Chen et al., 2014), and minor ginsenosides (Huq et al., 2016), all of which were proved to be valuable pharmacologically active products.

In this study, an intron-retained β-glucosidase gene transcript (bgl1B) from *Aspergillus niger* B2 strain was accidentally obtained and the catalytic properties of this β-glucosidase were compared with those of the normally spliced enzyme, aiming to illustrate the effect of intron retention on β-glucosidase. Their ability to hydrolyze geniposide to genipin was also investigated.

2. Materials and methods

2.1. Strain, culture condition and total RNA extraction

The strain *A. niger* B2 was screened out in terms of its high yield of β-glucosidase production in our previous work (Yue et al., 2012). For β-glucosidase induction, the medium containing wheat bran (smashed) 10 g/l, $(NH_4)_2SO_4$ 0.4 g/l, KH_2PO_4 0.3 g/l, $MgSO_4{\cdot}7H_2O$ 0.04 g/l, and $CaCl_2$ 0.05 g/l was used. The spore suspension of *A. niger* B2 was inoculated at a proportion of 1 % (v/v) to 50 ml of the above medium in 250-ml Erlenmeyer flasks. The flasks were incubated at 30 °C and 220 rpm. After 72 h incubation, the mycelia were harvested, washed thoroughly with DEPC-treated water, frozen in liquid nitrogen, and ground to a fine powder. Total RNA was isolated from powdered mycelia using TRIzol reagent (Invitrogen) according to the supplier's instructions.

2.2. β-Glucosidase gene cloning by RT-PCR

The Superscript III kit (Invitrogen) was used for synthesis of cDNA from 1 μg total RNA using oligo (dT) 20 (Invitrogen) for poly(A) priming. The synthesized cDNA was used for PCR template. Two specific primers (5′-ATGAGGTTCACTTTGATCGAGGCG-3′ for upstream and 5′-TTAGTGAACAGTAGGCAGAGACG-3′ for downstream) were synthesized on the basis of the published family 3 β-glucosidase cDNA sequence from *A. niger* (GenBank No. AJ132386 for bgl1 gene and CAB75696.1 for the protein) (Dan et al., 2000). DNA amplification was carried out through 25 cycles of denaturation (30 s at 94 °C), annealing (30 s at 53.7 °C), and extension (165 s at 72 °C) (plus 10 min extensions). The resulting PCR product was ligated into pMD19-T vector (Invitrogen) and subjected to DNA sequencing (Genewiz, Suzhou, China).

2.3. Transformation of Pichia pastoris

The obtained β-glucosidase gene (bgl1) was subcloned from pMD19-T vector into pPICZα-A vector (Invitrogen) at the sites of EcoRI and XbaI, with the deletion of the first 57-bp long fragment presumably encoding a signal peptide. *E. coli* DH5α was chemically transformed with the recombinant vector and then cultured at 37 °C on LB plates supplemented with 25 μg/ml Zeocin for selection of recombinants. The nucleotide sequence of the recombinant pPICZα-A-bgl1 plasmid was verified by restriction analysis and DNA sequencing. The correct pPICZα-A-bgl1 plasmid was digested with PmeI for linearization prior to further transformation. The *P. pastoris* GS115 strain (Invitrogen) was used as a host. Eighty μl of the competent *P. pastoris* GS115 cells mixed with 5 μg linearized pPICZα-A-bgl1 were transferred into an ice-cold 0.2-cm electroporation cuvette and incubated in an ice bath for 5 min. After electroporation at 1.5 kV, 25 μF, and 200 Ω for 5 ms, 1 ml of ice-cold 1 M sorbitol was quickly added to the cuvette, which was then incubated for 1–2 h at 30 °C without shaking. Aliquots of 200 μl were spread on YPDS (1.0 % yeast extract, 2.0 % peptone, 2.0 % glucose, 1 M sorbitol, and 2.0 % agar) plates supplemented with 100 μg/ml Zeocin, and the plates were incubated at 30 °C until colonies appeared. As a negative control, linearized pPICZα-A vector alone was transferred into *P. pastoris* GS115 cells. Gradiently increased Zeocin (150 μg/ml-300 μg/ml) was used for further selection of transformants with high-copy bgl1 integration. The transformants bearing the integration of the pPICZα-A-bgl1 plasmids were identified by PCR of the genomic DNA with bgl1 subcloning primers.

2.4. Expression and purification of BGL1

A single colony of transformants was grown in 50 ml BMGY medium (1 % (w/v) yeast extract, 2 % (w/v) peptone, 1.34 % YNB, 1 % glycerol, 0.4 μg/ml biotin, buffered with 1/10 volume potassium phosphate buffer pH 6.0) in a 250-ml flask at 30 °C, 250 rpm. When OD_{600} of the culture reached about 5.0 (approximately 18–20 h), the cells were harvested by centrifuging at 3000× g for 5 min and resuspended in 50 ml of BMMY medium (1 % (w/v) yeast extract, 2 % (w/v) peptone, 1.34 % YNB, 0.5 % methanol, 0.4 μg/ml biotin, buffered with 1/10 volume potassium phosphate buffer pH 6.0) in a 250-ml flask and incubated at 30 °C with shaking for induction of bgl1 gene expression. To keep bgl1 gene expressed, methanol (100 %) was added every 24 h to a final concentration of 1 %. The culture was subject to centrifugation to collect the supernatant when the highest BGL1 activity was obtained. The supernatant was applied to a nickel-charged resin column (Takara, Shiga, Japan). The column was washed with gradual washing solution containing 50-150-300 mM imidazole. The fraction with BGL1 activity was collected and dialyzed against 20 mM Tris–HCl buffer (pH 7.4). The purity of each fraction was assessed by SDS-PAGE (Laemmli, 1970). Only those fractions showing a single band were pooled and the concentration of the resulting purified sample was determined using the method of Bradford with bovine serum albumin as a standard (Bradford, 1976).

2.5. Enzyme assay

β-Glucosidase activity was assayed by measuring *p*-nitrophenol released from 4-nitrophenyl-β-D-glucopyranoside (PNPG). A reaction mixture of 200 μl contained 50 μl appropriately diluted enzyme, 50 μl 5 mM PNPG, and 100 μl 50 mM citrate buffer (pH 4.8). The mixture was incubated for 10 min at 50 °C and then the reaction was terminated by the addition of 3.0 ml of 0.2 M Na_2CO_3. The absorbance at 400 nm was measured and the amount of *p*-nitrophenol was calculated based on *p*-nitrophenol's extinction coefficient of 17.4 $cm^{-1}mM^{-1}$. One unit of β-glucosidase was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per min.

2.6. Kinetic analysis

To determine the kinetic parameters of β-glucosidase for PNPG, the initial reaction rates at varying final concentration of PNPG (0.05–2.0 mM) were determined. The reaction mixtures as aforementioned were incubated at 50 °C for 5 min. Each result was an average of at least three repetitions. K_m and V_{max} values were calculated from the Lineweaver-Burk double reciprocal plots.

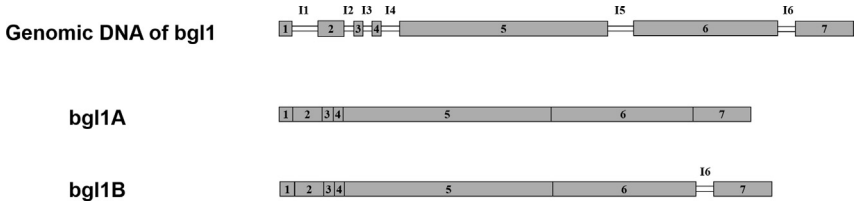

Fig. 1. Normally spliced transcript and intron-retained transcript from bgl1 gene. The number 1–7 represents the exon 1 to exon 7 respectively, and I1-I6 represents the intron 1 to intron 6 respectively.

2.7. Transformation of geniposide to genipin with the cell-bound β-glucosidase

The recombinant *P. pastoris* whole cells with bgl1 expression were used for the transformation of geniposide to genipin, since the cells retained nearly 75 % amount of recombinant enzymes which were supposed to secrete to the culture medium (see the detail in Results). Forty mg (wet weight) per ml of *P. pastoris* cell suspension was mixed with an equal volume of 1.0 % geniposide. The reaction tubes were incubated at 50 °C. At predetermined time intervals, reaction samples were taken out for determination of the amounts of geniposide and genipin. The thin layer chromatograph (TLC) method was used with silica gel as a solid support, and the same volume of samples was loaded on the TLC plates. The solvent system to develop the TLC plates was 6:7:2 chloroform: acetic acid: water. The detection solution was composed of 5 % (v/v) sulfuric acid and 95 % (v/v) methanol. After development, the plates were allowed to dry thoroughly and then were sprayed with the detection solution and heated to 80 °C till the color spots emerged.

3. Results

3.1. Cloning of β-glucosidase gene and intron-retained β-glucosidase gene from A. niger

With the known *A. niger* GH3 β-glucosidase gene (GenBank No. AJ132386) (Dan et al., 2000) as a reference, the homologous β-glucosidase gene was obtained from the strain *A. niger* B2 by RT-PCR. After cloned to pMD19-T vector, two pieces of PCR products were randomly selected to be sequenced. Unexpectedly we got two sequences with different lengths: one was 2583bp long, the same length with AJ132386, yet shared 93.6 % similarity with AJ132386 gene and 96.8 % identity with AJ132386 protein; the other one was 2634bp long, matching the former one perfectly except for a 51bp insertion after the 2349th bp site. Notably the insertion had the boundaries coinciding with the consensus for eukaryotic splice junctions (Mount, 1982), hinting that it might be an intron retained in the transcript during the pre-RNA splicing. By aligning the transcript with the genomic DNA of AJ132386, the insertion was confirmed to be the sixth (the last) intron of the β-glucosidase gene, which contained altogether 7 exons separated by 6 introns (Fig. 1). Here, the normal β-glucosidase gene was designated bgl1A (GenBank No. KJ739789), and the intron-retained one designated bgl1B (GenBank No. KJ866182). To see whether bgl1B appeared in the cDNAs occasionally, 8 more pieces of PCR products were randomly selected and sequenced, among which 3 turned out to be bgl1B, while 5 to be bgl1A. So bgl1B might exist in the cDNAs at a frequency of 40 %. We presumed that the IR form of alternative splicing occurred on the β-glucosidase gene in *A. niger* B2, resulting in the generation of the two splice variants. The intron-retained variant then became a good material for exploring whether the IR had any effect on the translated product β-glucosidase.

3.2. Production of BGL1A and BGL1B in P. pastoris

The genes of bgl1A and bgl1B were expressed through pPICZα-A vector in *P. pastoris* GS115 strain. As the polyhistidine tag had been added to the C terminus of the genes, the recombinant proteins secreted to the culture could be purified by nickel-affinity chromatography. SDS-PAGE proved that both BGL1A and BGL1B were homogeneous and the size of each protein was estimated to be about 130 kDa (Fig. 2), which was much larger than their

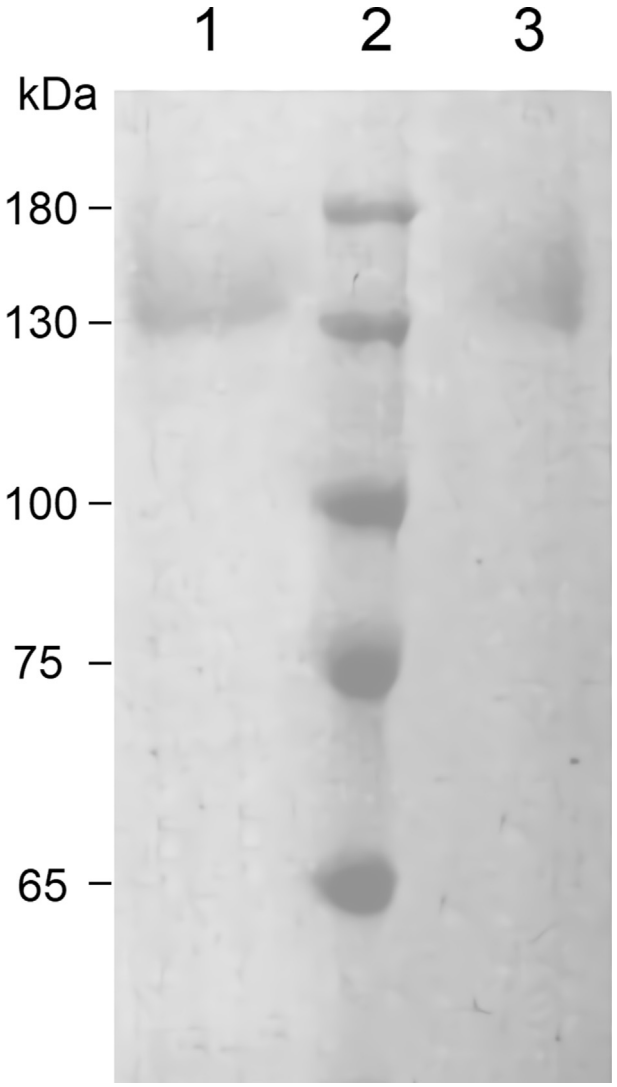

Fig. 2. SDS-PAGE analysis of purified BGL1A and BGL1B. Lane 1, BGL1A protein; Lane 2, Molecular mass markers; Lane 3, BGL1B protein.

theoretical sizes, 94.2 kDa for BGL1A and 96.2 kDa for BGL1B. The larger sizes of the recombinant enzymes might be attributed to overglycosylation of the heterologous proteins in *P. pastoris*, the phenomenon frequently occurred in *P. pastoris* host as reviewed by Looser et al. (2015).

3.3. Catalytic properties of BGL1A and BGL1B

As the intron retention in bgl1B gene didn't change its reading frame, the gene product BGL1B only had an extra 17-amino acid-long insertion after 783th residue compared with BGL1A. BGL1B's β-glucosidase function had been validated first, indicating that the insertion didn't alter its catalytic function. The hydrolysis catalytic of BGL1A and BGL1B were then compared in terms of their temperature and pH dependence.

As shown in Fig. 3A, BGL1A exhibited an optimal temperature of 50 °C, while BGL1B had a higher optimal temperature of 60 °C. When both enzymes were incubated at 50 °C for 60 min, BGL1B remained 63.0 % of the initial activity, higher than that of BGL1A (46.9 %). The half-life of BGL1B and BGL1A was estimated to be 115.5 min and 57.8 min, respectively. Both the optimal temperature and the half-life indicated that BGL1B had better thermostability than BGL1A. Fig. 3C showed the pH profiles of the two enzymes determined at 50 °C and citrate buffer with different pH. There was only a slight difference between their pH optima with 4.4 of BGL1A and 4.2 of BGL1B. As for their pH stability, both BGL1A and BGL1B exhibited a good pH stability with all above 90 % of initial activity retained after 30 min incubation at pH 3.0–6.0 citrate buffer, 37 °C (Fig. 3D). The temperature and pH dependent profiles of BGL1A were similar with the reference AJ132386's protein (Seidle et al., 2004), indicating that the family 3 BGL1 proteins from different *A. niger* strains were much conservative.

3.4. Kinetic analysis of BGL1A and BGL1B

The kinetic parameters of BGL1A and BGL1B for PNPG substrate were determined in the presence of 0.038 mg/ml each enzyme (Table 1). BGL1B exhibited higher K_m value than that of BGL1A, demonstrating the lower affinity for PNPG, yet BGL1B had a k_{cat} of 42.9 s^{-1}, larger than that of BGL1A (31.2 s^{-1}). Thus BGL1B and BGL1A showed an equivalent catalytic efficiency towards PNPG in terms of their k_{cat}/K_m values.

3.5. Investigation on the ability of BGL1A and BGL1B to transform geniposide to genipin

To test whether BGL1A and BGL1B could be applied to conversion of geniposide to genipin, the recombinant *P. pastoris* cells with bound BGL1A or BGL1B were used as the catalysts instead of purified enzymes, since we confirmed that 74.2 % of the whole heterologously expressed BGL1A activity and 73.9 % of that of BGL1B activity retained in the host cells and we further proved that the cell-associated enzyme existed in the insoluble cell debris, most likely in cell wall, not in cytoplasm (Table 2). The tendency of BGL1A and BGL1B to attach to the cells was probably due to the presence of the fibronectin type III-like domain (noted Fn3-like domain) at C-terminus of both enzymes (779–846 residues in BGL1A and 779–863 residues in BGL1B). Fn3-like domain has been proved to have roles in polymeric substrate recognition and in cell wall

Table 1
Kinetic parameters of BGL1A and BGL1B.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
BGL1A	0.94 ± 0.11	31.2 ± 2.0	33.2
BGL1B	1.35 ± 0.10	42.9 ± 2.8	31.8

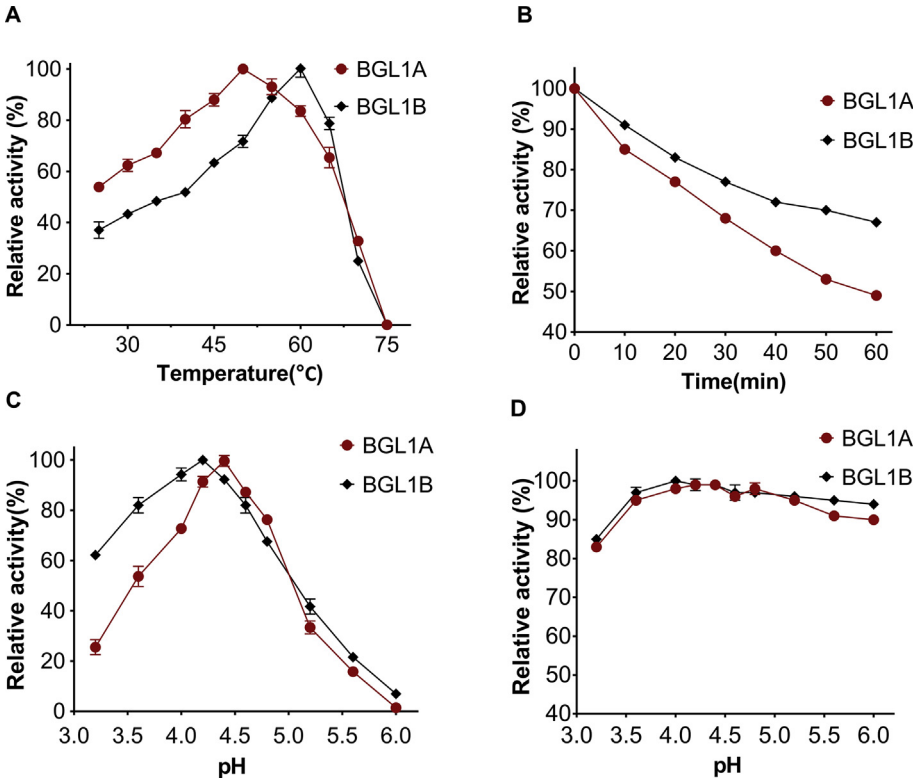

Fig. 3. Effects of pH and temperature on activity and stability of BGL1A and BGL1B. (A) Optimal temperature of BGL1A and BGL1B. (B) Stability of BGL1A and BGL1B at 50 °C. (C) Optimal pH of BGL1A and BGL1B. D) Stability of BGL1A and BGL1B at pH 3.0–6.0 citrate buffer.

Table 2
The distribution of β-glucosidase activity among different parts of *P. pastoris* cells (%)[a].

Recombinant strain with	Fermentation broth	Supernatant of broth	Cell suspension lysed by sonication	Supernatant of cell lysate	Insoluble cell debris
BGL1A	100	21.5	74.2	1.21	72.1
BGL1B	100	24.5	73.9	1.79	71.5

[a] The final volume of each preparation was adjusted to the same as that of the fermentation broth taken for the experiment before enzyme activity of each part was assayed. Taking the β-glucosidase activity (IU/ml) of the fermentation broth as 100 %, the relative activity of each part was calculated.

adhesion (da Silva et al., 2016; Lima et al., 2013; Marín-Navarro et al., 2011). Our results in terms of enzyme distribution were in good accordance with the proposed cell-adhesion function of Fn3-like domain. The resultant cell-bound enzymes, sort of immobilized enzymes, may have advantages in biotransformation, such as better stability of enzymes, easier separation of products, re-use of catalysts, etc.

Equal amount of *P. pastoris* cells (40 mg/ml) with bound BGL1A or BGL1B was mixed with 1.0 % geniposide. TLC analysis (Fig. 4) showed that the spot for genipin product appeared after 10-minute incubation in both reaction mixtures and the spot kept developing with incubation time, indicating that both BGL1A and BGL1B could efficiently hydrolyze geniposide. When comparing the 120-minute hydrolysates of the two cell-bound enzymes, BGL1B was observed to exhibit a more complete hydrolysis process, with nearly no geniposide substrate remained, while there was noticeable amount of residual geniposide untransformed in BGL1A's reaction mixture. As BGL1A and BGL1B displayed the same expression pattern in *P. pastoris*, we supposed that an equal amount of BGL1A and BGL1B was brought to the geniposide transformation system by the equal amount of *P. pastoris* cells, so the higher conversion rate BGL1B exhibited was probably attributed to BGL1B's higher k_{cat} towards geniposide, consistent to the higher k_{cat} towards PNPG compared with that of BGL1A.

4. Discussions

In this work a β-glucosidase gene bgl1A and its corresponding intron-retained gene bgl1B were cloned from *A. niger* B2 strain. The high frequency of bgl1B in cloning transformants proved that the transcript variant for bgl1B normally existed in the total RNA pool of *A. niger* B2, suggesting that there might exist the alternative splicing process presented as IR type on the β-glucosidase gene of *A. niger* B2, although further proof for alternative splicing is needed to verify the generation of the two different proteins from the splice variants in this strain. *A. niger* was previously reported to have alternative splicing on glucoamylase gene, resulting in the two mRNA variants with or without a 169-bp intervening sequence, and then creating G1 and G2 forms of glucoamylase (Boel et al., 1984). Here the β-glucosidase gene from *A. niger*, which most likely underwent alternative splicing presented as an IR form, added support to the observation that IR was the prevalent alternative splicing event in fungi (McGuire et al., 2008; Sieber et al., 2018).

The function of intron-retained transcripts was reported to be involved in gene expression regulation in higher eukaryotes, in which those incompletely spliced mRNAs were prevented from export to cytoplasm or existed in less stable state, both leading to down-regulation of gene expression (Yap et al., 2012; Ni et al., 2016). In addition, intron-retained transcripts might be introduced stop codons in frame with the initiation codon, causing inability to synthesize active proteins, as proved with the intron-retained transcripts of lignin peroxidase gene and manganese peroxidase gene in *P. chrysosporium* (Macarena et al., 2005). Yet, intron retention also is an important way to generate diverse proteins with varying functions, as evidenced by Boldo et al.'s work, in which the intron-retained chitinase was proposed to be a cell wall-linked chitinase, involved in cell wall remodeling, while the completely spliced one was secreted in order to degrade exogenous chitin (Boldo et al., 2010). In present work, we obtained a novel β-glucosidase BGL1B which resulted from a 51-bp intron retained in the RNA transcript. The comparison of catalytic properties of BGL1B with the completely spliced BGL1A showed that the intron retention didn't cause drastic effect on catalytic functions. However, noticeable changes due to intron retention could be observed, for example, BGL1B had a higher optimal temperature and a more thermostability, also it had a lower affinity but a higher k_{cat} towards PNPG, and it exhibited a more complete conversion from geniposide to genipin. We presumed that this kind of splice variants with distinct substrate preference or slightly different functions might benefit the fungus to grow competitively at various nutritional conditions. Also we speculated that intron retention type of alternative splicing was the source of enzyme diversity in fungi.

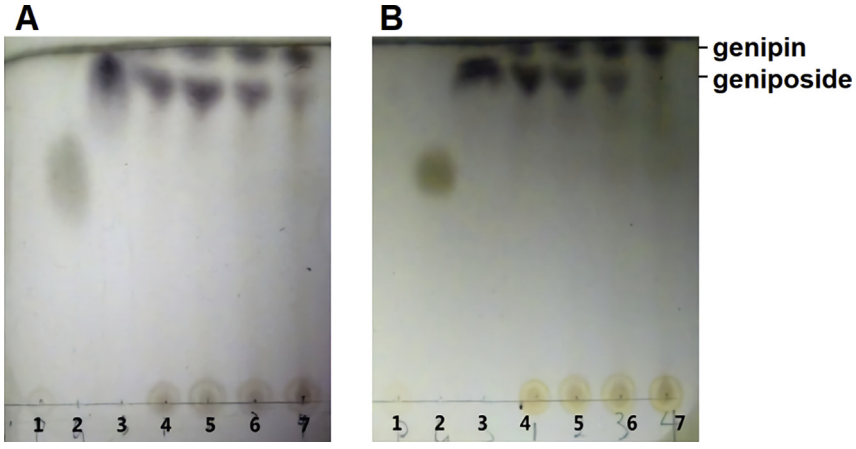

Fig. 4. TLC analysis of geniposide hydrolyzates catalyzed by cell-bound BGL1A (A) and BGL1B (B). Lane 1, Cell suspension only; Lane 2, Glucose standard (2 %); Lane 3, Geniposide standard (0.5 %); Lane 4, 5, 6, and 7, Sample taken from reaction mixture collected at 10, 30, 60, and 120 min, respectively.

Aligning BGL1B with proteins in PDB showed that BGL1B had the highest sequence identity of 81 % with *Aspergillus aculeatus* BGL1 (PDB ID:4IIB), while the homologous template for the 17-amino acid-long insertion could not be found, so the structure of BGL1B could not be built by homologous modeling. However, there are reasons to believe that the insertion does not obstruct the catalytic residues or the substrate-binding cleft of the enzyme. The further crystal structure investigation can provide more insights into the influence of the intron insertion fragment on BGL1B's catalytic functions.

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