Optimization of β-Glucuronidase Activity from *Lactobacillus delbrueckii* Rh2 and Its Use for Biotransformation of Baicalin and Wogonoside

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Scutellariae radix, the root of *Scutellaria baicalensis*, is a traditional medicinal herb containing baicalin and wogonoside as the main flavonoids. Before absorption, these flavonoids are metabolized into their corresponding aglycones, baicalein and wogonin, by β-glucuronidase produced by the intestinal microflora. To develop a method for efficient transformation of these flavonoids, optimal conditions for the production of β-glucuronidase from *Lactobacillus delbrueckii* Rh2 (Rh2) were determined. Addition of 4% galactose to basal medium increased the enzyme activity of Rh2 by about 8-fold. Optimal pH and temperature for the β-glucuronidase activity of cell extract were 5.0 and 50°C, respectively, and more than 95% of the enzyme activity was maintained at 60°C for 11 h. Under optimal conditions, more than 90% of the glycones were converted into their corresponding aglycones within 3 h. These results demonstrate that the transformation of baicalin and wogonoside from *S. baicalensis* by Rh2 would be useful for the efficient production of wogonin and baicalein.

Key words: baicalin, β-glucuronidase, *Lactobacillus*, scutellariae radix, wogonoside

Scutellariae radix, the root of *Scutellaria baicalensis*, is recognized in China, Korea, and Japan as a medicinal herb for the treatment of inflammation, fever, cough, dysentery, jaundice, hepatitis, tumor, and diarrhea [Chang and But, 1987; Ishimaru *et al.*, 1995]. Its main active compounds are flavonoid glycosides, such as baicalin and wogonoside (Fig. 1). These flavonoids have been reported to possess anti-inflammatory [Wakabayashi, 1999; Li *et al.*, 2000], antiviral [Kitamura *et al.*, 1998; Huang *et al.*, 2000; Wu *et al.*, 2001], antioxidant [Kimuya *et al.*, 1981; Gao *et al.*, 1999; Shieh *et al.*, 2000], and anticancer properties [Matsuzaki *et al.*, 1996; Ikemoto *et al.*, 2000; Chang *et al.*, 2002]. Orally administered flavonoid glycosides are hydrolyzed by β-glucuronidase of the intestinal microflora before absorption, and only the aglycones are detected in the plasma [Drasar and Hill, 1974; Nishioka *et al.*, 1992]. The importance of intestinal microbiota for the degradation of dietary flavonoids has been reported in a growing number of scientific papers [Drasar and Hill, 1974; Scalbert and Williamson, 2000] and reviewed in detail by Lampe and Chang [2007]. However, the intestinal flora and their enzyme patterns differ between each individual, which leads to the variation in the conversion and bio-availability of various flavonoid glycosides *in vivo*. Therefore, to improve the bioavailability of baicalin and wogonin glucuronide, transformation into their aglycone counterparts before oral administration is desirable. Consequently, β-glucuronidase gene from *Lactobacillus brevis* was cloned, over-expressed in *E. coli*, and used for the biotransformation of baicalin and wogonoside [Kim *et al.*, 2009]. However, genetically modified organism (GMO) and safety issues became the stumbling stone in using *E. coli* as expression host. For these reasons, the screening and optimization of enzyme production for food grade microorganisms are still pursued.

In the present study, 66 media with various carbon sources and concentrations were assessed for the optimal

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production of β-glucuronidase from Rh2. In addition, the effects of temperature and pH on the β-glucuronidase activity of the cell extracts were analyzed. Finally, the optimal conditions for the production of β-glucuronidase and enzyme reaction were applied to biotransform baicalin and wogonoside in scutellariae radix into their corresponding aglycosides.

Materials and Methods

Materials. Wogonin, baicalein, baicalin, and ρ-nitrophenyl-β-D-glucuronide were purchased from Sigma (St. Louis, MO). Chiroinositol and pinitol were generously donated by Amicogen Co. (Jinju, Korea), and other carbon sources were purchased from Sigma. Lactobacilli MRS broth, BHI medium, yeast extract, proteose peptone, and beef extract were purchased from BD (Sparks, MD). High-performance liquid chromatography (HPLC)-grade acetonitrile and water were obtained from J.T. Baker (Phillipsburg, NJ). Scutellariae radix was purchased from Kyoung-Dong Market in Seoul, Korea.

Isolation of wogonoside from Scutellariae radix. For the purification of wogonoside, the dried roots of Scutellariae radix were crushed and extracted with 20 times volume of methanol at room temperature for 24 h. After filtering, the residue was extracted twice more in the same manner. Methanol was removed from filtrate by evaporation (Eyela rotary evaporator N-100, Rikakikai Co. Ltd., Tokyo, Japan), and distilled water was added to collect the extracted samples, which were then freeze-dried. Subsequently, 50 mg freeze-dried extract was dissolved in 1 mL of distilled water and filtered through a 0.45-mm filter (Millipore Co. Ltd., Billerica, MA) before being loaded on a prep-HPLC column. Prep-HPLC was conducted twice using a reverse-phase column (21.2 mm Ø×250 mm; HIQ sil C18 HS-10, Kya Tech, kyoto, Japan), a fraction collector (FC203B, Gilson Inc., Middleton, WI), an HPLC pump (model ACME 9000, Younglin Instrument Co., Anyang, Korea), a UV detector (UV VIS detector, Younglin Instrument), and Autochro 3000 software (Gilson). Elution was performed at a rate of 20 mL/min for 70 min with a linear gradient of acetonitrile and water with the following temporal profile: 0~45 min, from 10% acetonitrile and 90% water to 65% acetonitrile and 35% water; 45~70 min, from 65% acetonitrile and 35% water to 10% acetonitrile and 90% water. The purity of the extracted wogonoside was estimated by HPLC (1P 608 HPLC pump Bio Nex, Anyang, Korea) fitted with a reverse-phase column (4.6 mm Ø×250 mm; Alltima HP C18 HL 5U, Alltech, Milano, Italy). The mixture of solvent A (acetonitrile) and solvent B [water containing 0.125% (v/v) formic acid] flowed at a rate of 1 mL/min for 70 min with a linear gradient profile (0-45 min, linear from 10% to 65% A; 45-70 min, linear from 65% to 10% A) and was detected by UV at 254 nm.

Growth conditions and enzyme assay. To assess the effect of various carbon sources on the growth and several glycosidase activities, 66 media were designed (Table 1). Various concentrations of carbon sources, including several carbohydrates, sugar alcohol, and phytic acid were added to four types of basal medium (NB, NB +2% dextrose, m-MRS, and Lactobacilli MRS broth (BD)). For the extensive comparison of bacterial growth, commercial media such as BHI and MRS (BD) were used as controls. Each medium was inoculated with 1% (v/v) of overnight culture of Rh2 and grown anaerobically at 37°C. Bacterial growth was optically measured with a spectrophotometer at 600 nm. After 18 h cultivation, 8 mL cells were harvested, washed, resuspended in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), and then mixed with 200 L of lysis solution (acetone:toluene=9:1, v/v). After vigorous mixing, 80 μL of the aqueous layer was obtained as the cell extract, which was mixed with 20 μL of 5 mM ρ-nitrophenyl-β-D-glucuronide, and incubated at 37°C for 30 min. The reaction was stopped by adding 100 μL of 0.5 M Na_2CO_3. The released pNP was measured at 405 nm in a microplate reader (Model Benchmark, Bio-Rad, Tokyo, Japan). One enzyme unit was defined as the quantity of enzyme releasing 1 μmol of pNP per min under the described conditions.

Determination of optimal pH, temperature and thermal stability of β-glucuronidase. To determine the optimal pH for β-glucuronidase activity, Rh2 was grown anaerobically in m-MRS containing 4% galactose at 37°C for 18 h. One milliliter of the cells were harvested and washed twice in PBS. The pellet was resuspended in 8 mL of 100 mM of acetate buffer at pH 4 and 5, or 8 mL of 100 mM phosphate buffer at pH 6, 7, and 8 at 37°C. β-Glucuronidase activity was measured as described above. For the determination of the optimal temperature for β-glucuronidase, 400 μL cell extract in 100 mM acetate buffer (pH 5.0) was incubated at various temperatures (25-80°C) with 100 μL of 5 mM ρ-nitrophenyl-β-D-glucuronide for 30 min, and the activity was measured as described above. For the assessment of the thermal stability of β-glucuronidase, the whole cells and the cell extracts in 100 mM acetate buffer (pH 5.0) were incubated in water bath adjusted to 20, 50, 60, or 70°C for 11 h. During the incubation period, 80 μL of the samples were collected at each time point (0, 1, 3, 5, 7, 9, and 11 h), kept on ice for 5 min, and warmed to 40°C for 5 min. Subsequently, 20 μL of 5 mM ρ-nitrophenyl-β-D-glucuronide was added and incubated at 37°C for 30 min to assess the

Table 1. Sixty-six different types of microbial medium tested

Basal medium	Medium numbers and added substrates					
NB[1)]	1	4% L-Sorbose	9	4% Fructose	17	4% Methyl-α-D-glucopyranoside
	2	4% L-Arabinose	10	4% Raffiose	18	4% α-methyl-D-mannoside
	3	4% Lactose	11	4% Rhamnose	19	4% α-L(-) fucose (6-deoxy-L-galactopyranose)
	4	4% Mannose	12	4% Ribose	20	4% Sucralose
	5	4% Melezitose	13	4% Galactose	21	4% Myoinositol
	6	4% Glucose	14	4% Sucrose	22	4% Pinitol
	7	4% Cellobiose	15	4% Maltose	23	4% Chiroinositol
	8	4% Xylose	16	4% Lactulose	24	4% Mannitol
NB+ 2% Dextrose	25	2% Melezitose	29	1% Myoinositol	33	2% Myoinositol
	26	2% Sucrose	30	1% Pinitol	34	2% Pinitol
	27	2% Rhamnose	31	1% Phytic acid	35	2% Phytic acid
	28	1% Chiroinositol	32	2% Chiroinositol		
m-MRS[2)]	36	2% Glucose	42	2% L-Arabinose	48	4% Myoinositol
	37	2% Maltose	43	2% Galactose	49	4% Pinitol
	38	2% Sucrose	44	2% Mannose	50	4% Mannitol
	39	2% Xylose	45	2% Rhamnose	51	4% Phytic acid
	40	2% Cellobiose	46	2% Raffinose		
	41	2% Lactose	47	4% Chiroinositol		
MRS broth (BD)	52	2% Melezitose	56	1% Chiroinositol	60	2% Chiroinositol
	53	2% Sucrose	57	1% Myoinositol	61	2% Myoinositol
	54	2% Raffinose	58	1% Pinitol	62	2% Pinitol
	55	2% Rhamnose	59	1% Phytic acid	63	2% Phytic acid
Controls	64	BHI (BD)	65	m-MRS	66	MRS (BD)

[1)]NB was composed of 0.5% yeast extract, 1% proteose peptone, 1% ammonium acetate, 0.05% manganese sulfate, 0.001% magnesium sulfate, 0.2% dipotassium phosphate, and 0.1% Tween80.
[2)]m-MRS has the same component as MRS broth (BD) without glucose.

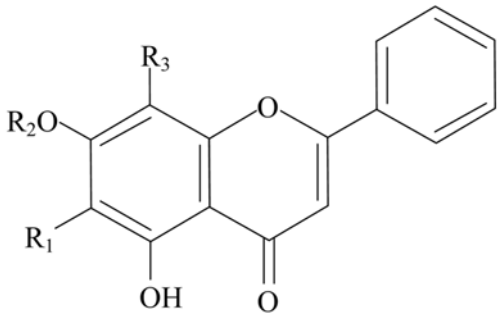
enzyme activity as described above.

Bioconversion of baicalin and wogonoside by cell extract. The cell extracts of Rh2 prepared in 100 mM acetate buffer (pH 5.0) as described above was used to transform baicalin and wogonoside. Two hundred microliters of the cell extracts were mixed with the same volume of 40 mM baicalin or 120 mM wogonoside dissolved in 100 mM acetate buffer (pH 5.0) and incubated at 50°C for 420 min. During incubation, 400 μL each of the samples were collected at each time point and centrifuged for 5 min at 16,000×g. The supernatants were used to analyze baicalin, baicalein, wogonoside, and wogonin by HPLC.

Statistical anaylsis. The results were expressed as the means±SD of three independent experiments. Significant differences between means were determined using Statistical Analysis System software package (SAS, Cary, NC) and a p value of <0.001 was regarded as statistically significant.

Results and Discussion

Growth and β-glucuronidase activity of Rh2 cultured in various media. For the determination of optimal growth medium for Rh2, 66 media with various carbon sources and concentrations were tested (Table 1). Among them, L-sorbose, L-arabinose, xylose, raffinose, sucrose, maltose, rhamnose, sucralose, myo-inositol, chiro-inositol, pinitol, methyl-α-D-glucopyranose, α-methyl-D-mannoside, and α-L(-) fucose (6-deoxy-L-galactopyranose) were ineffective as carbon sources for the growth of Rh2. In addition, Rh2 did not grow in BHI (number 64) or m-MRS (number 65). Among the various experimental carbon sources, glucose showed the best growth (numbers 6, 25-36, 52-63, and 66 in Fig. 2A). Pinitol and glucose exhibited a synergetic effect on the growth of Rh2 (numbers 30, 34, 58, and 62), whereas pinitol as the sole carbon source (numbers 22 and 49) had no effect on



Baicalin : R_1=OH, R_2=β-D-glucuronic acid, R_3=H
Baicalein : R_1=OH, R_2=H, R_3=H
Wogonoside : R_1=H, R_2=β-D-glucuronic acid, R_3=OCH_3
Wogonin : R_1=H, R_2=H, R_3=OCH_3

Fig. 1. Chemical structures of baicalin, baicalein, wogonoside, and wogonin.

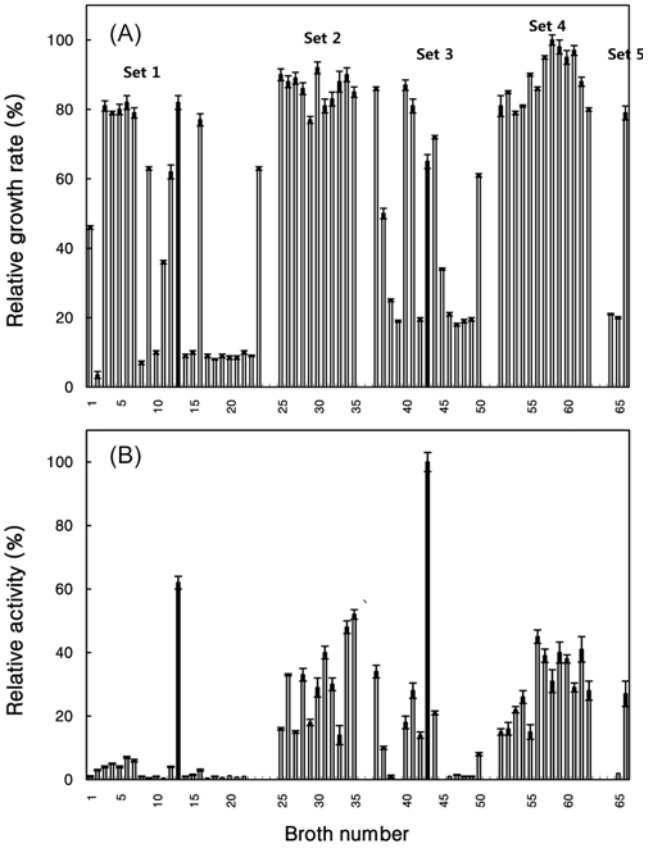


Fig. 2. Relative growth (A) and β-glucuronidase activity (B) of Rh2 grown in various culture media. Growth and β-glucuronidase activity are presented relative to those of cells grown in media numbers 58 and 43, respectively. Data are the averages of three assays. Error bars represent standard deviation.

bacterial growth. When the β-glucuronidase activity of each cell extract was measured, Rh2 grown in medium 13 (BM with 4% galactose; BM-Gal) and 43 (m-MRS with 2% galactose; m-MRS-Gal) containing galactose in common showed notably increased enzyme activity (Fig. 2B). The enzyme activity of cell extract from Rh2 cultured in m-MRS-Gal was approximately 4-fold higher than that in MRS (BD). It is well known that the production of the enzymes involved in carbohydrate hydrolysis and utilization can be repressed by the presence of glucose [Degnan and Macfarlane, 1994; 1995; Trindade *et al.*, 2003; Kullin *et al.*, 2006]. However, there has been no peer-reviewed paper concerning the enhanced production of β-glucuronidase using galactose. More detailed characterization of the relationships between β-glucuronidase induction and galactose at the genetic level should be assessed.

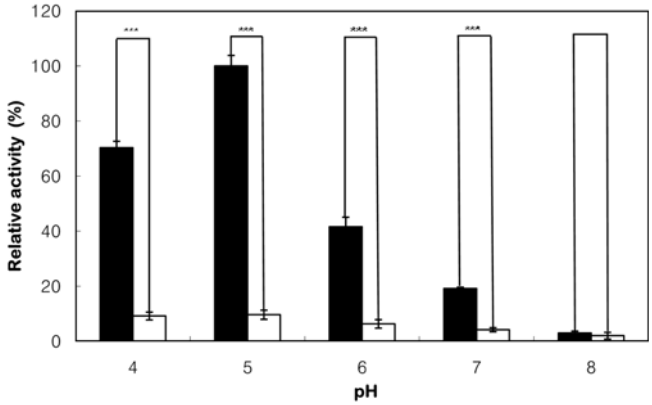


Fig. 3. Relative β-glucuronidase activity of crude microbial enzyme extracts at various pH values at 37°C. (■: m-MRS-Gal, □: MRS) Data are the averages of three assays, and error bars represent standard deviation. Bars with *** are significantly different between groups at $p<0.001$.

Physicochemical properties of β-glucuronidase (Optimal pH, temperature, and thermal stability). To determine the optimal pH, β-glucuronidase activity of cell extract was examined in buffers with pH values ranging from 4 to 8. The maximal β-glucuronidase activity of cell extract of Rh2 cultured in MRS and m-MRS-Gal were both observed at pH 5.0 (Fig. 3). On the other hand, the optimal pH for β-glucuronidase activity in *Lb. gasseri* ADH showed optimum activity at pH 6.0 at 65°C [Russell and Klaenhammer, 2001]. In general, *Lactobacillus* enzymes are more active at acidic pH values, because its native environment is generally acidic. Krahulec and Krahulcova [2007] reported that purified β-glucuronidase from *Streptococcus equi* subsp. *zooepidemicus* showed the optimal activity at 52°C and pH 5.6. When assayed at temperatures ranging from 25 to 80°C, the highest β-glucuronidase activity was observed at 50°C for Rh2 cultured in both MRS and m-MRS-Gal, although β-glucuronidase activity in Rh2 cultured in m-MRS-Gal was approximately 10-fold higher than that of Rh2 cultured in MRS (Fig. 4). The thermal stability of β-glucuronidase activity of cell extract Rh2 cultured in m-

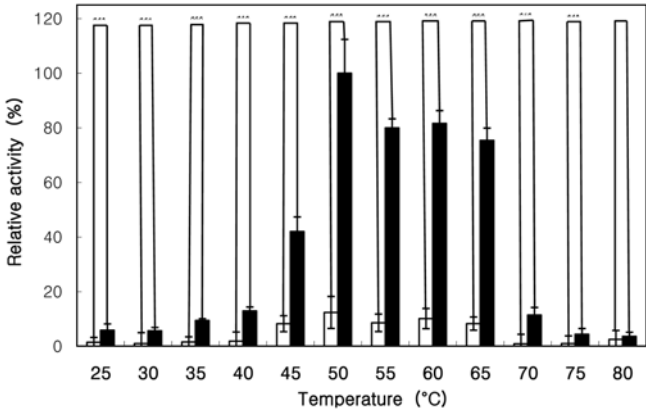


Fig. 4. Relative β-glucuronidase activity of cell extracts from m-MRS-Gal (■) and MRS (□) at various temperatures at pH 5.0. Data are the averages of three assays and error bars represent standard deviation. Bars with *** are significantly different between groups at $p<0.001$.

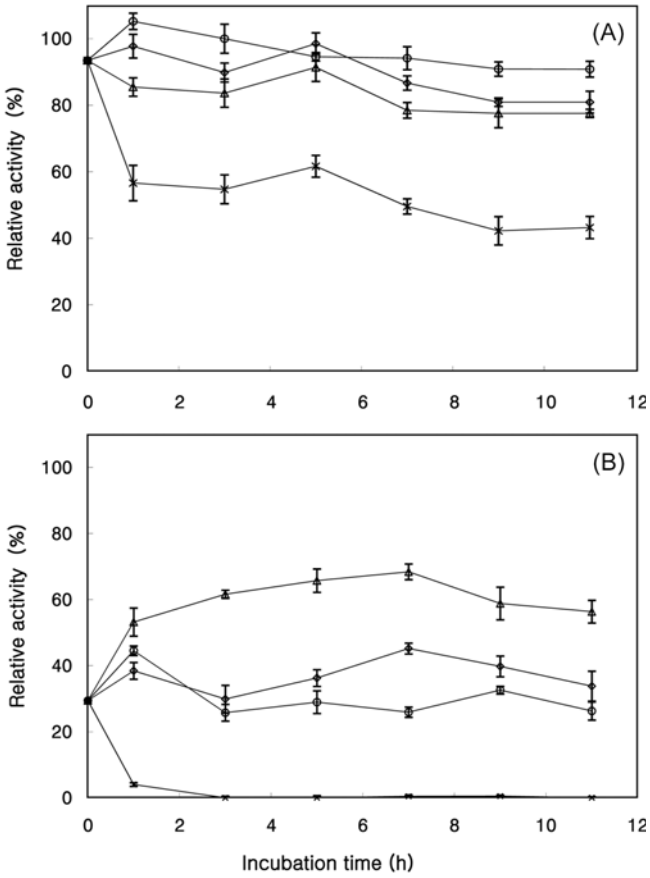


Fig. 5. Heat stability of β-glucuronidase in cell extract (A) and whole cell (B) suspensions. The remaining activities at each time point and temperature [20°C (○), 50°C (◇), 60°C (△), 70°C (×)] are presented with SD.

MRS-Gal was examined in disrupted and undisrupted cell suspensions at temperatures ranging from 20 to 70°C. More than 81 and 78% of the original activity remained after 11 h of incubation at 50 and 60°C, respectively (Fig. 5A). The β-glucuronidase activity of the undisrupted cell suspension increased up to 7 h at 50 and 60°C, and then gradually decreased at later time points (Fig. 5B). Production cost and time are very important factors in the industrial process. In the present study, undisrupted cells showed efficient conversion of wogonoside and baicalin (data not shown).

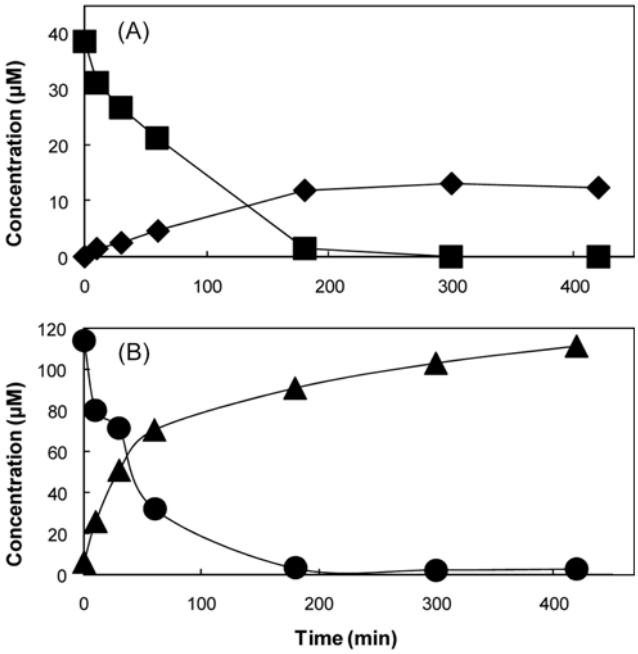


Fig. 6. Concentrations of baicalin, baicalein, wogonin and wogonoside during the bioconversion reaction in cell extract containing β-glucuronidase from Rh2 grown in m-MRS-Gal. A: baicalin (■), baicalein (◆); B: wogonoside (●), wogonin (▲).

Assay for the conversion of baicalin and wogonoside. At the optimal condition (pH 5, 50°C), more than 90% of the wogonoside and baicalin were transformed into wogonin and baicalein by the cell extracts of Rh2 grown in m-MRS-Gal within 3 h (Fig. 6). To the best of our knowledge, this is the first report on the bioconversion of wogonoside and baicalin using food-grade bacterial β-glucuronidase induced by galactose in the culture medium. To develop a food material with enhanced bioavailability of β-glucuronide compounds, the biotransformation of β-glucuronides and enhanced production of the β-glucuronidase from food-grade microorganisms need be pursued. However, only a few studies have been conducted on the optimization of culture condition for production of the β-glucuronidase from fungi [Chouiter *et al.,* 2008] and *E. coli* [Cenci *et al*., 1998; Caldini *et al*., 1999] which cannot be directly applied to food processing. In the present study, the production of β-glucuronidase from Rh2 was enhanced by using galactose as carbon source, and the optimal conditions for

pH and temperature have been established. These results will be applied in the efficient production of food materials containing wogonin and baicalein.

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