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Over-Expression of the *Thermobifida fusca* β-Glucosidase in a *Yarrowia lipolytica* Transformant to Degrade Soybean Isoflavones

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Abstract: A gene (*bgl*) encoding a β-glucosidase in thermophilic actinomycete *Thermobifida fusca* NTU 22 was cloned into a *Yarrowia lipolytica* expression system. Heterologous expression resulted in extracellular β-glucosidase production with activity as high as 630 U/mL in a Hinton flask culture filtrate. This recombinant β-glucosidase was purified 9.2-fold from crude culture filtrate by DEAE-Sepharose FF column chromatography as measured by its increase in specific activity. The overall yield of the purified enzyme was 47.5%. The molecular weight of the purified β-glucosidase estimated by SDS-PAGE was 45 kDa, which agreed with the predicted molecular weight based on the nucleotide sequence. About 15% enzyme activity loss was observed after the enzyme was heat-treated at 50 °C for 180 min. It was also found that the activity of the enzyme was inhibited by Hg^{2+}, Cu^{2+}, Ba^{2+}, Ag^{+}, *p*-chloromercuribenzene, and iodoacetate. The β-glucosidase from *T. fusca* had the most activity for daidzein-7-glucoside and genistein-7-glucoside among the tested flavonoid glycosides, but there was moderate or little activity for luteolin-7-glucoside, cyanidine-3-glucoside, and quercetin-3-glucoside. These properties are important for the soybean isoflavone applications of this β-glucosidase.

Keywords: *Thermobifida fuscal*; β-glucosidase; *Yarrowia lipolytica*; daidzin; genistin

1. Introduction

β-D-glucoside glucohydrolase (EC 3.2.1.21), generally called β-glucosidase, catalyzes the hydrolysis of β-glucosidic linkages of glucose or oligosaccharides conjugates [1]. This enzyme is commonly produced by a wide range of organisms, including bacteria, fungi, and plants. β-Glucosidases play pivotal roles in various biotechnological processes such as the reduction of cellobiose accumulation by the action of exo-glucanases to minimize the end-product inhibition [2] and the modification of cyanogenesis, glycolipids, and secondary metabolites [1]. They are involved in the bioconversion of lignocellulose to glucose, producing hydrogen with other exo- or endo-hydrolases and using glucose as an ingredient for fermentation [3,4]. They can also hydrolyze the β-glucosidic bonds of phenolic compounds conjugated to sugar residues via the hydroxyl groups [5–7].

Soybean ingredients are rich sources of flavonoids and phenolic compounds with biological activity [8]. Flavonoids, such as isoflavones, are mainly present in soybean foods, as glycosides. Soybean

isoflavone aglycones are estrogen-like compounds and are absorbed faster than their glucoside derivatives in humans [9]. In addition, the aglycone forms of soybean isoflavones have higher biological activities [10].

Actinomycetes, comprising multiple groups of Gram-positive, aerobic, and largely mycelial bacteria, have considerable ecological roles in the biodegradation of lignocellulose. Within this group of bacteria, the thermophilic actinomycetes are of particular note because they secrete a variety of thermostable hydrolytic enzymes involved in the degradation of nature lignocellulose [11]. The β-glucosidase activity of thermophilic actinomycetes (i.e., *Thermobifida fusca*, *Thermomonospora chromogena*, and *Thermomonospora curvata*) is mainly intracellular [12].

For economic reason, enzyme genes from thermophilic actinomycetes have been frequently cloned and expressed in the mesophilic systems to reduce the energy cost of cultivation [13]. *Yarrowia lipolytica* is mesophilic, generally viewed as a safe (GRAS) yeast used as a host for heterologous protein over-production [14]. Many molecular biological tools are available for use in heterologous expression in this yeast [15].

The thermophilic actinomycete *Thermobifida fusca* NTU 22 was isolated from compost samples collected in South Taiwan [11]. The raw-starch-digesting amylase and acetylxylan esterase genes of *T. fusca* were successfully heterologously expressed in a *Y. lipolytica* expression system [16,17]. The β-glucosidase gene (*bgl*) from *T. fusca* NTU 22 was also cloned and expressed in *Escherichia coli*. This study aimed to constitutively overexpress the *bgl* gene in a *Y. lipolytica* expression system. The enzyme was further purified and its properties were studied. Various flavonoid glycosides were also investigated as substrates.

2. Results

2.1. Amplification and Construction of the bgl Gene in a Y. lipolytica Expression System

The *bgl* gene was cloned into a pYLSC1 vector using *Sfi*I and *Xba*I restriction sites as described in Section 4, theoretically placing the *bgl* coding sequence in frame. The transformant (pYLSC1-*bgl*) that displayed the highest and most stable β-glucosidase activity was selected for further experiments. Alignment of the *bgl* coding sequence with entries in the NCBI database resulted in a 99.65% identity with the gene sequence of *T. fusca* YX (NCBI accession number Q47RE2). A mismatch in the amino acid sequences of Q47RE2 and *bgl* occurred only at the 137th amino acid, where *bgl* has an arginine instead of a histidine. Molecular modeling of β-glucosidase deduced from the *bgl* gene is shown in Figure 1. Two glutamic acids, including Glu 182 and Glu 388, were present in the predicted active center of the β-glucosidase, and the 137th amino acid was not the active site. Therefore, we assume that the variation at 137th amino acid has no specific means.

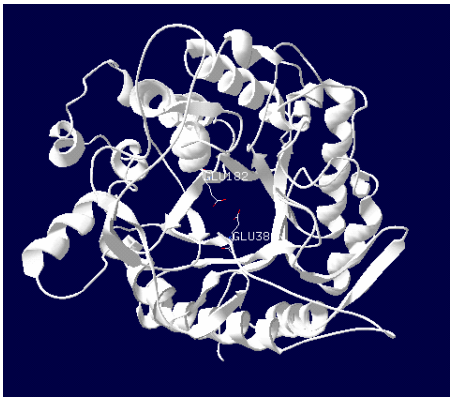

Figure 1. Molecular modeling of β-glucosidase deduced from *bgl* gene. Center for biological sequence analysis. http://www.cbs.dtu.dk/. CPHmodels 2.0: X3M a Computer Program to Extract 3D Models.

2.2. Constitutive Expression of the bgl Gene in a Y. lipolytica Transformant

Figure 2 shows that the transformant (pYLSC1-*bgl*) logarithmically grew at 28 °C from 24 to 36 h and then moved toward a stationary phase. The OD_{600} value was 62 after 36 h of incubation. Extracellular β-glucosidase rapidly accumulated along with the increase in OD_{600} value. The maximum activity (630 U/mL) in the culture filtrate was observed after 60 h of incubation. Little β-glucosidase activity was detected in the control strain, *Y. lipolytica* (pYLSC1), under the same conditions (data not shown).

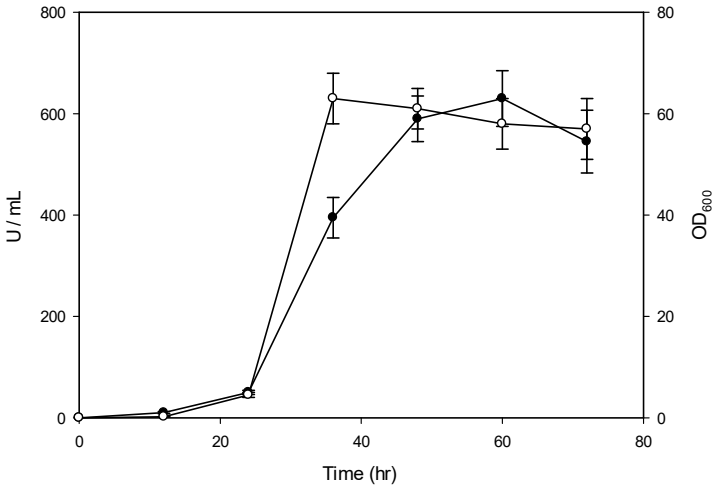

Figure 2. Time course of β-glucosidase activity in a *Y. lipolytica* transformant (pYLSC1-*bgl*). (-●-) extracellular β-glucosidase activity; (-○-), OD 600 nm. The culture conditions were: temperature 28 °C; shaking speed 200 rpm.

2.3. Purification and Characterization of β-Glucosidase from the Y. lipolytica Transformant (pYLSC1-bgl)

The purification of β-glucosidase was performed as described in the Materials and Methods section. The culture filtrate was concentrated by Pellicon ultrafiltration and then applied to the DEAE-Sepharose™ FF column chromatography. The DEAE-Sepharose™ FF column chromatography elution profile is shown in Figure 3. The purified β-glucosidase exhibited 41.8% of its total initial activity and a 9.2-fold increase in specific activity over that of the crude culture filtrate solution. The outcomes of the total purification are summarized in Table 1.

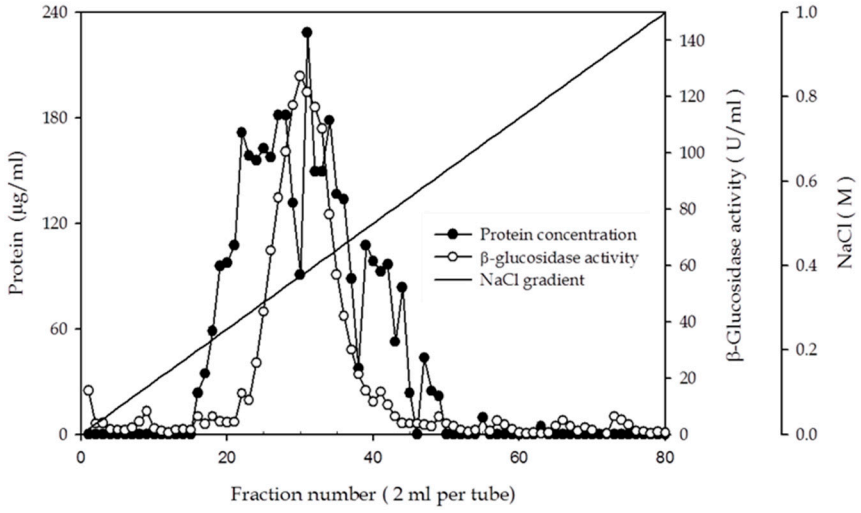

Figure 3. DEAE-Sepharose FF chromatography. (-●-), protein; (-○-), β-glucosidase activity; (-) NaCl gradient. Column: 1.13 × 8-cm; flow rate: 60 mL/h.

Table 1. Purification steps of β-glucosidase from a *Y. lipolytica* transformant (pYLSC1-*bgl*).

	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Culture Filtration	40,176	2730	0.068	1.0	100
Pellicon ultrafiltration	16,797	2402	0.143	2.1	88
DEAE-sepharose FF	1840	1141	0.62	9.2	41.8

As shown in Figure 4A, the purified β-glucosidase demonstrated a single significant protein band on native PAGE (10%). The significant single protein band was shown to be a β-glucosidase by staining with *p*-nitrophenyl-β-glucopyranoside. The molecular weight of the subunit of the purified protein was estimated to be 45 kDa from its mobility on SDS-PAGE relative to those of standard proteins (Figure 4B).

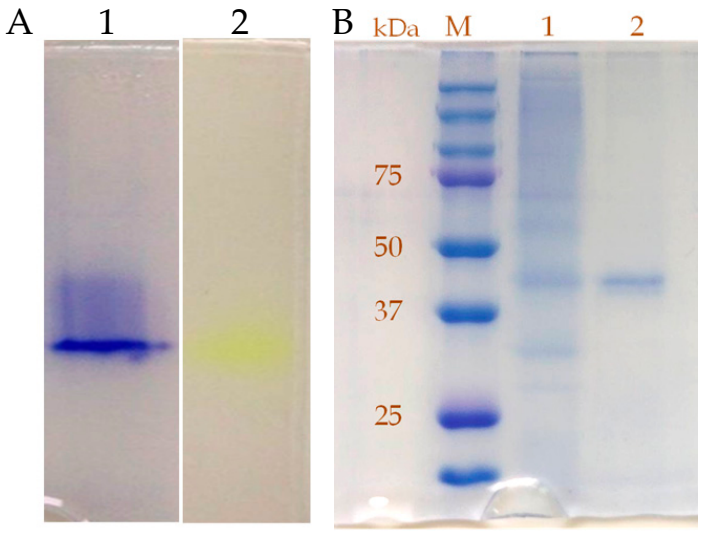

Figure 4. Polyacrylamde gel electrophoresis of the β-glucosidase purified from *T. fusca* NTU22. (**A**) Native polyacrylamide gel electrophoresis (PAGE) of the purified enzyme. Lane 1: Protein stained by Coomassie Brilliant Blue R-250. Lane 2: Activity stained by *p*-nitrophenyl-β-glucopyranoside. (**B**) SDS-PAGE. Lane M: Protein molecular mass standard; Lane 1: Crude culture filtrate; Lane 2: Purified β-glucosidase. Electrophoresis conditions: 150 V, 1 h.

The optimal pH and temperature values of the β-glucosidase activity from the *Y. lipolytica* transformant (pYLSC1-*bgl*) were 6.0 and 60 °C, respectively. Approximately 90% of the original β-glucosidase activity remained after the enzyme had received a 50 °C heat treatment for 180 min. The enzyme activity was very unstable at 70 °C. The β-glucosidase purified from the *Y. lipolytica* transformant was stable over a pH range from 6.0 to 10.0 at 4 °C for 24 h.

Testing the enzyme with metal salts and chemical reagents indicated that the β-glucosidase activity was completely inhibited by 1 mM Hg^{2+} (Table 2). Cu^{2+}, Ba^{2+}, and Ag^{+} ions also inhibited about approximately 80% of the β-glucosidase activity. The enzyme activity was extremely inhibited by *p*-chloromercuribenzene (PCMB) and iodoacetate.

According to the results in Table 3, the β-glucosidase hydrolyzed substrates containing β-1,4 glycosidic linkage. Especially, the enzyme had much higher activity for *p*-nitrophenyl-β-D-glucopyranoside and cellobiose in comparison with others. The β-glucosidase was also able to hydrolyze β-D-galactosidic bond but did not show activity against the β-D-xylopyranosidic bond.

Table 2. Effect of metal salts and chemical reagents on the activity of recombinant β-glucosidase from a *Y. lipolytica* transformant (pYLSC1-*bgl*).

Metal Salt/Chemical Reagent [a]	Relative Activity (%) [b]
Control	100
$CoCl_2$	105
$MnCl_2$	105
$FeCl_3$	100
$CaCl_2$	93
$ZnCl_2$	90
$MgCl_2$	87
$AgNO_3$	22
$BaCl_2$	17
$CuCl_2$	14
$HgCl_2$	0
2-Mercaptoethanol	96
EDTA	93
DTT [c]	85
PMSF [c]	84
Iodoacetate	7
PCMB [c]	0

[a] The metal salt/chemical reagent concentrations were 1 mM. [b] The reaction mixtures were composed of β-glucosidase and various metal salt/chemical reagents in 750 μL of 100 mM phosphate buffer (pH 7.0) and were incubated at 50 °C for 10 min. The residual activities were determined. [c] DTT: dithiothreitol; PCMB: *p*-chloromercuribenzene; PMSF: phenylmethylsulphonyl fluorophosphate.

Table 3. The decomposition rates of flavonoids (as substrates).

Substrate	Specific Activity [a]
Cellobiose [b]	22.03
Lactose	2.70
Maltose	0.41
Sucrose	0.21
α-Cellulose	0.20
Carboxymethyl cellulose	0.23
p-Nitrophenyl-β-D-glucopyranoside [c]	28.12
p-Nitrophenyl-β-D-galactopyranoside	19.29
p-Nitrophenyl-β-D-xylopyranoside	0.28
p-Nitrophenyl-phosphate	1.86

[a] Micromoles per milliliter of enzyme solution per minute, released in 1.0 mL of 100 mM sodium phosphate (pH 7.0) at 50 °C. [b] Measured by release of glucose. [c] Measured by release of *p*-nitrophenol.

2.4. Substrate-Specific Characterization of the β-Glucosidase from the Y. lipolytica Transformant (pYLSC1-bgl)

The chemical structures of the flavonoids used in this study are shown in Figure 5. The relative activities of the hydrolysis of various flavonoid glucoside substrates by the β-glucosidase from the *Y. lipolytica* transformant (pYLSC1-*bgl*) are presented in Table 4. The enzyme efficiently hydrolyzed the β-glycosidic linkages of 7-glucoside of genistein, and 7-glucoside of daidzein to genistein and daidzein. The conversion rates were 80.5% and 50.0%, respectively. The enzyme also hydrolyzed a 3-glucoside of cyanidine, but the aglycone product was not found. Both a 7-glucoside of luteolin and 3-glucoside of quercetin were not hydrolyzed by the purified enzyme.

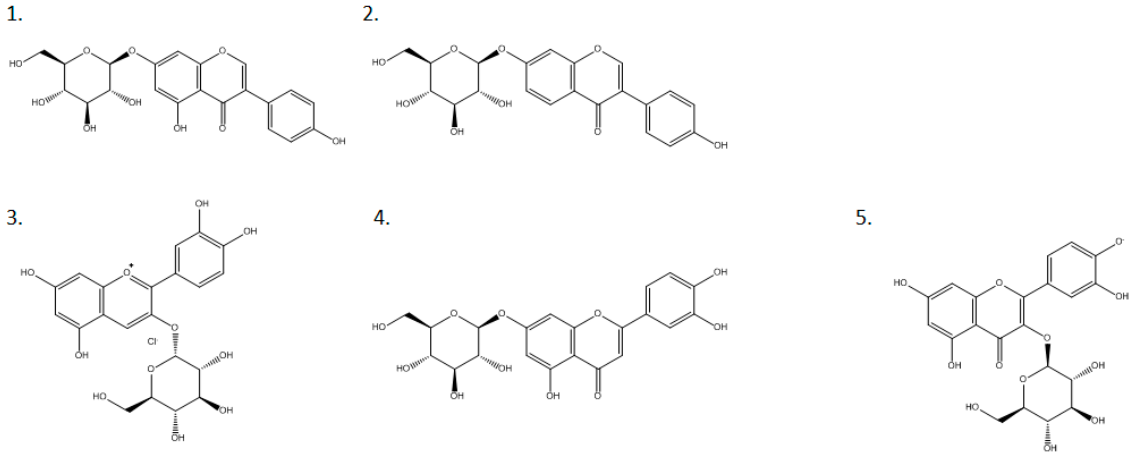

Figure 5. Chemical structures of the flavonoids. 1, Genistin; 2, Daidzin; 3, Cyanidin 3-*O*-glucoside chloride; 4, Luteolin 7-glucoside; 5, Quercetin 3-β-glucoside.

Table 4. The conversion rates of flavonoids (as substrates).

Substrate	Conversion Rate (μmol/min/unit)
Genistein-7-glucoside	50.0
Daidzein-7-glucoside	80.5
Cyanidin-3-glucoside	24.4
Luteolin-7-glucoside	0
Quercetin-3-glucoside	0

3. Discussion

Y. lipolytica has a high secretory capacity and was recently used as a heterologous expression host for thermostable enzymes. The β-glucosidase gene (*bgl*) from *T. fusca* NTU22 has also been heterologously expressed in an *E. coli* BL21 (DE3)–pET32a host–vector expression system. The *E. coli* transformant was cultured in 50 mL of M9 medium in 500 mL Hinton flasks and shaken (125 rpm) at 37 °C for 16 h. The maximum β-glucosidase activity was still 2.62 U/mL. In this study, the β-glucosidase gene (*bgl*) from *T. fusca* NTU22 was successfully constitutively expressed in *Y. lipolytica.* The maximum enzyme activity (630 U/mL) in the culture filtrate of a *Y. lipolytica* transformant (pYLSC1-*bgl*) was approximately 240 times higher than that in the cell-free extracts of *E. coli* transformants.

The pH- and temperature-dependent properties of the β-glucosidase (BglC) of *T. fusca* YX purified from the *E. coli* transformants were similar to those of the β-glucosidase (BGL) of *T. fusca* NTU22 purified from the *Y. lipolytica* transformant [12]. The optimum temperature of the β-glucosidases from *Lactobacillus acidophilus*, *Lactobacillus casein*, and *Aspergillus oryzae* were 37 °C, 35 °C, and 50 °C, respectively [18–20]. The recombinant β-glucosidase (BglC) from thermophilic actinomycetes had excellent thermo-property.

Metal ions inhibited the highly glucose-tolerant β-glucosidase from *A. oryzae*. Significant inactivation effects were observed with Hg^{2+}, Cu^{2+}, Ag^{+}, Zn^{2+}, and Fe^{3+} [20]. The β-glucosidase (GmlCHG) from the roots of soybean (*Glycine max*) seedlings was only inhibited by $Hg2^{+}$ [21]. However, the Mn^{2+} increased the β-glucosidase activity (67.4%) of the *L. acidophilus* [18]. The activity of the β-glucosidase (BglC) from *T. fusca* YX was not modulated by Ca^{2+}, Mg^{2}+, or EDTA [12]. However, the effects of other chemical reagents or metal ions were not investigated. We tested their effects and found that Hg^{2+}, Cu^{2+}, Ba^{2+}, and Ag^{+} ions significantly inhibited enzyme activity. These two enzymes exhibited very similar metal ion inhibition effects.

β-Glucosidases are an extensive group of enzymes that hydrolyze a vast variety of glycosides, including aryl and alkyl-β-D-glycosides [22]. They are produced intracellularly by friendly

microorganisms and demonstrate broad substrate specificity [23], and their physiological functions vary greatly depending on their source and substrate specificity [22]. Three aglycons (daidzein, genistein, and glycitein) and glucoside, malonyl, and acetyl derivatives of isoflavones exist in soybeans and soybean foods [24]. Daidzin (7-glucosides of daidzein) and genistin (7-glucosides of genistein) are their main isoflavones. The β-glucosidase from *Aspergillus niveus* had better substrate specificity for genistin. However, the β-glucosidase synthesized by *Aspergillus awamori* and *Aspergillus niger* may hydrolyze β-glycosidic linkages of polyphenol glycosides other than genistin [7]. *Bifidobacterium animalis*, *L. acidophilus*, and *L. casei* have a greater ability to biotransform genistin into genistein than daidzin into daidzein [25].

The deglycosylation of flavonoids by human cytosolic β-glucosidase is an important first step in their uptake and metabolism. The aglycones forms of flavonoids are likely to be more important biologically than the glycoside forms [26]. However, deglycosylation rates depend on the structure of the flavonoids and the position of the sugar substitutions [27]. This study showed that the β-glucosidase from the *Y. lipolytica* transformant (pYLSC1-*bgl*) had a high substrate selectivity towards the 7-glucosides of genistein and the 7-glucosides of daidzein. These properties are important for the soybean isoflavone applications of this β-glucosidase.

4. Materials and Methods

4.1. Microorganisms and Vectors

Thermobifida fusca NTU22 was the source of chromosomal DNA in this study [11]. *Yarrowia lipolytica* P01g and pYLSC1 were purchased from Yeastern Biotech Co., Ltd. (Taipei, Taiwan). *E. coli* TOP10 F′ was obtained from Invitrogen Co., Ltd. (San Diego, CA, USA).

4.2. Construction of the β-Glucosidase Expression Plasmid

The β-glucosidase gene was amplified from the chromosomal DNA of *T. fusca* NTU22 by PCR using the primers 5′-T TTG GCC GTT CTG GCC ATG GTG ACC TCG CAA TCG ACG AC-3′ (*Sfi*I site is underlined) and 5′-TTT CTA GAG TCA TTC CTG TCC GAA GAT TCC ACC GTT GCG CA-3′ (*Xba*I site is underlined) according to the gene sequence of *T. fusca* YX (NCBI accession number Q47RE2). The PCR-amplified fragment was digested with *Sfi*I and *Xba*I, and then ligated with *Sfi*I-*Xba*I-treated pYLSC1 to generate the expression plasmid pYLSC1-*bgl* (Figure 6) for the production of protein in *Y. lipolytica* P01g.

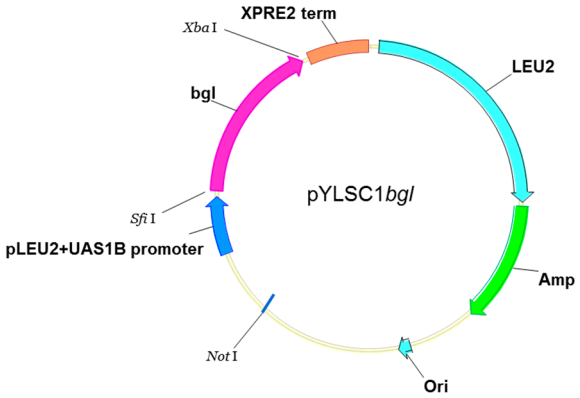

Figure 6. Plasmid map of pYLC1-*bgl*.

4.3. Transformation and Screening of Y. lipolytica Transformant

The pYLSC1-*bgl* plasmid was linearized with *Not*I and then transformed into *Y. lipolytica* P01g [17]. The transformants were selected at 28 °C on YND agar plates (6.7 g/L yeast nitrogen base without

amino acids, 20 g/L glucose, 15 g/L agar, pH 4.0) for 2–4 days. The transformants were cultured in YPD medium (Y1375, Sigma-Aldrich, St. Louis, MO, USA) in Hinton flasks and shaken (200 rpm) at 28 °C. Transformants with good β-glucosidase activity were selected for further analysis; integration of the construct into the *Y. lipolytica* P01g genome was verified by genomic PCR.

4.4. β-Glucosidase Activity Assay

β-Glucosidase activity was measured with *p*-nitrophenyl-β-glucopyranoside [28]. One unit of β-glucosidase activity is defined as the amount of the enzyme releasing 1 mmol of *p*-nitrophenol per min at 25 °C.

4.5. Expression of β-Glucosidase in Hinton Flask

The high-β-glucosidase-activity transformant was incubated in 50 mL of YPD medium in 500-mL Hinton flasks. Cultivation was performed on a reciprocal shaker at 200 rpm and 28 °C for 24 h. After several days of culturing, the culture broth was centrifuged at 10,000× *g* and 4 °C for 30 min, and the culture filtrate was collected as a crude enzyme solution.

4.6. Enzyme Purification

The purification procedures were processed in phosphate buffer (20 mM, pH 6.0) at 4 °C unless otherwise stated. The culture filtrate was concentrated by Pellicon ultrafiltration (Pellicon XL, Biomax 10 K, Merck KGaA, Billerica, MA, USA). The concentrated solution was applied to a DEAE-Sepharose™ FF column (1.13 × 8 cm) preequilibrated with phosphate buffer (pH 7.0). After the column was washed with phosphate buffer (100 mM, pH 7.0), the enzyme was eluted with a linear gradient from 0.0 to 1.0 M NaCl (flow rate: 60 mL/h) in buffer. The enzyme activity was found within the range of 0.3–0.4 M NaCl. All manipulations followed the manufacturer's instructions.

4.7. Hydrolysis of Flavonoid

Each flavonoid was mixed with β-glucosidase to result in a final flavonoid concentration of 100 μM. Samples were incubated at 50 °C for up to 120 min, and control samples of heat-inactivated (100 °C, 30 min) enzyme solution were run in parallel. The sample was extracted with 80% methanol and then analyzed by HPLC.

4.8. Detection of Flavonoid with HPLC

Flavonoids were analyzed by Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) using a prepacked 4.6 mm × 150 mm (5-μm) Zorbax XDB-C18 column (Agilent Technologies Industries Co., Ltd., Santa Clara, CA, USA) and an ultraviolet (UV) detector set at 262 nm. Mobile phase solvents A (0.1% acetic acid, pH 3.4) and B (80% acetonitrile) were run at a flow rate of 1.6 mL/min using a gradient from 15% to 70% B (25 min).

4.9. Statistical Analysis

All measurements were performed at least three times. The data are expressed as the mean ± SD. The mean values were compared to the appropriate control using Dunnett's test. *p*-values less than 0.05 indicated statistically significant differences.

5. Conclusions

The β-glucosidase gene (*bgl*) of *T. fusca* NTU 22 was successfully expressed in a *Y. lipolytica* expression system. The maximum enzyme activity of the culture filtrate of a *Y. lipolytica* transformant (pYLSC1-*bgl*) was approximately 240 times higher than that of the cell-free extract of the *E. coli* transformants. The enzyme was purified 9.2-fold from crude culture filtrate by DEAE-Sepharose

FF column chromatography. The β-glucosidase from *T. fusca* exhibited the most activity against daidzein-7-glucoside and genistein-7-glucoside of the flavonoid glycosides that we tested.

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Author Contributions: Chao-Hsun Yang, Wen-Hsiung Liu, and Yu-Chun Huang designed the study. Wei-Lin Chen and Chao-Hsun Yang wrote the paper. Wei-Lin Chen, Yo-Ming Yang, Gui-wen Guo, and Cheng-Yu Chen performed the enzymatic and gene experiments. Chao-Hsun Yang, Yu-Chun Huang, Keh-Feng Huang, and Wei-Lin Chen supervised the study. All authors made substantial contributions to the discussion of data and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hsieh, M.C.; Graham, T.L. Partial purification and characterization of a soybean β-glucosidase with high specific activity towards isoflavone conjugates. *Phytochemistry* **2001**, *58*, 995–1005. [CrossRef]
2. Pei, X.; Yi, Z.; Tang, C.; Wu, Z. Three amino acid changes contribute markedly to the thermostability of β-glucosidase BglC from *Thermobifida fusca*. *Bioresour. Technol.* **2011**, *102*, 3337–3342. [CrossRef] [PubMed]
3. Kumar, A.; Gautam, A.; Dutt, D. Co-Cultivation of *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 as a Cost-Effective Method to Produce Cellulases for the Hydrolysis of Pearl Millet Stover. *Fermentation* **2016**, *2*, 12. [CrossRef]
4. Brethauer, S.; Wyman, C.E. Review: Continuous hydrolysis and fermentation for cellulosic ethanol production. *Bioresour. Technol.* **2010**, *101*, 4862–4874. [CrossRef] [PubMed]
5. Wang, L.; Wu, Y.; Liu, Y.; Wu, Z. Complex Enzyme-Assisted Extraction Releases Antioxidative Phenolic Compositions from Guava Leaves. *Molecules* **2017**, *22*, 1648. [CrossRef] [PubMed]
6. Mai, Z.; Su, H.; Zhang, S. Characterization of a Metagenome-Derived β-Glucosidase and Its Application in Conversion of Polydatin to Resveratrol. *Catalysts* **2016**, *6*, 35. [CrossRef]
7. Georgetti, S.R.; Vicentini, F.T.M.C.; Yokoyama, C.Y.; Borin, M.F.; Spadaro, A.C.C.; Fonseca, M.J.V. Enhanced in vitro and in vivo antioxidant activity and mobilization of free phenolic compounds of soybean flour fermented with different β-glucosidase-producing fungi. *J. Appl. Microbiol.* **2009**, *106*, 459–466. [CrossRef] [PubMed]
8. McCue, P.; Horii, A.; Shetty, K. Mobilization of phenolic antioxidants from defatted soybean powders by *Lentinus edodes* during solid-state bioprocessing is associated with enhanced production of laccase. *Innov. Food Sci. Emerg. Technol.* **2004**, *5*, 385–392. [CrossRef]
9. Chien, H.L.; Huang, H.Y.; Chou, C.C. Transformation of isoflavone phytoestrogens during the fermentation of soymilk with lactic acid bacteria and bifidobacteria. *Food Microbiol.* **2006**, *23*, 772–778. [CrossRef] [PubMed]
10. Lin, C.H.; Wei, Y.T.; Chou, C.C. Enhanced antioxidative activity of soybean koji prepared with various filamentous fungi. *Food Microbiol.* **2006**, *23*, 628–633. [CrossRef] [PubMed]
11. Liu, W.H.; Yang, C.H. The isolation and identification of a lignocellulolytic and thermophilic actinomycete. *Food Sci. Agric. Chem.* **2002**, *4*, 89–94.
12. Spiridonov, N.A.; Wilson, D.B. Cloning and biochemical characterization of BglC, a β-glucosidase from the cellulolytic actinomycete *Thermobifida fusca*. *Curr. Microbiol.* **2001**, *42*, 295–301. [CrossRef] [PubMed]
13. Zamost, B.L.; Nielsen, H.K.; Starnes, R.L. Thermostable enzymes for industrial application. *J. Ind. Microbiol.* **1991**, *8*, 71–82. [CrossRef]
14. Madzak, C.; Treton, B.; Blanchin-Roland, S. Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. *J. Mol. Microbiol. Biotechnol.* **2000**, *2*, 207–216. [PubMed]
15. Madzak, C.; Gaillardin, C.; Beckerich, J.M. Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*. *J. Biotechnol.* **2004**, *109*, 63–81. [CrossRef] [PubMed]
16. Yang, C.H.; Huang, Y.C.; Chen, C.Y.; Wen, C.Y. Heterologous expression of *Thermobifida fusca* thermostable alpha-amylase in *Yarrowia lipolytica* and its application in boiling stable resistant sago starch preparation. *J. Ind. Microbiol. Biotechnol.* **2010**, *37*, 953–960. [CrossRef] [PubMed]

17. Huang, Y.C.; Chen, Y.F.; Chen, C.Y.; Chen, W.L.; Ciou, Y.P.; Liu, W.H.; Yang, C.H. Production of ferulic acid from lignocellulolytic agricultural biomass by *Thermobifida fusca* thermostable esterase produced in *Yarrowia lipolytica* transformant. *Bioresour. Technol.* **2011**, *102*, 8117–8122. [CrossRef] [PubMed]
18. Coulon, S.; Chemardin, P.; Gueguen, Y.; Arnaud, A.; Galzy, P. Purification and characterization of an intracellular β-glucosidase from *Lactobacillus casein* ATCC 393. *Appl. Biochem. Biotechnol.* **1998**, *74*, 105–114. [CrossRef]
19. Li, K.B.; Chan, K.Y. Production and properties of β-Glucosidase from *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* **1983**, *46*, 1380–1387. [PubMed]
20. Riou, C.; Salmon, J.; Vallier, M.; Gunata, Z.; Barre, P. Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae*. *Appl. Environ. Microbiol.* **1998**, *64*, 3607–3614. [PubMed]
21. Suzuki, H.; Takahashi, S.; Watanabe, R.; Fukushima, Y.; Fujita, N.; Noguchi, A.; Yokoyama, R.; Nishitani, K.; Nishino, T.; Nakayama, T. An isoflavone conjugate-hydrolyzing β-glucosidase from the roots of soybean (*Glycine max*) seedlings: Purification, gene cloning, phylogenetics, and cellular localization. *J. Biol. Chem.* **2006**, *281*, 30251–30259. [CrossRef] [PubMed]
22. Berrin, J.G.; Czjzek, M.; Kroon, P.A.; Mclauchlan, W.R.; Puigserver, A.; Williamson, G.; Juge, N. Substrate (aglycone) specificity of human cytosolic β-glucosidase. *Biochem. J.* **2003**, *373*, 41–48. [CrossRef] [PubMed]
23. Wolosowska, S.; Synowiecki, J. Thermostable β-glucosidase with a broad substrate specifity suitable for processing of lactose-containing products. *Food Chem.* **2004**, *85*, 181–187. [CrossRef]
24. Wang, H.; Murphy, P.A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **2009**, *42*, 1666–1673. [CrossRef]
25. Otieno, D.O.; Ashton, J.F.; Shah, N.P. Evaluation of enzymic for biotransformation of isoflavone phytoestrogen in soymilk by *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus casei*. *Food Res. Int.* **2006**, *39*, 394–407. [CrossRef]
26. Williamson, G.; Plumb, G.W.; Uda, Y.; Price, K.R.; Rhodes, M.J.C. Dietary quercetin glycosides: Antioxidant activity and induction of the anticarcinogenic phase II marker enzyme quinone reductase in Hepalclc7 cells. *Carcinogenesis* **1996**, *17*, 2385–2387. [CrossRef] [PubMed]
27. Day, A.J.; DuPont, M.S.; Ridley, S.; Rhodes, M.; Rhodes, M.J.C.; Morgan, M.R.A.; Williamson, G. Deglycosylation of favonoid and isofavonoid glycosides by human small intestine and liver β-glucosidase activity. *FEBS Lett.* **1998**, *436*, 71–75. [CrossRef]
28. Schmidt, S.; Rainieri, S.; Witte, S.; Matern, U.; Martens, S. Identification of a *Saccharomyces cerevisiae* glucosidase that hydrolyzes flavonoid glucosides. *Appl. Environ. Microbiol.* **2011**, *77*, 1751–1757. [CrossRef] [PubMed]