A DISSERTATION FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

Cloning of the β-Glucosidase Genes from *Bifidobacterium* Strains and Their Heterologous Expression in *B. bifidum* BGN4

Bifidobacterium 유래 β-glucosidase 클로닝과

Bifidobacterium bifidum BGN4에서의 발현

February, 2013

So Youn Youn

Department of Food and Nutrition

Graduate School

Seoul National University

Cloning of the β-Glucosidase Genes from *Bifidobacterium* Strains and Their Heterologous Expression in *B. bifidum* BGN4

Advisor : Geun Eog Ji

So Youn Youn

2013

Department of Food and Nutrition

Graduate School

Seoul National University

Abstract

β-Glucosidase is necessary for the bioconversion of glycosidic phytochemicals in food. Two *Bifidobacterium* strains (*Bifidobacterium animalis* subsp. *lactis* SH5 and *B. animalis* subsp. *lactis* RD68*)* with relatively high β-glucosidase activities were selected among 46 lactic acid bacteria. A β-glucosidase gene (*bbg572*) from *B. lactis* was shotgun cloned, fully sequenced, and analyzed for its transcription start site, structural gene, and deduced transcriptional terminator. The structural gene of *bbg572* was 1,383 bp. Based on amino sequence similarities, *bbg572* was assigned to family 1 of the glycosyl hydrolases. To overexpress *bbg572* in *Bifidobacterium*, several bifidobacteria expression vectors were constructed by combining several promoters and terminator sequence from different bifidobacteria. The maximum activity of recombinant Bbg572 was achieved when it was expressed under its own promoter and terminator. Its enzyme activity increased 31-fold compared with those of its parental strains. The optimal pH for Bbg572 was pH 6.0. Bbg572 was stable at 37-40°C. Bbg572 hydrolyzed isoflavones, quercetins and disaccharides with various β-glucoside linkages. Bbg572 converted the ginsenoside Rb1 and Rb2. These results suggest that this new β-glucosidase-positive *Bifidobacterium* transformant can be utilized for the production of specific aglycone products.

To characterize and overexpress the β-glucosidases of *Bifidobacterium*, two novel β-glucosidase encoding genes (*bbg504* and *bbg1176*) from *Bifidobacterium pseudocatenulatum* Int57 and SJ32 were identified and cloned. These cloned β-glucosidase genes (*bbg504* and *bbg1176*) were fully sequenced and analyzed for the transcription start site, structural genes, and putative transcriptional terminator. The structural genes of *bbg504* and *bbg1176* contained 2,247 bp open reading

frame (ORF) encoding a protein containing 729 amino acids (molecular weight of 82.39 kDa) and 1,176 bp ORF encoding a protein containing 392 amino acids (molecular weight of 43.12 kDa), respectively. Based on their amino sequence similarities to the well characterized carbohydrate hydrolases, Bbg504 and Bbg572 were assigned to family 3 and family 1 of the glycosyl hydrolases, respectively. Several *Bifidobacterium* expression vectors were constructed by combining various promoters and a terminator sequence from different *Bifidobacterium*. Bp504bbg504t with its own promoter and terminator exhibited the highest β-glucosidase activity. Additionally, Bbg1176 also showed high β-glucosidase activity in *B. bifidum* BGN4 when cloned into *p504* promoter-mediated expression system (Bp504bbg1176). These β-glucosidase activities of Bp504bbg504t and Bp5041176 were about 321- fold and 30-fold greater than those of their respective parental strains. Furthermore, Bbg504 exhibits similar pH optimum (pH 6.0) and thermostability to those of Bbg1176. Bbg504 and Bbg1176 showed substrate specific activities across a broad substrate range. Interestingly, the two β-glucosidases, Bbg504 and Bbg1176, showed different hydrolysis patterns with ginsenosides. Bbg504 hydrolyzed ginsenoside Rb1, F2 and Rg3(S), while Bbg1176 hydrolyzed Rd, Rb1, Rb2, Rg1, Rg3(S) and Rg3(R). This is the first research on cloning and expression of β-glucosidase from *B. pseudocatenulatum*. Based on these results, two novel β-glucosidases from *Bifidobacterium* transformants Bp504bbg504 and Bp504bbg1176 converted glycosides more efficiently and can be utilized for the production of specific aglycone products.

Key words : β-glucosidase, *Bifidobacterium*, promoter, terminator

Student Number : 2007-30440

Table of Contents

List of Tables

List of Figures

1. Literature review

Bifidobacterium and carboxyl hydrolase

1.1 *Bifidobacterium*

Bifidobacterium is among the prevalent groups of culturable anaerobic bacteria within the human and animal gastrointestinal tract, and among the first to colonize the human GIT, where they are thought to exert health-promoting actions, such as protective activities against pathogens via production of antimicrobial agents (e.g. bacteriocins) and/or blocking of adhesion of pathogens, and modulation of the immune response [39]. Certain bifidobacteria are, because of these perceived health-promoting activities, commercially used as probiotic microorganisms. Growth and metabolic activity of probiotic bacteria, including bifidobacteria, can be selectively enhanced by various dietary carbohydrates, which for that reason are called "prebiotics" [11, 53]. In this respect it is important to mention that over 8% of the identified genes in most studied bifidobacterial genomes are predicted to be involved in carbohydrate metabolism. [26, 46, 54, 57].

Bifidobacteria are saccharolytic and are believed to play an important role in carbohydrate fermentation in the colon. Although the ability to metabolize particular carbohydrates is species- and strain-dependent, physiological data confirm that bifidobacteria can indeed ferment various complex carbon sources such as xylo-oligosaccharides, (trans)-galactooligosaccharides, soy bean oligosaccharides, malto-oligosaccharides, fructo-oligosaccharides and other plant derived-oligosaccharides [15].

1.2 Carbohydrate-modifying enzymes in *Bifidobacterium* sp.

Glycoside hydrolases (EC 3.2.1.x) are a widespread group of enzymes, which hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety in the presence of water. Glycosyl hydrolases (also called glycoside hydrolases) appear to be the most critical group of enzymes for bifidobacteria, which allows them to adapt to and exist in the host environment through hydrolysis of complex dietary and host-produced carbohydrates. In this respect, bifidobacterial carbohydrate-degrading enzymes is not only important in order to identify and understand preferable bifidobacterial substrates, but also from a perspective of discovering novel selective (bifidogenic) prebiotics.

Among them, β-glucosidase constitues a major group among glycosyl hydrolase enzymes. β-Glucosidases constitute a group of well characterized, biologically important enzymes that hydrolyze glycosidic bonds to release nonreducing terminal glucosyl residues from glycosides and oligosaccharides. In bacteria and fungi, β-glucosidases are mainly a part of the cellulase enzyme system and are responsible for the hydrolysis of short chain oligosaccharides and cellobiose (resulting from the synergistic action of endoglucanases and cellobiohydrolases) into glucose in a rate-limiting step. The enzyme activity decreases as the glucose chain length increases [2, 27].

In general, β-glucosidases from different orders and kingdoms appear to differ in their specificities for the aglycone part linked to the glycosyl group. The identification of amino acid residues occurring at the enzyme active site is of considerable importance for revealing the structure-function relationship.

A substantial amount of work has been done on cloning structural genes of these versatile biocatalysts from a variety of microorganisms and plants into high-yielding mesophilic expression systems such as *Escherichia coli*, *Saccharomyces cerevisiae,* and filamentous fungi, in a few cases.

1.3 Classification and structure of β-Glucosidase

In general, a classification scheme based on sequence and folding similarities was proposed for glycosidases by Henrissat and co-workers [16, 17]. In this method, those enzymes with overall amino acid sequence similarity and well-conserved sequence motifs are grouped into the same family. In this system, β-glucosidases along with other carbohydrases have been assigned to various families under the glycosylhydrolase category, β-gloucosidases have been placed in family 1 and family 3.

The β-glucosidases, with the exception of glucosylceramidase (acid β-glucosidase) placed in family 30, are placed in either family 1 or family 3 of glycosylhydrolases. Family 1 of glycosylhydrolases includes β-glucosidases from archaebacteria, plants, and mammals. The crystal structures have been solved for a few family 1 β-glucosidases, which are also designated as members of the 4/7 super family with a common eight fold β/α barrel motif, consisting of similar amino acid sequences at the active site [23].

Family 1 comprises nearly 62 β-glucosidases from archaebacteria, plants, mammals, and also includes 6-phosphoglycosidases and thioglucosidases. Family 3 comprises β-glucosidases of some bacterial, mold, and yeast origin. Family 3 of glycosylhydrolases consists of nearly 44 β-glucosidases and hexosaminidases of bacterial, mold, and yeast origin.

Most family 1 enzymes also show significant β-galactosidase activity. In glycoside hydrolase family 1, The conserved glutamate residue in NEP and ENG motif has been identified as potential acid/base catalyst and active site nucelophile respectively. In glycoside hydrolase family 3, D in conserved SDW motif is the

putative active site and H in KHF is the proposed H^+ doner. In family 3 β-glucosidases, E/D is the active site nucleophile, while H/E is the putative proton donor (Table1.1).

β-Glucosidases have various structures, but the overall fold of the catalytic domain is similar in each GH family. The families GH1, GH5, and GH30 belong to the Clan GH-A, and they all have similar $(\beta/\alpha)_8$-barrel domains that contain their active site. In contrast, GH3 enzymes have two domains contributing to their active site. GH9 enzymes have $(\alpha/\alpha)_6$-barrel structures, while the GBA2 family shows weak homology to proteins with this $(\alpha/\alpha)_6$ structure as well (Fig. 1.1).

The clan GH-A enzymes of families GH1, GH5, and GH30 all have a common $(\beta/\alpha)_8$-barrel structure and their active sites contain two conserved carboxylic acid residues on β-strands 4 and 7, serving as the catalytic acid/base and nucleophile, respectively [18, 23].

The lengths and subunit masses of these GH1 enzymes vary considerably, depending on the presence of auxiliary domains and redundant GH1 domains.

The GH3 β-glucosidases and exoglucanases have a two-domain structure, a $(\beta/\alpha)_8$-barrel followed by an α/β sandwich comprising a 6-stranded β-sheet sandwiched between three α-helices on either side [52]. The active site of GH3 enzymes is situated between the $(\beta/\alpha)_8$ and $(\alpha/\beta)_6$ sandwich domains, each of which contributes one catalytic carboxylate residue (Fig. 1.2). The catalytic nucleophile for barley Exo I is an aspartate at residue D285, which resides on the loop after β-strand 7 of the $(\beta/\alpha)_8$ barrel, while the catalytic acid/base is glutamate E491, which is on a long loop extending from the $(\alpha/\beta)_6$ sandwich domain [20].

Table 1.1 Amino acid sequence similarity of β-glucosidases in family 1 and family 3 of glycosylhydrases [47, 56]

Glycoside hydrolase family 1						
	Residue	Sequence	Residue	Residue	Sequence	Residue
Clostridium thermocellum Bgl A	162	FTH**NEP**GVV	170	353	VIS**ENG**AAF	361
Bacillus polymyxa Bgl B	163	NII**NEP**YCA	171	354	LIT**ENG**AAM	362
Candida wickerhamii Bgl B	320	IIF**NEP**---	328	511	FIT**EFG**FDE	519
Pyrococcus furiosus Cel B	203	STM**NEP**NVV	211	369	IIT**ENG**MA-	377
Humicola grisea Bgl 4	163	ITF**NEP**WCS	171	375	YUT**ENG**TS-	383
Trifolium repens Bgl	200	STL**NEP**EVF	208	420	SIT**ENG**MNE	428
Costus sp. Bgl	257	ITL**NEP**LSL	265	471	IVT**ENG**TAE	479
Agrobacterium sp.	167	ATF**NEP**WCA	175	357	YIT**ENG**ACY	365
Glycoside hydrolase family 3						
Aspergillus aculeatus Bgl 1	201	ATA**KHY**ILN	209	298	FVM**SDW**GAH	306
Saccharomycopsis fibuligera Bgl 1	201	ACU**KHF**IGN	209	294	FVV**SDW**GAQ	302
Saccharomycopsis fibuligera Bgl 2	203	ACU**KHF**IGN	211	298	FVV**SDW**AAQ	306
Pichia anomala Bgl	200	STA**KHL**IGN	208	298	FVM**TDW**GAL	306
Phaenerochaete chrysosporium CBGL	154	ACA**KHF**INN	162	239	YVM**SDW**WAT	247
Candida pelliculosa Bgl	201	STA**KHL**IGN	209	295	FVM**TDW**GAL	303
TTrichoderma reesei Bgl 1	201	ATA**KHY**ILN	209	298	YUM**TDW**NAQ	306
Aspergillus kawachii Bgl A	201	ATA**KHY**IAE	209	298	FVM**SDW**AAH	306

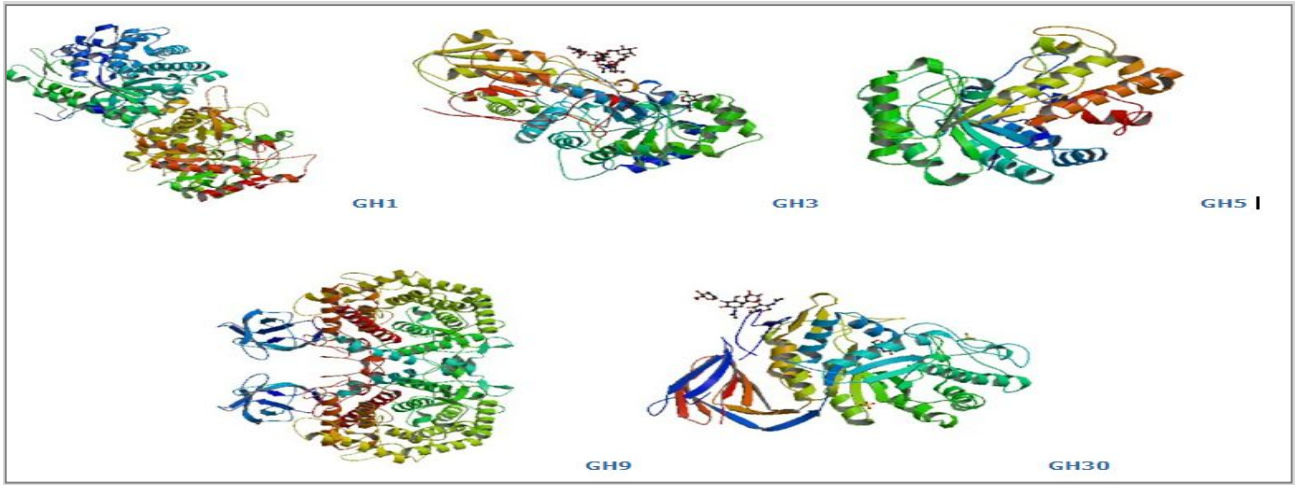
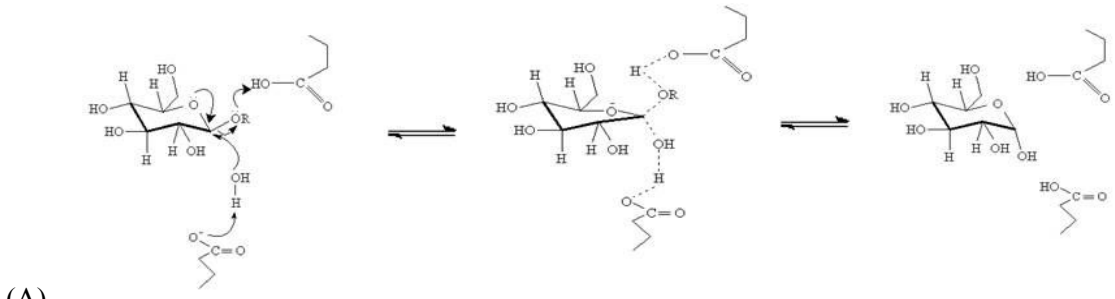


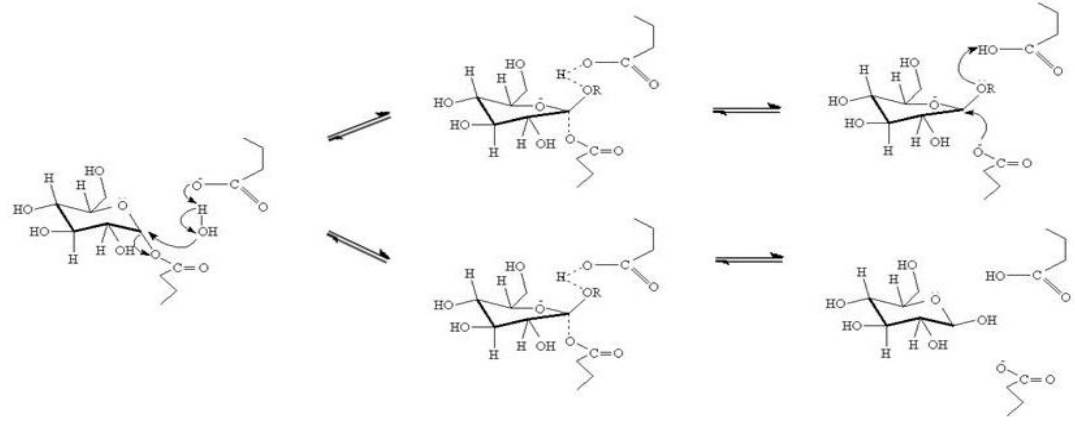
Fig. 1.1 Structures of β-glucosidases from different GH families. (1) These include β-glucosidases or related enzymes GH1 (Zeamays ZmGlu1, PDB code 1E1E), (2) GH3 (Hordeum vulgare ExoI β-glucan glucohydrolase, PDB code 1EX1), (3) GH5 (Candida albicans exo-β-(1,3)-glucanase Exg exoglucanase, PDB code 1CZ1), (4) GH9 (Vibrio parahaemolyticus, putative exoglucanase, PDB code 3H7L) and (5) GH30 (Homo sapiens, acid β-glucosidase/glucocerebrosidase GBA1, PDB cod 2V3D). The structurals are colored in a spectrum from blue to red from their N- to C-termini, with the catalytic nucleophile and acid-base residues shown in stick for those enzymes in which they are known. The ligands shown are glucose in the GH3 barley ExoI and N-butyl-deoxynojirimycin in the GH30 human GBA1 [22].

1.4 Catalytic mechanisms

Glycoside hydrolases perform catalysis using two mechanisms, one with inversion and one with retention of chirality at the anomeric carbon [38]. The GH9 β-glucosidases use an inverting mechanism, in which an activated water molecule makes a direct nucelophilic attack on the anomeric carbon to displace the aglycone in a single step, as shown in Fig 2A. Most β-glucosidases that have been characterized (i.e., GH1, GH3, and GH30 enzymes) are retaining enzymes, and they perform catalysis in two steps, glycosylation and deglycosylation (Fig. 2B).



(A)



(B)

Fig. 1.2 Retaining catalytic mechanisms of inverting and retaining β-glucosidases. A. Inverting Mechanism; B. Retaining mechanism [7, 44].

1.5 Mechanism of substrate binding and specificity

Although the residues responsible for the hydrolytic mechanism are well characterized, how β-glycosidases recognize and interact with their substrates, which in large part determines their diverse functions, is less clear.

The complexes of several of these enzymes with substrates, inhibitors, and covalent intermediates are available, allowing in-depth analysis of residues likely to be critical to substrate and transition state binding [6].

Marana analyzed GH1 specificity and concluded that a conserved glutamate, which bridges the glycone hydroxyl groups 4 and 6 in enzymes with β-glucosidase and β-galactosidase activities but is replaced in 6-phosphoglycosidases, is critical for the distinction between enzymes [32]. However, it still remains to be determined how GH1 enzymes can behave primarily as β-glucosidases or β-mannosidases or show different ranges of allowed glycones, even though they bind the sugar with the same conserved residues [5, 19, 28]. It is worth noting that binding of the aglycone has also been observed to affect the sugar binding position [6, 55], so residues more distant in the substrate binding pocket cannot be excluded from playing roles in glycone specificity.

1.6 Sources and characteristics of recombinant β-glucosidases

The β-glucosidase genes from a large number of bacteria, mold, yeast, plant, and animal systems have been cloned and expressed in both *E. coli* and eukaryotic hosts such as *S. cerevisiae* and filamentous fungi. Cloning has been performed by two methods, either by (1) formation of a genomic DNA library followed by selection of the recombinant clones by screening for β-glucosidase production, or (2) starting with a cDNA library (or a genomic library), screening of recombinant clones by specific nucleotide probes designed from *a priori* knowledge of the polypeptide sequence. Detailed properties of cloned β-glucosidases from various bacteria are presented in Table 1.2.

Table 1.2 Cloned β-glucosidases from various bacteria

Source of β-glucosidase	Gene	Host	Enzyme localization	Substrate specificity	Glycosyl transferase activity	Classification	Reference
Agrobacterium tumefaciens	*cbg1*	*E. coli*	Intracellular	coniferin	-	Family 3	[4]
Azospirilum irakense	*salA*,	*E. coli*	Periplasmic space	pNPG, salicin arbutin	-	Family 3 (sub-family B)	[7]
	salB	*E. coli*	Periplasmic space	pNPG, salicin arbutin	-	Family 3 (sub-family AB)	
Bacillus sp.	*bglA*	*E. coli*	Intracellular	ρNPG, cellobiose, gellan, trisaccharides	-	Family 1	[15]
	bglB	*E. coli*	Intracellular	ρNPG	-	Family 3	
Bacillus circulans sub sp. *alkalophilus*	*bglA*	*E. coli*	Intracellular	cellobiose ρNPG, lactose	G3 synthesis from cellobiose	Family 1	[36]
Bacillus polymyxa	*bglA*	*E. coli* *S. cerevisiae*	Intracellular	ρNPG, cellobiose	G3 synthesis from cellobiose	Family 1	[37]
	bglB	*E. coli*	Intracellular	-	-	Family 1	[10]
Bacillus subtilis	*bglA*	*E. coli* JM83	Mostly periplasmically localized	cellobiose, CMC	-	Family 1	[49]
Butyrivibrio fibrisolvens	*bglA*	*E. coli* C600	Cytoplasm	cellobiose, cellotriose, cellotetraose, cellopentaose	-	Family 3	[30]
Cellovibrio gilvus	*bgl*	*E. coli* WL66	Intracellular	MUG, ρNPG, cellobiose, cellodextrins	-	Family 3	[24]
Cellovibrio mixtus	*lam2*	*E. coli* JM109	Intracellular	Laminarin, ρNPG, cellobiose	-	-	[45]
Clostridium thermocellum	*bglA*	*E. coli*	-	ρNPG, ρNPX, cellobiose	-	Family 1	[14]
Clostridium thermocellum	*bglA*	*E. coli*	Intracellular	ρNPG, cellobiose, sophorose	-	Family 3	[13], [43]
Erwinia chrysanthemi	*bglx*	*E. coli* DH5α	Periplasmic space	ρNPG, ρNPX	-	Family 3	[58]

Source of β-glucosidase	Gene	Host	Enzyme localization	Substrate specificity	Glycosyl transferase activity	Classification	Reference
Erwinia herbicola	*bglA*	*E. coli* DH5α *E. coli* HB101	Mostly periplasmically localized	salicin, phloridzin, arbutin, cellobiose	-	Family 1	[33]
Flavobacterium meningosepticum		*E. coli*	-	aryl-glucosides	-	-	[29]
Microspora bispora	*bglB*	*E. coli*	Intracellular	cellobiose, aryl-glucosides	-	-	[59]
Prevotella ruminicola	*cdxA*	*E. coli*	Membrane associated	ρNPG, arbutin, cellobiose, cellodextrins	-	Family 3	[60]
Pseudomonas fluorescens subsp. *cellulosa*	*cdxD*	*E. coli*	Cell envelope	cellobiose, cellodextrin, ρNPG,MUG	-	Family 3	[41]
Pyrococcus furiosus	*celB*	*E. coli* JM109	Intracellular	ρNPG	Synthesis of various glucosides	Family 1	[56]
Pyrococcus horikoshii		*E. coli*	Memebrane bound	Laminaribiose, cellobiose, gentioiose, alkyl-glucosides	-	Family 1	[35]
Ruminococcus albus	*bgl*	*E. coli*	Extracellular	cellobiose, ρNPG, cellodextrins	-	Family 3	[50]
Thermoanaerobacter brockii	*cglT*	*E. coli* *B. subtilis*	Intracellular	Laminaribiose, cellobiose, ρNP fucoside, ρNPG, cellodextrins	-	Family 1	[3]
Thermotoga maritima	*bglA*	*E. coli*	Intracellular	cellobiose, ONPG, ρNPG, ρNPGal		Family 1	[9]
Thermotoga neapolitana	*bglB*	*E. coli* DH5α	Intracellular	ρNPG, ρNPX, laminaribiose	-	Family 3	[61]
Thermophhilic anaerobic		*E. coli* C600	Cytosol	ρNPG, cellobiose, laminaribiose, cellodextrins	-	-	[48]

1.7 Functional roles and applications based on hydrolytic activity of β-glucosidases

Although various researches have been done on β-glucosidases from microorganisms, most of them has focused on their application rather than their endogenous function. As such, most of the enzymes that have been studied in the about their natural function are those involved in bioconversion to produce glucose. (Fig. 1.3 and Fig. 1.4)

The identification and production of β-glucosidases, especially those with high glucose tolerance, has been of interest, and applicable β-glucosidases have been isolated from bacteria and fungi [12, 21, 31, 51]. Enzymes from the source plants or other sources may be added to foods and beverages to enhance food quality. Additionally, foods, feeds, and beverages may be improved nutritionally by release of vitamins, antioxidants, and other beneficial compounds from their glycosides.

The role of β-glucosidase to cellulose hydrolysis is significant because cellobiose is an inhibitor of both endo- and exo-glucanases, and it must be removed to allow efficient and complete saccharification of cellulose.

The other important candidates for hydrolytic actions by β-glucosidases are flavonoids and isoflavonoid glucosides. These are phenolic and phytoestrogen glucosides that occur naturally in fruits, vegetables, tea, red wine, and soyabeans. The aglycone moiety, released as a result of hydrolytic activity of β-glucosidases, has potent biological activity, with several uses in the field of medicine as antitumor agents, in general biomedical research, and in the food industry. The hydrolysis of daidzin and genistin to daidzein and genistein, respectively, with the release of glucose was demonstrated with the *Lactobacillus casei* subspecies

rhamnosus enzyme, thus reducing the undesirable bitter and astringent isoflavonoid glucosides from soybean cooked syrup (SCS) [34]. Similarly, phloridzin was hydrolyzed to liberate the aglycone moiety, which is a precursor of melanin. The latter reduce the risk of skin cancer and promotes dark color of hair [40]. In the food industry, hydrolytic activity of β-glucosidases may be useful for the production of low-viscosity gellan foods. For instance, β-glucosidases produced by *Bacillus* sp. catalyze cleavage of the trisaccharide glycosylrhamnosyl-glucose to release glucose and rhamnosyl-glucose, thereby reducing viscosity [15]. β-Glucosidases play an important role in removal of bitterness from citrus fruit juices by catalyzing the hydrolysis of the naringin (4,5,7-trihydroxyflavanone-7-rhamnoglucoside) into the prunin [42].

Interestingly, β-glucosidases from bacterial sources, such as *Cellovibrio mixtus* [45], *Thermoanaerobacter brockii* [3], *Thermotoga neopolitana* [61], can be used with laminarinase to act at β1→3 glucan hydrolysis and release glucose from laminaridextrins and laminaribiose. This property is desirable in the production of yeast extract and the bioconversion of algal biomass to fermentable sugars.

Genistein-7-β-glucoside
Monoterpenyl-glucoside
Genistin
Apigenin 7-O-β-glucoside
Oleuropein
Coumaryl alcohol 4-β-glucoside
Indoxyl β-glucoside
Strictosidine
Phloridzin
Coniferin
Daidzin
Lactobacillus casei
Candida wickerhamii
H2O

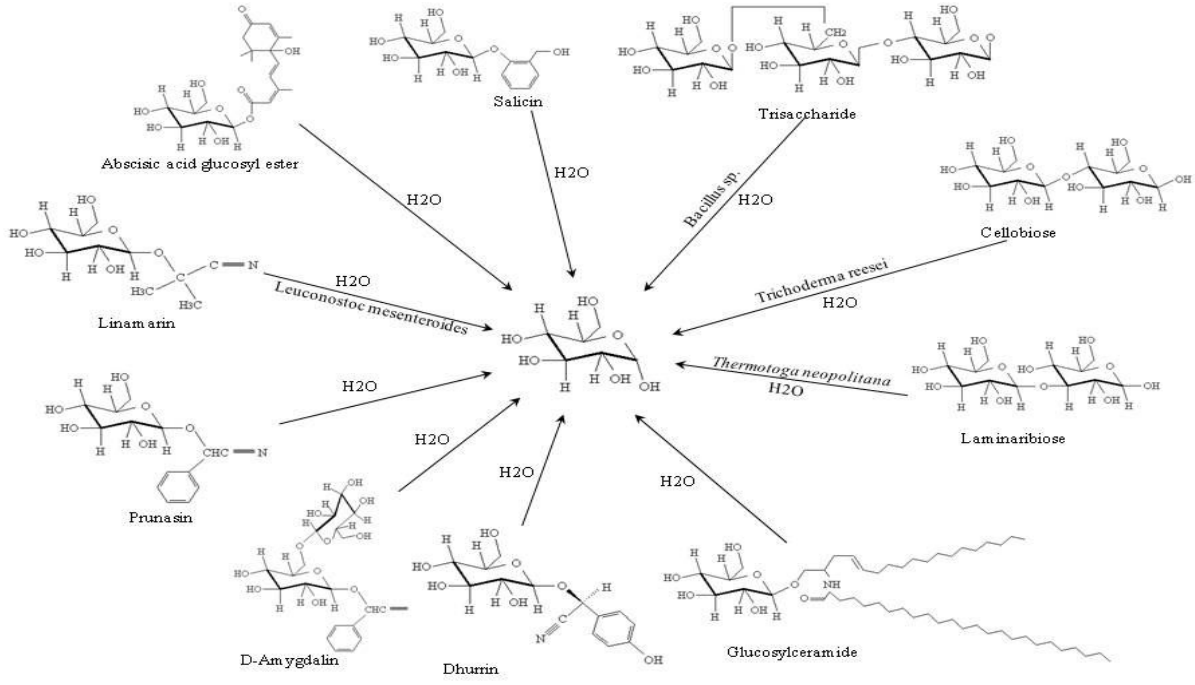


Fig. 1.3 Applications based on hydrolytic activities of β-glucosidase. The enzyme source for each reaction is shown on the arrow [1, 22].

References

1. **Bhatia, Y., S. Mishra, and V. S. Bisaria.** 2002. Microbial β-glucosidases: cloning, properties, and applications. Crit. Rev. Biotechnol. **22:**375-407.
2. **Bisaria, V. S. and S. Mishra.** 1989. Regulatory aspects of cellulase biosynthesis and secretion. CRC Crit. Rev. Biotechnol. **9:**61-103.
3. **Breves, R., K. Bronnenmeier, N. Wild, F. Lottspeich, W. L. Staudenbauer, and J. Hofemeister.** 1997. Genes encoding two different β-glucosidases of *Thermoanaerobacter brockii* are clustered in a common operon. Appl. Environ. Microbiol. **63:**3902-3910.
4. **Castle, L. A., K. D. Smith, and R. O. Morris.** 1992. Cloning and sequencing of an *Agrobacterium tumefaciens* β-glucosidase gene involved in modifying a vir inducing plant signal molecule. J. Bacteriol. **174:**1478-1486.
5. **W. Chuenchor, S. Pengthaisong, R. C. Robinson, J. Yuvaniyama, W. Oonanant, D. R. Bevan, A. Esen, C. J. Chen, R. Opassiri, J. Svasti, and J. R. Ketudat Cairns.** 2008. Structural insights into rice BGlu1 b-glucosidase oligosaccharide hydrolysis and transglycosylation. J. Mol. Biol. **377:**1200–1215.
6. **M. Czjzek, M. Cicek, V. Zamboni, D. R. Bevan, B. Henrissat, and A. Esen.** 2000. The mechanism of substrate (aglycone) specificity in b-glucosidases is revealed by crystal structures of mutant maize b-glucosidase-DIMBOA, -DIMBOAGlc, and dhurrin complexes. Proc. Natl. Acad. Sci. USA **97:**13555–13560.

7. **G. J. Davies, V. M. A. Ducros, A. Varrot, and D. L. Zechel.** 2003. Mapping the conformation itinerary of β-glucosidases by X-ray crystallography. Biochem. Soc. Trans. **31:**523-527.

8. **D. Faure, J. Desair, V. Keijers, M. A. Bekri, P. Proost, B. Henrissat, and J. Vanderleyden.** 1999. Growth of *Azospirillum irakense* KBC 1 on the aryl β-glucoside salicin requires either Sal A or Sal B. J. Bacteriol. **181:**3003-3009.

9. **Gabelsberger, J., W. Liebl, and K. H. Schleifer.** 1993. Purification and properties of a recombinant β-glucosidase of the hyperthermophilic bacterium *Thermotoga maritima*. Appl. Microbiol. Biotechnol**. 40:**44-52.

10. **L. Gonzales-Candelas, D. Ramon, and J. Polaina.** 1990. Sequence and homology analysis of two genes encoding β-glucosidases from *Bacillus polymyxa. alkalophilus*: ability to form long polymeric assemblies. J. Struct. Biol. **129:**69-79.

11. **G. R. Gibson.** 2008. Prebiotics as gut microflora management tools. J. Clin. Gastroenterol. **42:**S75-S79.

12. **H. J. Gilbert, H. Stalbrand, H. Brumer.** 2008. How the walls come tumbling down: recent structural biochemistry of plant polysaccharide degradation. Curr. Opin. Plant Biol. **11:**338-348.

13. **F. Graebnitz, K. P. Ruecknagel, M. Seiss, and W. L. Staudenbauer.** 1989. Nucleotide sequence of the *Clostridium thermocellum bglB* gene encoding thermostable β-glucosidase B: homology to fungal β-glucosidases. Mol. Gen. Genet. **217:**70-76.

14. **Graebnitz, F., M. Seiss, K. P. Ruecknagel, and W. L. Staudenbauer.** 1991. Structure of the β-glucosidase gene *bglA* of *Clostridium thermocellum*. Sequence analysis reveals a uperfamily of cellulases and β-glycosidases including human lactase/phlorizin hydrolase. Eur. J. Biochem. **200:**301-309.

15. **Hashimoto, W., H. Miki, H. Nankai, N. Sato, S. Kawai, and K. Murata.** 1998. Molecular cloning of two genes for β-D-glucosidase in *Bacillus* sp. GL1 and identification of one as a gellan degrading enzyme. Arch. Biochem. Biophys. **360:**1-9.

16. **Henrissat, B.** 1991. A classification of glycosyl-hydrolases based on amino acid sequence similarities. Biochem. J. **293:**781-788.

17. **Henrissat, B., and A. Bairoch.** 1996. Updating the sequence-based classification of glycosyl hydrolases. Biochem. J. **316:**695-696.

18. **Henrissat, B., I. Callebaut, S. Fabrega, P. Lehn, J. P. Mornon, G. Davies.** 1995. Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proc. Natl. Acad. Sci. USA **92:**7090-7094.

19. **Hrmova, M., A. J. Harvey, J. Wang, N. J. Shirley, G. P. Jones, B. A. Stone, P. B. Hoj, and G. B. Fincher.** 1996. Barley b-D-glucan exohydrolases with b-D-glucosidase activity. J. Biol. Chem. **271:**5277-5286.

20. **M. Hrmova, J. N. Varghese, R. De Gori, B. J. Smith, H. Driguez, and G. B. Fincher.** 2001. Catalytic mechanisms and reaction intermediates

along the hydrolytic pathway of a plant beta-D-glucan glucohydrolase. Structure **9:**1005-1016.

21. **Igarashi, K., T. Tani, R. Kawal, and M. Samejima.** 2003. Family 3 b-glucosidase from cellulose-degrading culture of the white-rot fungus Phanerochaete chrysosporium. J. Biosci. Bioeng. **95:**572-576.

22. **James, R., C. Ketudat, E. Asim.** 2010. β-Glucosidases. Cell. Mol. Life Sci. **67:**3389-3405.

23. **Jenkins, J., L. L. Leggio, G. Harris, and R. Pickersgill.** 1995. β-Glucosidase, β-galactosidase, family A cellulases, family F xylanases and two barley glycanases form a superfamily of enzymes with a 8-fold α/β architecture and with two conserved glutamates near the carboxy-terminal ends of β-strands four and seven. FEBS Lett. **362:**281-285.

24. **Kashiwagi, Y., C. Iijima, T. Sasaki, and H. Taniguchi.** 1991. Characterization of a β-glucosidase encoded by a gene from *Cellovibrio gilvus*. Agric. Biol. Chem. **55:**2553-2559.

25. **Kleerebezem, M., and E. E. Vaughan.** 2009. Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. Annu. Rev. Microbiol. **63:**269-290.

26. **Konstantinidis K. T., and J. M. Tiedje.** 2004. Trends between gene content and genome size in prokaryotic species with larger genomes. Proc. Natl. Acad. Sci. USA. **101:**3160-3165.

27. **Kubicek, C. P., R. Messner, F. Gruber, R. L. Mach, and E. M. Kubicek-Pranz.** 1993. The Trichoderma cellulase regulatory puzzle: from

the interior life of a secretory fungus. Enz. Microb. Technol. **15:**90-99.

28. **Kuntothom, T., S. Luang, A. J. Harvey, G. B. Fincher, R. Opassiri, M. Hrmova, and J. R. Ketudat Cairns.** 2009. Rice family GH1 glycosyl hydrolases with b-D-glucosidase and b-D-mannosidase activities. Arch. Biochem. Biophys. **491:**84-95

29. **Li, Y. K. and J. A. Lee.** 1999. Cloning and expression of β-glucosidase from *Flavobacteruim meningosepticum*: a new member of family B β-glucosidase. Enz. Microb. Technol. **24:**144-150.

30. **Lin, L. L., E. Rumbak, H. Zappe, J. A. Thompson, and D. R. Woods.** 1990. Cloning, sequencing and analysis of expression of a *Butyrivibrio fibrisolvens* gene encoding a β-glucosidase. J. Gen. Microbiol. **136:**1567-1576.

31. **Lymar, E. S., B. Li, and V. Renganathan.** 1995. Purification and characterization of a cellulose-binding b-glucosidase from cellulose degrading cultures of Phanerochaete chrysosporium. Appl. Environ. Microbiol. **61:**2976-2980.

32. **Marana, S. R.** 2006. Molecular basis of substrate specificity in family 1 glycoside hydrolases. IUBMB Life **58:**63-73

33. **Marri, L., S. Valentini, and D. Venditti.** 1995. Cloning and nucleotide sequence of *bglA* from *Erwinia herbicola* and expression of β-glucosidase activity in *Escherichia coli*. FEMS Microbiol. Lett. **128:**135-138.

34. **Matsuda, S., F. Norimoto, Y. Matsumoto, R. Ohba, Y. Teramoto, N. Ohta, and S. Veda.** 1994. Solubilization of a novel isoflavone glycoside

hydrolyzing β-glucosidase from *Lactobacillus casei* subsp. *rhamnosus*. J. Ferment. Bioeng. **77:**439-441.

35. **Matsui, I., Y. Sakai, E. Matsui, M. Kikuchi, Y. Kawarabayasi, and K. Honda.** 2000. Novel substrate specificity of a membrane-bound β-glycosidase from the hyperthermophilic archeon *Pyrococcus horikoshii*. FEBS Lett. **467:**195-200.

36. **Paavilainen, S., J. Hellman, and T. Korpela.** 1993. Purification, characterization, gene cloning and sequencing of a new β-glucosidase from *Bacillus circulans* subsp. *alkalophilus*. Appl. Environ. Microbiol. **59:**927-932.

37. **Painbeni, E., S. Valles, J. Poliana, and A. Flors.** 1992. Purification and characterization of a *Bacillus polymyxa* β-glucosidase expressed in *Escherichia coli*. J. Bacteriol. **174:**3087-3091.

38. **Park, J. K., L. X. Wang, H. V. Patel, and S. Roseman.** 2002. Molecular cloning and characterization of a unique b-glucosidase from Vibrio cholorae. J. Biol. Chem. **277:**29555-29560.

39. **Rastall, R. A., G. R. Gibson, H. S. Gill, F. Guarner, T. R. Klaenhammer, B. Pot, G. Reid, I. R. Rowland, and M. E. Sanders.** 2005. Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: an overview of enabling science and potential applications. FEMS Microbiol. Ecol. **52:**145-152.

40. **Ridgway, T., G. Tucker, and H. Wiseman.** 1997. Novel bioconversions

for the production of designer antioxidant and colourant flavonoides using polyphenol oxidases. Biotechnol. Genet. Eng. Rev. **14:**165-190.

41. **Rixon, J. E., L. M. A. Ferreira, A. J. Durrant, J. I. Laurie, P. J. Hazlewood, and H. J. Gilbert.** 1992. Characterization of the gene *CelD* and its encoded product 1,4 β-D-glucan glucohydrolase D from *Pseudomonas fluorescens* subsp. *cellulosa*. Biochem. J. **285:**947-955.

42. **Roitner, M., T. Schalkhammer, and F. Pittner.** 1984. Characterization of naringinase from *Aspergillus niger*. Monatsh. Chem. **115:**1255-1267.

43. **Romaniec, M. P. M., N. Huskisson, P. Barker, and A. L. Demain.** 1993. Purification and properties of the *Clostridium thermocellum bglB* gene product expressed in *Escherichia coli*. Enz. Microb. Technol. **15:**393-400.

44. **Rye, C. S., and S. G. Withers.** 2000. Glycosidase mechanisms. Curr. Opin Chem Biol **4:**573-580.

45. **Sakellaris, H., J. M. Manners, and J. M. Pemberton.** 1997. A gene encoding an exo- β-glucosidase from *Cellovibrio mixtus*. Curr. Microbiol. **35:**228-232.

46. **Schell, M. A., M. Karmirantzou, B. Snel, D. Vilanova, B. Berger, G. Pessi, M. C. Zwahlen, F. Desiere, P. Bork, M. Delley, R. D. Pridmore, and F. Arigoni.** 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc. Natl. Acad. Sci. USA. **99:**14422-14427.

47. **Skory, C. D. and S. N. Freer.** 1995. Cloning and characterization of a gene encoding a cell-bound, extracellular β-glucosidase in the yeast Candida

wickerhamii. Appl. Environ. Microbiol. **61:**518-525.

48. **Sota, H., P. Arunwanich, O. Kurita, N. Vozumi, H. Honda, S. Iijma, and T. Kobayashi.** 1994. Molecular cloning of thermostable β-glucosidase gene from a thermophilic anaerobe NA10 and its high expression in *Escherichia coli*. J. Ferment. Bioeng. **77:**199-201.

49. **Srivastava, K. K., P. K. Verma, and R. Srivastava.** 1999. A recombinant cellulolytic *Escherichia coli*: cloning of the cellulase gene and characterization of a bifunctional cellulase. Biotechnol. Lett. **21:**293-297.

50. **Takano, M., R. Moriyama, and K. Ohmiya.** 1992. Structure of a β-glucosidase gene from *Ruminococcus albus* and properties of the translated product. J. Ferment. Bioeng. **73:**79-88.

51. **Tsukada, T., K. Igarashi, M. Yoshida, M. Samejima.** 2006. Molecular cloning and characterization of two intracellular b-glucosidases belonging to glycoside hydrolase family 1 from the basidiomycete Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol. **73:**807-814.

52. **Varghese J. N., M. Hrmova, G. B. Fincher.** 1999. Three-dimensional structure of a barley b-D-glucan exohydrolase; a family 3 glycosyl hydrolase. Structure **7:**179-190.

53. **Ventura, M., M. O'Connell-Motherway, S. Leahy, J. A. Moreno-Munoz, G. F. Fitzgerald, D. Sinderen.** 2007. From bacterial genome to functionality; case bifidobacteria. Int. J. Food Microbiol. **120:**2-12.

54. **Ventura, M., S. O'Flaherty, M. J. Claesson, F. Turroni, T. R. Klaenhammer, D. Sinderen, P. W. O'Toole.** 2009. Genome-scale analyses

of health-promoting bacteria: probiogenomics. Nat. Rev. Microbiol. **7:**61-71.

55. **Verdoucq, L., M. Czjzek, J. Moriniere, D. R. Bevan, A. Esen.** 2003. Mutational and structural analysis of aglycone specificity in maize and sorghum b-glucosidases. J. Biol. Chem. **278:**25055-25062.

56. **Voorhorst, W. G. B., R. I. L. Eggen, E. J. Lensink, and W. M. D. E. Vos.** 1995. Characterization of celB gene encoding for β-glucosidase from the hyperthermophilic archeon Pyrococcus furiosus and its expression and site directed mutation in *Escherichia coli*. J. Bacteriol. **177:**7105-7111.

57. **Vrese, M., and J. Schrezenmeir.** 2008. Probiotics, prebiotics, and synbiotics. *Adv Biochem Eng Biotechnol*. **111:**1-66.

58. **Vroemen, S., J. Heldens, C. Boyd, B. Henrissat, and N. T. Keen.** 1995. Cloning and characterization of the *bgxa* gene from *Erwinia chrysanthemi* D1 that encodes a β-glucosidase / xylosidase enzyme. Mol. Gen. Genet. **246:**465-477.

59. **Wright, R. M., M. D. Yablonsky, Z. P. Shalita, A. K. Goyal, and D. E. Eveleigh.** 1992. Cloning, characterization, and nucleotide sequence of a gene encoding *Microspora bispora BglB*, a thermostable β-glucosidase expressed in *Escherichia coli*. Appl. Environ. Microbiol. **58:**3455-3465.

60. **Wulff-Strobel, C. R. and D. B. Wilson.** 1995. Cloning, sequencing, and characterization of a membrane associated *Prevotella ruminicola* B_1 4 β-glucosidase with cellodextrinase and cyanoglycosidase activities. J. Bacteriol. **177:**5884-5890.

61. **Zverlow, V. V., I. Y. Volkov, T. V. Velikodvorskaya, and W. H. Schwarz.** 1997. *Thermotoga neopolitana bglB* gene, upstream of *lamA*, encodes a highly thermostable β-glucosidase that is a laminaribiase. Microbiology **143:**3537-3542.

2. Identification of the β-Glucosidase Gene from *Bifidobacterium animalis* subsp. *lactis* and Its Expression in *B. bifidum* BGN4

2.1 Introduction

β-Glucosidase is an important enzyme that hydrolyzes β-glucosides by cleaving the β-D-glucosidic linkages and liberating glucose moieties [8]. β-Glucosidases are widely distributed in living organisms and play vital roles in many biological processes, such as the degradation of cellulosic biomass [11], cyanogenesis [24], the cleavage of glucosylated flavonoids [25], and the production of fuel ethanol from lignocelluloses [23]. A number of biologically active components of natural products, such as ginsenoside, isoquercetin, daidzein, and genistein, are glycosides that are important functional ingredients of natural food materials. Interestingly, the biological effects of many glycosides are not attributable to their glycoside forms, but to their aglycones [15, 19]. Aglycones are highly bioactive because of their unimpeded intestinal absorption, unlike the corresponding glycosides, which are not absorbed across enterocytes because of their greater hydrophilicity and higher molecular weights [3, 34, 38]. When glycosides are ingested as food components, their bioavailability can be enhanced by the hydrolysis of their sugar moieties by the various glycosidases of the intestinal microflora. Intestinal microflora differ between individuals, so the bioavailabilities of the glycosidic phytochemicals differ. To overcome these individual differences in the bioavailability of phytochemicals in food materials, the bioconversion of phytochemicals by glycosidases before oral administration has been studied.

The genus *Bifidobacterium* is recognized by the World Health Organization and the Food and Agriculture Organization as one of the most important probiotic microorganism [18], except for *B. scardovii* and *B. dentium* which might cause clinical infection and dental caries [7, 36]. Several groups of bacteria are known to

express β-glucosidase activity, including species of *Bifidobacterium* [13], which are a major component of the human gastrointestinal tract microflora. Previously, β-glucosidase was expressed in *Escherichia coli* [20, 27, 28, 29] and used in various fields related to carbohydrate chemistry. However, safety problems must be addressed before recombinant enzymes are used in the food industry. Because it is generally nonpathogenic, *Bifidobacterium* may be advantageous as the host cells to produce various recombinant food-grade enzymes.

For this purpose, Kim *et al* [22] cloned structural gene of β-glucosidase from *B. lactis* AD011 at the downstream of 16S rRNA promoter and ribosome binding site (RBS) of bifiobacterial expression vector, pBES16PR, and expressed in *E. coli*. However, they did not show any detectable β-glucosidase activity in *B. bifidum* BGN4.

To overcome our previous study, we have cloned, sequenced, and sucessfully overexpressed bifidobacterial β-glucosidase gene in *B. bifidum* BGN4 by constructing several expression vector systems using bifidobacterial promoters, a signal sequence, and a terminator. The pH and temperature stability and substrate specificity of the overexpressed β-glucosidase were characterized.

2.2 Materials and methods

2.2.1 Bacterial strains and culture conditions

The β-glucosidase-deficient *Escherichia coli* XL1 blue MR, *E. coli* DH5α, and *B. bifidum* BGN4 were used as cloning, subcloning, and expression hosts, respectively. *E. coli* XL1 blue MR and *E. coli* DH5α were cultured aerobically in Luria–Bertani (LB) broth (Becton Dickinson, Sparks, MD, USA) at 37°C for 15 h with vigorous shaking and supplemented with 100 μg/ml ampicillin (LBA; Sigma, St Louis, MO, USA), if necessary. *Bifidobacterium* species were cultured anaerobically in brain heart infusion (BHI) broth (Becton Dickinson) supplemented with 0.05% (v/w) L-cysteine·HCl (Sigma), at 37°C for 16 h. The transformed *B. bifidum* BGN4 cells were grown in modified Transgalactooligosaccharide propionate (TP) [17] broth supplemented with 1% (w/v) glucose instead of transgalactooligosaccharides (TOS), and 3.6 μg/ml chloramphenicol (BioBasic, Markham, Ontario, Canada) was added if necessary. The bacteria and plasmids used in this study are listed in Table 2.1.

Table 2.1 Bacterial strains and plasmids.

Strain or vector	Relevant characteristics or genotype	Source or reference
Bacterial strains		
SH5	*B. lactis* SH5, wild type; Glu+ (original host of *bbg572*)	
RD68	*B. lactis* RD68, wild type; Glu+	
BGN4	*B. bifidum* BGN4, wild type; Glu-; Transformation host	
DH5α	*E. coli* DH5α , *F-(80dlacZ M15) (lacZYA-argF)U169hsdR17(r-m+) recA1 endA1 relA1 deoR*; Cloning host	[12]
XL1 blue MR	*E. coli* XL1 blue MR, Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*; Cloning host	Stratagene
Bpamyss572	*B. bifidum* BGN4 harboring pamyss572	
Bp919ss572	*B. bifidum* BGN4 harboring p919ss572	
Bp572ss572	*B. bifidum* BGN4 harboring p572ss572	
Bp919bbg572	*B. bifidum* BGN4 harboring p919bbg572	
Bp572bbg572	*B. bifidum* BGN4 harboring p572bbg572	
Bp572bbg572t	*B. bifidum* BGN4 harboring p572bbg572t	
Plasmids		
SuperCos1 Cosmid	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
pUC18	Amp^r	
pBES2	Amp^r, Cm^r; *E. coli-Bifidobacterium* shuttle vector	[32]
pBESAF2	Amp^r, Cm^r; *E. coli-Bifidobacterium* shuttle vector	[31]
pUC572PT	Amp^r: pUC18 with 2.276 kb insert of *B. lactis* DNA containing the *bbg572pt*	

2.2.2 Screening of lactic acid bacteria for their β-glucosidase activities

Various lactic acid bacteria, including 19 *Bifidobacterium* spp., nine *Lactobacillus* spp., eight *Bacillus* spp., two *Lactococcus* spp., two *Enterococcus* spp., one *Leuconostoc* sp., one *Weissella* spp., and one *Pediococcus* sp., were used in this study (Table 2. 2).

These bacteria were cultivated in BHI broth supplemented with 0.05% L-cysteine·HCl for 16 h at 37°C. An aliquot (1.0 ml) of culture broth was centrifuged at 6,000 × g for 10 min at 4°C and washed twice with 500 μl of 50 mM phosphate buffer (PB buffer, pH 6.0). The cells were resuspended in 500 μl of the same buffer and disrupted by sonication (VCX 400; Sonics & Materials Inc., Newton, CT, USA). The cell-free extracts were obtained by centrifugation at 6,000 × g for 10 min at 4°C. The substrate specificity of each cell-free extract was determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as the substrate (Sigma). An aliquot (90 μl) of each cell-free extract was mixed with 10 μl of 10 mM *p*NPG substrate and incubated at 37°C for the appropriate time. The reaction was stopped with 100 μl of 1 M Na_2CO_3 and the absorbance was measured at 405 nm. One unit of enzyme activity corresponded to the amount of enzyme that liberated 1 μmol of *p*-nitrophenol (*p*NP) per minute at 37°C. The amount of liberated *p*NP was calculated using a standard *p*NP solution (Sigma) and the protein concentration was measured with the Bradford method using bovine serum albumin (Bio-Rad, Piscataway, NJ, USA) as the standard, according to the manufacturer's instructions.

Table 2.2 Lactic acid bacterias used for the screening as β-glucosidase producing strains

No.	Microorganism	No.	Microorganism
1	*Bifidobacterium pseudocatenulatum* INT57	13	*Bifidobacterium* sp. RD03
2	*Bifidobacterium* sp. RD02	14	*Bifidobacterium* sp. RD47
3	*Bifidobacterium* sp. RD6	15	*Bifidobacterium* sp. RD57
4	*Bifidobacterium thermophilum* KCCM 12097	16	*Bifidobacterium dentium* KCTC 3222
5	*Bifidobacterium pseudocatenulatum* SJ32	17	*Bifidobacterium thermophilum* KCCM 12097
6	*Bifidobacterium animalis* subsp. *lactis* SH5	18	*Bifidobacterium breve* KCTC 3220
7	*Bifidobacterium* sp. RD01	19	*Bifidobacterium longum* KCCM 11953
8	*Bifidobacterium* sp. RD07	20	*Lactobacillus casei* KFRI 00127
9	*Bifidobacterium* sp. RD10	21	*Lactobacillus casei* KFRI 00129
10	*Bifidobacterium bifidum* BGN4	22	*Lactobacillus casei* KFRI 00196
11	*Bifidobacterium* sp. RD86	23	*Lactobacillus casei* KFRI 00346
12	*Bifidobacterium* sp. RD68	24	*Lactobacillus casei* KFRI 00699

No.	Microorganism	No.	Microorganism
25	*Lactobacillus casei* KCTC 3600	35	*Bacillus subtilis* sponge1
26	*Lactobaillus acidophilus* KCTC 3150	36	*Bacillus subtilis* sponge2
27	*Lactobaillus acidophilus* KCTC 3154	37	*Bacillus subtilis* G15
28	*Lactobacillus bulgaricus* KCTC 3188	38	*Bacillus subtilis* D17
29	*L*actococcus *lactis* subsp. *cremoris* ATCC *19257*	39	*Bacillus natto* MUCILAGES
30	*Lac*tococcus *lactis* subsp. *lactis* ATCC 11454	40	*Bacillus natto* C16a
31	*E*nterococcus *faecalis ATCC 19433*	41	*Bacillus natto* D25
32	*E*nterococcus *faecium* ATCC 27270	42	*Bacillus natto* G14
33	*Leu*conostoc *mesenteroides* subsp. *mesenteroides* ATCC 27258	43	*Pediococcus acidilactisi* ATCC 3334
34	*Weissella confusa* ATCC 10881		

2.2.3 Construction of cosmid library of *Bifidobacterium*

Chromosomal DNA was extracted from *Bifidobacterium* species with the method of Choi *et al*. [19] and partially digested with *Sau*3AI (Promega, Madison, WI, USA). The SuperCos 1 cosmid vector (Stratagene, La Jolla, CA, USA) was digested with *Bam*HI (Promega) and treated with calf intestinal alkaline phosphatase (Promega), then ligated to the DNA fragments using a T4 DNA Ligation Kit (Stratagene). The ligation mixture was packaged *in vitro* using the Gigapack III Gold Packaging Extract (Stratagene) and transformed into *E. coli* XL1 blue MR according to the manufacturer's instructions. To select the bacterial clones expressing β-glucosidase activity, 5-bromo-4-chloro-3-indolyl β-D-glucopyranoside (Wako Pure Chemical Industries, Osaka, Japan)-containing plates supplemented with ampicillin (Biobasic Inc., Toronto, Ontario, Canada) were used. Blue colonies were isolated as positive clones and used for further analysis. Plasmids were isolated from *E. coli* with the Midi Plus™ Ultrapure Plasmid Extraction System (Viogene, Taipei, Taiwan). The transformed *E. coli* cells were used to construct a genomic library.

2.2.4 Cloning of the *Bifidobacterium* β-glucosidase genes and sequence analysis

DNA fragments encoding β-glucosidase were isolated from a cosmid genomic library using Midi Plus (Viogene) and partially digested with *Bam*HI. These were subcloned into the cloning vector pUC18, digested with *Bam*HI, and used to transfect *E. coli* DHα as the cloning host, according to standard procedures [33].

The positive colonies showing β-glucosidase activity were isolated and the plasmids were purified and subjected to restriction and sequence analyses. The nucleotide sequences were determined with the BigDye® Terminator v 3.1 Kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3730xl Genetic Analyzer (Applied Biosystems) in Bionicsro (Seoul, Korea). The promoter regions were predicted using the program at http://www.fruitfly.org/seq_tools/promoter.html and the terminator regions were predicted with the program at http://linux1.softberry.com.

2.2.5 Analysis of transcription start sites

The transcriptional start site of *bbg572* was determined by 5′-rapid amplification of cDNA ends (5′-RACE) using a RACE kit (Clontech, Palo Alto, CA, USA). mRNA from the *Bifidobacterium* species was reverse transcribed using SMARTScribe reverse transcriptase and 5′-CDS PrimerA provided by the manufacturer. After first-strand cDNA synthesis, the cDNA was tailed and amplified by PCR using a universal primer provided in the 5′-RACE kit and combined with the *bbg572*-gene-specific primer (5′-GGAATGACGCGCGGCACGCCAATCGAG-3′). PCR was performed under the following conditions: 5 min denaturation at 94°C, followed by 25 cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 3 min; and the reaction was completed with a final extension at 72°C for 10 min. The PCR product was cloned into the pGEM-T Easy vector (Promega) and confirmed by sequencing.

2.2.6 Construction of a vector encoding β-glucosidase and transformation of *Bifidobacterium*

The target genes were amplified using *PfuUltra* II Fusion HS DNA polymerase (Stratagene) and each primer set (Table 2. 3), according to the manufacturer's instruction manual.

First, the structural gene of the β-glucosidase, *bbg572*, was PCR amplified from the cosmid library using the appropriate primer set for each gene (Table 2. 3) and cloned into the *Bifidobacterium* shuttle vector pBES2 [32] to construct pBES-bbg572. *B. bifidum* BGN4, a β-glucosidase-negative host, was transformed with this plasmid, according to the method of Kim *et al* [21].

Second, to study the regulatory effects of the promoter and terminator sequence, regions of different gene promoters (*pamy*, *p919* and *p572*) and the region of the gene terminator (*572t*) were amplified from each template using the primer sets for each gene (Table 2. 2). The amplified putative promoter sequences were digested with restriction enzymes and then cloned into pBES-bbg572 digested with the same restriction enzymes to incorporate the promoter upstream from the β-glucosidase gene. *B. bifidum* BGN4 was then transformed with the constructs individually. The amplified signal sequence (*ssamy*) was fused between the promoter and the structural gene in the corresponding vectors. The amplified putative terminator sequence of the β-glucosidase gene was digested with restriction enzymes and then cloned downstream from the structural gene in p572bbg572. *B. bifidum* BGN4 was then transformed with each construct individually. The 7 constructs are described in Table 2. 4.

Table 2.3 Primers for amplifying β-glucosidase gene and putative promoters from *Bifidobacterium*.

Primer name	Sequences of primer		Product	Reference
	Forward (5→3)	Reverse (5→3)		
primerbbg572pt	ggatccATGCTGCTCCTTATGTGTC	gaattcGCTCCCGCAGCTTCG	*bbg572pt*	
primerbbg572p	tctagaATGCTGCTCCTTATGTGTC	gaattcCTACTTGGCGGAGTGCTC	*bbg572p* (*p572+bbg572*)	
primerbbg572	ggatccATGACGATGACGTTCCC[a] agtactATGACGATGACGTTCCC[b]	gaattcCTACTTGGCGGAGTGCTC	*bbg572*	
primerbbg572t	ggatccATGACGATGACGTTCCC[a] agtactATGACGATGACGTTCCC[b]	gaattcGCTCCCCGCAGCTTCG	*bbg572t* (*bbg572+572t*)	
primerPamy	tctagaGAAATACCGCAATGCACG	ggatccGGCTCCTTTATTCCTTTTC	*pamy*	31
primer919P	tctagaTGAAGTGTGTCGTGTGG	ggatccTGGTGTACCTTTTCTTG	*p919*	37
primer572P	tctagaATGCTGCTCCTTATGTGTC	ggatccTGCTGATTCCTCC	*p572*	
primerSSamy	ggatccATGAAACATCGGAAACC	agtact GGCCTGTGCTGCGG	*ssamy*	

Restriction enzyme sites were indicated by small letter: XbaI tctaga, BamHI ggatcc, EcoRI gaattc, ScaI agtact. a) Primers were used to construct p919bbg572, p572bbg572 and p572bbg572t. b) Primers were used to construct pamyss572, p919ss572, p504ss572 and p572ss572.

Table 2.4 Constructed vectors with cloned β-glucosidase gene.

Promoter			Signal sequences	ORF	Terminator	Plasmid
pamy	*p919*	*p572*	*ssamy*	*bbg572*	*572t*	
	o			o		p919bbg572
		o		o		p572bbg572
		o		o	o	p572bbg572t
o			o	o		pamyss572
	o		o	o		p919ss572
			o	o		p504ss572
		o	o	o		p572ss572

2.2.7 Analysis of β-glucosidase activity in recombinant *B. bifidum* BGN4

The β-glucosidase activities of recombinant *B. bifidum* BGN4 were analyzed using *p*NPG as the substrate to compare the strengths of the promoters and terminator.

The recombinant *B. bifidum* BGN4 strains were cultured overnight at 37°C in modified TP broth supplemented with 3.6 μg/ml chloramphenicol. An aliquot (80 μl) of each culture broth was transferred to 8 ml of modified TP broth containing 3.6 μg/ml chloramphenicol and incubated for 16 h. The β-glucosidase activities were determined with *p*NPG, as described previously.

2.2.8 Optimal pH, temperature, and time of the β-glucosidase

The optimal pH for the recombinant β-glucosidase was determined with 10 mM *p*NPG as the substrate in 50 mM PB buffer at pH 3.0–8.0. *p*NPG (10 μl) was added to 90 μl of cell-free extract in each buffer (pH 2.0–8.0; 50 mM glycine-HCl (pH 2.0), 100 mM citric acid–citrate buffer (pH 3.0–6.0), 50 mM Tris-HCl buffer (pH 7.0–8.0)) and incubated at 37°C for 30 min. The reaction was stopped with 100 μl of 1 M Na_2CO_3 and the absorbance was measured at 405 nm. .

To evaluate the optimal temperature for enzyme activity, the cell-free extract in 50 mM PB buffer (pH 6.0) was maintained at 10–80°C for 30 min, and the remaining activity was determined as described above.

The specific activity of β-glucosidase according to the culture time was determined over a time range of 0–32 h. Each cell-free extract in 50 mM PB buffer (pH 6.0) was maintained at 37°C for 30 min, and the activity was determined as described above.

2.2.9 Substrate specificity of β-glucosidase

Cellobiose, sophorose, laminaribiose, gentiobiose, daidzin, daidzein, genistin, glycitin, glycitein, and isoquercetrin were purchased from Sigma. Quercetin-3,4-di-O-β-D-glucoside and quercetin-7-O-β-D-glucoside were purchased from Extrasynthese (Genay, France). Genistein and quercetin-7-glucoside were purchased from Chengdu Biopurify Phytochemicals Ltd (Chengdu, China). Ginsenoside standards such as compound K, Rb1, Rb2, Rc, Rd, F1, Rg3, and Rh1 were purchased from Cogon Chemical (Chengdu, China); F2, Rg2(S), and Rh2(s) were from LKT Laboratories (St Paul, MN, USA); Rg1 was from Wako Pure Chemical Industries; Rg3 (S) and Re were from BTGin Co. Ltd (Daejeon, Korea). All standard ginsenosides were dissolved in water (containing 3% Tween 80) as individual solutions and saccharides were dissolved in water. Other substrates were dissolved in methanol (J.T. Baker, Phillipsburg, NJ, USA).

The cell-free extract from the overnight culture of the recombinant *B. bifidum* BGN4 was prepared in 50 mM PB buffer (pH 6.0), as described above. Reaction mixtures (200 μl) containing 180 μl of cell-free extract and 1 mg/ml ginsenosides were incubated at 37°C with shaking. Samples were harvested at 24 h and analyzed with thin-layer chromatography (TLC), according to Chi *et al* [4].

For the hydrolysis of disaccharides, 180 μl of cell-free extract in 50 mM PB buffer (pH 6.0) was mixed with 20 μl of 100 mM disaccharide in the same buffer and incubated at 37°C. At 24 h, the samples were analyzed with TLC using *n*-butanol:formic acid:water (4:8:1) as the developer. They were visualized by spraying with 10% H_2SO_4 and drying at 120°C for 10 min.

For the hydrolysis of the flavonoid glycosides, 180 μl of cell-free extract in 50 mM

PB buffer (pH 6.0) was mixed with 20 µl of 100 mM quercetin and incubated at 37°C. After 24 h, the samples were analyzed with TLC using *n*-butanol:acetic acid:water (7:1:2) as the developer. The samples were visualized by spraying them with 10% H_2SO_4 and drying at 120°C for 10 min.

For the hydrolysis of the isoflavone glycosides, 180 µl of cell-free extract in 50 mM PB buffer (pH 6.0) was mixed with 20 µl of 100 mM isoflavone and incubated at 37°C. After 24 h, the samples were analyzed with TLC using chloroform:methanol:water (65:35:10) as the developer. The isoflavones were visualized under a UV lamp at 254 nm.

The Hybrid LC/MS/MS System was measured for two kinds of transformed ginsenoside (Rb1 and Rb2 with Bp504bbg504t) by National Instrumentation Center for Environmental Management (NICEM). For full scan MS analysis, the spectra were recorded in the m/z range from 260 to 960.

2.3 Results and discussion

2.3.1 Sequence analysis of *Bifidobacterium* β-glucosidase genes

Two *B. lactis* strains (SH5 and RD68) showing relatively high β-glucosidase activities were selected from 43 experimental lactic acid bacterial strains (Fig. 2.1). After the construction of cosmid libraries in *E. coli* using the genomic DNAs from these two strains, about 30 clones with β-glucosidase activity were isolated for each construct. One clone from each screened colony showing the greatest enzyme activity was selected. The fragment insert in the cosmid was digested with *Bam*HI and subcloned into pUC18, and the β-glucosidase-positive clones were isolated. Sequence analysis of the two cloned β-glucosidase genes showed that the β-glucosidase gene from *B. lactis* SH5 was identical to that of *B. lactis* RD68 with respect to its open reading frame (ORF), putative promoter region, and putative terminator region. As a consequence, β-glucosidase gene from *B. lactis* SH5 was designated as *bbg572pt* and used for further study (Fig. 2.2). *bbg572pt* was comprised of 2,276 bp and contained one ORF. An putative terminator sequence was located downstream of ORF. The putative ribosome binding site (RBS), AGGAGGA, was detected (gray box; Fig. 2.2) in the upstream of ORF, which is similar to the RBS of *B. longum* MG1 [30]. The deduced amino acid sequence contained 460 residues, with a molecular mass of 50.71 kDa, with 85%, 68%, 70%, and 69% amino acid sequence identities with the β-glucosidases from *B. breve* clb, *B. breve* CECT7263, *B. dentium* ATCC27679, and *B. longum* subsp. *infantis* ATC15697, respectively (Fig. 2.3). Based on these amino acid sequence similarities, the β-glucosidase encoded by *bbg572* was assigned to glycosyl hydrolase family 1. The overall GC content of *Bifidobacterium* is generally 55.0%–67.0%, which is

higher than those of other bacteria [35]. The GC contents of *bbg572* was 63%, which is slightly lower than that of the β-glucosidase from *B. breve* clb (65.1%) [28].

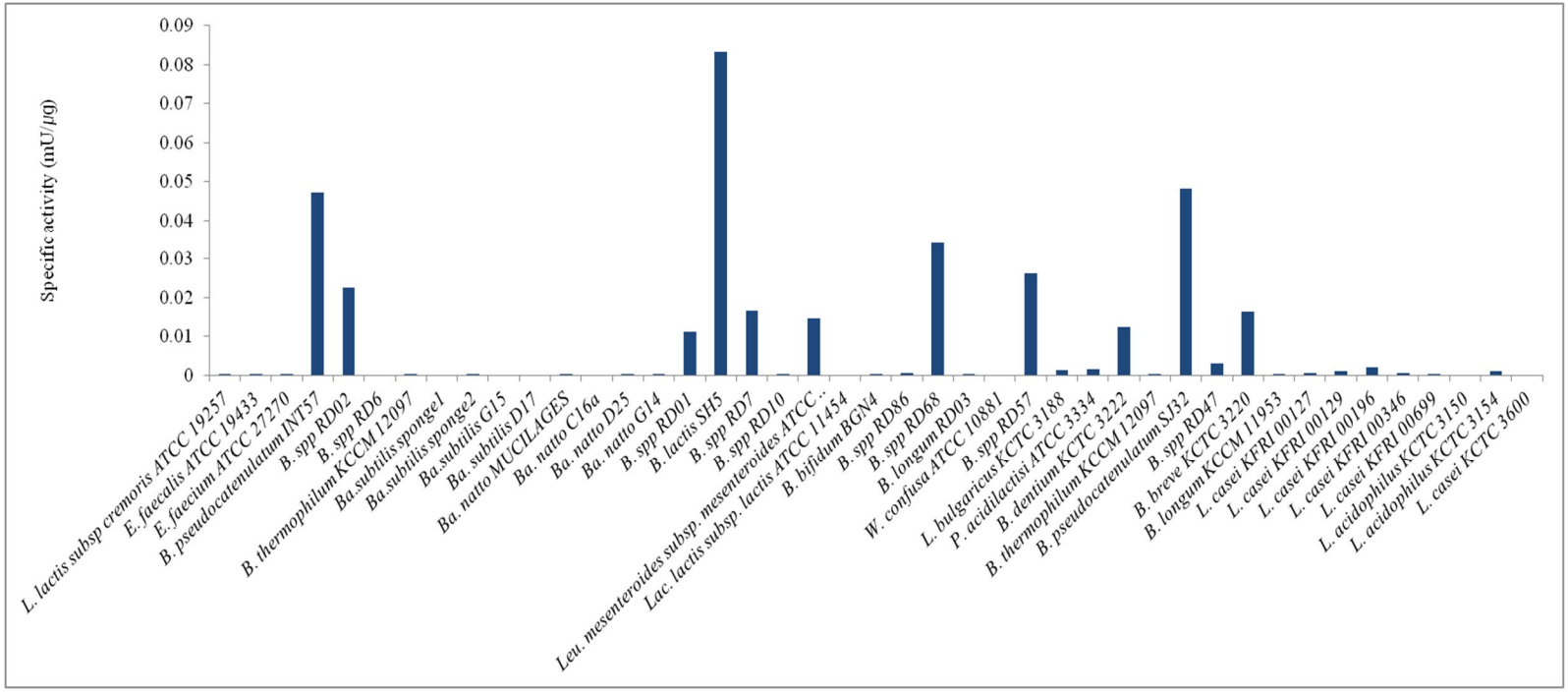


Fig. 2.1 β-Glucosidase activities of the various experimental lactic acid bacterias.

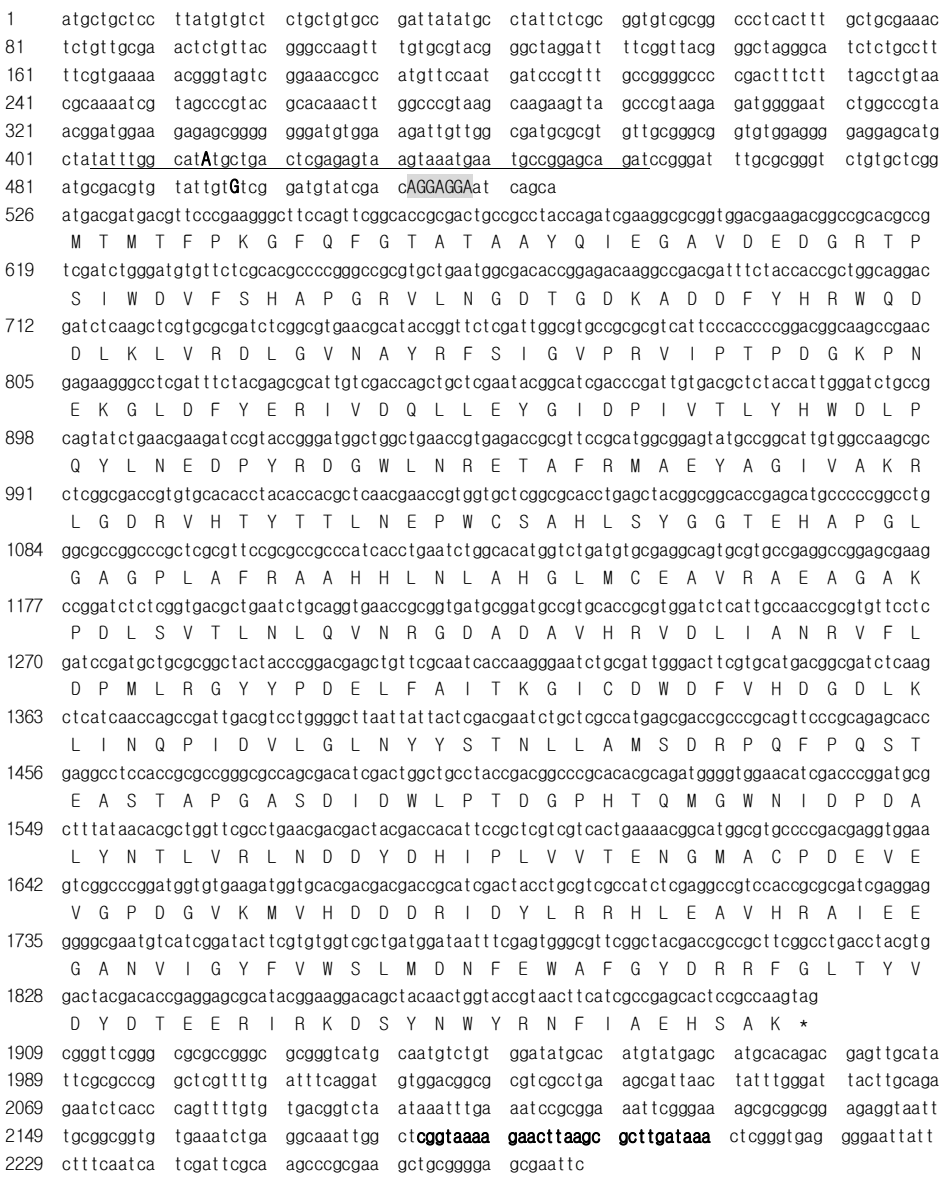


Fig. 2.2 Nucleotide and deduced amino acid sequences of the *bbg572pt* in pUC18 (pUC572PT). An putative terminator sequence is indicated by bold letters. Putative ribosomal binding site (RBS) is indicated in gray box and capitalized. Transcription start points are in bold and capitalized. Predicted promoter region is underlined. Translational termination site is marked by asterisk. The sequence data is available in GenBank under accession number JX274651

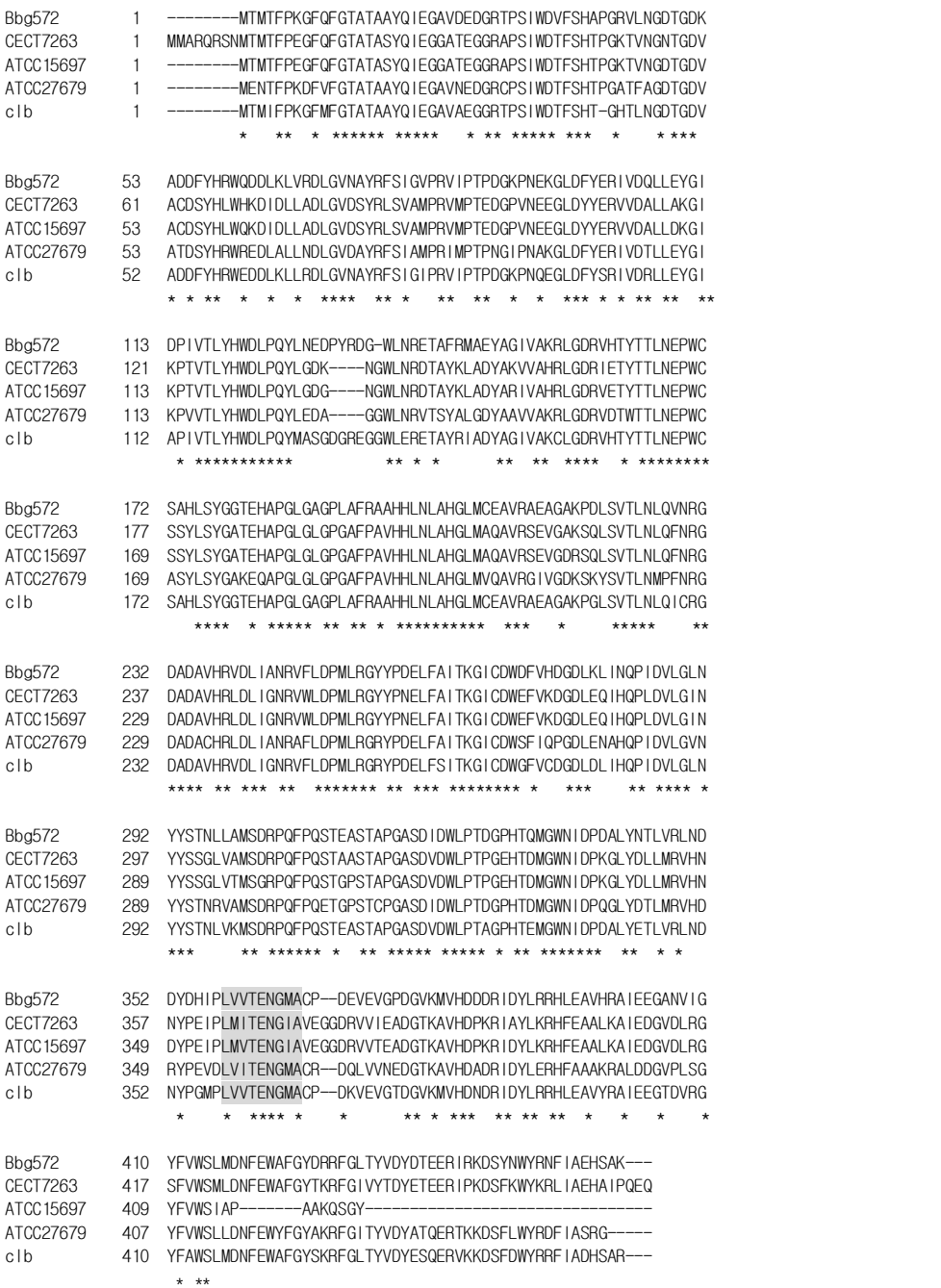


Fig. 2.3 Multiple sequence alignment of β-glucosidases from *Bifidobacterium* : Bbg572, *Bifidobacterium lactis* SH5 and RD68; CECT7263, *Bifidobacterium breve* CECT 7263; ATCC15697, *Bifidobacterium longum* subsp. *infantis* ATCC 15697; ATCC27679, *Bifidobacterium dentium* ATCC 27679; clb, *Bifidobacterium breve* clb. A region conserved in glycosyl hydrolase family 3 is gray boxed. Conserved amino acid residues are indicated as asterisk.

2.3.2 Analysis of the transcription start point

Putative promoter was predicted upstream from *bbg572* (Fig. 2.2) and designated as *p572*. The putative –10 and –35 position in promoter region showed homology with the consensus sequence of *E. coli* and this promoter induced successful expression of *bbg572* in *E. coli* DH5α (data not shown). 5′-RACE analysis revealed two potential transcription start points (*tsp*s) for *bbg572* (the G and A residues in Fig. 2.4). The –10 region (tgtatt) and –35 region (ttgcgc) upstream from the G residue displayed 66.7% and 50% identity, respectively, to the corresponding *E. coli* consensus sequences. The –10 region (tatatt*)* and –35 region (ttgcgg) upstream from the A residue displayed 83.3% and 50% sequence identity, respectively, to the corresponding *E. coli* consensus sequences.

The divergence in sequence homology in the –35 regions is consistent with many previous studies that have shown that transcription readily occurs in *E. coli* even in the absence of absolute similarity in this region [6].

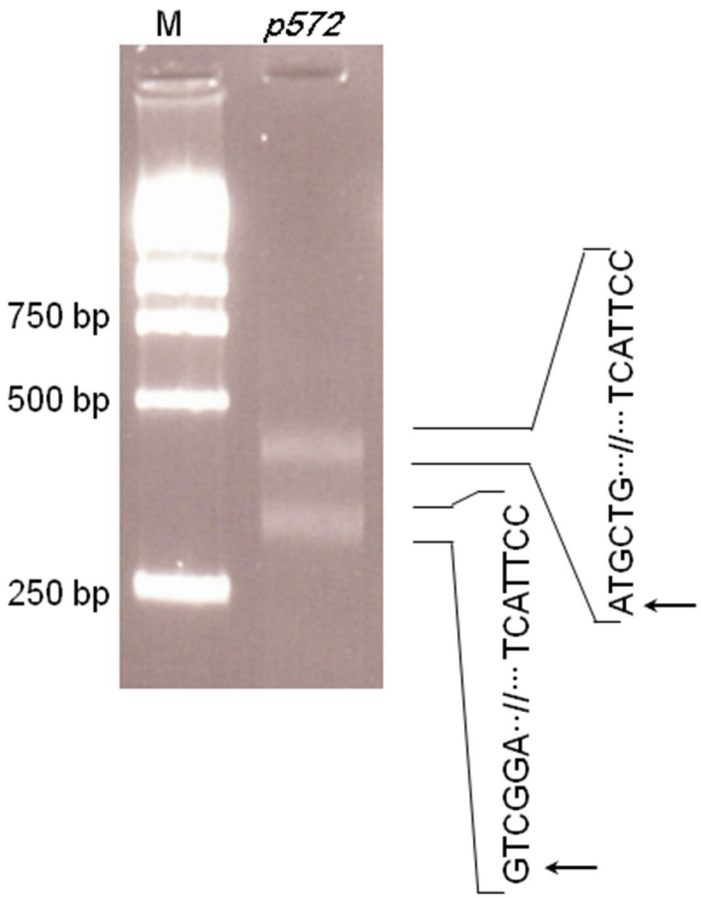


Fig. 2.4 5'-rapid amplification of cDNA ends (RACE) result of the *bbg572*. Lane M, 1 kb DNA ladder. A labeled 27-mer oligonucleotide primer complementary to nucleotide 756-782 (257 bp from the initiation codon) of the *bbg572pt* was utilized to define the transcription start points (*tsp*s) indicated in lane 2nd. The *tsp*s are indicated by arrows.

2.3.3 Expression of β-glucosidase in *B. bifidum* BGN4

Strong promoters are required for the enhanced expression of a target gene. In our previous studies, we have successfully expressed foreign genes in *B. longum* using several promoters, including *p919* from *B. bifidum* BGN4 [37] and *pamy* from the α-amylase gene of *B. pseudocatenulatum* INT57 [31]. To compare the strengths of these promoters, several vectors were constructed with combinations of promoters and reporter (*bbg572*) and transformed into the β-glucosidase-negative strain *B. bifidum* BGN4 as the host. Promoter strength was analyzed by measuring the β-glucosidase activity using *p*NPG.

When the signal sequence of the amylase gene (*ssamy*) was inserted between the promoter and reporter gene to induce the extracellular expression of *bbg572*, all of the transformants containing *ssamy* showed unexpectedly low β-glucosidase activities in *B. bifidum* BGN4 (Fig. 2.5). The *ssamy* also failed to export the *bbg572* products expressed in *B. bifidum* BGN4. The intracellular β-glucosidase activity of Bp572ss572 was 1,186-fold higher than its extracellular activity (data not shown). This might be attributable to the incompatibility between the transformation host and the promoters and *ssamy* used. We assumed that the change in transformation host affected the activities of the promoters and *ssamy*, causing low protein expression and secretion. Many previous studies have shown that the –10 and –35 sequences affect the promoter strength [26]. Other factors, such as the TG motif [14], spacers, UP elements [9], and the promoter's three-dimensional structure, are also reported to affect the efficiency of transcription [16]. In the expression system for *bbg572*, *p572* was stronger promoter than *pamy*, or

p919 (Fig. 2.5). In the expression systems for *bbg572*, the expression of β-glucosidase was markedly enhanced by the deletion of the *ssamy*. The removal of *ssamy* resulted in a 24.35-fold increase in Bp572bbg572 (1.5 mU/μg protein) and a 137-fold increase in that of Bp919bbg572 (0.78 mU/μg protein) (Fig. 2.5).

It is well known that the transcription terminator enhances the expression of a gene [1]. Putative transcription terminator was found downstream of *bbg572* and designated as *572t*. It was added downstream of the *bbg572* in p572bbg572 to produce p572bbg572t (Fig. 2.6). The addition of the terminator caused a 25.4% increase in the specific β-glucosidase activity in *B. bifidum* BGN4 (Fig. 2.5). The β-glucosidase activity of *B. bifidum* BGN4 harboring p572*bbg572t* was 1.89 mU/μg, which was much higher than those of *B. lactis* SH5 (31.32-fold) and *B. lactis* RD68 (15.88-fold) (Fig. 2.5).

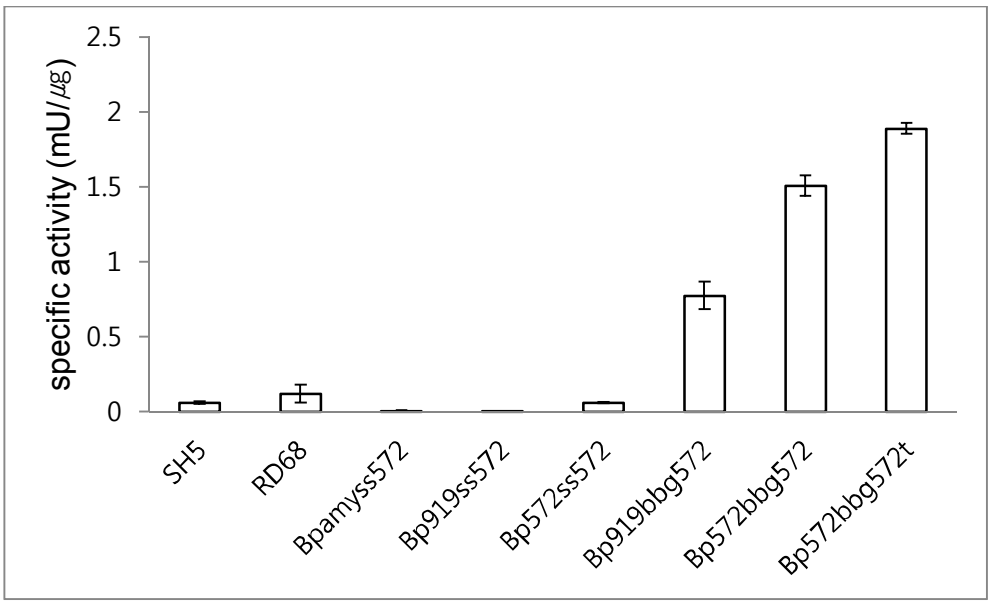


Fig. 2.5 Specific β-glucosidase activities of wild type *Bifidobacterium* spp. and recombinant *B. bifidum* BGN4 with various vector constructs. The total β-glucosidase activity was determined at 37°C in the 50 mM Tris-HCl, pH 6.0, with ρNPG as a substrate. SH5, *B. lactis* SH5; RD68, *B. lactis* RD68; others, *B. bifidum* BGN4 harboring different constructs.

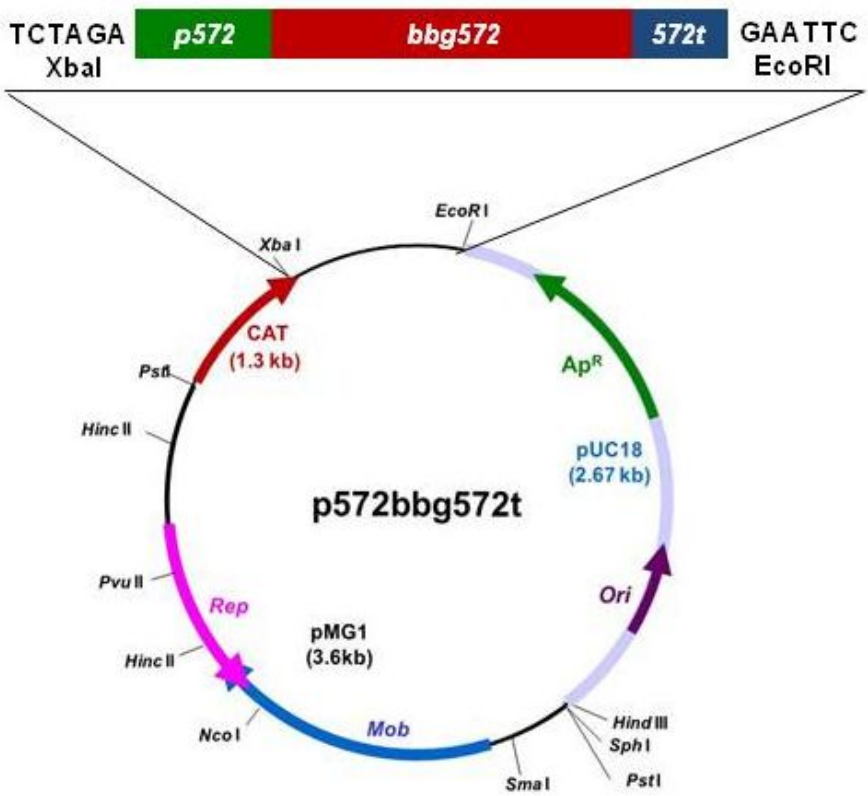


Fig. 2.6 Schematic map of the p572bbg572t. The chloramphenicol acetyl transferase gene (CAT) is red, and the *B. longum* replicon pMG1 predicted replication proteins Rep is pink, and *E. coli* origin of replication (*Ori*) is violet. The cloning site is expanded and annotated with restriction sites (XbaI and BamHI) plus *bbg572pt* containing promoter (*p572*), ORF (*bbg572*) and terminator (*572t*) above the plasmid.

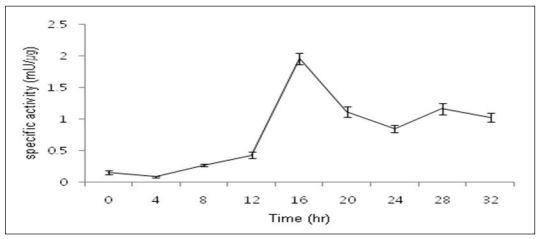
2.3.4 Biochemical characterizations of the β-glucosidase activity of cell extracts from transformants harboring p572bbg572t

The β-glucosidase activity of Bp572bbg572t has been analyzed according to culture time, pH, and temperature. As shown in Figure 2.7A, the specific activity increased according to the culture time, peaking at 16 h and decreasing slowly thereafter.

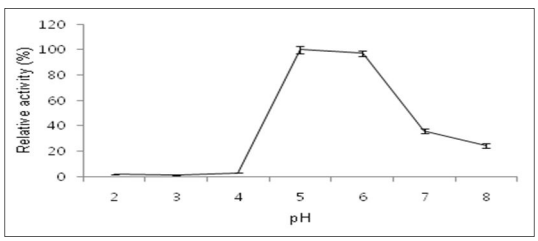
The optimal pH for Bp572bbg572t was pH 6.0 (Fig. 2.7B), which is consistent with those of most bacterial β-glucosidases, which have pH optima in slightly acidic or neutral pH ranges [10]. In previous studies, the optimal pHs of the β-glucosidases from *B*. *breve* 203, β-D-glucosidase I and β-D-glucosidase II, were 6.0 and 5.5, respectively [20], and that of the β-glucosidase of *B*. *breve* clb was pH 5.5 [28].

When the enzyme activity was investigated in the temperature range of 20–80°C, Bp572bbg572t exhibited more than 95% of its maximal activity at 30–50°C, with a sharp decrease above 50°C (Fig. 2.7C).

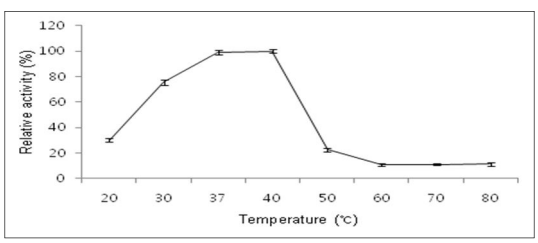
In the previous study, the optimal temperatures of the β-glucosidase enzymes from *B*. *breve* 203, β-D-glucosidase I and β-D-glucosidase II, were 45°C and 40°C, respectively [20], and that of the β-glucosidase from *B*. *breve* clb was 45°C [28].



(A)



(B)



(C)

Fig. 2.7 Specific β-glucosidase activities of Bp572bbg572t **(A)** according to the culture time and relative β-glucosidase activities of Bp572bbg572t at pH range from 2 to 8 and (B) temperature range from 20 to 80°C (C) the cell free extract of Bp572bbg572t was used as enzyme and ρNPG as a substrate.

2.3.5 Substrate specificity of the recombinant β-glucosidase from *B. bifidum* BGN4

The substrate range of Bp572bbg572t was investigated using various disaccharides, isoflavones, quercetins, and ginsenosides (Tables 2. 5 and 2.6). As control, cell extract of *B. bifidum* BGN4 harboring pBESAF2 [31] showed no detectable glycosidic activity on various substrates (data not shown).

Bp572bbg572t were able to hydrolyze β-1,2 (sophorose), β-1,3 (laminaribiose), β-1,4 (cellobiose) and β-1,6 (gentiobiose) linkages between two glucose molecules. It showed β-glucosidase activities on the ginsenoside Rb1, Rb2 and seven flavonoides (daidzin, genistin, glycitin, isoquercetrin, spiraeoside, quercetin-3,4-di-O-β-D-glucoside, and quercetin-7-O-β-D-glucoside). (Fig. 2.8-2.11) No release of hydrolysis products by Bp572bbg572t was observed for the protopanaxadiol-type ginsenosides compound K, Rc, and Rd, or the protopanaxatriol-type ginsenosides F1, Re, Rg1, Rg2(S), Rg3(S), Rg3(R), Rh1, and Rh2(S).

For the verification of the transformed ginsenoside Rb1 and Rb2, LC/MS/MS analysis were performed. Two ginsenosides used as substrates were biotransformed to ginsenoside Rd (Fig. 2.12 and Fig. 2.13).

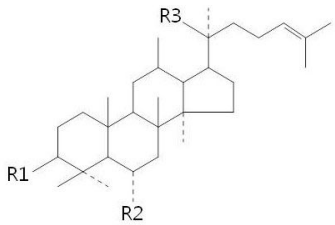
β-Glucosidases are used to hydrolyze phenolic compounds and phytoestrogen glucosides to improve their biological activity, and have several applications in the field of medicine, and in the food industry [2]. In this study, we focused on the various glycoside specificities of recombinant β-glucosidase (Bp572bbg572t) and its full potential exploitation to increase the activity of β-glucosidase. *bbg572* encodes enzyme of glycosyl hydrolase family 1 and selectively catalyze the cleavage of glucosidic bonds.

In this study, we found that the highest expression level of *bbg572* in *B. bifidum* BGN4 was achieved when using its own promoter and terminator. Furthermore, the expression level was 31.32-fold higher than that of the original strain *B. lactis* SH5. Furthermore, our results of bioconversion of several glycosidic phytochemical suggest that these new β-glucosidase-positive transformants of *B. bifidum* BGN4 can be utilized for the production of specific aglycone products for food industry and many biotechnological applications.

Table 2.5 Substrate specificities of Bbg572.

Substrate	Linkage	Enzyme activity
		Bbg572
Disaccharides		
Cellobiose	*O*-β-D-glucosyl-(1→4)-D-glucose	O
Sophorose	*O*-β-D-glucosyl-(1→2)-D-glucose	O
Laminaribiose	*O*-β-D-glucosyl-(1→3)-D-glucose	O
Gentiobiose	*O*-β-D-glucosyl-(1→6)-D-glucose	O
Isoflavone		
Daidzin	Daidzein 7-*O*-β-D-glucoside	O
Genistin	Genistein 7-*O*-β-D-glucoside	O
Glycitin	Glycitein 7-*O*-β-D-glucoside	O
Quercetin		
Isoquercetrin	Quercetin-3-*O*-β-D-glucoside	O
Spiraeoside	Quercetin-4-*O*-β-D-glucoside	O
	Quercetin-3,4-di-*O*-β-D-glucoside	O
	Quercetin-7-*O*-β-D-glucoside	O

Table 2.6 Substrate specificity of Bbg572 on various ginsenosides.

Ginsenoside	R1	R2	R3	Enzyme activity Bbg572
				
20(S)-Protopanaxadiol type				
Compound K	OH	H	O-Glc[a]	X
F2	O-Glc	H	O-Glc	X
Rb1	O-Glc^{2}-^{1}Glc[a]	H	O-Glc^{6}-^{1}Glc	O
Rb2	O-Glc^{2}-^{1}Glc[a]	H	O-Glc^{6}-^{1}Arap[a]	O
Rc	O-Glc^{2}-^{1}Glc[a]	H	O-Glc^{6}-^{1}Araf[a]	X
Rd	O-Glc^{2}-^{1}Glc[a]	H	O-Glc[a]	X
20(S)-Protopanaxatriol type				
F1	OH	OH	O-Glc[a]	X
Re	OH	O-Glc^{2}-^{1}Rha[a]	O-Glc[a]	X
Rg1	OH	O-Glc[a]	O-Glc[a]	X
Rg2(S)	OH	O-Glc^{2}-^{1}Rha[a]	OH	X
Rg3(S)	O-Glc-Glc[a]	H	H	X
Rg3(R)	O-Glc-Glc[a]	H	H	X
Rh1	OH	O-Glc[a]	OH	X
Rh2(S)	O-Glc[a]	H	H	X

a)Arap, α-L-arabinopyranosyl; Araf, α-L-arabinofuranosyl; Glc, β-D-glucopyranosyl; Rha, α-L-Rhamnopyranosyl

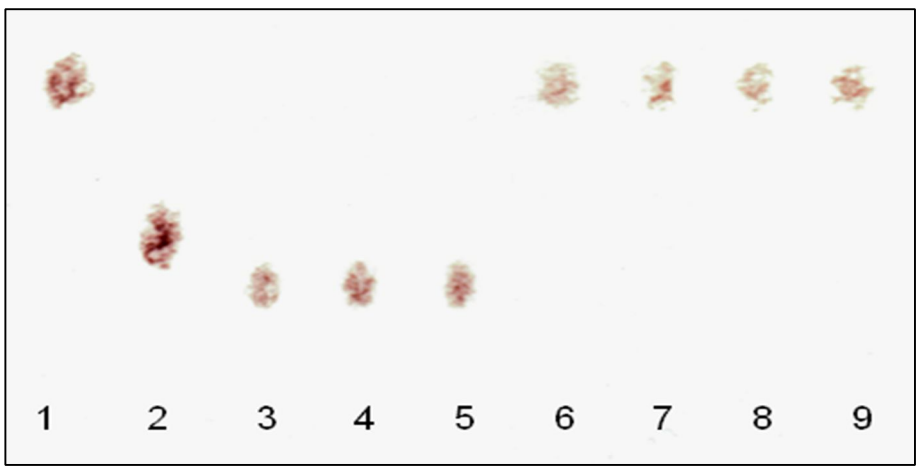


Fig. 2.8 TLC profile of hydrolysis product of various disaccharides using cell free extract of Bp572bbg572t. 1-glucose; 2-cellobiose; 3-sophorose; 4-laminaribiose; 5-gentiobiose; Lanes 6 to 9, hydrolysis products of cellobiose (lane 6), sophorose (lane 7), laminaribiose (lane 8) and gentiobiose (lane 9).

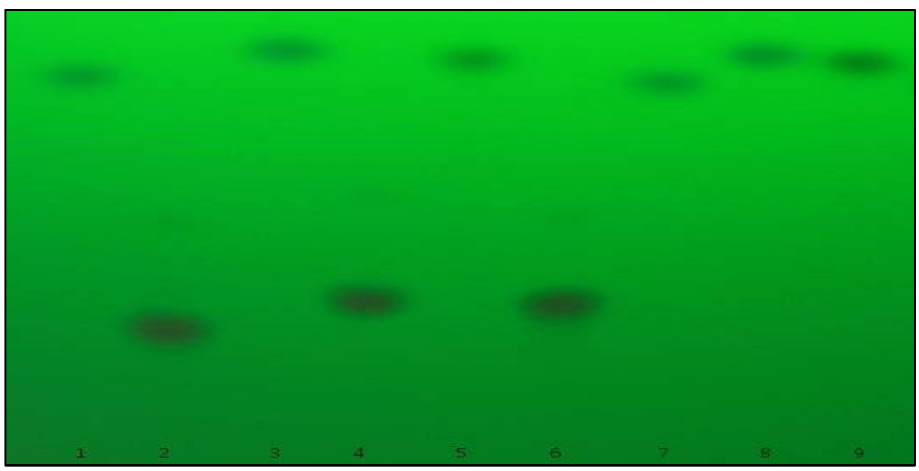


Fig. 2.9 TLC profile of hydrolysis product of various isoflavones using cell free extract of Bp572bbg572t. 1-daidzein; 2-daidzin; 3-glycitein; 4-glycitin; 5-genistein; 6-genistin; Lanes 7 to 9, hydrolysis products of daidzin (lanes 7), glycitin (lanes 8), and genistin (lanes 9).

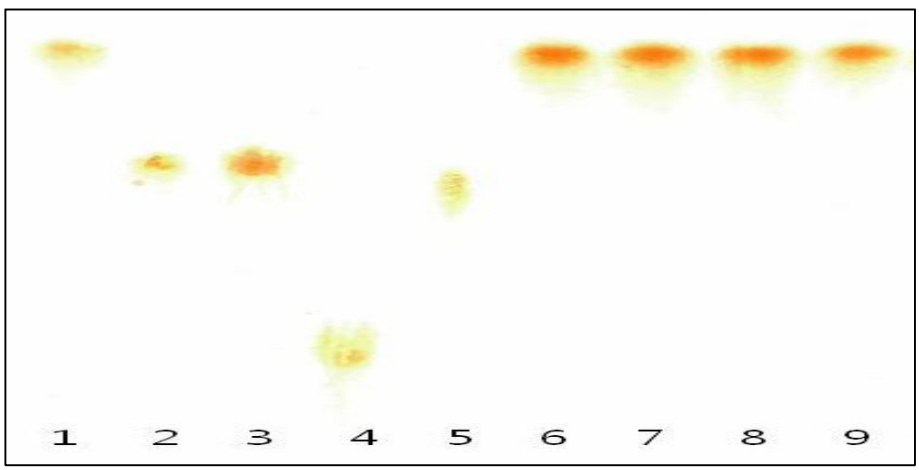


Fig. 2.10 TLC profile of hydrolysis product of various quercetins using cell free extract of Bp572bbg572t. 1-quercetin; 2-isoquercetrin; 3-spiraeoside; 4-isoquercitrin; 5-quercetin-7-*O*-β-D-glucoside; Lanes 6 to 9, hydrolysis products of isoquercetrin (lanes 6), spiraeoside (lane 7), isoquercitrin (lane 8) and quercetin-7-O-β-D-glucoside (lane 9).

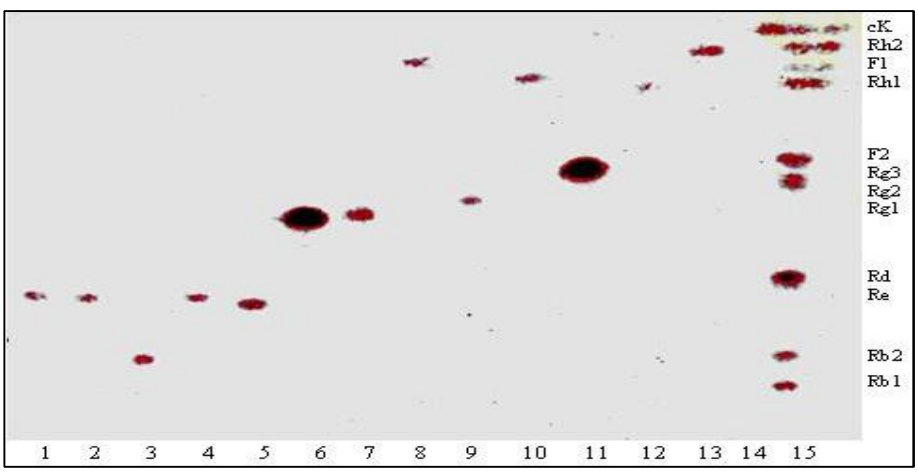


Fig. 2.11 TLC profile of hydrolysis product of various ginsenosides using cell free extract of Bp572bbg572t. Hydrolysis of Rb1 (lane 1) ; Rb2(lane 2) ; Rc(lane 3) ;Rd(lane 4) ; Re(lane 5) ; Rg1(S) (lane 6); Rg2 (lanes 7); Rg3(S) (lane 8); Rg3(R) (lane 9); F1 (lane 10); F2 (lane 11); Rh1(S) (lane 12); Rh2(S) (lane 13); cK (lane 14).

.

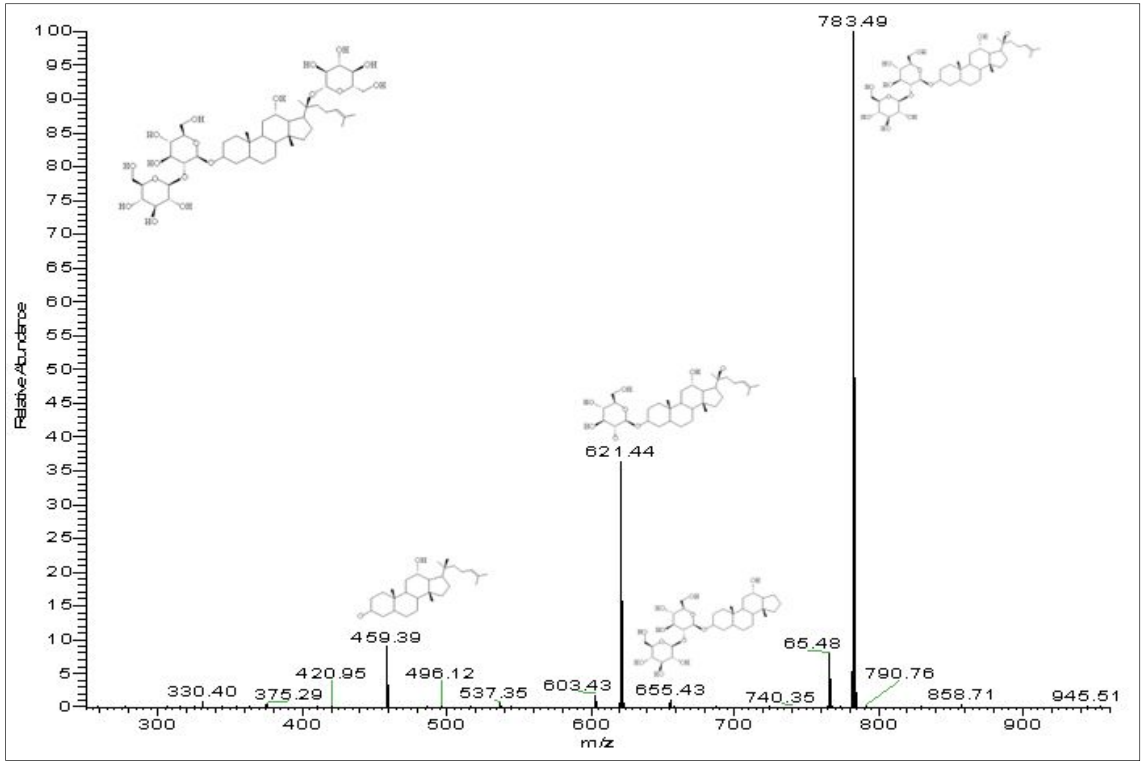


Fig. 2.12 LC-MS/MS analysis of transformed ginsenoside Rb1 with Bp504bbg504t. MS/MS product-ion scan from the molecular ion m/z 945.55. Fragmentation pattern and t_R are identical to ginsenoside Rd.

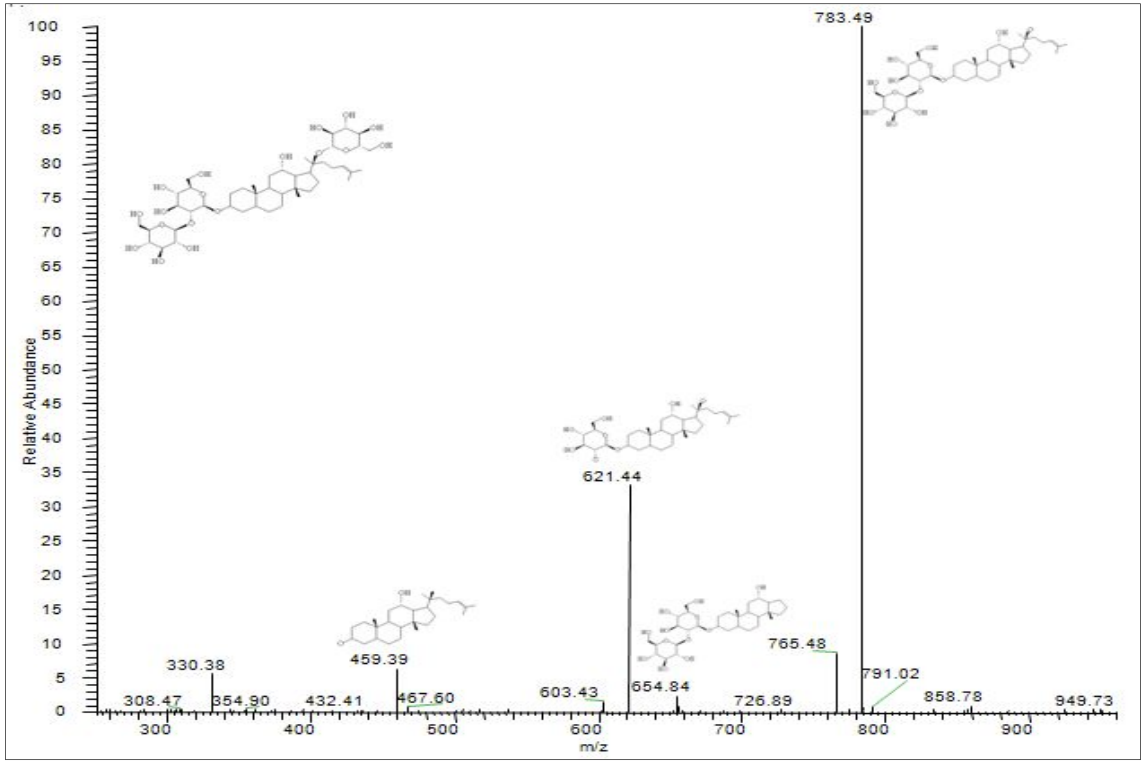


Fig. 2.13 LC-MS/MS analysis of transformed ginsenoside Rb2 with Bp504bbg504t. MS/MS product-ion scan from the molecular ion m/z 945.55. Fragmentation pattern and t_R are identical to ginsenoside Rd.

References

1. **Aiba, H., A. Hanamura, and H. Yamano.** 1991. Transcriptional terminator is a positive regulatory element in the expression of the *Escherichia coli* crp gene. J. Biol. Chem. **266:**1721-1727.
2. **Bhatia, Y., S. Mishra, and V. S. Bisaria.** 2002. Micorbial β-glucosidases: cloning, properties and applications. Crit. Rev. Biotechnol. **22:**375-407.
3. **Brown, J. P.** 1998. Hydrolysis of glycosides and esters. In Role of the gut flora in toxicity and cancer, Academic Press, San Diego.
4. **Chi, H., and G. E. Ji.** 2005. Transformation of ginsenosides Rb1 and Re from Panax ginseng by food microorganisms. Biotechnol. Lett. **27:**765-771.
5. **Choi, Y. O., J. M. Seo, and G. E. Ji.** 2008. Modulatory activity of CpG oligonucleotides from *Bifidobacterium longum* on immune cells. Food Sci. Biotechnol. **17:**1131-1395.
6. **Collado-Vides, J., B. Magasanik, and J. D. Gralla.** 1991. Control site location and transcriptional regulation in *Escherichia coli*. Microbiol. Rev. **55:**371-394.
7. **Mahlen1, S. D., and J. E. Clarridge.** 2009. Site and clinical significance of *Alloscardovia omnicolens* and *Bifidobacterium* species isolated in the clinical laboratory. *J. Clin. Microbiol*. **47:**3289-3293.
8. **Esen, A.** 1993. β-Glucosidase, In β-Glucosidase: Biochemistry and Molecular Biology, pp. 1-13. American Chemical Society, Washinton D.C.
9. **Estrem, S. T., T. Gaal, W. Ross, and R. L. Gourse.** 1998. Identification of

an UP element consensus sequence for bacterial promoters. Proc. Natl. Acad. Sci. USA **95:**9761-9766.

10. **Gekas, V., and M. H. Lopez-Levia.** 1985. Hydrolysis of lactose. *Process Biochem*. **20:**2-12.

11. **Ghosh, P., N. B. Pamment, and W. R. B. Martin.** 1982. Simultaneous saccharification and fermentation of cellulose: Effect of beta-D-glucosidase activity and ethanol inhibition of cellulases. Enzyme Microb. Technol. **4:**425-430.

12. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.

13. **Hawkswor, G., B. S. Drasar, and M. J. Hill.** 1971. Intestinal bacteria and hydrolysis of glycosidic bonds. J. Med. Microbiol. **4:**451-459.

14. **Helmann, J. D.** 1995. Compilation and analysis of *Bacillus subtilis* sigma A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23:**2351-2360.

15. **Hendrich, S.** 2002. Bioavailability of isoflavones. *J. Chromatogr. B* **777:**203-210.

16. **Jensen, P. R., and K. Hammer.** 1998. The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. Appl. Environ. Microbiol. **64:**82-87.

17. **Ji, G. E., S. K. Lee, and I. H. Kim.** 1994. Improved selective medium for isolation and enumeration of *Bifidobacterium* sp. Korean J. Food Sci.

Technol. **26:**526-531.

18. Joint FAO/WHO Expert Consultation. 2001. Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.

19. **Kawakami, Y., W. Tsurugasaki, S. Nakamura, and K. Osada.** 2005. Comparison of regulative functions between dietary soy isoflavones aglycone and glucoside on lipid metabolism in rats fed cholesterol. J. Nutr. Biochem. **16:** 205-212.

20. **Kenji, S., T. Takashi, K. Hidehiko, and T. Tatsurokuro.** 1986. Isolation and characterization of two β-D-glucosidases from *Bifidobacterium breve* 203. Agric. Biol. Chem. **50:**2287-2293.

21. **Kim, J. Y., Y. Wang, M. S. Park, and G. E. Ji.** 2010. Improvement of transformation efficiency through in vitro methylation and SacII site mutation of plasmid vector in *Bifidobacterium longum* MG1. J. Microbiol. Biotechnol. **20:**1022-1026.

22. **Kim, J. Y., Y. Wang, S. J. Park, M. S. Park, and G. E. Ji.** 2012. Cloning of expression of β-glucosidases from *Bifidobacterium lactis* AD011. Food Sci. Biotechnol. **21:**731-738.

23. **Le, T. M., and N. T. Vu.** 2010. Cloning of a β-glucosidase gene (BGL1) from traditional starter yeast *Saccharomycopsis fibuligera* BMQ 908 and expression in *Pichia pastoris*. *Int. J. Biol. Life Sci*. **6:**83-87.

24. **Lei, V., W. K. Amoa-Awua, and L. Brimer.** 1999. Degradation of cyanogenic glycosides by *Lactobacillus plantarum* strains from

spontaneous cassava fermentation and other microorganisms. Int. J. Food Microbiol. **53:**169-184.

25. **Marotti, I., A. Bonetti, B. Biavati, P. Catizone, and G. Dinelli.** 2007. Biotransformation of common bean (*Phaseolus vulgaris* L.) flavonoid glycosides by *Bifidobacterium* species from human intestinal origin. J. Agric. Food Chem. **55:**3913-3919.

26. **McCracken, A., M. S. Turner, P. Giffard, L. M. Hafner, and P. Timms.** 2000. Analysis of promoter sequences from *Lactobacillus* and *Lactococcus* and their activity in several *Lactobacillus* species. Arch. Microbiol. **173:**383-389.

27. **Nunoura, N., K. Ohdan, K. Tanaka, H. Tamaki, T. Yano, M. Inui, H. Yukawa, K. Yamamoto, and H. Kumagai.** 1996. Cloning and nucleotide sequence of the beta-D-glucosidase gene from *Bifidobacterium breve* clb, and expression of beta-D-glucosidase activity in *Escherichia coli*. Biosci. Biotechnol. Biochem. **60:**2011-2018.

28. **Nunoura, N., K. Ohdan, T. Yano, K. Yamamoto, and H. Kumagai.** 1996. Purification and characterization of beta-D-glucosidase (beta-D-fucosidase) from *Bifidobacterium breve* clb acclimated to cellobiose. Biosci. Biotechnol. Biochem. **60:**188-193.

29. **Nunoura, N.,K. Ohdan, K. Yamamoto, and H. Kumagai.** 1997. Expression of the β-D-glucosidase I gene in *Bifidobacterium breve* 203 during acclimation to cellobiose. J. Ferment. Bioeng. **83:**309-314.

30. **Park, M. S., B. Kwon, J. J. Shim. C. S. Huh, and G. E. Ji.** 2008.

Heterologous expression of cholesterol oxidase in *Bifidobacterium longum* under the control of 16S rRNA gene promoter of bifidobacteria. Biotechnol. Lett. **30:**165-172.

31. **Park, M. S., J. M. Seo, and J. Y. Kim.** 2005. Heterologous gene expression and secretion in *Bifidobacterium longum*. Lait **85:**1-8.

32. **Park, M. S., H. W. Moon, and G. E. Ji.** 2003. Molecular characterization of plasmid from *Bifidobacterium longum*. J. Microbiol. Biotechnol. **12:**457-462.

33. **Sambrook, J., E. F. Frietsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 3rd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

34. **Setchell, K. D. R., N. M. Brown, L. Zimmer-Nechemias, W. T. Brashear, B. E. Wolfe, A. S. Kirschner, and J. E. Heubi.** 2002. Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. Am. J. Clin. Nutr. **76:**447-453.

35. **Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt.** 1986. Bergey's manual of systematic bacteriology, The Williams & Wilkins Co.

36. **Ventura, M, F. Turroni, A. Zomer, E. Foroni, V. Giubellini, F. Bottacini, C. Canchaya, M. J. Claesson, F. He, M. Mantzourani, L. Mulas, A. Ferrarini, B. Gao, M. Delledonne, B. Henrissat, P. Coutinho, M. Oggioni, R. S. Gupta, Z. Zhang, D. Beighton, G. F. Fitzgerald, P. W. O'Toole, and V. Sinderen.** 2009. The *Bifidobacterium dentium* Bd1

Genome Sequence Reflects Its Genetic Adaptation to the Human Oral Cavity. PLos Genet. **5:** e1000785.

37. **Wang, Y., J. Y. Kim, M. S. Park, and G. E. Ji.** 2012. Novel *Bifidobacterium* promoters selected through microarray analysis lead to constitutive high level expression. J. Microbiol. **50:**638-643.

38. **Xu, X., K. S. Harris, H. J. Wang, P. A. Murphy, and S. Hendrich.** 1995. Bioavailability of soybean isoflavones depends upon gut microflora in women. J. Nutr. **125:**2307-2315.

3. Molecular Cloning, Characterization and Heterologous Expression of β-Glucosidases from *Bifidobacterium pseudocatenulatum* in *B. bifidum* BGN4.

3.1 Introduction

Bifidobacteria that live in the human intestinal tract play an important role in maintaining health such as the prevention of diarrhea [36], reduction of cholesterol level [2], immunostimulation [28], anticarcinogenicity [4, 27, 37], and production of vitamins [7]. Certain bifidobacteria are, because of these perceived health-promoting activities, commercially exploited as probiotic microorganisms, and are increasingly being used in functional foods and pharmaceutical products [9, 33].

Glycosyl hydrolases appear to be the most critical group of enzymes for bifidobacteria. Over 8% of the identified genes in bifidobacterial genomes are predicted to be involved in carbohydrate metabolism, which is about 30% more than what the majority of other gastrointestinal tract microorganisms apply towards utilization of such compounds [20, 21, 31, 34].

β-Glucosidase catalyze the hydrolysis of the linkage between the glucose or glucose-substituted molecules. This enzyme plays an important role in biological pathways, such as cleavage of structural polysaccharides, mediation of cellular, as well as in a number of biotechnological applications, in particular for improving dietary bioavailability [3]. Especially, the bioavailabilities of the glycosidic compounds differ between individuals with different intestinal microflora. Therefore, to overcome these individual differences in the bioavailability of glycosides in food materials, the efficient bioconversion of the various glycosides by glycosidases is needed.

Bifidobacterium pseudocatenulatum play an important role in maintaining and promoting human health by eliciting a number of beneficial properties, such as amelioration of atopic eczema, enhancement of antigen presentation to dendritic

cells and activation of macrophages [10, 11]. In a previous study, *B. pseudocatenulatum* INT57 and SJ32 were used for the biotransformation of various ginsenosides [5]. However, little is known about identification and biochemical characterize of β-glucosidase enzymes from *B. pseudocatenulatum* INT57 and SJ32, although these strains have a strong β-glucosidase activity.

Since the beneficial effects of *B. bifidum* BGN4, such as anti-inflammatory activity against bowel diseases, modulatory activity on immune cells, antiallergic and anticancergenic effects, are strain-dependent, improvement of the strain property of *B. bifidum* BGN4 is warranted as the newly constructed recombinant with β-glucosidase gene cloned is of value [18, 19, 22, 38].

The development of an expression vector system for *Bifidobacterium* is also interesting because *Bifidobacterium* is a prospective delivery system into the human intestinal tract for the delivery of the heterologous gene products such as antigens for the vaccine development.

The present study aimed to firstly identify and characterize two different β-glucosidase genes, *bbg504* and *bbg1176*, from the genomes of *B. pseudocatenulatum* INT57 and SJ32, clone and overexpress than in *B. bifidum* BGN4.

3.2 Materials and methods

3.2.1 Bacterial strains, plasmids, media, and culture conditions

Bifidobacterium species were cultured anaerobically in *Lactobacilli* MRS broth (Becton Dickinson, Sparks, MD, USA) supplemented with 0.05% (v/w) L-cysteine·HCl (Sigma, St Louis, MO, USA), at 37°C for 16 h.

The β-glucosidase-deficient *E. coli* XL1 blue MR, *E. coli* DH5α, and *B. bifidum* BGN4 were used as cloning, subcloning, and expression hosts, respectively. *E. coli* XL1 blue MR and *E. coli* DH5α were cultured aerobically in Luria–Bertani (LB) broth (Becton Dickinson) at 37°C for 15 h with vigorous shaking. For selection of recombinants, 100 μg/ml ampicillin (LBA; Sigma) was added to LB broth. The recombinant *B. bifidum* BGN4 was grown in modified transgalactooligosaccharide propionate (TP) broth [15] supplemented with 1 % (w/v) glucose instead of transgalactooligosaccharides (TOS), and 3.6 μg/ml chloramphenicol (BioBasic, Markham, Ontario, Canada) was added. The bacteria and plasmids used in this study are listed in Table 3.1.

Table 3.1 Bacterial strains and plasmids

Strain or vector	Relevant characteristics or genotype	Source or reference
Bacterial strains		
INT57	*B. pseudocatenulatum* INT57, wild type; Glu+	
SJ32	*B. pseudocatenulatum* SJ32, wild type; Glu+ (original host of *bbg504*)	
BGN4	*B. bifidum* BGN4, wild type; Glu-; Transformation host	
DH5α	*E. coli* DH5α , *F-(80dlacZ M15) (lacZYA-argF)U169hsdR17(r-m+) recA1 endA1 relA1 deoR*; Cloning host	[11]
XL1 blue MR	*E. coli* XL1 blue MR, *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*; Cloning host	Stratagene
Bpamyssbbg504	*B. bifidum* BGN4 harboring pamyss504	
Bp919ssbbg504	*B. bifidum* BGN4 harboring p919ss504	
Bp504ssbbg504	*B. bifidum* BGN4 harboring p504ss504	
Bp572ssbbg504	*B. bifidum* BGN4 harboring p572ss504	
Bp504bbg504	*B. bifidum* BGN4 harboring p504bbg504	
Bp504bbg504t	*B. bifidum* BGN4 harboring p504bbg504t	
Bpamybbg1176	*B. bifidum* BGN4 harboring pamybbg1176	
Bp504bbg1176	*B. bifidum* BGN4 harboring p504bbg1176	
Plasmids		
SuperCos1 Cosmid	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
pUC18	Amp^r	
pBES2	Amp^r, Cm^r; *E. coli-Bifidobacterium* shuttle vector	[18]
pBESAF2	Amp^r, Cm^r; *E. coli-Bifidobacterium* shuttle vector	[19]
pUC504PT	Amp^r, pUC18 with 3.585 kb insert of *B. pseudocatenulatum* DNA containing the *bbg504pt*	
pUC1176	Amp^r, pUC18 with 1.176 kb insert of *B. pseudocatenulatum* DNA containing the *bbg1176*	

3.2.2 Assessment of β-glucosidase for the various experimental bacteria and recombinant *B. bifidum* BGN4

For the screening of lactic acid bacteria with strong β-glucosidase activities, various lactic acid bacteria, including 19 *Bifidobacterium* spp., nine *Lactobacillus* spp., eight *Bacillus* spp., two *Lactococcus* spp., two *Enterococcus* spp., one *Leuconostoc* sp., one *Weissella* spp., and one *Pediococcus* sp., were used in this study (data not shown). β-Glucosidase activities of the 43 kinds of bacteria and recombinant *B. bifidum* BGN4 were determined with *p*-nitrophenol glucopyranoside (*p*NP) as the substrate and measured according to the method of Youn *et al* [39]. One unit of enzyme activity corresponded to the amount of enzyme which liberated 1 μmol of *p*-nitrophenol (*p*NP) per minute at 37℃.

3.2.3 Construction of cosmid library of *Bifidobacterium pseudocatenulatum*

Genomic DNA from *Bifidobacterium pseudocatenulatum* was extracted according to the method of Choi *et al* [6], which was used to construct a genomic DNA library of *B. pseudocatenulatum*. The library was amplified in *Escherichia coli* XL-1 Blue MR according to the Supercos 1 Cosmid Library (Stratagene, La Jolla, CA, USA) instruction manual. To select the bacterial clones expressing β-glucosidase activity, 5-bromo-4-chloro-3-indolyl β-D-glucopyranoside (Wako Pure Chemical Industries, Osaka, Japan)-containing plates supplemented with ampicillin (Biobasic Inc., Toronto, Ontario, Canada) were used. β-Glucosidase positive colonies were isolated and used for further analysis. Plasmids were isolated from *E. coli* with the Midi Plus™ Ultrapure Plasmid Extraction System (Viogene, Taipei, Taiwan). The transformed *E. coli* cells were used to construct a genomic library.

3.2.4 Cloning of the *Bifidobacterium* β-glucosidase genes and sequence analysis

To obtain β-glucosidase gene (*bbg504*), DNA fragments encoding β-glucosidase was isolated from a cosmid genomic library using Midi Plus (Viogene, Taipei, Taiwan) and partially digested with *Bam*HI. These were subcloned into the cloning vector pUC18 (pUC504PT), digested with *Bam*HI, and used to transform *E. coli* DH5α as the cloning host, as described by Sambrook *et al* [30].

For the amplification of β-glucosidase gene (*bbg1176*) from the genome of *B. pseudocatenulatum*, PCR primers were designed based on the β-glucosidase gene sequence obtained from the annotated genome data of *B. adolescentis* ATCC15703 (accession no. NC_008618). The primers 1176-F and 1176-R (Table 3.2) were designed to incorporate the restriction enzyme sites KpnI and BamHI respectively at their 5' ends, allowing the directional cloning of PCR product into the plasmid expression vector pUC18 (pUC1176). The PCR product was digested with restriction enzymes BamHI and KpnI and recovered from the agarose gel, and ligated to pUC18 (pUC1176), and recombinant plasmid were transformed into *E. coli* DH5α. The PCR conditions used were: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 5 min, annealing at 60°C for 90 sec, extension at 72°C for 90 sec, and final extension at 72°C for 10 min.

The positive colonies showing β-glucosidase activity, pUC504PT and pUC1176, were isolated and the plasmids were purified and sequence analysis. The nucleotide sequences were determined with the BigDye® Terminator v 3.1 Kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3730xl Genetic Analyzer (Applied Biosystems) in Bionicsro (Seoul, Korea). The promoter regions were predicted using the format at http://www.fruitfly.org/seq_tools/promoter.html and the

terminator regions were predicted with the format at http://linux1.softberry.com.

Table 3.2 Primers for amplifying β-glucosidases genes and putative promoters from *Bifidobacterium*

Primer name	Sequences of primer		Product	Reference
	Forward (5→3)	Reverse (5→3)		
primerbbg504pt	caattgGCGATTTTCTGCAGG	caattgAGTGTGCGAATGACG	*bbg504pt*	
primerbbg504p	tctagaGCGATTTTCTGCAGG	caattgCTACTTGGTGAGTTCAGC	*bbg504p*	
primerbbg504	ggtaccATGAGCGAAAAAACCTATCCTTC[a] agtactATGAGCGAAAAAACCTATCCTTC[b]	caattgCTACTTGGTGAGTTCAGC	*bbg504*	
primerbbg504t	ggtaccATGAGCGAAAAAACCTATCCTTC[a] agtactATGAGCGAAAAAACCTATCCTTC[b]	caattgAGTGTGCGAATGACGTG	*bbg504t*	
primerbbg1176	ggtaccATGAAAGAACAATACGAGTTTCC	ggatccCTATCTGGCCATGAC	*bbg1176*	
primerPamy	tctagaGAAATACCGCAATGCACG	ggtaccGGCTCCTTTATTCCTTTTC	*pamy*	[19]
primer919P	tctagaTGAAGTGTGTCGTGTGG	ggatccTGGTGTACCTTTTCTTG	*p919*	[29]
primer504P	tctagaGGGCGATTTTCTGCAG	ggtaccACGTTCCTTTCGC	*p504*	
primerSSamy	ggtaccATGAAACATCGGAAACC	agtact GGCCTGTGCTGCGG	*ssamy*	

Restriction enzyme sites were indicated by small letter : MfeI caattg, XbaI tctaga, KpnI ggtacc, ScaI agtact. a) Primers were used to construct p504bbg504 and p504bbg504t. b) Primers were used to construct pamyss504, p504ss504, p572ss504.

3.2.5 Analysis of transcription start sites

The transcription start site of the β-glucosidase gene from *B. lactis* (*bbg504*) was determined by primer extension. The total RNA was extracted from *B. pseudocatenulatum* after it was grown for 9 h at 37°C in BHI broth, using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. The purified RNA was resuspended in sterile distilled RNase-free water and its concentration was estimated by measuring the optical densities of the solution at 260 nm. Primer extension analysis was performed as follows : *bbg504*-specific oligonucleotide primer complementary to the 5′ end of the genes was synthesized (5′-gtggccgagttggacttgcgcaggccg-3′). Primer extension analysis was performed with [γ-^{32}P]-dATP and reverse transcriptase (Invitrogen). Sequencing ladders were run in parallel using the same primer and corresponding templates, with the dideoxynucleotide sequencing reaction.

3.2.6 Construction of a vector encoding β-glucosidase and transformation of *Bifidobacterium*

The target genes were amplified using *PfuUltra* II Fusion HS DNA polymerase (Stratagene) and each primer set (Table 3. 2), according to the manufacturer's instruction manual.

The structural genes of the β-glucosidase, *bbg504* and *bbg1176*, were PCR amplified from the cosmid library and *bbg1176* from genomic DNA of *B. pseudocatenulatum* INT and SJ32 using the appropriate primer set for each gene (Table 3. 2). These were cloned into the *Bifidobacterium* shuttle vector pBES2 [25] to construct pBES-bbg504 and pBES-bbg1176. *B. bifidum* BGN4, a β-glucosidase-negative host, was transformed individually with these plasmids.

To study the regulatory effects of the promoter and terminator sequences, regions of different gene promoters (*pamy*, *p919*, *p504* and *p572*) and the regions of the gene terminators (*504t*) were amplified from each template using the primer sets for each gene (Table 3.2). The amplified putative promoter sequences were digested with restriction enzymes and then cloned into pBES-bbg504 and pBES-bbg1176 digested with the same restriction enzymes to incorporate the promoter upstream from the β-glucosidase gene. *B. bifidum* BGN4 was then transformed with the constructs individually. The amplified putative terminator sequences of the β-glucosidase genes (*bbg504*) were digested with restriction enzymes and then cloned downstream from the structural genes in p504bbg504. The amplified signal sequence (*ssamy*) was fused between the promoter and the structural gene in the corresponding vectors. *B. bifidum* BGN4 was then transformed with each construct individually. The 8 constructs are described in Table 3.3.

Table 3.3 Constructed vector with cloned β-glucosidase genes

Promoter				Signal sequences	ORF		Terminator	Plasmid
pamy	*p919*	*p504*	*p572*	*ssamy*	*bbg504*	*bbg1176*	*504t*	
		o			o			p504bbg504
		o			o		o	p504bbg504t
	o			o	o			p919ssbbg504
o				o	o			pamyssbbg504
		o		o	o			p504ssbbg504
			o	o	o			p572ssbbg504
o						o		pamyssbbg1176
		o				o		p504bbg1176

3.2.7 Optimal temperature and pH and thermal stability of the β-glucosidases

The optimal pH for the recombinant β-glucosidases was determined with 10 mM *p*NPG as the substrate in 50 mM PB buffer at pH 3.0–8.0. *p*NPG (10 µl) was added to 90 µl of each cell-free extract in each buffer (pH 3.0–8.0) and incubated at 37°C for 30 min. The reaction was stopped with 100 µl of 1 M Na_2CO_3 and the absorbance was measured at 405 nm. The cell-free extraction buffers were 100 mM citric acid–citrate buffer (pH 3.0–6.0) and 50 mM Tris-HCl buffer (pH 7.0–8.0).

To evaluate the thermal stability for each enzyme activity, each cell-free extract in 50 mM PB buffer (pH 6.0) was maintained at 37°C, 50°C and 65°C for 30 min, and the activity remaining in each sample was determined as described above.

3.2.8 Substrate specificity of β-glucosidase

Cellobiose, sophorose, laminaribiose, gentiobiose, daidzin, daidzein, genistin, glycitin, glycitein, and isoquercetrin were purchased from Sigma Chemical Co.. Quercetin-3,4-di-O-β-D-glucoside and quercetin-7-O-β-D-glucoside were purchased from Extrasynthese (Genay, France). Genistein and quercetin-7-glucoside were purchased from Chengdu Biopurify Phytochemicals Ltd (Chengdu, China). Ginsenoside standards such as compound K, Rb1, Rb2, Rd, F1, Rg3, and Rh1 were purchased from Cogon Chemical (Chengdu, China); F2, Rg2(S), and Rh2(s) were from LKT Laboratories (St Paul, MN, USA); Rg1 was from Wako Pure Chemical Industries; Rg3 (S) and Re were from BTGin Co. Ltd (Chungnam, Korea). Saccharides were dissolved in water and other substrates were dissolved in methanol (J.T. Baker, Phillipsburg, NJ, USA).

The cell-free extracts of recombinant *B. bifidum* BGN4 transformants were prepared in 50 mM PB buffer (pH 6.0). Reaction mixtures (200 μl) containing 180 μl of cell-free extract and substrates were incubated at 37°C with shaking. Samples were harvested at 24 h and analyzed with thin-layer chromatography (TLC).

Samples containing ginsenosides were analyzed by method of Chi *et al* [5]. For the hydrolysis of disaccharides, flavonoid glycosides, and isoflavone glycosides, TLC were determined as described by Youn *et al* [39].

The Hybrid LC/MS/MS System was measured for verification of three kinds of transformed ginsenoside (Rb1 with Bp504bbg504t or Bp504bbg1176 and Rb2 with Bp504bbg1176) by National Instrumentation Center for Environmental Management (NICEM). For full scan MS analysis, the spectra were recorded in the m/z range from 260 to 960.

3.3 Results and discussion

3.3.1 Identification and sequence analysis of *Bifidobacterium* β-glucosidase genes

Although most *Bifidobacterium* can produce β-glucosidases which enable them to utilize complex carbohydrates in their natural habitats, glucosidases from only 3 kinds of *Bifidobacterium*, *B. lactis*, *B. breve*, *B. longum*, have been expressed in *E. coli*. [16, 17, 24, 39].

In this study, two strong β-glucosidase positive strains, *B. pseudocatenulatum* INT57 and SJ32, were screened from 43 experimental lactic acid bacterial strains. After the construction of cosmid libraries in *E. coli* using the total genomic DNAs from these two strains, about 20 clones with β-glucosidase activity were isolated for each strain. The clone showing the greatest enzyme activity was selected for the relative strain. The insert in the cosmid was digested with *Bam*HI and subcloned into pUC18 (pUC504PT).

The *bbg504pt* (Fig. 3.1) cloned from *B. pseudocatenulatum* SJ32 contained 3,595 bp and one ORF. Upstream from the ORF, putative ribosome binding sites (RBSs) AAGAAAA were detected (gray boxes; Fig. 3.1), which are similar to the RBS of *B. longum* MG1[30]. The promoter region was predicted using BPROM (http://www.fruitfly.org/seq_tools/promoter.html). A putative terminator sequence was located downstream of the ORF. (Fig. 3.1). The amino acid sequence deduced from *bbg504* contained 749 residues, with a molecular mass of 82.39 kDa. A database search for the ORF with the FASTA program showed that it had 87%, 82%, 82%, and 81% amino acid sequence identities with the β-glucosidases from *B.*

dentium ATCC27679, *B. breve* CECT7263, *B. longum* subsp. *infantis* ATCC 15697, and *B. longum* subsp. *longum* KACC 91563, respectively (Fig. 3.3).

Based on these amino acid sequence similarities, the β-glucosidase encoded by *bbg504* was assigned to glycosyl hydrolase family 3. The overall GC content of *Bifidobacterium* is generally 55.0%–67.0%, which is higher than most of the other bacteria. The GC contents of *bbg504* were 61%, which are slightly lower than that of the β-glucosidase from *B. breve* clb (65.1%) [25] and *B. lactis* SH5 and RD68 [39].

To obtain the other β-glucosidase gene, the primers, primerbbg1176F and primerbbg1176R, were designed on the basis of the β-glucosidase gene (GenBank nucleotide sequence database under the accession no. NC_008618), and β-glucosidase gene (*bbg1176*) was cloned from the *B. pseudocatenulatum* INT57 and SJ32 by PCR amplification. The PCR product was 1176 bp and no other nonspecific bands were found. Positive clones (pUC1176), screened by ampicillin and 5-bromo-4-chloro-3-indolyl β-D-glucopyranoside and detected by blue colony, were obtained.

The β-glucosidase from *B. pseudocatenulatum* INT57 and SJ32 (*bbg1176*) exhibited 92 % homology to that of *B. adolescentis* ATCC15703.

Figure 3.2 shows the complete nucleotide sequence of the insert in pUC1176. One open reading frame of 1176 bp encoding the protein composed of 392 amino acid residues with a molecular mass of 43.12 kDa were identified. The amino acid sequence is 92% and 88% identical to that of *B. adolescentis* ATCC15703 and *B. dentium* Bd1 (Fig. 3.4). Based on these amino acid sequence similarities, the β-glucosidase encoded by Bbg1176 was assigned to glycosyl hydrolase family 1.

Unexpectedly, sequence analysis of the two cloned β-glucosidase genes, *bbg504pt* and *bbg1176*, showed that the β-glucosidase genes from *B*. *pseudocatenulatum* INT57 was identical to that of *B*. *pseudocatenulatum* SJ32 with respect to its open reading frame (ORF), putative promoter region (*p504*), and putative terminator region (*504t*). Consequently, it was evident that *B*. *pseudocatenulatum* INT57 and *B*. *pseudocatenulatum* SJ32 contained identical β-glucosidase gene.

gcgattttct gcagggaagt gccgtaatag ccgagcgtcc caaacagttc gacggccgcg ttgagaatct ccgcccttcg
gtcgacttct tcgttggcca tgatgattcc ctctcttgcc cttcattgat ggcgattcat gtgcgctgca atgcttatga
atcgtcattt ctctcaagtt gtaaatactg cagttacttt acggtcggtt aattgatttt tcaaagaaga ttggcgtgtt
gcggtgttgg ttttgataaa gtacctaCca accggttagt agagatttcg gggttgaggt ctcatgcaga tcggttaacc
gatacgaact caagaaaacg atgcgaaaga ggaacgt

atgagcgaaaaaacctatccttccatcaatgatctgacgcttgaagagaaggcatccctgacttccggcggcgacgcttggcatctgcag
M S E K T Y P S I N D L T L E E K A S L T S G G D A W H L Q
ggtgttgaagccaagggcattcccggctatatgatcaccgatggccccacggcctgcgcaagtccaactcggccaccaccggcgaagtc
G V E A K G I P G Y M I T D G P H G L R K S N S A T T G E V
gatttgaacaactgcgtgcctgccacctgcttcccgccggcggctggtctgtccagctcctggaatccggaactcatccaccaggttggc
D L N N C V P A T C F P P A A G L S S S W N P E L I H Q V G
gaagccatggccgaagagtgcattcaggagaaggtcgccgtaatcctcggcccgggcgtgaacatcaagcgcaacccgcttggcggccgt
E A M A E E C I Q E K V A V I L G P G V N I K R N P L G G R
tgcttcgaatactggtccgaagacccgtacctggcgggtcatgaagccatcggcatcgtcgccggcgtgcaatccaagggcgtgggcact
C F E Y W S E D P Y L A G H E A I G I V A G V Q S K G V G T
tcgctcaagcatttcgccgccaacaatcaggaaaccgatcgtctgcgcatcagcgccaacatctcccagcgtgcgctgcgcgaaatctac
S L K H F A A N N Q E T D R L R I S A N I S Q R A L R E I Y
ttcccggcgttcgagcacatcgtcaaggaagcccagccgtggaccatcatgtgcgcatacaactgcatcaacggcgtgcatgccgcccag
F P A F E H I V K E A Q P W T I M C A Y N C I N G V H A A Q
gaccactggctgctcaccgacgtgctgcgcgacgaatggggtttccaaggcatcgtcatgagcgactggggcgccgaccatgatcgcgtc
D H W L L T D V L R D E W G F Q G I V M S D W G A D H D R V
gcatccctcaacgcgggcctcaacctcgaaatgccgccaagctacaccgacgaccaaatcgtgtatgcggcacgcgacggtcgcatccag
A S L N A G L N L E M P P S Y T D D Q I V Y A A R D G R I Q
ccggcacagctcgaccgcatggcacaaggcatgatcgacctggtcaacaagacgcgcgcagccatgagcatcgaaaactatcgtttcgac
P A Q L D R M A Q G M I D L V N K T R A A M S I E N Y R F D
atcgaagctcacgacgaagtcgcacatcaggcggccatcgaatccatggtgctgctcaagaacgatgatgccatccttccgatcgccggc
I E A H D E V A H Q A A I E S M V L L K N D D A I L P I A G
gatgccaaggtcacggtcatcggcgaattcgcacgcactccgcgctaccagggcggcggctcctcgcacatcaccccaaccaagatgacc
D A K V T V I G E F A R T P R Y Q G G G S S H I T P T K M T
agcttcctcgacaccctcaccgaacgcggcgtggacgccaagttcgccccaggcttcaccctcgacctcgaaccggccgatccggcactc
S F L D T L T E R G V D A K F A P G F T L D L E P A D P A L
gaagccgaagccgtcgacgctgccaagggcgccgacgtggtgctcatgttcctggggctccccgaagcagccgaatccgaaggcttcgac
E A E A V D A A K G A D V V L M F L G L P E A A E S E G F D
cgtgagaccctcgacatgccggccaagcagatcgcactgctcgaagccgtcgcagccgaaaacaagaacgttgtggtcgtactctccaac
R E T L D M P A K Q I A L L E A V A A E N K N V V V V L S N
ggctccgtcgtcaccgtggcaccgtgggccaagaacgccaagggcatcctcgaatcctggctgctcggccagtccggcggtccggcgctc
G S V V T V A P W A K N A K G I L E S W L L G Q S G G P A L
gctgacgtgctgttcggcaaggtgagcccgtccggcaagctcgcgcagaccattccgttcgacatcaacgacgatccgagcaccatcaac
A D V L F G K V S P S G K L A Q T I P F D I N D D P S T I N
tggccgggcgaagaggggcatgtcgactacggcgaaggcgtgttcgtgggctaccgctattacgacacctacaacaaggccgtcgactac
W P G E E G H V D Y G E G V F V G Y R Y Y D T Y N K A V D Y
ccgttcggattcggcctgagctacgccacgttcgaagtgagcgacgtgaaggctgtcaagaccggtgcctgtaccgcatccgtcagcgca
P F G F G L S Y A T F E V S D V K A V K T G A C T A S V S A
gtggtcaagaacacctcgaacgttgacgccgccgaaaccgtgcaggtgtacgtggctcctggcaaggccgacgtagttcgcccgaaacac
V V K N T S N V D A A E T V Q V Y V A P G K A D V V R P K H
gagcttaagggcttcaagaaggtattcctcaaggctggcgaatccgcagaagtctccttcgagctcgacgatcgcgccttgcctactgg
E L K G F K K V F L K A G E S A E V S F E L D D R A F A Y W
tctgagaggttcaatgactggcacgtggagagcggcgaatacaccatcgaggtcggcacgtccagccgtgacattgccggttccgcggtg
S E R F N D W H V E S G E Y T I E V G T S S R D I A G S A V
gtggagctcgacggcgacggcaaggcccagccgctcaccgaatggtccaacttcatggaatggcgtaaggatccgctcggttccaaggtg
V E L D G D G K A Q P L T E W S N F M E W R K D P L G S K V
cttgaaatcctgcgtgccgagggcgaagccggccgcatgccggtggtgcccgacaacgacatgacccgcctgttcctcgattccatgccg
L E I L R A E G E A G R M P V V P D N D M T R L F L D S M P
atcaacagcatgtcggtgctgatgggcgccgatggcaagcagatcttcgaatacatgcttgagaagtacgctgaactcaccaa gtag
I N S M S V L M G A D G K Q I F E Y M L E K Y A E L T K *

tttcgtagga ttcaaggctt aaggccgagg gattcgatcc gttaacgctg aaccgatgcg gatctgattc ctcggccttt
tttgtcgcgg acgatgagat gcgcgacacg ctaatcgttg catgtacaac agttgtatat gcaacggttt agcatgtgtg
gtgtgacaga agtgacgaat gaacaataca aggaaccgtc aggttcacga cttgccgaag tctgggaaag tggcctgacc
aagcctccca gcgtggccat tcgctcgctg cacaacgtgg tcaaccggta tttgcgcgtc acccgtcccg aagaggcgga
agacgtcagc aacggcaacg ttgacatcat cgtgtacttg gcaagacatg ataacgagga gatcttccct caagacatcg
agcaacgttt cggcgtgacc cgatccacat catgccgagt gttgggactg atggagcaga aggggctgat cgcccgcgag
tcggtgaagc gtgacgcacg tctgaaaaag atcgtattga cggacaaatc gaggcatatt gccgaagtgc tgcacgacaa
tgcgaaaaac atggaacgaa tcctgttgga aggtcttgat gacgaccaga tccgccaatt catgcacatg cttgacgtca
tgcagacgaa tctggtgaaa acaggtttga tcggagacga aagccgatat tcaagcttgg aaatacagga acaggatttt
ggcgaatcgc aggtttcgca aaagccgcag gtttcgcaag aatcgcaaga atcatgcgat gtacggaatc cgcataatcg

cgattctcat gatgaatacg tttcgcgcat actgcgaatg tcgcaggaaa tggcgatcgc aaacaacctc aggtaacaca
cgcaagcaag taagtacgaa aaagaaagga caaggagtga cagcggcaaa cgcaacgacg tccacaacgt caaaaccaaa
gcaacacgtc attcgcacac t

Fig. 3.1 Nucleotide and putative amino acid sequences of the *bbg504pt* in pUC18 (pUC504PT). The deduced terminator sequence is indicated by bold letters. Putative ribosomal binding site (RBS) is indicated in gray box. Transcription start point is in bold and capitalized. Predicted promoter region is underlined. Translational termination site is marked by asterisk. The sequence data is available in GenBank under accession number JX274650.

```
atgaaagaacaatacgagtttccgccggaattcatttggggcgcgtccacagccgcgcatcagatcgaggggaacaacgtcgcgagcgac
  M  K  E  Q  Y  E  F  P  P  E  F  I  W  G  A  S  T  A  A  H  Q  I  E  G  N  N  V  A  S  D
tggtgggccagggagcatgccgaacgcacggacctcaacgaaccgtctggagacgcggccgacagctaccatcgctacggcgaggacatc
  W  W  A  R  E  H  A  E  R  T  D  L  N  E  P  S  G  D  A  A  D  S  Y  H  R  Y  G  E  D  I
agaatgctcgccgacgccggattgcgcatgtaccgcttctccatcgaatgggcccgcatcgagcctgcggagggatgcttctccaaagcg
  R  M  L  A  D  A  G  L  R  M  Y  R  F  S  I  E  W  A  R  I  E  P  A  E  G  C  F  S  K  A
cagctgctgcactaccgccatatgatcgacgtgtgccatcagaacggcgtcgagcccatggtcacgctgaaccacatgacactgccgcag
  Q  L  L  H  Y  R  H  M  I  D  V  C  H  Q  N  G  V  E  P  M  V  T  L  N  H  M  T  L  P  Q
tggcttgcggccaagggcggatggctcaacgccgatgccgtcgactacttcgctaggtacgtgcggtacgtgatgacgatactccacgac
  W  L  A  A  K  G  G  W  L  N  A  D  A  V  D  Y  F  A  R  Y  V  R  Y  V  M  T  I  L  H  D
gtcacctgggtatgcaccatcaacgagccgaacatggtcgcgctgacccgtggcgggaccgaaggcagcgatttcgtggccgcatcgcta
  V  T  W  V  C  T  I  N  E  P  N  M  V  A  L  T  R  G  G  T  E  G  S  D  F  V  A  A  S  L
cccgcacccgatccggacatctccgttacactcgtcgaggcgcacaggaaggctcgggaaatactgtccgggaaccctcggatcaagtcc
  P  A  P  D  P  D  I  S  V  T  L  V  E  A  H  R  K  A  R  E  I  L  S  G  N  P  R  I  K  S
ggatggaccatcgcctgccaggcgttccatgccatgccgggatgcgaacaggagatggaggaataccagtatccgcgcgaggactacttc
  G  W  T  I  A  C  Q  A  F  H  A  M  P  G  C  E  Q  E  M  E  E  Y  Q  Y  P  R  E  D  Y  F
accgaagccgcggccggggacgacttcatcggcgtgcaggcctatctgaggacctttatcggcaaggaagggccagtgccggtccccgag
  T  E  A  A  A  G  D  D  F  I  G  V  Q  A  Y  L  R  T  F  I  G  K  E  G  P  V  P  V  P  E
gacgcggaacgcaccctcaccggctgggagtatttcccgccggcgttgggcatcgccatccggcacacatgggacgtcgccgggcacacg
  D  A  E  R  T  L  T  G  W  E  Y  F  P  P  A  L  G  I  A  I  R  H  T  W  D  V  A  G  H  T
ccgataatcgtcaccgaaaacggcatcgccaccgcggacgaccgccggaggatcgactacaccttcgacgccatagccggcatgcgcgac
  P  I  I  V  T  E  N  G  I  A  T  A  D  D  R  R  R  I  D  Y  T  F  D  A  I  A  G  M  R  D
gcgatggacgacggcatcgacgtgaggggatacctccactggtcgttgctcgacaactacgagtgggggtcgttcgctccgacgttcggt
  A  M  D  D  G  I  D  V  R  G  Y  L  H  W  S  L  L  D  N  Y  E  W  G  S  F  A  P  T  F  G
ctggcttcttgggacaaggacaccttcgaacgtcatccgaagccatccctggactggctggggtcaattgcgagaacgggggtcatggcc
  L  A  S  W  D  K  D  T  F  E  R  H  P  K  P  S  L  D  W  L  G  S  I  A  R  T  G  V  M  A
agatag
  R  *
```

Fig. 3.2 Nucleotide and putative amino acid sequences of the *bbg1176* in pUC18 (pUC1176). Translational termination site is marked by asterisk. The sequence data is available in GenBank under accession number KC13710.

```
Bbg2247         ---------------------------------MSEKTYPSINDLTLEEKASLTSGGDAW
ATCC27679       ---------------------------------MSESTYPEVNDLTLEEKASLTSGGDAW
CECT7263        ---------------------------------MSEAIYPSVKDLTLEEKASLTSGGDAW
ATCC15697       MEEPRTTARQSGRIGANAYRTTAQLKKLKERGIMSESTYPSVKDLTLEEKASLTSGGDSW
KACC91563       ---------------------------------MSEAIYPSVKDLTLEEKASLTSGGDAW
                                                  ***  **   *************** *

Bbg2247         HLQGVEAKGIPGYMITDGPHGLRKSNSATTGEVDLNNCVPATCFPPAAGLSSSWNPELIH
ATCC27679       HLQGVEAKGIPGYMITDGPHGLRKSLASSTGETDLNDSVPATCFPPAAGLSSSWNPELIH
CECT7263        HLQGAEAKGIPGYMITDGPHGLRKSLASSTGETDLDDSVPATCFPPAAGLSSSWNPDLIH
ATCC15697       HLQGVESKGIPGYMITDGPHGLRKSLASSTGETDLNNSVPATCFPPAAGLSSSWNPELIH
KACC91563       HLQGVESKGIPSYMITDGPHGLRKSLASSAGETDLDDSVPATCFPPAAGLSSSWNPELIH
                **** * **** *************      ** **   ****************** ***

Bbg2247         QVGEAMAEECIQEKVAVILGPGVNIKRNPLGGRCFEYWSEDPYLAGHEAIGIVAGVQSKG
ATCC27679       QVGEAMAEECIQEKVAVILGPGVNIKRNPLGGRCFEYWSEDPYLAGHEAIGIVAGVQSKG
CECT7263        KVGEAMAEECIQEKVAVILGPGVNIKRNPLGGRCFEYWSEDPYLAGHEAIGIVEGVQSKG
ATCC15697       KVGEAMAEECIQEKVAVILGPGVNIKRNPLGGRCFEYWSEDPYLAGHEAVGIVAGVQSKG
KACC91563       KVGEAMAEECIQEKVAVILGPGVNIKRNPLGGRCFEYWSEDPYLAGHEAIGIVEGVQSKG
                 ********************************************** *** ******

Bbg2247         VGTSLKHFAANNQETDRLRISANISQRALREIYFPAFEHIVKEAQPWTIMCAYNCINGVH
ATCC27679       IGTSLKHFAANNQETDRLRVSANISQRALREIYFPAFEHIVKEAQPWTIMCSYNRINGVH
CECT7263        VGTSLKHFAANNQETDRLRVDARISPRALREIYFPAFEHIVKKAQPWTIMCSYNRINGVH
ATCC15697       VGTSLKHFAANNQETDRLRVDARISQRALREIYLPAFEHIVKTAQPWTIMCSYNRINGVH
KACC91563       VGTSLKHFAANNQETDRLRVDARISPRALREIYFPAFEHIVKKAQPWTIMCSYNRINGVH
                :****************** * ** ******* ******** ******** ** *****

Bbg2247         AAQDHWLLTDVLRDEWGFQGIVMSDWGADHDRVASLNAGLNLEMPPSYTDDQIVYAARDG
ATCC27679       SAQNHWLLTDVLRDEWGFEGIVMSDWGADHDRVASLNAGLNLEMPPSYTDDQIVYAARDG
CECT7263        SAQNHWLLTDVLRDEWGFDGIVMSDWGADHDRGASLNAGLNLEMPPSYTDDQIVYAVRDG
ATCC15697       SAQNHWLLTDVLRDEWGFEGIVMSDWGADHDRVASLNAGLNLEMPPSYTDDQIVYAARDG
KACC91563       SAQNHWLLTDVLRDEWGFDGIVMSDWGADHDRGASLNAGLNLEMPPSYTDDQIVYAVRDG
                 ** ************** ************* ********************** ***

Bbg2247         RIQPAQLDRMAQGMIDLVNKTRAAMSIENYRFDIEAHDEVAHQAAIESMVLLKNDDAILP
ATCC27679       RIQPEQLDRMAQGMIDLVNKTRAAMSVEGYRFDVDAHDEVAHQAAVESMVLLKNDDAILP
CECT7263        RITPAQLDRMAQGMIDLVNKTRAAMSIDNYRFDVDAHDEVAHQAAIESIVMLKNDDAILP
ATCC15697       RIAPAQLDRMAQGMIDLINKACAAMSIDGYRFDVDAHDEVAHQAAIESIVLLKNDDAILP
KACC91563       LITPAQLDRMAQGMIDLVNKTRAAMSIDNYRFDVDAHDEVAHQAAIESIVMLKNDDAILP
                 * * ************ **  ****   ****  ********** ** * *********

Bbg2247         IAGD---------AKVTVIGEFARTPRYQGGGSSHITPTKMTSFLDTLTERGVDAKFAPG
ATCC27679       VASD---------AKVAVIGEFARTPRYQGGGSSHITPTKMTSFLDTLAARGVDAKFAPG
CECT7263        LNAGPVANPSATPQKIAVIGEFARTPRYQGGGSSHITPTKMTSFLDTLAERGIKADFAPG
ATCC15697       LNADPAA-----ARKIAVIGEFARTPRYQGGGSSHITPTKMTSFLDTLAERGIKADFAPG
KACC91563       LNAGPVANPSATPQKIAVIGEFARTPRYQGGGSSHITPTKMTSFLDTLAERGIKADFAPG
                          *  ******************************  **  * ****

Bbg2247         FTLDLEPADPALEAEAVDAAKGADVVLMFLGLPEAAESEGFDRETLDMPAKQIALLEAVA
ATCC27679       FTLDLEPADAAMAADAVEVAKGADVVLMFLGLPEAAESEGFDRETLDIPAKQVELLEAVA
CECT7263        FTLDLEPADPALESEAVETAKNADVVLMFLGLPEAAESEGFDRDTLDMPAKQITLLEQVA
ATCC15697       FTLDLEPADPALESEAVETAKNADVVLMFLGLPEAAESEGFDRDTLDMPAKQIALLEQVA
KACC91563       FTLDLEPADPALESEAVETAKNADVVLMFLGLPEAVESEGFDRDTLDMPAKQIALLEQVA
                ********* *    **  ** ************* ******* *** ****  *** **

Bbg2247         AENKNVVVVLSNGSVVTVAPWAKNAKGILESWLLGQSGGPALADVLFGKVSPSGKLAQTI
ATCC27679       AENKNIVVVLSNGSVVSVAPWADNAKGILESWLLGQAGGPALADVIFGNVSPSGKLAQTV
CECT7263        AANQNVVVVLSNGSVITVAPWAKNAKGILESWLLGQSGGPALADVIFGQVSPSGKLAQSI
ATCC15697       AANQNVVVVLSNGSVVSVAPWAKNAKGILESWLLGQSGGPALADVIFGQVSPSGKLAQSI
KACC91563       AANQNVVVVLSNGSVVSVAPWAKNAKGILESCLLGQAGGPALADVIFGQVSPSGKLAQSI
                * * * *********  ***** ******** **** ******** ** *********

Bbg2247         PFDINDDPSTINWPGEEGHVDYGEGVFVGYRYYDTYNKAVDYPFGFGLSYATFEVSDVKA
ATCC27679       PMDINDDPSMINWPGEEGHVDYGEGVFVGYRYYDTYDKAVDYPFGYGLSYATFEVSDVKV
```

```
CECT7263        PLDINDDPSMLNWPGEEGHVDYGEGVFVGYRYYDTYGKAVDYPFGYGLSYATFEITGVAV
ATCC15697       PLDINDDPSMLNWPGEEGHVDYGEGVFVGYRYYDTYGKSVDYPFGYGLSYATFEIADVAA
KACC91563       PLDISDDPSTLNWPGEEGHVDYGEGVFVGYRYYDTYGKVVDYPFGYGLSYATFEIDDVAA
                * ** ****  ************************* * ****** ********   *

Bbg2247         VKTGACTASV----SAVVKNTSNVDAAETVQVYVAPGKADVVRPKHELKGFKKVFLKAGE
ATCC27679       AKTGANTASV----SAVVKNTSDVDAAETVQVYVAPGKSAVARPIHELKGFRKVFLKAGE
CECT7263        AKTGANTATVN----ATVTNTSDVDAAETVQVYVVPGKADVARPKHELKGFTKVFLKAGE
ATCC15697       AKTGANTATVTATVTATVTNTSDVDAAETVQVYVAPGKADVARPKHELKGFTKVFLKAGE
KACC91563       AKTGANTATVT----ATVTNTSDVDAAETVQVYVAPGKADVARPKHELKGFTKVFLKAGE
                 **** ** *    * * *** ********** ***  * ** ****** ********

Bbg2247         SAEVSFELDDRAFAYWSERFNDWHVESGEYTIEVGTSSRDIAGSAVVELDGDGKAQPLTE
ATCC27679       SAEVSFDLDERAFAYWSEKFDDWHVESGEYAIEVGTSSRDIAGTGVVELDGDGKAEPLTE
CECT7263        SKTVTIDLDERAFAYWSEKYNDWHVEAGEYAIEVGVSSRDIADTVAVALDGDGKTQPLTE
ATCC15697       SKSVTIDLDERAFAYWSEKYNDWHVESGEYAIEVGVSSRDIADTVVVTLEGDGKSQPLTE
KACC91563       SKTVTIDLDERAFAYWSEKYNDWHVEAGEYAIEVGVSSRDIADTVAVALDGDGKTQPLTE
                *  *   ** ********   ***** *** **** ******    * * ****  ****

Bbg2247         WSNFMEWRKDPLGSKVLEILRAEGEAGRMPVVPDNDMTRLFLDSMPINSMSVLMGADGKQ
ATCC27679       WSTFGEWSDDPVGSKIVASVYAEGEAGNLPKMPDNDMMRMFLRSMPINSMPMLMSEGGKK
CECT7263        WSTYGEWEADPFGAKIVAAVAAAGEAGELPKLPDNAMMRMFLNSMPINSLPTLLGEGGKK
ATCC15697       WSTYGEWEADPFGAKIVAAVAAAGEAGELTKLPDNAMMRIFLNSMPINSLSTLLGEGGKK
KACC91563       WSTYGEWEADPFGAKIVAAVAAAGEAGELTKLPDNAMMRMFLNPMPINSLPTLLGEGGKK
                **   **  ** * *      * ****     *** * * **  *****   *    **

Bbg2247         IFEYMLEKYAELTK
ATCC27679       ITAFMLDEYAKVTE
CECT7263        IAQFMVDEYAKLSK
ATCC15697       IAKFMVDEYAKLAK
KACC91563       IAQFMVDEYAKLAK
                *   *   **
```

Fig. 3.3 Multiple sequence alignment of β-glucosidase (Bbg504) : CECT7263, *B. breve* CECT7263; KACC91563, *B. longum* subsp. *longum* KACC91563; ATCC15697, *B. longum* subsp. *infantis* ATCC15697; Bbg2247, *B. pseudocatenulatum* INT57 and SJ32; ATCC27679, *B. dentium* ATCC27679; ATCC15703. Conserved sequence motifs among family 3 glycosyl hydrolases are gray boxed. Conserved amino acid residues are indicated as asterisk.

```
Bbg1176         MKEQYEFPPEFIWGASTAAHQIEGNNVASDWWAREHAERTDLNEPSGDAADSYHRYGEDI
ATCC15703       MKEQYEFPPEFIWGASTAAHQIEGNNVASDWWAREHAERTDLSEPSGDAADSYHRYGEDI
Bd1             MRETYEFPQEFIWGASTAAHQIEGNNVASDWWAREHAECADLSEPSGDAADSYHRYGEDI
                * * **** ****************************  ** ******************

Bbg1176         RMLADAGLRMYRFSIEWARIEPAEGCFSKAQLLHYRHMIDVCHQNGVEPMVTLNHMTLPQ
ATCC15703       RMLADAGLGMYRFSIEWARIEPAEGCFSKAQLLHYRQMIDACHENGVEPMVTLNHMTLPL
Bd1             RMLADAGLGMYRFSIEWARIEPAEGCFSKAQLLHYRHMIDACHENGIEPMVTLNHMTLPL
                ******** ************************** *** ** ** ************

Bbg1176         WLAAKGGWLNADAVDYFARYVRYVMTILHDVTWVCTINEPNMVALTRGGTEGSDFVAASL
ATCC15703       WLATKGGWLNADAVDYFARYVRYVMPILHDVTWVCTINEPNMVALTRGGTEGSDFVAASL
Bd1             WLAVKGGWLNDGAVDYFDRYVRYLMPILHDVTWVCTINEPNMVALTRGGTEGSDFVSASL
                *** ******  ***** ***** * ***************************** ***

Bbg1176         PAPDPDISVTLVEAHRKAREILSGNPRIKSGWTIACQAFHAMPGCEQEMEEYQYPREDYF
ATCC            PAPDPDISATLVKAHRKAREILSENPRIKSGWTIACQAFHAMPGCEREMEEYQYPREDYF
Bd1             PAPDLDISAALVEAHREARGILSENPRIKSGWTIACQAFHAMPGCEQEMEEYQYPREDYF
                **** ***  ** *** ** *** ********************** *************

Bbg1176         TEAAAGDDFIGVQAYLRTFIGKEGPVPVPEDAERTLTGWEYFPPALGIAIRHTWDVAGHT
ATCC15703       TEAAAGDDFIGVQAYLRTFIGKDGPVPVPEDAERTLTGWEYFPPALGIAIRHTWNVAKRT
Bd1             TEAAAGDDFIGVQAYLRTFIGKDGPVPVPEDAERTLTGWEYFPPALGIAIRHTWNVAGHT
                ********************** ****************************** **  *

Bbg1176         PIIVTENGIATADDRRRIDYTFDAIAGMRDAMDDGIDVRGYLHWSLLDNYEWGSFAPTFG
ATCC15703       PIIVTENGIATADDRRRIDYTFDAIAGMHDAMDDGIDVRGYLHWSLLDNYEWGSFAPTFG
Bd1             PIIVTENGIATADDRRRIDYTFGAIAGMHDAMADGVDVRGYLHWSLLDNYEWGSFAPTFG
                ********************** ***** *** ** ************************

Bbg1176         LASWDKDTFERHPKPSLDWLGSIARTGVMAR
ATCC15703       LASWDRDTFERHPKPSLDWLGSIARKGVMAR
Bd1             LACWDKDTFERHPKPSLNWLGMIAKTGVMSR
                ** ** *********** *** **  *** *
```

Fig. 3.4 Multiple sequence alignment of β-glucosidase (Bbg1176) from *Bifidobacterium pseudocatenulatum*: Bbg1176, *B. pseudocatenulatum* INT57 and SJ32; ATCC15703, *B. pseudocatenulatum* ATCC15703; Bd1, *B. dentium* Bd1. Conserved sequence motifs among family 1 glycosyl hydrolases are gray boxed. Conserved amino acid residues are indicated as asterisk

3.3.2 Analysis of the transcription start point

Putative promoter was predicted upstream from *bbg504* (Fig. 3.5) and designated as *p504*. The inserted sequence of the putative –10 and –35 positions in each promoter region showing homology with the consensus sequence of *E. coli* and *p504* induced the successful expression of each corresponding β-glucosidase gene in *E. coli* DH5α (data not shown). Analysis of the transcription start point (*tsp*) revealed that the transcription of *p504* was initiated at a single C residue. The –10 (taaagt) and –35 (ttggcg) sequences upstream from this point showed 66.7% identity to the typical –10 (tataat) and –35 (ttgaca) consensus sequences of *E. coli*.

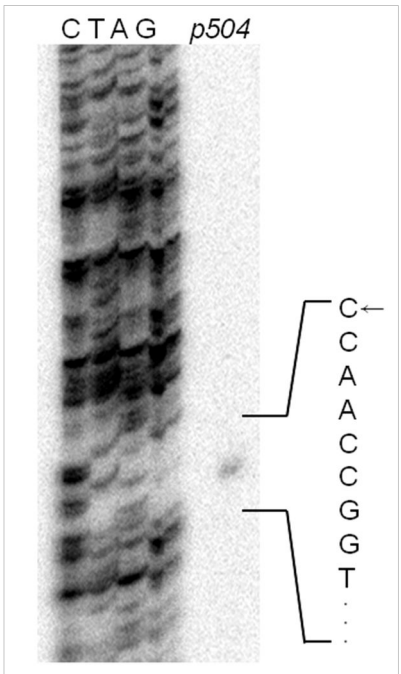


Fig. 3.5 Primer extension analysis of the *bbg504* gene. A labeled 27 mer oligonucleotide primer complementary to nucleotide 498-524 (230 bp from the initiation codon) of the *bbg504pt* gene was used to define the transcription start point (*tsp*) indicated by an arrow.

3.3.3 Expression of β-glucosidase in *B. bifidum* BGN4

Strong promoters are required for the enhanced expression of a target gene. In our previous studies, we have successfully expressed foreign genes in *Bifidobacterium* using several promoters, including *p919* from *B. bifidum*, *p572* from β-glucosidase gene of *B. lactis* and *pamy* from the α-amylase gene of *B. pseudocatenulatum* INT57. To compare the strengths of these promoters, several vectors bearing various combinations of promoters and reporter genes were constructed and transformed into the β-glucosidase-negative strain *B. bifidum* BGN4 as the host. The signal sequence of the amylase gene (*ssamy*) was inserted between the promoter and reporter gene to induce the extracellular expression of *bbg504*.

In the expression of *bbg504*, *p504* originated from the own promoter of *bbg504*, induced a stronger promoter activity than *pamy*, *p919* or *p572*. The β-glucosidase activity of Bp504ss504 (0.35 mU/μg) was about 2,761 times stronger than that of pamyssbbg504. However, the expression of β-glucosidase was markedly reduced by the insertion of the signal sequence (Fig. 3.6). Moreover, the signal sequence also failed to export the *bbg504* products extracellulary in *B. bifidum* BGN4. The intracellular β-glucosidase activity of Bp504ss504 was 0.34 mU/μg, which was about 712-fold higher than its extracellular activity. This phenomenon might be attributable to the incompatibility between the transformation host, the promoters and signal sequence used. Many previous studies have shown that the promoter strength and transcription efficiency is affected by various factors, such as the –10 and –35 sequences [23], TG motif [13], spacers, UP elements [8], and the promoter's three-dimensional structure [14]. Therefore, we assumed that the change of the transformation host affected the activities of the promoters and signal

sequence, causing low protein expression and secretion in *B. bifidum* BGN4.

It is well known that the transcription terminator enhances the expression of a gene [1]. A putative transcription terminators were found downstream from each ORF (*bbg504*) and designated as *504t*. It was added downstream from the ORF in p504bbg504 to produce p504bbg504t. The addition of the terminators resulted in a 28.2% increase in the specific activity of the Bbg504 in *B. bifidum* BGN4. The β-glucosidase activity of *B. bifidum* BGN4 harboring p504bbg504t was about 3.02 mU/ μg, which was higher than those of *B. pseudocatenulatum* INT57 (157.1-fold) and *B. pseudocatenulatum* SJ32 (98.1-fold) (Fig. 3.6).

Additionally, *bbg1176* from *B. pseudocatenulatum* SJ32 was cloned into pBES2 and *pamy* and *p504* was inserted to upstream of *bbg1176* to construct Bpamyssbbg1176 and Bp504bbg1176. As a result, Bp504bbg1176 expressed about 30 fold higher activity than its parental strain, but showed lower β-glucosidase activity than Bp504bbg504.

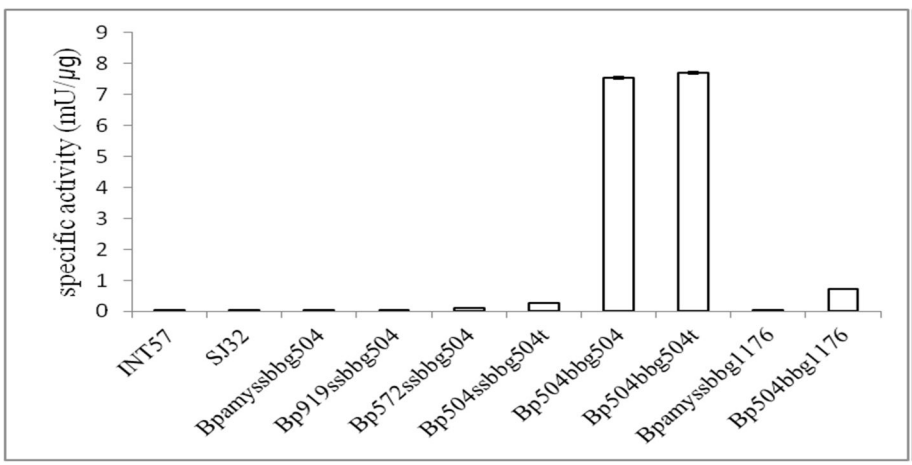


Fig. 3.6 Specific β-glucosidase activities of the wild type *Bifidobacterium pseudocatenulatum* and recombinant *B. bifidum* BGN4 with various vector constructs. The specific β-glucosidase activity was determined at 37°C in the 50 mM Tris-HCl buffer, pH 6.0, with ρNPG as a substrate. INT57, *B. pseudocatenulatum* INT57; SJ32, *B. pseudocatenulatum* SJ32; others, *B. bifidum* BGN4 harboring different constructs.

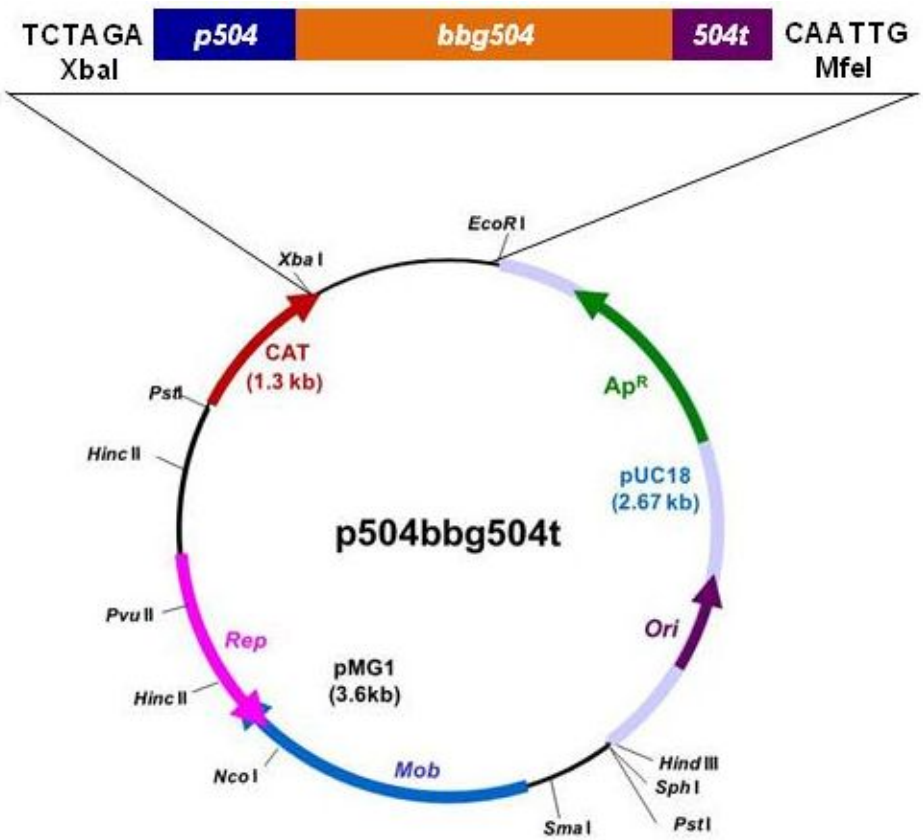


Fig. 3.7 Schematic map of the p504bbg504t. The chloramphenicol acetyl transferase gene (CAT) is red, and the *B. longum* replicon pMG1 predicted replication proteins Rep is pink, and *E. coli* origin of replication (*Ori*) is violet. The cloning site is expanded and annotated with restriction sites (XbaI and BamHI) plus *bbg504pt* containing promoter (*p504*), ORF (*bbg504*) and terminator (*504t*) above the plasmid.

3.3.4 Biochemical characterizations of the β-glucosidase activity of Bp504bbg504t and Bp504bbg1176

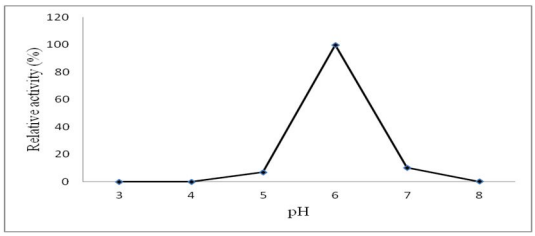
The intracellular β-glucosidases were obtained from Bp504bbg504t and Bp504bbg1176. The β-glucosidase activities of these recombinant BGN4 transformants were analyzed according to pH and thermal stability.

The optimal pH of the β-glucosidase (Bp504bbg504t and Bp504bbg1176) was pH 6.0. (Fig. 3.8A and Fig. 3.8B).

In previous studies, the β-glucosidase activities of *B. lactis* AD011, E893, and E141 peaked at pH 4.5, and the optimal pHs of the β-glucosidases from *B. breve* 203, β-D-glucosidase I and β-D-glucosidase II, were 6.0 and 5.5, respectively, and that of the β-glucosidase of *B. breve* clb was pH 5.5.

Bp504bbg504t and Bp504bbg1176, Bp504bbg1176 showed thermal stability at 37°C and 50°C without notable loss of activity. However, both of them considerably lost their activity over 65°C (Fig. 3.9A and Fig. 3.9B).

(A)



(B)

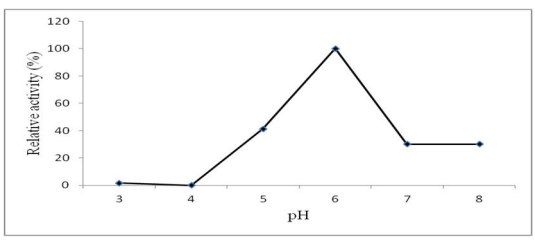
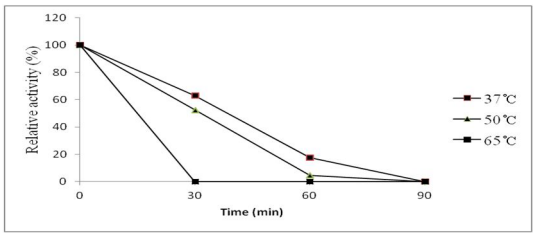


Fig. 3.8 Effect of pH on the specific activities of (A)Bp504bbg504t and (B)Bp504bbg1176 at various pH ranges. The optimal pH was determined at 37°C for 30 min in the 50 mM Tris-HCl buffer, pH 6.0, with ρNPG as a substrate.

(A)



(B)

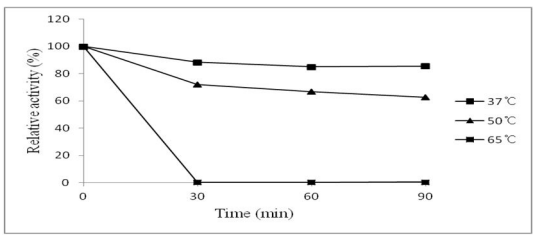


Fig. 3.9 Thermal stability of (A)Bp504bbg504t and (B)Bp504bbg1176. The enzyme activity was determined at 37°C, 50°C and 65°C in 50 mM Tris-HCl buffer, pH 6.0, with ρNPG as a substrate.

3.3.5 Substrate specificity of the recombinant β-glucosidases from *B. bifidum* BGN4

The substrate specificities of Bp504bbg504t and Bp504bbg1176 were investigated using various disaccharides, isoflavones, quercetins, and ginsenosides (Tables 3.4 and 3.5). A cell extract of wild type *B. bifidum* BGN4 and *B. bifidum* BGN4 harboring pBESAF2 [12] showed no detectable glycosidic activity on the experimental substrates (data not shown).

Both Bp504bbg504t and Bp504bbg1176 were able to hydrolyze the β-1,2 (sophorose) and β-1,3 (laminaribiose) linkages between two glucose molecules. Although Bbg572 from *B. lactis* hydrolyzed gentiobiose and cellobiose [39], these enzymes, Bbg504 and Bbg1176, did not hydrolyze cellobiose (β-1,4 linkage) and gentiobiose (β-1,6 linkage) (Fig. 3.9).

Both enzymes hydrolyzed the various substrates (daidzin, genistin, glycitin, isoquercetrin, spiraeoside, quercetin-3,4-di-O-β-D-glucoside, and quercetin-7-O-β-D-glucoside) (Fig. 3.11 and Fig. 3.12).

When various ginsenosides were used as substrates, the recombinant β-glucosidases from *B. lactis* AD011 expressed in *E. coli*, E141 and E893 converted Rb1 to Rd and E141 produced Rd from Rb2 [17]. However, Bp504bbg504t and Bp504bbg1176 exhibited different patterns. Interestingly, Bp504bbg504t converted Rb1, F2, Rh2(S) and Rg3(S), whereas Bp504bbg1176 transformed Rb1, Rb2, Rd, Rg1, Rg3(S) and Rg3(R) (Fig. 3.10).

For the verification of the transformed ginsenoside Rb1 and Rb2, LC/MS/MS analysis were performed. Two kinds of ginsenosides used as

substrates were biotransformed to ginsenoside Rd and compound O. Rb1 with Bp504bbg504t and Rb1 with Bp504bbg1176 transformed to ginsenoside Rd. Especially, Rb2 converted to compound O by Bp504bbg1176. Compound O is known as minor ginsenoside. Therefore, Bbg504 is a valuable enzyme for producing compound O (Fig. 3.13-Fig. 3.15).

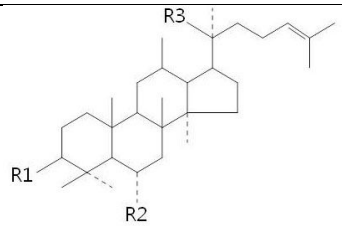
No release of hydrolysis products by either enzyme was observed for the protopanaxadiol-type ginsenosides compound K and Rc, or the protopanaxatriol-type ginsenosides F1, Re, Rg2(S), and Rh1.

Conclusively, two novel β-glucosidases from *B. pseudocatenulatum* INT57 and SJ32 were identified and characterized. Bbg504 and Bbg1176 encode enzymes of glycosyl hydrolase families 3 and 1, respectively, and selectively catalyze the cleavage of glucosidic bonds. Bbg504 and Bbg1176 hydrolyzed various complex natural substrates to enhance the bioavailabilities of the various glycosides.

Table 3.4 Substrate specificities of Bbg504 and Bbg1176

Substrate	Linkage	Enzyme activity	
		Bbg504	Bbg1176
Disaccharides			
Cellobiose	*O*-β-D-glucosyl-(1→4)-D-glucose	X	X
Sophorose	*O*-β-D-glucosyl-(1→2)-D-glucose	O	O
Laminaribiose	*O*-β-D-glucosyl-(1→3)-D-glucose	O	O
Gentiobiose	*O*-β-D-glucosyl-(1→6)-D-glucose	X	X
Isoflavone			
Daidzin	Daidzein 7-*O*-β-D-glucoside	O	O
Genistin	Genistein 7-*O*-β-D-glucoside	O	O
Glycitin	Glycitein 7-*O*-β-D-glucoside	O	O
Quercetine			
Isoquercetrin	Quercetin-3-*O*-β-D-glucoside	O	O
Spiraeoside	Quercetin-4-*O*-β-D-glucoside	O	O
	Quercetin-3,4-di-*O*-β-D-glucoside	O	O
	Quercetin-7-*O*-β-D-glucoside	O	O

Table 3.5 Ginsenoside specificities of Bbg504 and Bbg1176

Ginsenoside	R1	R2	R3	Enzyme activity	
				Bbg504	Bbg1176
					
20(S)-Protopanaxadiol type					
Compound K	OH	H	O-Glc[a]	X	X
F2	O-Glc	H	O-Glc	O	X
Rb1	O-Glc[2]-[1]Glc[a]	H	O-Glc[6]-[1]Glc	O	O
Rb2	O-Glc[2]-[1]Glc[a]	H	O-Glc[6]-[1]Arap[a]	X	O
Rd	O-Glc[2]-[1]Glc[a]	H	O-Glc[a]	X	O
20(S)-PROTOPANAXATRIOL TYPE					
F1	OH	OH	O-Glc[a]	X	X
Re	OH	O-Glc[2]-[1]Rha[a]	O-Glc[a]	X	X
Rg1	OH	O-Glc[a]	O-Glc[a]	X	O
Rg2(S)	OH	O-Glc[2]-[1]Rha[a]	OH	X	X
Rg3(S)	O-Glc-Glc[a]	H	H	O	O
Rg3(R)	O-Glc-Glc[a]	H	H	X	O
Rh1	OH	O-Glc[a]	OH	X	X
Rh2(S)	O-Glc[a]	H	H	X	X

a)Arap, α-L-arabinopyranosyl; Araf, α-L-arabinofuranosyl; Glc, β-D-glucopyranosyl; Rha, α-L-Rhamnopyranosyl

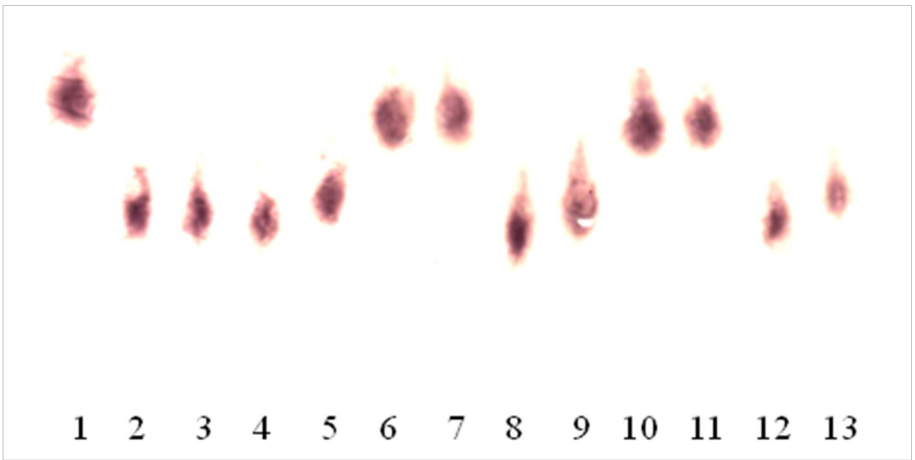


Fig. 3.10 TLC profile of hydrolysis product of various disaccharides ginsenosides using cell free extract of Bp504bbg504t and Bp504bbg1176. Glucose (lane 1), sophorose (lane 2), laminaribiose (lane 3), cellobiose(lane 4), gentiobiose (lane 5). Hydrolysis products of sophorose (lane 6 and 10), laminaribiose (lane 7 and 11), cellobiose (lane 8 and 12) and gentiobiose (lane 9 and 13).

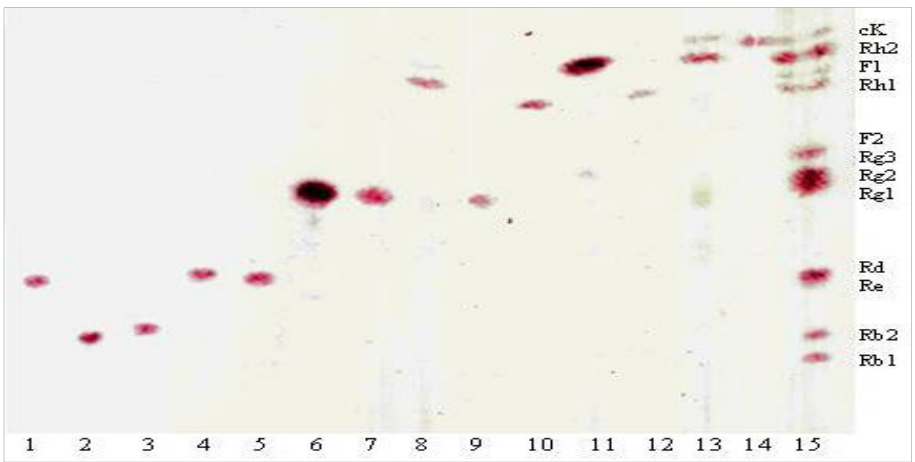


Fig. 3.11 TLC profile of hydrolysis product of various ginsenosides using cell free extract of Bp504bbg504t. Lane 1 to 14 contained hydrolysis products obtained from the incubation of Bp504bbg504t with Rb1 (lane 1), Rb2 (lane 2), Rc (lane 3), Rd (lane 4), Re (lane 5), Rg1(S) (lane 6), Rg2 (lanes 7), Rg3(S) (lane 8), Rg3(R) (lane 9), F1 (lane 10), F2 (lane 11), Rh1(S) (lane 12), Rh2(S) (lane 13) and cK (lane 14). Lane 15, ginsenoside standards listed on the right panel.

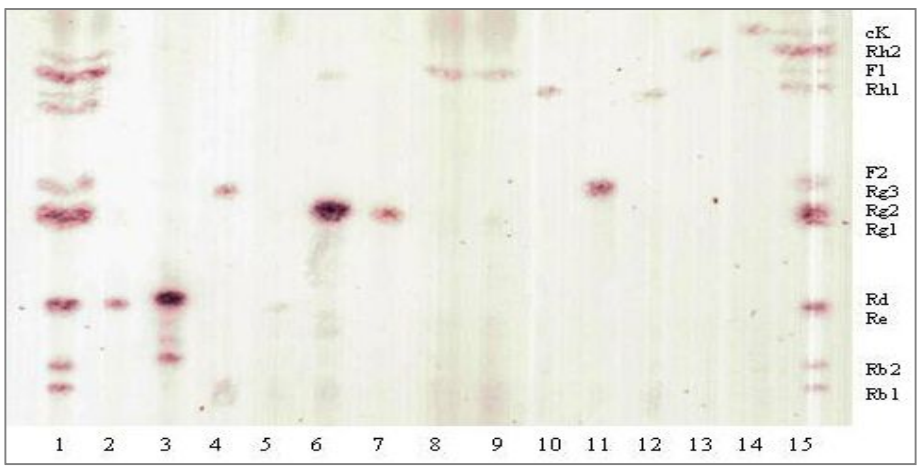


Fig. 3.12 TLC profile of hydrolysis product of various ginsenosides isoflavones using cell free extract of Bp504bbg1176. Lane 1 and 15, ginsenoside standards listed on the right and left panel. Lane 2 to 14 contained hydrolysis products obtained from the incubation of Bp504bbg1176 with Rb1 (lane 2), Rb2 (lane 3), Rd (lane 4), Re (lane 5), Rg1(S) (lane 6), Rg2 (lanes 7), Rg3(S) (lane 8), Rg3(R) (lane 9), F1 (lane 10), F2 (lane 11), Rh1(S) (lane 12), Rh2(S) (lane 13) and cK (lane 14).

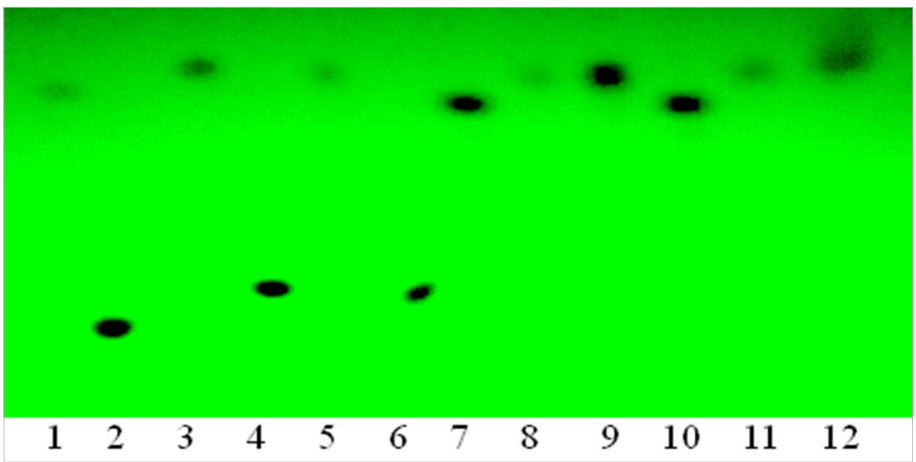


Fig. 3.13 TLC profile of hydrolysis product of various isoflavones using cell free extract of Bp504bbg504t and Bp504bbg1176. Hydrolysis product of various isoflavones using cell free extract of Bp504bbg504t (lane 7 to 9) and Bp504bbg1176 (lane 10 to 12). Daidzein (lane 1), daidzin (lane 2), glycitein (lane 3), glycitin (lane 4), genistein (lane 5), genistin (lane 6). Hydrolysis products of daidzin (lane 7 and 10), glycitin (lane 8 and 11), genistin (lane 9 and 12).

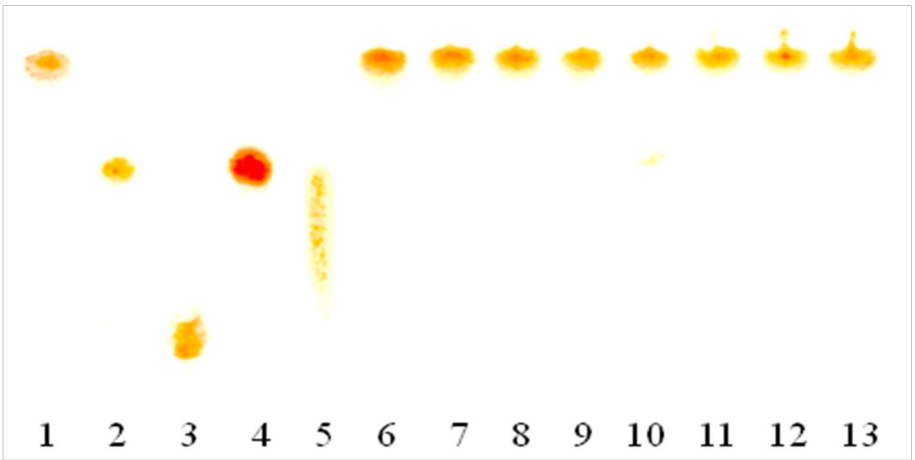


Fig. 3.14 TLC profile of hydrolysis product of various quercetins using cell free extract of Bp504bbg504t and Bp504bbg1176. Hydrolysis product of various quercetins using cell free extract of Bp504bbg504t (lane 6 to 9) and Bp504bbg1176 (lane 10 to 13). Quercetin (lane 1), isoquercetrin (lane 2), spiraeoside (lane 3), isoquercitrin (lane 4), quercetin-7-*O*-β-D-glucoside (lane 5). Hydrolysis products of isoquercetrin (lanes 6 and 10), spiraeoside (lane 7 and 11), isoquercitrin (lane 8 and 12) and quercetin-7-O-β-D-glucoside (lane 9 and 13).

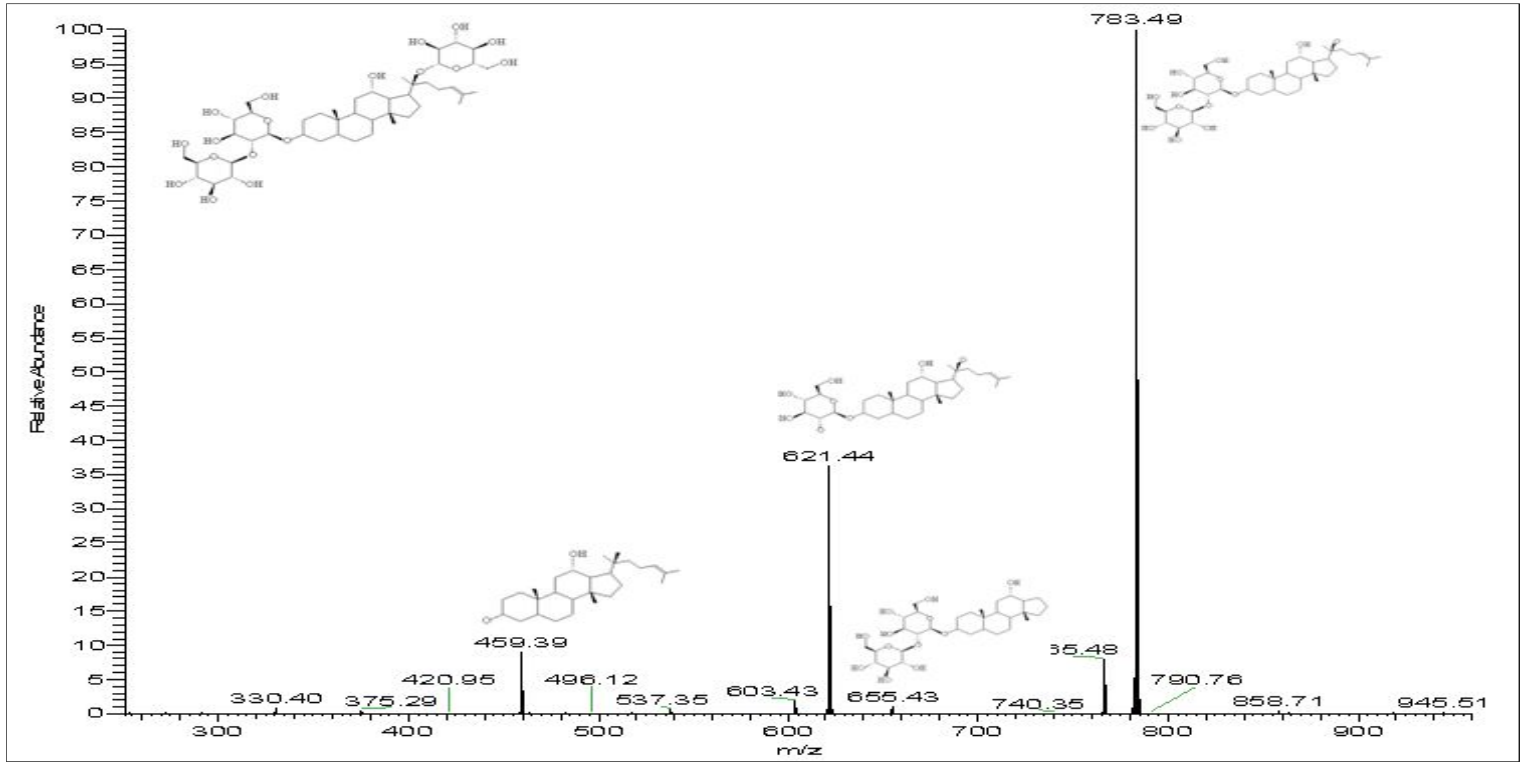


Fig. 3.15 LC-MS/MS analysis of transformed ginsenoside Rb1 with Bp504bbg504t. MS/MS product-ion scan from the molecular ion m/z 945.51. Fragmentation pattern and t_R are identical to ginsenoside Rd.

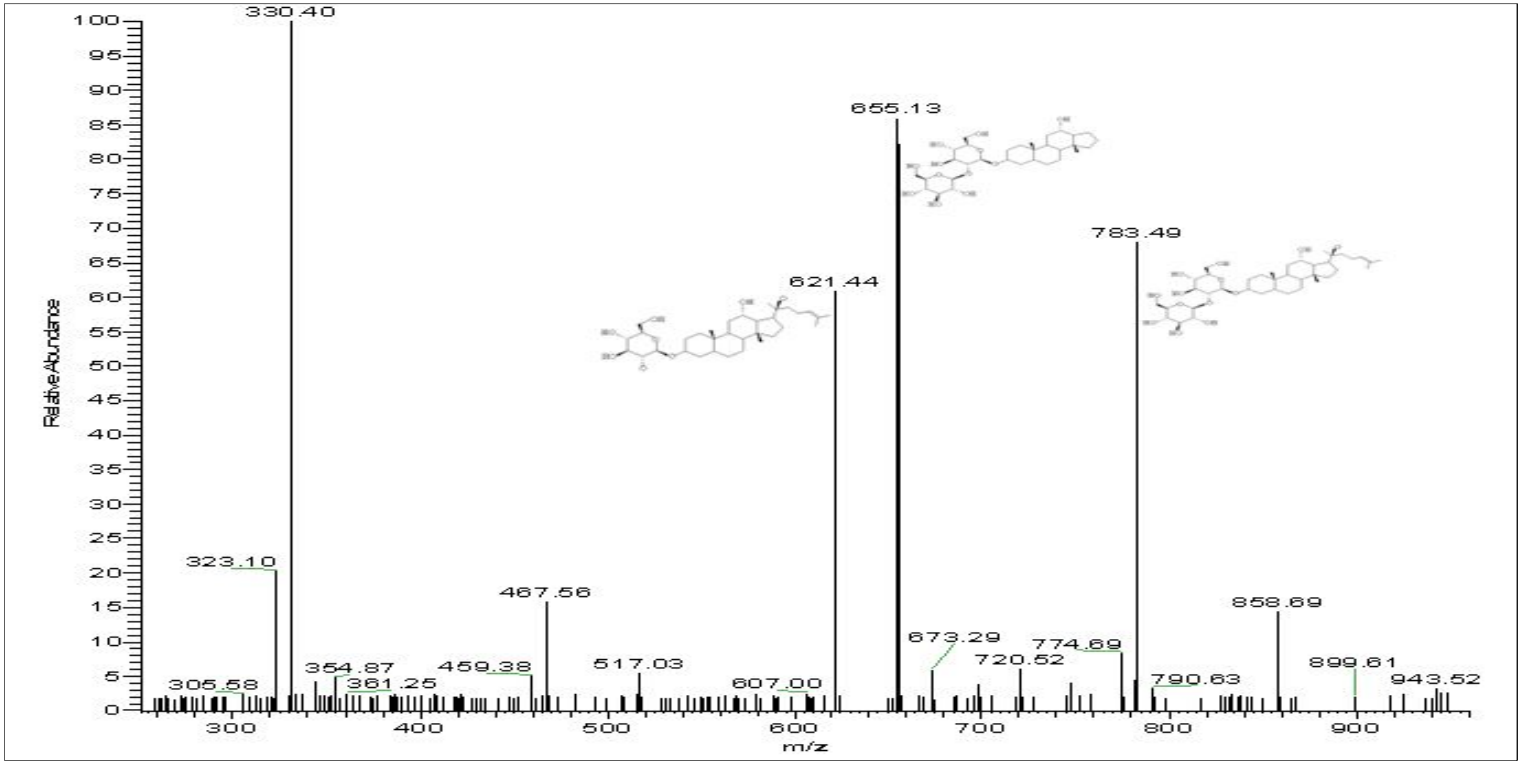


Fig. 3.16 LC-MS/MS analysis of transformed ginsenoside Rb1 with Bp504bbg1176. MS/MS product-ion scan from the molecular ion m/z 945.54. Fragmentation pattern and t_R are identical to ginsenoside Rd.

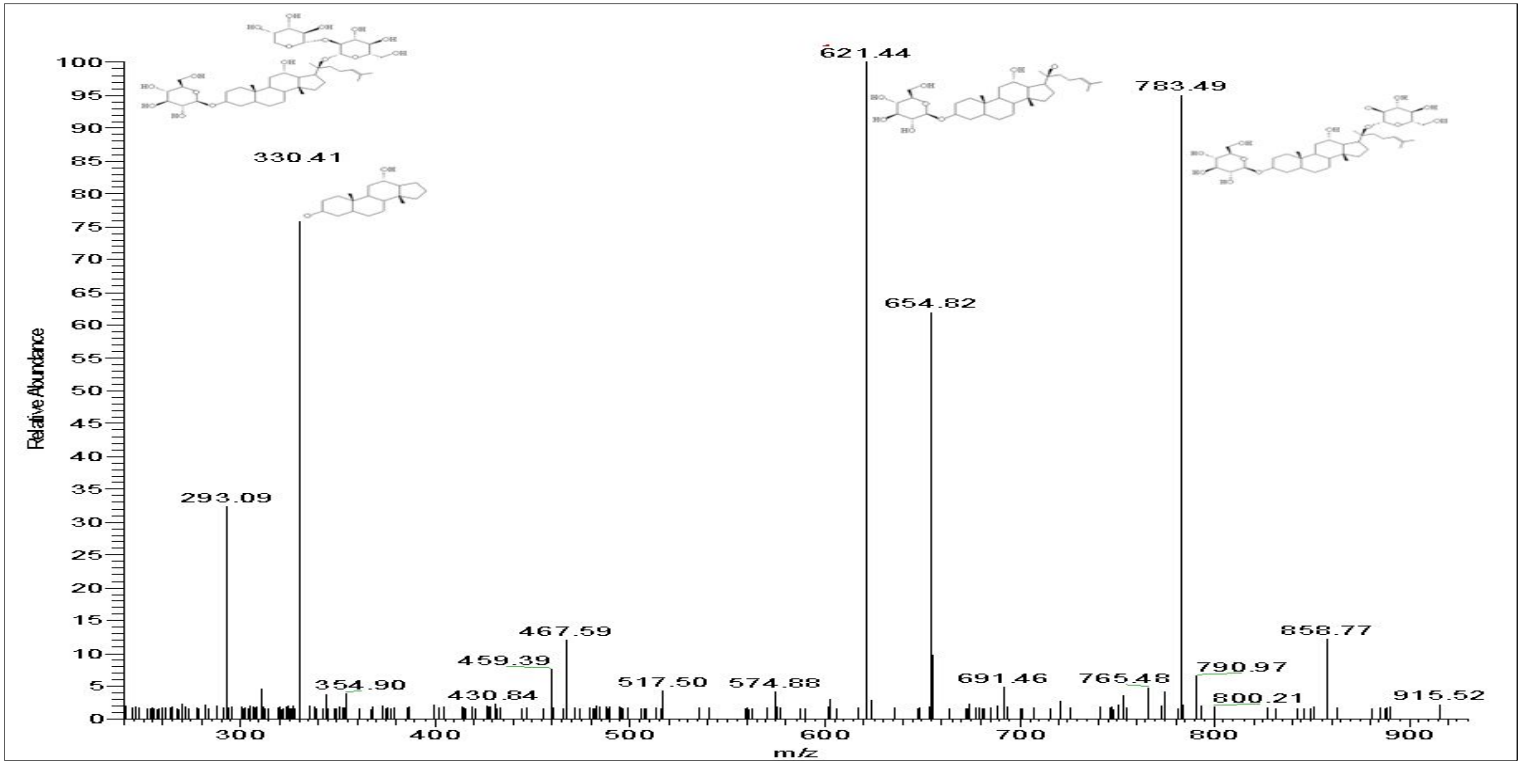


Fig. 3.17 LC-MS/MS analysis of transformed ginsenoside Rb2 with Bp504bbg1176. MS/MS product-ion scan from the molecular ion m/z 915.53. Fragmentation pattern and t_R are identical to ginsenoside Compound O.

References

1. **Aiba, H., A. Hanamura, and H. Yamano.** 1991. Transcriptional terminator is a positive regulatory element in the expression of the *Escherichia coli* crp gene. J. Biol. Chem. **266:**1721-1727.
2. **Beena, A., and V. Prasad.** 1997. Effect of yoghurt and bifidus yoghurt fortified with skim milk powder, condensed whey and lactose-hydrolyzed condensed whey on serum cholesterol and triacylglycerol concentrations in rat. J. Dairy Res. **64:**453-457.
3. **Bhatia, Y., S. Mishra, and V. S. Bisaria.** 2002. Micorbial β-glucosidases: cloning, properties and applications. *Crit. Rev. Biotechnol*. **22:**375-407.
4. **Burns, A. J., and I. R. Rowland.** 2000. Anti-carcinogenicity of probiotics and prebiotics. Curr. Issues Intestinal Microbiol. **1:**13–24.
5. **Chi, H., and G. E. Ji.** 2005. Transformation of ginsenosides Rb1 and Re from Panax ginseng by food microorganisms. Biotechnol. Lett. **27:**765-771.
6. **Choi, Y. O., J. M. Seo, and G. E. Ji.** 2008. Modulatory activity of CpG oligonucleotides from *Bifidobacterium longum* on immune cells. Food Sci. Biotechnol. **17:**1131-1395.
7. **Deguchi, Y., T. Morishita, and M. Mutai.** 1985. Comparative studies on synthesis of water-soluble vitamins among human species of Bifidobacteria. Agric. Biol. Chem. **49:**13–19.
8. **Estrem, S. T., T. Gaal, W. Ross, and R. L. Gourse.** 1998. Identification of an UP element consensus sequence for bacterial promoters. Proc. Natl.

Acad. Sci. USA **95:**9761-9766.

9. **Gibson, G. R.** 2008. Prebiotics as gut microflora management tools. J Clin Gastroenterol. **42:**S75-S79.

10. **Gore, C., K. Munro, C. Lay, R. Bibiloni, J. Morris, A. Woodcock, A. Custovic, and G. W. Tannock.** 2007. *Bifidobacterium pseudocatenulatum* is associated with atopic eczema: a nested case-control study investigating the fecal microbiota of infants. J Allergy Clin Immunol. **121:**135-140.

11. **Han, S. H., K. H. Cho, C. K. Lee, Y. C. Song, S. H. Park, N. J. Ha, and K. J. Kim.** 2005. Enhancement of Antigen Presentation Capability of Dendritic Cells and Activation of Macrophages by the Components of *Bifidobacterium pseudocatenulatum* SPM 1204. J Appl Pharmacol. **13:**174-180.

12. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.

13. **Helmann, J. D.** 1995. Compilation and analysis of *Bacillus subtilis* sigma A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23:**2351-2360.

14. **Jensen, P. R., and K. Hammer.** 1998. The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. Appl. Environ. Microbiol. **64:**82-87.

15. **Ji, G. E., S. K. Lee, and I. H. Kim.** 1994. Improved selective medium for isolation and enumeration of *Bifidobacterium* sp. Korean J. Food Sci.

Technol. **26:**526-531.

16. **Jung, I. H., J. H. Lee, Y. J. Hyun, and D. H. Kim.** 2012. Metabolism of ginsenoside Rb1 by human intestinal microflora and cloning of its metabolizing β-D-glucosidase from *Bifidobacterium longum* H-1. Biol Pharm Bull. **35:**573-581.

17. **Kim, J. Y., Y. Wang, S. J. Park, M. S. Park, and G. E. Ji.** 2012. Cloning of expression of β-glucosidases from *Bifidobacterium lactis* AD011. Food Sci. Biotechnol. **21:**731-738.

18. **Kim, N. J., and G. E. Ji.** 2006. Modulatory activity of *Bifidobacterium* sp. BGN4 cell fractions on immune cells. J Mocriobiol. Biotechnol., **16**: 584-589.

19. **Kim, N. J., J. Kunisawa, M. N. Kweon, G. E. Ji, H. Kiyono.** 2007. Oral feeding of *Bifidobacterium bifidum* (BGN4) prevents CD4(+) CD45RB(high) T cell-mediated inflammatory bowel disease by inhibition of disordered T cell activation. Clin Immunol. **123:**30-39.

20. **Kleerebezem, M., and E. E. Vaughan.** 2009. Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. Annu Rev Microbiol. **63:**269–290.

21. **Konstantinidis, K. T., and J. M. Tiedje.** 2004. Trends between gene content and genome size in prokaryotic species with larger genomes. Proc Natl Acad Sci USA. **101:**3160–3165.

22. **Lee, S. Y., S. J. Oh, S. W. Park, G. R. Jeon, J. Y. Kim, S. Y. Yoon, and G. E. Ji.** 2006. Evaluation of anti-allergic effect of Bifidobacteria in

murine model of peanut allergy. Pediatr Allergy Respir Dis. **16:**131-141.

23. **McCracken, A., M. S. Turner, P. Giffard, L. M. Hafner, and P. Timms.** 2000. Analysis of promoter sequences from *Lactobacillus* and *Lactococcus* and their activity in several *Lactobacillus* species. Arch. Microbiol. **173:**383-389.

24. **Nunoura, N., K. Ohdan, T. Yano, K. Yamamoto, and H. Kumagai.** 1996. Purification and characterization of beta-D-glucosidase (beta-D-fucosidase) from *Bifidobacterium breve* clb acclimated to cellobiose. Biosci. Biotechnol. Biochem. **60:**188-193.

25. **Park, M. S., H. W. Moon, and G. E. Ji.** 2003. Molecular characterization of plasmid from *Bifidobacterium longum*. J. Microbiol. Biotechnol. **12:**457-462.

26. **Park, M. S., J. M. Seo, and J. Y. Kim.** 2005. Heterologous gene expression and secretion in *Bifidobacterium longum*. Lait **85:**1-8.

27. **Reddy, B. S.** 1998. Prevention of colon cancer by pre- and probiotics: Evidence from laboratory studies. Brit. J. Nutr. **80:**S219-S223.

28. **Roller, M., G. Rechkemmer, and B. Watzl.** 2004. Prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* modulates intestinal immune functions in rats. J. Nutr. **134:**153-156.

29. **Saavedra, J. M., N. A. Bauman, I. Oung, J. A. Perman, and R. H. Yolken.** 1994. Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhea and shedding

of rotavirus. Lancet. **344:**1046-1049.

30. **Sambrook, J., E. F. Frietsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 3rd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

31. **Schell, M. A., M. Karmirantzou, B. Snel, D. Vilanova, B. Berger, G. Pessi, M. C. Zwahlen, F. Desiere, P. Bork, M. Delley, R. D. Pridmore, and F. Arigoni.** 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci USA. **99:**14422-14427.

32. **Turnbaugh, P. J., and J. I. Gordon.** 2009. The core gut microbiome, energy balance and obesity. J Physiol **587:**4153-4158.

33. **Ventura, M., M. O'Connell-Motherway, S. Leahy, J. A. Moreno-Munoz, G. F. Fitzgerald, D. Sinderen.** 2007. From bacterial genome to functionality; case bifidobacteria. Int J Food Microbiol. **120:**2-12.

34. **Ventura, M., S. O'Flaherty, M. J. Claesson, F. Turroni, T. R. Klaenhammer, D. Sinderen, and P. W. O'Toole.** 2009. Genome-scale analyses of health-promoting bacteria: probiogenomics. Nat Rev Microbiol. **7:**61-71.

35. **de Vrese, M., J. Schrezenmeir.** 2008. Probiotics, prebiotics, and synbiotics. Adv Biochem Eng Biotechnol **111:**1-66.

36. **Wang, Y., J. Y. Kim, M. S. Park, and G. E. Ji.** 2012. Novel *Bifidobacterium* promoters selected through microarray analysis lead to constitutive high level expression. *J. Microbiol*. **50:** 638-643.

37. **Wollowski, I., G. Rechkemmer, and B. L. Pool-Zobel.** 2001. Protective role of probiotics and prebiotics in colon cancer. Am. J. Clin. Nutr. **73:**451S-455S.

38. **You, H. J., D. K. Oh, G. E. Ji.** 2004. Anticancerogenic effect of a novel chiroinositol-containing polysaccharide from *Bifidobacterium bifidum* BGN4. FEMS Microbiol Lett. **240:**131-136.

39. **Youn, S. Y., M. S. Park, G. E. Ji.** 2012. Identification of the β-glucosidase gene from *Bifidobacterium animalis* subsp. *lactis* and its expression in *B. bifidum* BGN4. J. Microbiol. Biotechnol. **22:**1714-1723.

4. Conclusion

Conclusion

Identification of the β-Glucosidase Genes from *Bifidobacterium animalis* subsp. *lactis* and their Expression in *B. bifidum* BGN4

In order to search for a β-glucosidase gene from *B. animalis* subsp. *lactis* with no genome information available, we applied shot-gun cloning technologies. In order to discover the exact sequence of the start codon region, as well as the upstream promoter region, the sequences of *bbg572pt* was analyzed, from which we were also able to identify the the putative upstream promoter region. And *bbg572pt* gene has been applied to uncover the 3'-region of the *bbg572* gene, which successfully completed the remaining 3'-sequences (*572t*). As a result, the full DNA sequence of the *bbg572* gene contained 1383 bp (GenBank: JX274651) and the corresponding Bbg572 protein consisted of 461 amino acid with the expected molecular weight of 50.71 kDa.

The amino acid sequence of the identified Bbg572 protein showed 85%, 68%, 70%, and 69% amino acid sequence homology, respectively, to those of β-glucosidases from *B. breve* clb, *B. breve* CECT7263, *B. dentium* ATCC27679, and *B. longum* subsp. *infantis* ATC15697, upon sequence alignments by ClustealW2 (EMBL-EBI). Based on these amino acid sequence similarities, the β-glucosidase encoded by *bbg572* was assigned to glycosyl hydrolase family 1.

In order to express the Bbg572 protein, we amplified the full open reading frame (ORF) of *bbg572* gene by PCR with two primers deliberately designed to contain the BamHI and EcoRI sites. The amplified DNA was digested with BamHI and

EcoRI before being ligated into the pBES2 vector with corresponding cohesive ends. Colonies with ligated plasmids were chosen and DNA sequencing was performed to verify the successful cloning of the *bbg572* gene into pBES2 to give pBES-bbg572. β-Glucosidase was used as a reporter to evaluate the expression level of each promoter (*p572*, *pamy* and *p919*). Each putative promoter region was inserted into the upstream of β-glucosidase gene in pBES2-bbg572 vector and transferred to *B. bifidum* BGN4. DNA sequence analysis was performed to confirm correct cloning by using the Applied Biosystems 3730 DNA Analyzer in the Genome Research Facility in Seoul National University.

As expected, all promoters were shown to produce β-glucosidase when *B. bifidum* BGN4 was used as the expression host whereas promoterless vector and wild type *B. bifidum* BGN4 did not show β-glucosidase activity. The promoter *p572* showed the strongest activity among the promoters examined in *B. bifidum* BGN4 (Bp504bbg504), while the others were weaker than β-glucosidase activity of the original host *B. animalis* subsp. *lactis* SH5 and *B. animalis* subsp. *lactis* RD68. And Bp504bbg504 with terminator (Bp504bbg504t) showed higher β-glucosidase activity than Bp504bbg504.

To investigate substrate specificities, substrates were incubated with intracellular enzyme of Bp572bbg572t at 37°C for 24 hr. The substrate range of Bp572bbg572t was investigated using various disaccharides (sophorose, laminaribiose, cellobiose, gentiobiose), flavonoides (daidzin, genistin, glycitin, isoquercetrin, spiraeoside, quercetin-3,4-di-O-β-D-glucoside, and quercetin-7-O-β-D-glucoside) and ginsenosides (Rb1, Rb2 and Rg3(S)) (Fig. 4.1-Fig. 4.4)

Molecular Cloning, Characterization and Comparison of β-glucosidases from *Bifidobacterium pseudocatenulatum* in *B. bifidum* BGN4

Bifidobacteria genome database revealed a number of putative glycosyl hydrolase genes. For the amplification of β-glucosidase gene (*bbg1176*) from the genome of *B. pseudocatenulatum*, PCR primers were designed based on the β-glucosidase gene sequence obtained from the annotated genome data of B. adolescentis ATCC 15703 (accession no. NC-008618). The size of β-glucosidase (bbg1176) was 1176 bp. The Bbg1176 contained 392 amino acids, with a calculated molecular weight of 43.12 kDa. The deduced amino acid sequence of Bbg1176 was used to compare with other amino acid sequences deposed in NCBI database. The Bbg1176 exhibits high identity score with *B. adolescentis* ATCC15703 (92%) and *B. dentium* Bd1 (88%). And Bbg1176 are grouped in glycosyl hydrolase family 1. To overexpress Bbg1176, *bbg1176* was ligated into the expression vector pBES2 with *p504*. As a result, β-glucosidase activity of Bp504bbg1176 was about 30 fold higher than its parental strain.

To Investigate other β-glucosidase gene from *B. pseudocatenulatum* with no genome information available, shot-gun cloning technologies was used. In order to discover the exact sequence of the start codon region, as well as the upstream promoter region, *bbg504pt* gene sequencing was carried out. The analysis of *bbg504pt* gene uncovered the 3'-region (981 bp, *504t*) of the *bbg504* gene, which also revealed the remaining 3'-sequences. The full DNA sequence of the *bbg504* gene is 2247 bp (GenBank: JX274650) and the corresponding Bbg504 protein consisted of 461 amino acid with the expected molecular weight of 82.39 kDa.

The amino acid sequence of the identified Bbg504 protein showed 87%, 82%, 82%, and 81% amino acid sequence homology, respectively, to those of β-glucosidases from *B. dentium* ATCC27679, *B. breve* CECT7263, *B. longum* subsp. *infantis* ATCC 15697, and *B. longum* subsp. *longum* KACC 91563. The Bbg504 are grouped in glycosyl hydrolase family 3 based on a unique sequence signature.

β-Glucosidases, *bbg504* and *bbg1176*, were used as a reporter to evaluate the expression level of each promoter (*p504*, *p572*, *pamy* and *p919*). Each putative promoter region was inserted into the upstream of β-glucosidase gene in pBES2-bbg504 vector and transferred to *B. bifidum* BGN4. All promoters were shown to produce β-glucosidase when *B. bifidum* BGN4 was used as the expression host. The promoter *p504* showed the strongest activity among the promoters examined in *B. bifidum* BGN4, while the others were weaker than β-glucosidase activity of the parental host *B. pseudocatenulatum* INT57 and *pseudocatenulatum* SJ32. The addition of transcription terminator further enhanced the expression of Bbg504. Therefore, the addition of the terminators (Bp504bbg504t) was about 28.2% higher than Bp504bbg504.

To investigate substrate specificities, substrates were incubated with intracellular enzyme of Bp504bbg504t and Bp504bbg1176 at 37°C for 24 hr. As a result, Bp504bbg504t and Bp504bbg1176 hydrolyzed the glucosidic bonds of the disaccharides (sophorose, laminaribiose), flavonoides (daidzin, genistin, glycitin, isoquercetrin, spiraeoside, quercetin-3,4-di-O-β-D-glucoside, and quercetin-7-O-β-D-glucoside) and ginsenosides (Bbg504: Rb1, F2, Rh2(S) and Rg3(S); Bbg1176: Rb1, Rb2, Rd, Rg1, Rg3(R) and Rg3(S)) (Fig. 4.1 - 4.4).

These results suggest that these new β-glucosidase-positive *Bifidobacterium*

transformants can be utilized for the production of specific aglycone products.

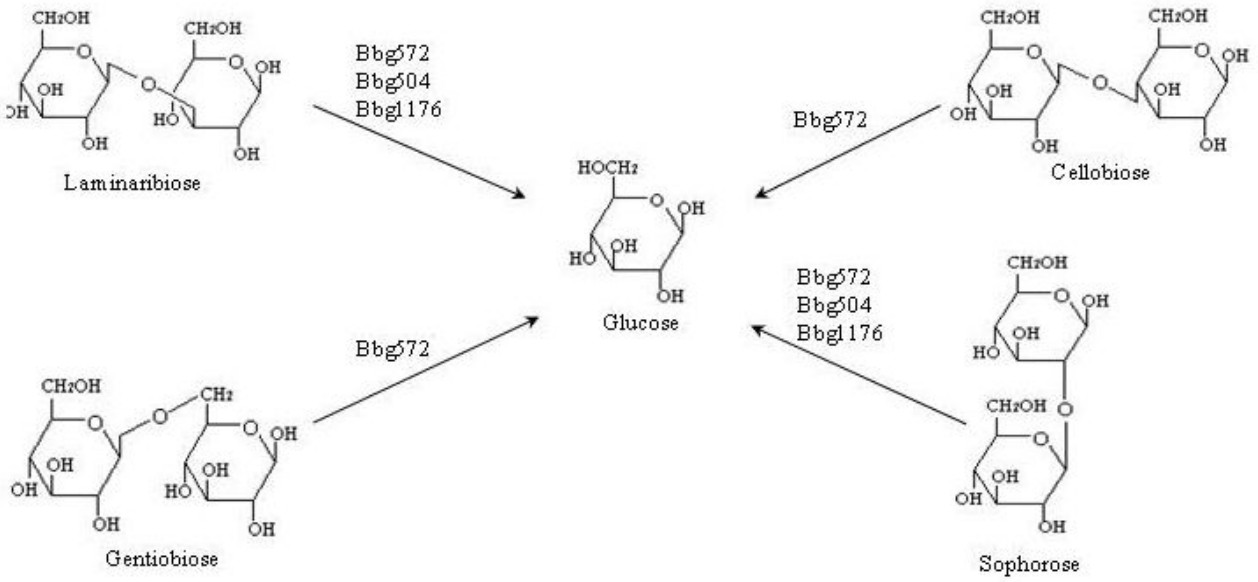


Fig. 4.1 Transformation pathways of disaccharides by recombinant *B. bifidum* BGN4

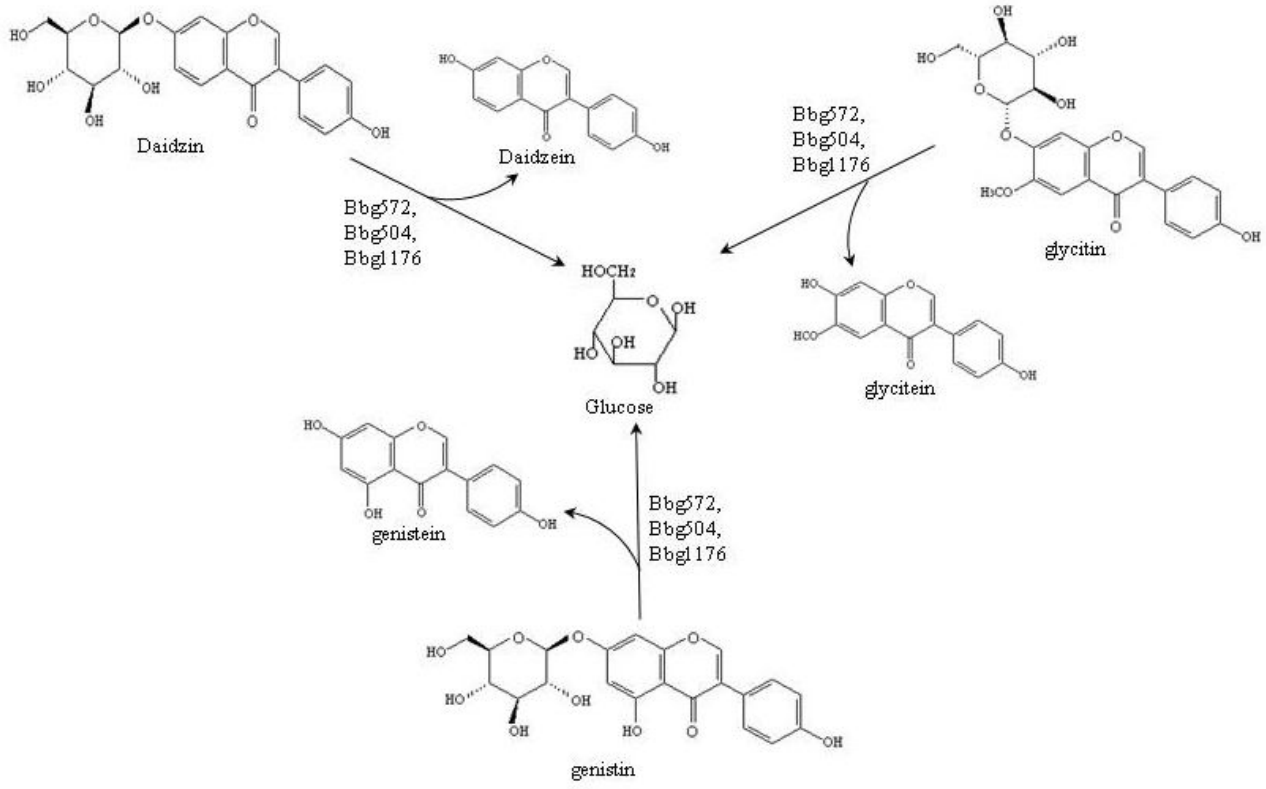


Fig. 4.2 Transformation pathways of isoflavones by recombinant *B. bifidum* BGN4

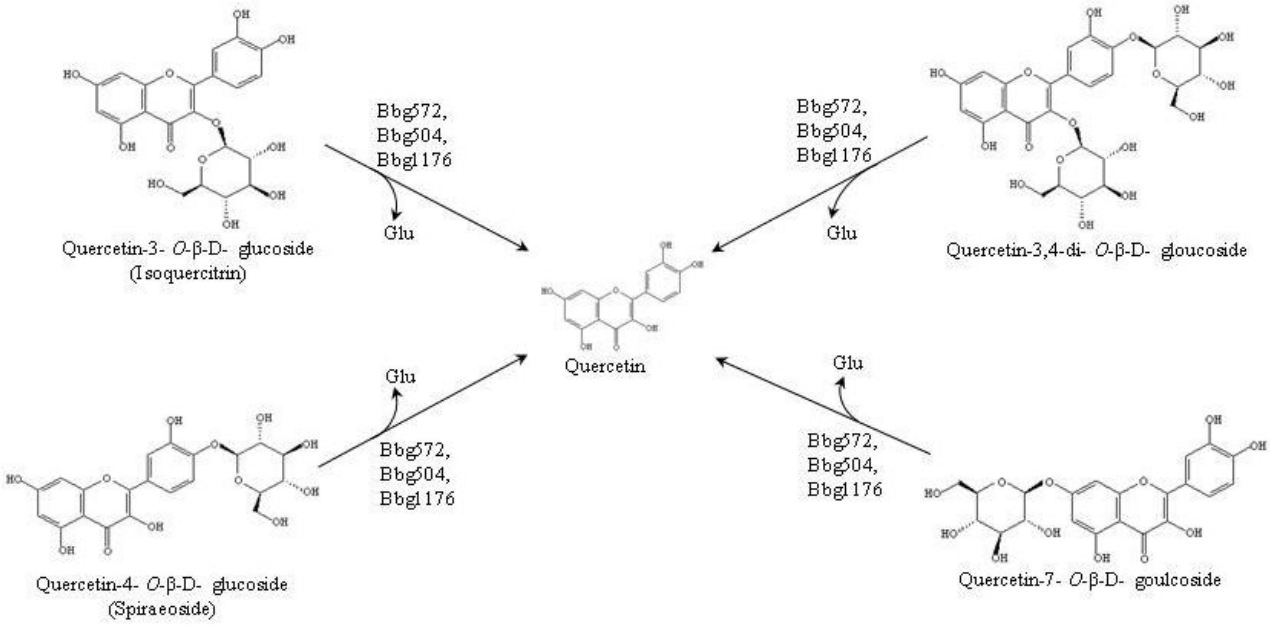


Fig. 4.3 Transformation pathways of quercetin glycosides by recombinant *B. bifidum* BGN4

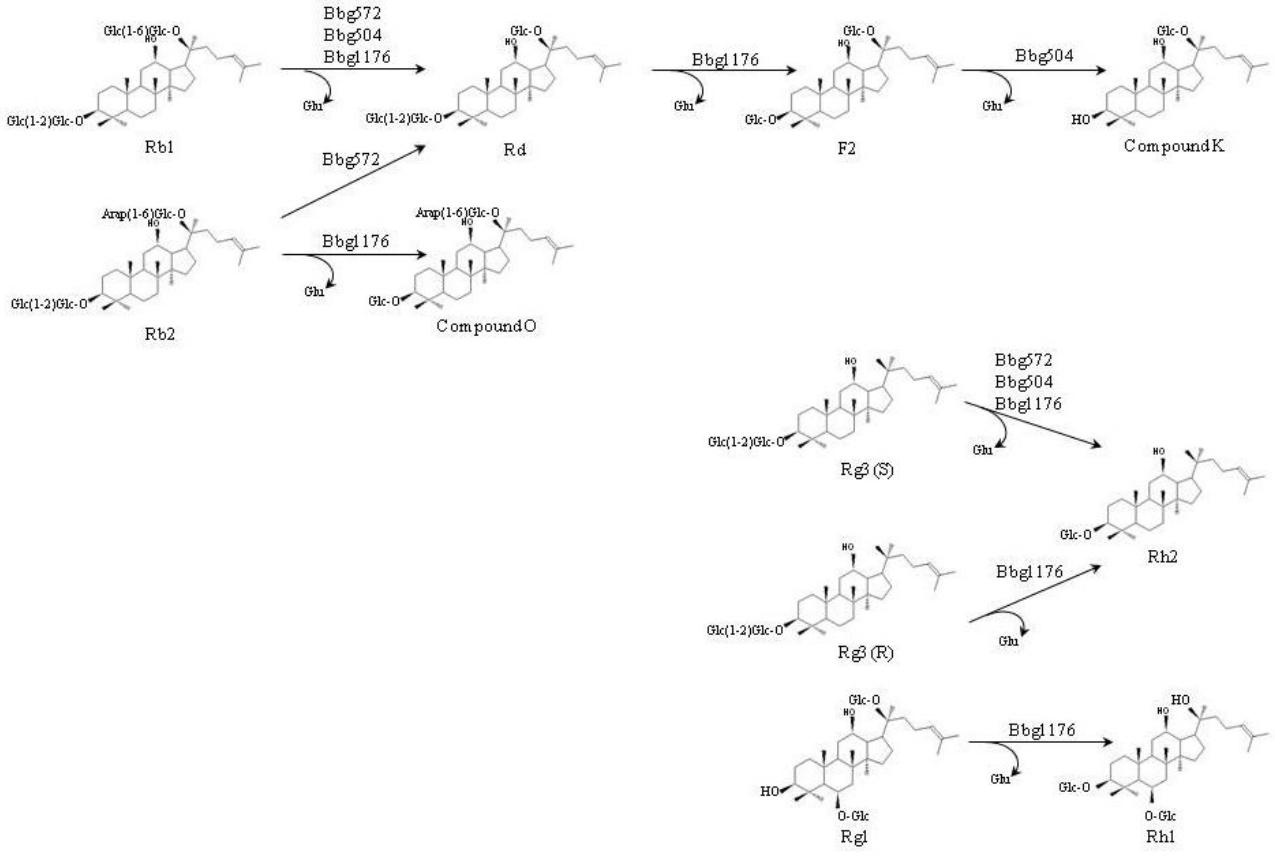


Fig. 4.4 Transformation pathways of ginsenosides by recombinant *B. bifidum* BGN4

국문 초록

일반적으로 자연계의 유용 성분은 배당체 형태를 이루고 있는 것들이 많다. 그러나 이러한 배당체 형태로 된 성분들은 쉽게 흡수되지 못하므로 장내 미생물에 의해 비배당체 형태로 바뀐 후 소화 과정이 일어난다. 소화 과정이 일어나기 위해서는 각각의 배당체를 비배당체로 만들기 위한 알맞은 장내 미생물을 가지고 있어야만 한다. 그러나 개인마다 이러한 장내 미생물들의 구성 함량들이 다르기 때문에, 각각의 배당체에 대한 장내 미생물들을 적게 가지고 있는 경우에는 많은 배당체를 섭취하게 되더라도 제대로 대사되지 못하며, 과량 섭취 시 오히려 소화 장애를 일으킬 수 있다. 이러한 문제점을 해결하기 위하여 배당체를 비배당체로 만들 수 있는 효소를 미생물로부터 클로닝한 후 발현시키면 배당체 소화를 위한 장내 미생물 효소를 가지고 있지 않더라도 비배당체의 소화 흡수를 도울 수 있을 것이다.

*Bifidobacterium*은 항종양 효과, 비타민 B_2, B_6, B_{12} 합성, 병원균 억제, 설사 및 변비 예방, 혈청 콜레스테롤 감소, 소화 과정 지원을 하며, GRAS (generally recognized as safe) 식품으로 안전성을 인정받고 있는 장내 유용 미생물이다. 이러한 유용 미생물인 *Bifidobacterium*으로부터 유래한 vector 를 이용함으로써 기존의 대장균 유래 vector 를 사용함으로 인한 안정성 문제 등도 해결할 수 있을 것이다. Host 또한 대장균이 아닌 *Bifidobacterium*을 사용하면 정제 과정을 거치지 않고도 균체 자체를 그대로 사용함으로써 앞으로 안정성과 더불어 효율성 또한 더욱 높아질 것이

다.

본 연구에서는 각 식품에 β-D-glucoside 구조를 이루고 있는 배당체를 비배당체로 전환시켜 주기 위하여 여러 개의 균주로부터 β-D-glucosidase를 *Bifidobacterium* 유래 shuttle vector와 *Bifidobacterium* host를 이용하여 발현하고, β-D-glucosidase들의 기질 특이성을 알아보았다.

우선, 43종의 유산균 (19 *Bifidobacterium* spp., 9 *Lactobacillus* spp., 8 *Bacillus* spp., 2 *Lactococcus* spp., 2 *Enterococcus* spp., 1 *Leuconostoc* sp., 1 *Weissella* spp., and 1 *Pediococcus* sp.) 중 β-D-glucosidase 활성이 높은 균주 4가지를 선발하여 클로닝을 수행하였다.

그 중 *B*. a*nimalis* subsp. *lactis* strain인 *B*. *lactis* SH5과 *B*. *lactis* RD68를 shot-gun cloning을 실행하고, 염기 서열을 분석한 결과 promoter (*p572*), ORF (*bbg572*), terminator (*572t*) 가 동일하였으며, Bbg572가 glycoside hydrolase family3 (GH3)에 속함을 알 수 있었다. *B*. *bifidum* BGN4에서 *B*. *lactis* β-glucosidase (*bbg572*) 활성을 높이기 위하여 여러 가지 promoter를 비교한 결과, *bbg572*의 upstream sequence에 있는 *p572*가 존재하는 vector system (p572bbg572t) 으로 발현하였을 때 가장 높은 활성을 보였으며, terminator 존재 시 (p572bbg572t) 활성이 더 높아짐이 보였다. 그러나, 분비 시스템을 구축하기 위하여 signal sequence를 첨가하였을 때 오히려 활성이 낮아졌으며, 분비 역시 되지 않음을 볼 수 있었다. 또한 β-glucosidase (Bbg572)는 이당류 (cellobiose, sophorose, laminaribiose, gentiobiose), isoflavone (daidzin, genistin, glycitin), isoquercetrin, spiraeoside, Quercetin-3,4-di-O-β-D-

glucoside, Quercetin-7-O-β-D-glucoside에서는 모두 β-glucosidase linkage에서 분해 반응을 보였으나, ginsenoside의 경우 Rb1, Rb2, Rg1에서만 다른 ginsenoside로 전환되는 반응이 일어나는 것을 볼 수 있었다.

또한, 선별된 4균주 중 나머지 2가지 균주인 *B. pseudocatenulatum* INT57과 *B. pseudocatenulatum* SJ32를 shot-gun 방법과 기존에 알려진 β-glucosidase 염기 서열을 이용한 PCR 등 2가지 방법으로 β-glucosidase cloning을 진행하였다. 그 결과 2개의 ORF을 동정하게 되었으며, 염기 서열 분석 결과 *B. pseudocatenulatum* INT57과 *B. pseudocatenulatum* SJ32의 promoter (*p504*), ORF (*bbg504*, *bbg1176*), terminator (*504t*) 가 동일하였다. 또한 아미노산 서열 다중 분석을 통하여 Bbg504는 glycoside hydrolase family1 (GH1), Bbg1176은 glycoside hydrolase family3 (GH3)에 속함을 밝힐 수 있었다. *bbg504*를 reporter gene으로 사용하고, 여러 가지 promoter들을 비교하였을 때 β-glucosidase 활성은 *bbg504*의 upstream sequence에 존재하는 promoter(*p504*)를 사용하였을 때 (p504bbg504) 가장 활성이 높았으며, terminator를 *bbg504*의 downstream sequence에 첨가하였을 때 (p504bbg504t) *B. bifidum* BGN4에서의 활성이 더 높아짐을 알 수 있었다. 또한 *bbg1176*의 경우 *p504*를 promoter로 사용하였을 때 β-glucosidase 활성이 p504bbg504보다는 낮았으나, 모균주보다 높아짐을 볼 수 있었다. 기질 특이성을 Bbg572와 비교하였을 때 Bbg504와 Bbg1176 모두 이당류인 sophorose와 laminaribiose는 분해하였으나, gentiobiose와 cellobiose는 분해하지 못하였으며, ginsenoside에서는 Bbg504는 Rb1, F2, Rg3(S), Bbg1176은

Rb1, Rb2, Rd, Rg1, Rg3(S), Rg3(R))에서 반응을 나타냈다. 특히, Bbg1176은 Rb2를 미량의 ginsenoside인 compound O로 전환함으로써 산업적으로 더 큰 의미를 가지고 있다.

이 실험에서는 *B. lactis*로부터 분리된 β-glucosidase (Bbg572)와 *B. pseudocatenulatum*로부터 분리된 β-glucosidase (Bbg504 와 Bbg1176) 등 총 3가지의 β-glucosidase를 동정하고, 그 특성을 살펴보았다. 그 결과, 동정된 β-glucosidase들은 자신의 promoter와 terminator를 가지고 있을 때 *B. bifidum* BGN4에서 가장 높은 활성을 보였고, 모균주보다 활성이 높았다. 또한 각각의 β-glucosidase는 다른 기질 특이성을 가지고 있기 때문에 특정 배당체를 얻고자 할 때 유용하게 이용될 수 있을 것으로 기대된다.

주요어 : 비피도박테리움, 비배당체, 클로닝, 발현, 베타 글루코시데이즈

학 번 : 2007-30440