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Deglycosylation of Isoflavones in Isoflavone-Rich Soy Germ Flour by *Aspergillus oryzae* KACC 40247

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ABSTRACT: *Aspergillus oryzae* KACC 40247 was selected as an efficient daidzein-producing fungus from strains of the genus *Aspergillus* by using 5% (w/v) soy germ flour (SGF) as an isoflavone-glycoside-rich medium. The culture conditions, including SGF concentration, agitation speed, initial pH, temperature, and time, were optimized as follows: 7% (w/v) SGF, initial pH 6.0, 33 °C, 300 rpm, and 24 h in a 100 mL baffled flask. The determined amount of isoflavone aglycons in SGF using 50% ethyl acetate was the highest among the solvent systems tested and it was 3.7-fold higher than that using 70% methanol. Under the optimized conditions, the content and concentration of daidzein were 134 mg/g of SGF and 9.4 g/L, respectively, with a productivity of 391 ± 2.8 mg/L/h, and those of isoflavone aglycons were 165 mg/g of SGF and 11.5 g/L, respectively, with a productivity of 479 mg/L/h. Optimization of culture conditions increased the content, concentration, and productivity of isoflavone aglycons by 3.1-, 3.0-, and 3.7-fold, respectively. To our knowledge, this is the highest production of isoflavone aglycons reported to date.

KEYWORDS: *isoflavone hydrolysis, Aspergillus oryzae KACC 40247, soybean germ flour, isoflavone aglycon*

INTRODUCTION

Soybean is an isoflavone-rich grain. Isoflavones, which act as phytoestrogens, are naturally occurring beneficial secondary metabolites. The 12 forms of isoflavones are classified into two groups: the glycosides group, including daidzin, genistin, glycitin, 6″-*O*-acetyldaidzin, 6″-*O*-acetylgenistin, 6″-*O*-acetylglycitin, 6″-*O*-malonyldaidzin, 6″-*O*-malonylgenistin, and 6″-*O*-malonylglycitin, and the aglycons group, including daidzein, genistein, and glycitein. Isoflavones have valuable biological and pharmaceutical properties, including osteoporosis[1] and cardiovascular diseases prevention;[2] menopausal symptom reduction;[3] and anticarcinogenic,[4] antioxidant,[5] anti-inflammatory,[6] and antimicrobial activities.[7] Many studies about the biotransformation for increasing the concentration of isoflavone aglycons in soy products have been performed because isoflavone aglycons possess higher pharmaceutical activity than isoflavone glycosides. The higher activity of isoflavone aglycons results from their greater similarity of chemical structure with the hormone estrogen. Isoflavone aglycons are more easily and quickly absorbed into intestine than glycoside forms and function as active compounds.[8,9]

Raw soybeans contain a lower content of isoflavone aglycons (2−3%),[10] whereas processed soy products have a higher percentage of aglycon forms.[11] For example, cooked soybeans and soy-milk powder contain almost 5% aglycon forms.[12] Soy products fermented by lactic acid bacteria[13,14] and fungi,[15,16] such as fermented soy milk, miso, tempeh, natto, and soy sauce, exhibit much higher ratios of isoflavone aglycons to total isoflavones, as high as 20 or 40% because microorganisms in the fermented soy products convert isoflavone glycosides to isoflavone aglycons via their β-glucosidase activities. During the past decade, new soybean products such as soybean flour and soy germ flour (SGF) have been developed for higher contents of isoflavones.[11,17−19] These new products have been obtained by removing fats and carbohydrates from soybeans and soy germ.

Several methods for the transformation of isoflavone glycosides to isoflavone aglycons, including acid hydrolytic, microbial, and enzymatic transformation techniques, have been attempted. Although the acidic hydrolysis method has advantages, including low cost, simple technology, and high hydrolytic percentage,[20] this method produces side reactions such as hydration and hydroxylation, causes high purification cost, and generates environmental pollution. Isoflavone aglycons have been obtained from isoflavone glycosides by hydrolysis reactions of microorganisms, including *Aspergillus awamori*, *Aspergillus oryzae*, *Aspergillus sojae*, *Rhizopus azygosporus*,[15] *Bacillus subtilis*,[21] *Bifidobacterium animalis*,[22] *Enterococcus durans*, *Streptococcus salivarius*, *Weissella confuse*,[23] *Lactobacillus paraplantarum*,[24] *Rhizopus oligosporus*, *Rhizopus oryzae*,[25] and *Streptococcus thermophilus*.[26] The enzymatic hydrolysis of isoflavone glycosides has been achieved using microbial β-glucosidases from *A. oryzae*,[27] *B. subtilis*,[28] *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus casei*,[29] *Dictyoglomus turgidum*,[30] *Escherichia coli*,[31] *Paecilomyces thermophila*,[32] *Pseudomonas* sp.,[33] *Pyrococcus furiosus*,[34] *Sulfolobus solfataricus*,[35] *Thermoanaerobacter ethanolicus*,[36] and *Thermotoga maritima*.[37] Recently, immobilized β-glucosidase systems have been applied to the hydrolysis of isoflavone glycosides of black soymilk in a highly efficient manner.[38,39] The immobilized enzyme systems enhanced the stability of the enzyme and enabled the reuse of the enzyme, indicating that these systems provide an economic method to prepare the aglycon-rich black soymilk.

The production of isoflavone aglycons by microorganisms has been performed using soybean products such as soy meal, soy milk, and black bean with low contents of isoflavone glycosides

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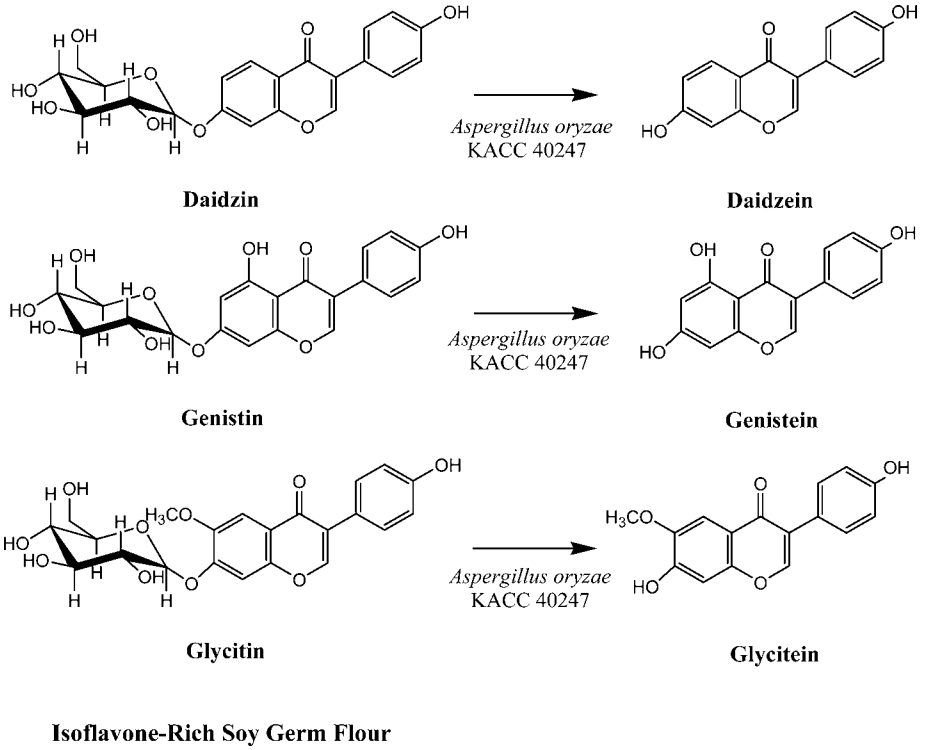

Figure 1. Biotransformation of isoflavone glycosides in SGF as an isoflavone-glycoside-rich medium to isoflavone aglycons by *A. oryzae* KACC 40247.

Table 1. Daidzein Production from Daidzin in 5% SGF by *Aspergillus* Strains[a]

strain	time (h)	daidzein (mg/g)	daidzein (mg/L)	productivity (mg/L/h)
A. ficuum KCTC 6175	24	11.3 ± 0.6	566 ± 0.01	23.6
A. flavus KCTC 16682	24	9.2 ± 0.23	462 ± 0.00	19.2
A. kawachii KCCM 32819	30	11.9 ± 0.56	594 ± 0.01	19.8
A. oryzae KACC 40232	24	5.5 ± 0.03	273 ± 0.00	11.4
A. oryzae KACC 40233	24	1.4 ± 0.02	70 ± 0.00	2.9
A. oryzae KACC 40244	24	8.6 ± 0.16	431 ± 0.01	17.9
A. oryzae **KACC 40247**	**30**	**44.7 ± 1.23**	**2235 ± 0.02**	**74.5**
A. oryzae KACC 40250	30	6.2 ± 0.02	312 ± 0.00	10.4
A. oryzae KACC 41403	30	10.0 ± 0.54	499 ± 0.01	16.6
A. oryzae KACC 41406	30	9.8 ± 0.20	492 ± 0.00	16.4
A. oryzae KACC 44836	24	6.4 ± 0.13	321 ± 0.00	13.4
A. oryzae KACC 44860	48	5.6 ± 0.03	279 ± 0.00	5.8
A. oryzae KACC 44847	24	7.7 ± 0.13	387 ± 0.00	16.1
A. oryzae KACC 44967	48	4.2 ± 0.08	212 ± 0.00	4.4
A. saitoi KCTC 6908	24	21.6 ± 0.82	1079 ± 0.02	44.9

[a]Data are the mean of triplicate experiments, and errors represent the standard deviations ($p < 0.05$).

(less than 4 mg/g).[15,21,22,24,26,40,41] The high concentration of isoflavone glycosides in SGF (2.1 and 1.8 g/L) with high contents of isoflavone glycosides in SGF (11 and 350 mg/g, respective) were used to produce the high concentrations of isoflavone aglycons (1.3 and 0.8 g/L, respective) by β-glucosidases.[42,43] To obtain the higher concentrations of isoflavone aglycons, the use of the higher concentrations of isoflavone glycosides is required.

In this study, *Aspergillus oryzae* KACC 40247 was selected as an efficient daidzein-producing fungus, and SGF concentration, agitation speed, temperature, and pH were optimized to increase the production of isoflavone aglycons. Under the optimized conditions, the high concentrations of isoflavone aglycons were obtained from the high concentrations of isoflavone-glycoside-rich SGF using the strain (Figure 1).

MATERIALS AND METHODS

Materials. SGF, potato dextrose medium, and agar were purchased from Bioland (Cheonan, Korea), MB Cell (Los Angeles, CA), and Daejung (Siheung, Korea), respectively. The isoflavone standards genistin, glycitin, genistein, and glycitein were purchased from Sigma-Aldrich (St. Louis, MO). Daidzin and daidzein were purchased from LC Laboratories (Woburn, MA) and Alfa Aesar (Ward Hill, MA), respectively. High-performance liquid chromatography (HPLC)-grade acetonitrile and acetic acid were purchased from J. T. Baker (Center Valley, PA), and Samchun Chemical (Pyeongtaek, Korea), respectively. Dimethyl sulfoxide (DMSO) and ethyl acetate were purchased from Duksan Pure Chemicals (Ansan, Korea). All other reagents were purchased from Sigma-Aldrich.

Determination of Isoflavones in SGF. Distilled water and 80% (v/v) and 100% (v/v) solutions of acetone, acetonitrile, DMSO, ethanol, ethyl acetate, and methanol were mixed with 5% (w/v) SGF at a

1:1 (v/v) ratio at room temperature for 2 min for determining the amount of isoflavones. As an alternative method, 80% (v/v) methanol was mixed with 5% SGF at a 7:1 (v/v) ratio at 80 °C with shaking at 200 rpm for 3 h.[34] The mixture of each solvent was centrifuged for 30 min at 30 °C at 13 000*g*, and the aqueous supernatant was collected. Each solvent was added to the residual pellets, and the supernatant was collected again using the same procedure. The solvent was removed from the collected supernatant using a centrifugal vacuum concentrator at room temperature. The sample obtained was resuspended in an equal volume of DMSO and then filtered through a 0.45 μm-pore membrane before HPLC analysis.

Microbial Strains and Culture Conditions. *Aspergillus ficuum* KCTC 6134; *Aspergillus flavus* KCTC 16682; *Aspergillus kawachii* KCCM 32819; *Aspergillus oryzae* KACC 40232, 40233, 40244, 40247, 40250, 41403, 41406, 44836, 41860, 44847 and 44967; and *Aspergillus saitoi* KCTC 6908 were screened to select an efficient daidzein-producing fungal strian. Spores of each fungus were incubated under dark conditions at 27 °C for 72 h on plates containing potato dextrose agar. Spores and mycelia were harvested by cutting pieces (10 × 10 mm) of agar from the plate. Ten pieces were inoculated into a 100 mL baffled flask containing 25 mL of 5% SGF, and the flask was incubated at 27 °C and an initial pH of 5.3 with agitation at 200 rpm for 48 h. A sample was withdrawn at 18, 24, 30, 36, and 48 h, and the concentration of daidezein was measured. The culture time was determined to be the time at the highest concentration of daidezein.

Optimization of Culture Conditions for Daidzein Production. Culture conditions were optimized for maximizing daidzein production by varying the SGF concentration from 1 to 10% (w/v) at an initial pH of 5.3, 200 rpm, and 27 °C; the agitation speed was varied from 200 to 350 rpm with 7% SGF at initial pH of 5.3 and 27 °C; the temperature was varied from 24 to 40 °C with 7% SGF at an initial pH of 5.3 and 300 rpm; and the initial pH was varied from 4.5 to 6.5 with 7% SGF at 300 rpm and 33 °C for 42 h. The pH of the medium was adjusted by the addition of 2 N NaOH or 1 N H_2SO_4 solution.[44] Every experiment was performed in triplicate in 100 mL baffled flasks.

HPLC Analysis. Isoflavones were analyzed using an HPLC system (Agilent 1100, Santa Clara, CA) equipped with a UV detector at 254 nm and a hydrosphere C18 reverse-phase column (50 × 4.6 mm, YMC, Kyoto, Japan) with a guard cartridge column (23 × 4.0 mm, YMC, Kyoto, Japan). The column was run with a gradient of solvent A [3% acetic acid/water (v/v)] and solvent B [water/acetonitrile/acetic acid, 50:50:3 (v/v/v)] at ratios ranging from 75:25 to 40:60 for 12 min and then from 40:60 to 75:25 for 3 min.[34] The flow rate was 1.5 mL/min, and the elution temperature was 35 °C. The substrates daidzin, glycitin, and genistin were detected on the basis of retention times of 2.7, 3.0, and 4.5 min, respectively. The products daidzein, glycitein, and genistein were detected on the basis of the retention times of 7.9, 8.8, and 11.3 min, respectively. The isoflavones in SGF were identified on the basis of retention times, which were the same as those of the isoflavone standards. The amounts of isoflavones using 5% SGF were determined using linear calibration curves and correlating the peak areas to the concentrations of isoflavone standards.

Statistical Analysis. The mean and standard error for all experiments, including determination of isoflavones, evaluation of reaction conditions, and HPLC analysis, were calculated from triplicates. One-way analysis of variance (ANOVA) was carried out using Tukey's method with a significance level of $p < 0.05$ using SigmaPlot 10.0 (Systat Software, Chicago, IL).

RESULTS AND DISCUSSION

Selection of an Efficient Daidzein-Producing Fungus from *Aspergillus* Strains Using SGF as a Medium. Isoflavone aglycons have been produced from isoflavone glycosides in soy products by *Aspergillus* strains,[15,16] which originated from fermented soy foods such as meju, soy sauce, and natto. Especially, *A. oryzae* strains were mainly used for screening because the reported activity of *A. oryzae* β-glucosidase was higher than those of *Aspergillus awamori*, *Aspergillus sojae*,

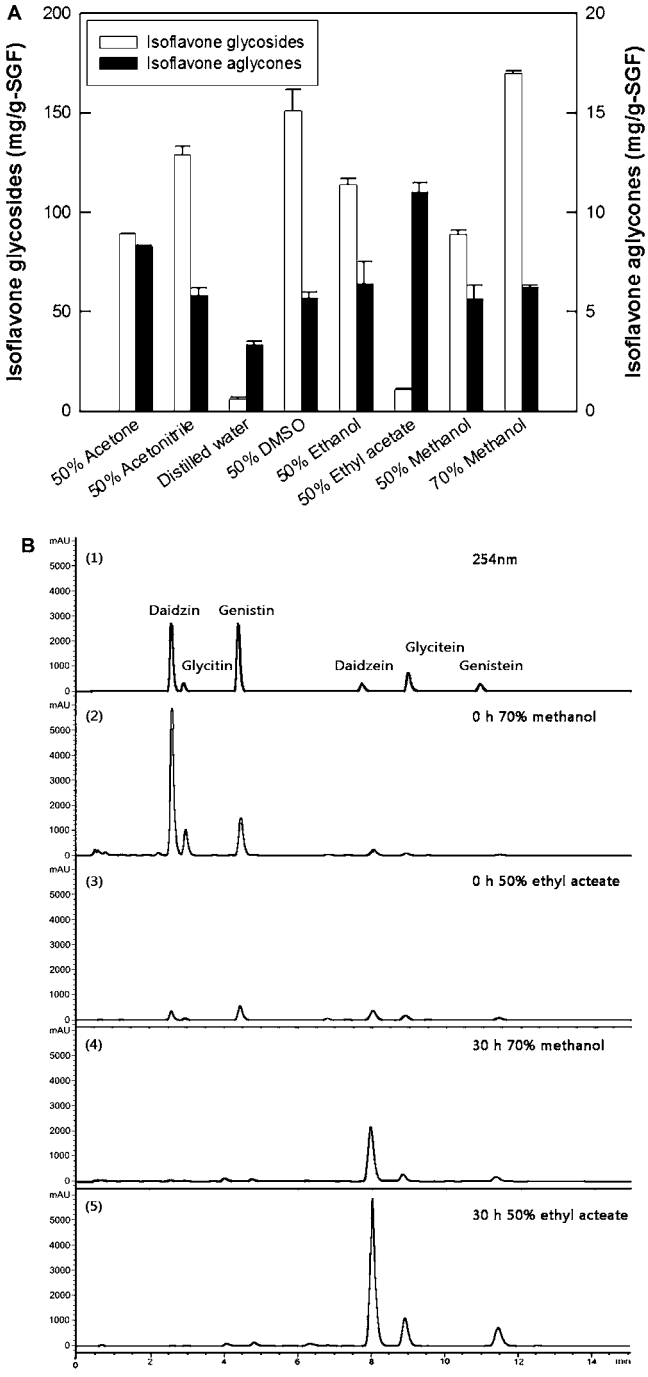

Figure 2. (A) Contents of isoflavone glycosides and isoflavone aglycons in 5% SGF determined using various solvent systems. Solvent systems, except for 70% methanol, were treated at room temperature for 2 min; 70% methanol was treated at 80 °C with shaking at 200 rpm for 3 h. Data represent the mean of three experiments, and error bars represent the standard deviation. (B) HPLC chromatograms of isoflavones at 254 nm. (1) Isoflavone standards. The retention times of daidzin, glycitin, genistin, daidzein, glycitein, and genistein were 2.7, 3.0, 4.5, 7.9, 8.8, and 11.3 min, respectively. (2) Isoflavone profile of 5% SGF using 70% methanol. (3) Isoflavone profile of 5% SGF using 50% ethyl acetate extract. (4) Isoflavone profile of the culture broth of *A. oryzae* KACC 40247 using 70% methanol in a 100 mL baffled flask containing 25 mL of 5% SGF at 27 °C and an initial pH of 5.3 with agitation at 200 rpm for 30 h. (5) Isoflavone profile of the culture broth of *A. oryzae* KACC 40247 using 50% ethyl acetate in a 100 mL baffled flask containing 25 mL of 5% SGF at 27 °C and an initial pH of 5.3 with agitation at 200 rpm for 30 h. Data are the mean of triplicate experiments, and error bars represent the standard deviations ($p < 0.05$).

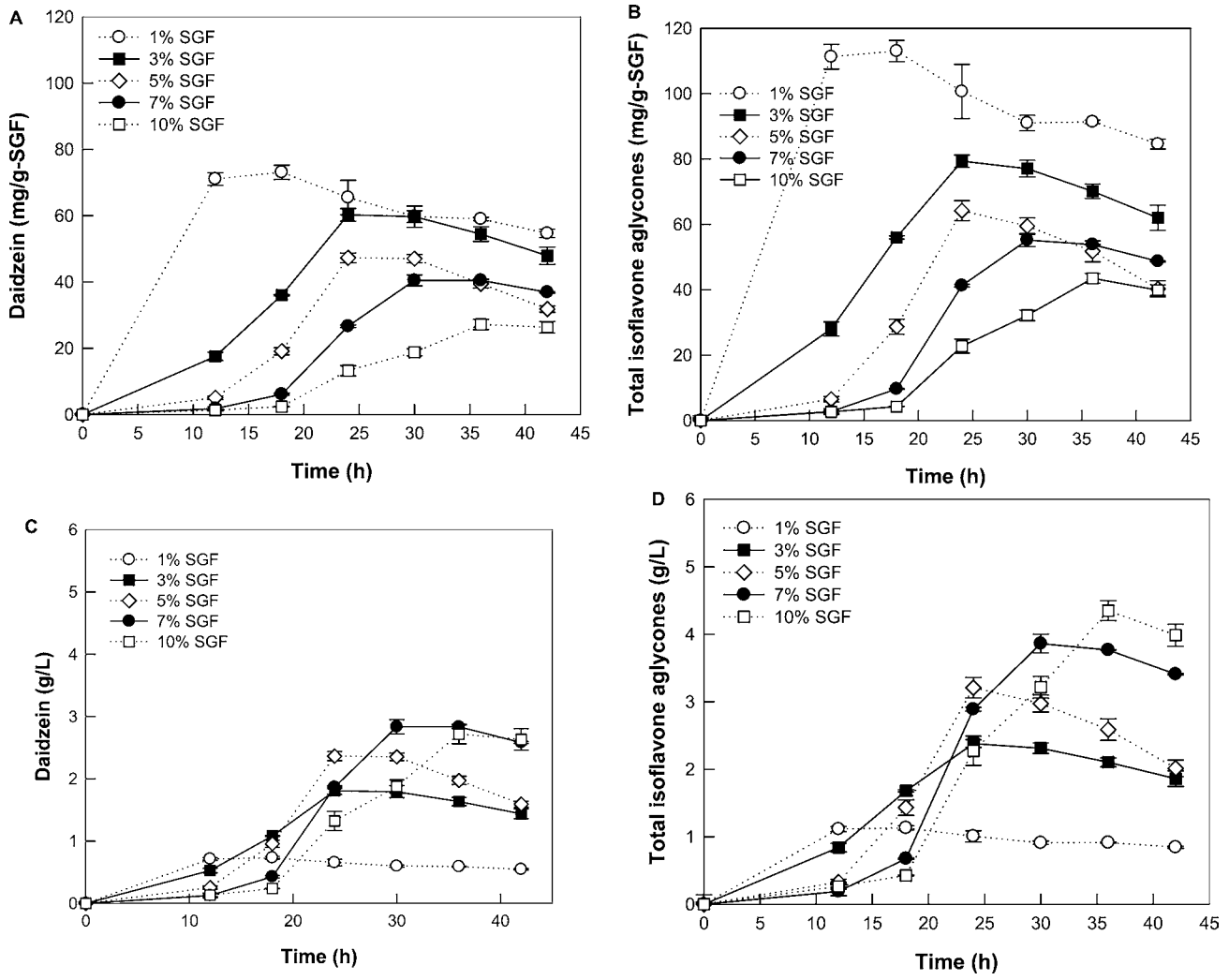

Figure 3. Effect of concentration of SGF on the production of isoflavone aglycons by *A. oryzae* KACC 40247. The reactions were performed at an initial pH of 5.3, 200 rpm, and 27 °C in a 100 mL baffled flask for 42 h. Data represent the mean of three experiments, and error bars represent the standard deviation. (A) Daidzein content, (B) isoflavone aglycons content, (C) daidzein concentration, and (D) isoflavone aglycons concentration. The samples were analyzed by GC/FID. Data are the mean of triplicate experiments, and error bars represent the standard deviations ($p < 0.05$).

Rhizopus azygosporus, and *Rhizopus* sp.[15] To select an efficient daidzein-producing fungus, the 15 *Aspergillus* strains were cultured at 27 °C, an initial pH of 5.3, and 200 rpm for 48 h in a 100 mL baffled flask containing 25 mL of 5% SGF, and 50% ethyl acetate was used for determining the amount of isoflavone aglycons from SGF. Among the *Aspregillus* strains, *A. oryzae* KACC 40247, which originated from the fermented soy food meju, provided the highest content (44.7 mg/g of SGF), concentration (2235 mg/L), and productivity (74.5 mg/L/h) of daidzein after 30 h (Table 1). Thus, *A. oryzae* KACC 40247 was selected as an efficient daidzein-producing fungus and was used in all subsequent experiments for the production of isoflavone aglycons.

Selection of an Effective Solvent System for Determining the Amount of Isoflavone Glycosides or Isoflavone Aglycons from SGF. SGF is made from soy germ passing through defatting, drying, and grinding processes. The isoflavone content of soy germ is 7.5-fold higher than that of whole soy bean.[45] Thus, SGF is a good source for the production of isoflavone aglycons. Distilled water; 50% solutions of acetone, acetonitrile, DMSO, ethanol, ethyl acetate, and methanol; and a 70% methanol solution were used for determining the amount of isoflavone glycosides and isoflavone aglycons from 5% SGF (Figure 2A). The determined content of isoflavone glycosides using 70% methanol was the highest among the solvent systems tested (170 ± 1.1 mg/g of SGF) and 17-fold higher than that using 50% ethyl acetate (10.7 ± 0.5 mg/g). However, the determined content of isoflavone aglycons using 50% ethyl acetate was the highest among the solvent systems tested (11.5 ± 0.6 mg/g of SGF) and approximately 2-fold higher than that using 70% methanol (6.2 ± 0.1 mg/g of SGF).

A. oryzae KACC 40247 was incubated in a 100 mL baffled flask containing 25 mL of 5% SGF at 27 °C and an initial pH of 5.3 with agitation at 200 rpm for 30 h. The isoflavones were determined using 70% methanol and 50% ethyl acetate. The isoflavone glycosides and isoflavone aglycons were analyzed by HPLC using a hydrosphere C18 reverse-phase column. Six typical isoflavones were detected with the same retention times as daidzin, glycitin, genistin, daidzein, glycitein, and genistein (Figure 2B). The isoflavone glycosides daidzin, glycitin and genistin were converted to the isoflavone aglycons daidzein, glycitein, and genistein, respectively, by *A. oryzae* KACC 40247. The isoflavone profile of 5% SGF using 70% methanol showed high intensities of the isoflavone glycosides, whereas that using

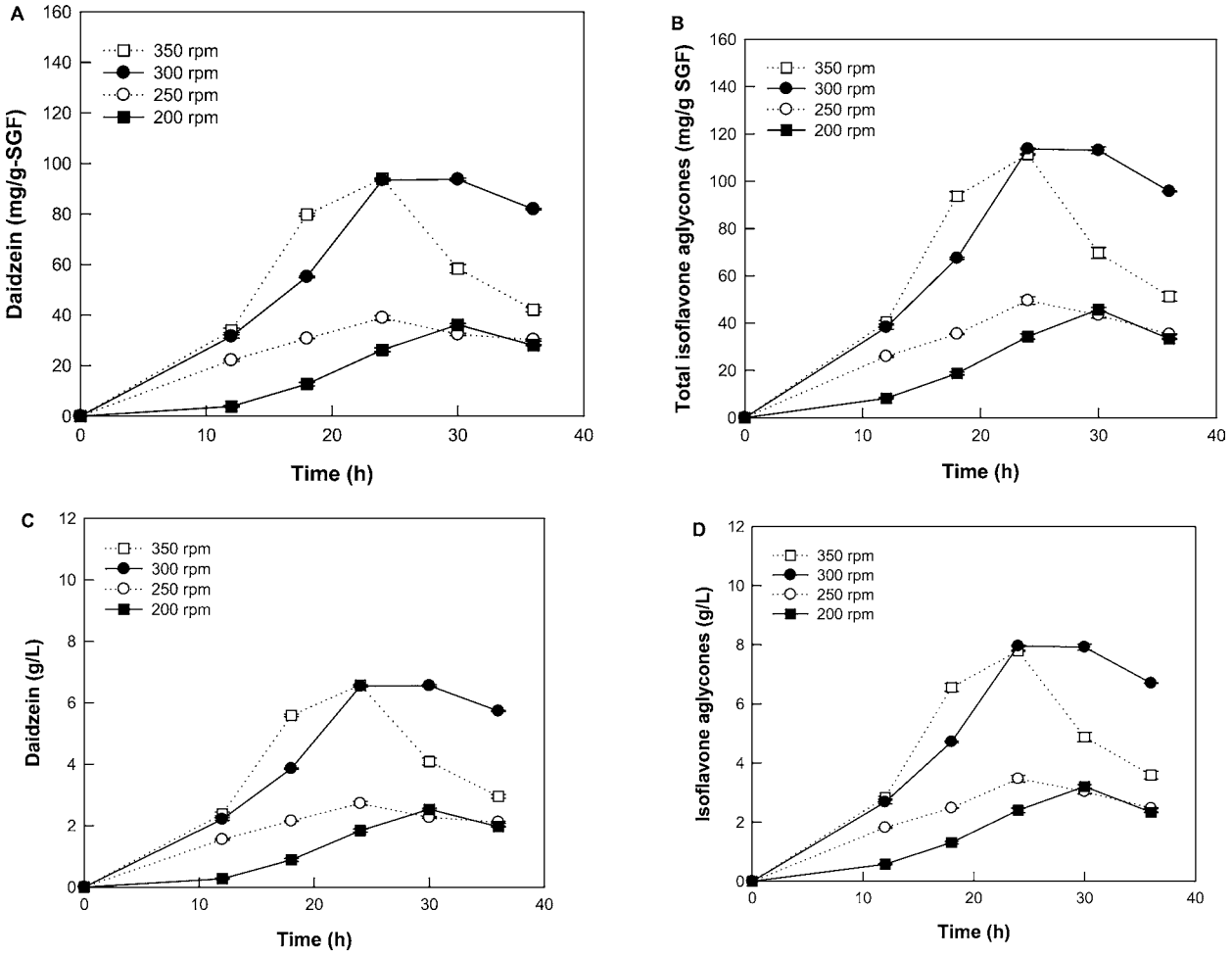

Figure 4. Effect of agitation speed on the production of isoflavone aglycons by *A. oryzae* KACC 40247. The reactions were performed with 7% SGF at an initial pH of 5.3, 200 rpm, and 27 °C in a 100 mL baffled flask for 42 h. Data represent the mean of three experiments, and error bars represent the standard deviation. (A) Daidzein content, (B) isoflavone aglycons content, (C) daidzein concentration, and (D) isoflavone aglycons concentration. Data are the mean of triplicate experiments, and error bars represent the standard deviations ($p < 0.05$).

50% ethyl acetate showed low intensities (Figure 2B2,3). The isoflavone profile of the culture broth using 70% methanol showed low intensities of the isoflavone aglycons, whereas that using 50% ethyl acetate showed high intensities (Figure 2B4,5). Thus, 70% methanol and 50% ethyl acetate were selected as effective solvent systems for determining the amount of isoflavone glycosides and isoflavone aglycons, respectively.

Although 70% methanol has been used as an efficient solvent system for determining the amount of soy isoflavone,[34,46,47] the results presented here show that 70% methanol was most effective for determining the amount of isoflavone glycosides, but not of isoflavone aglycons. Total isoflavone aglycons extracted from soybean seeds followed the order water > 53% (v/v) acetonitrile > 53% (v/v) ethanol > 53% (v/v) methanol.[48] The isoflavones in defatted cotyledon soy flour were extracted using acetone, ethanol, acetonitrile, and water. The amount of isoflavone aglycons using 50% (v/v) acetone was the highest.[49] However, 50% ethyl acetate was more effective for determining the amount of isoflavone aglycons from SGF than 70% methanol, distilled water, and 50% acetone. Recently, ethyl acetate has been applied for increasing the purity of the isoflavone aglycons because isoflavone aglycons were very soluble in ethyl acetate.[50]

The relative polarities of acetone, acetonitrile, DMSO, ethanol, ethyl acetate, and methanol were 35.5, 46.0, 44.4, 65.4, 22.8, and 76.2%. Among the organic solvents tested, methanol exhibited the highest polarity whereas ethyl acetate exhibited the lowest polarity. The relative polarity may explain the results that 70% methanol and 50% ethyl acetate are the effective solvents for determining the amount of isoflavone glycosides and isoflavone aglycons, respectively.

Effect of SGF Concentration on the Production of Isoflavone Aglycons by *A. oryzae* KACC 40247. The effect of SGF concentration on the production of isoflavone aglycons by *A. oryzae* KACC 40247 was investigated by varying the SGF concentration from 1 to 10% at 27 °C, an initial pH of 5.3, and 200 rpm for 42 h. The contents of daidzein and isoflavone aglycons decreased as the concentration of SGF increased (Figure 3A,B). High concentrations of isoflavone glycosides inhibited the activity of β-D-glucosidase from *A. oryzae*, which converted isoflavone glycosides to isoflavone aglycons.[43] High concentrations of glucose, which is formed from high concentrations of isoflavone glycosides by hydrolysis reaction, may inhibit the activity of β-D-glucosidase.[51,52] Thus, a high concentration of SGF might result in the reduced content of isoflavone aglycons in SGF. The concentration of isoflavone glycosides was 3.08 g/L at 1% SGF and 30.8 g/L at 10% SGF. Daidzin was soluble below the concentration of approximately 0.1 g/L at room temperature. Thus, the low solubility of

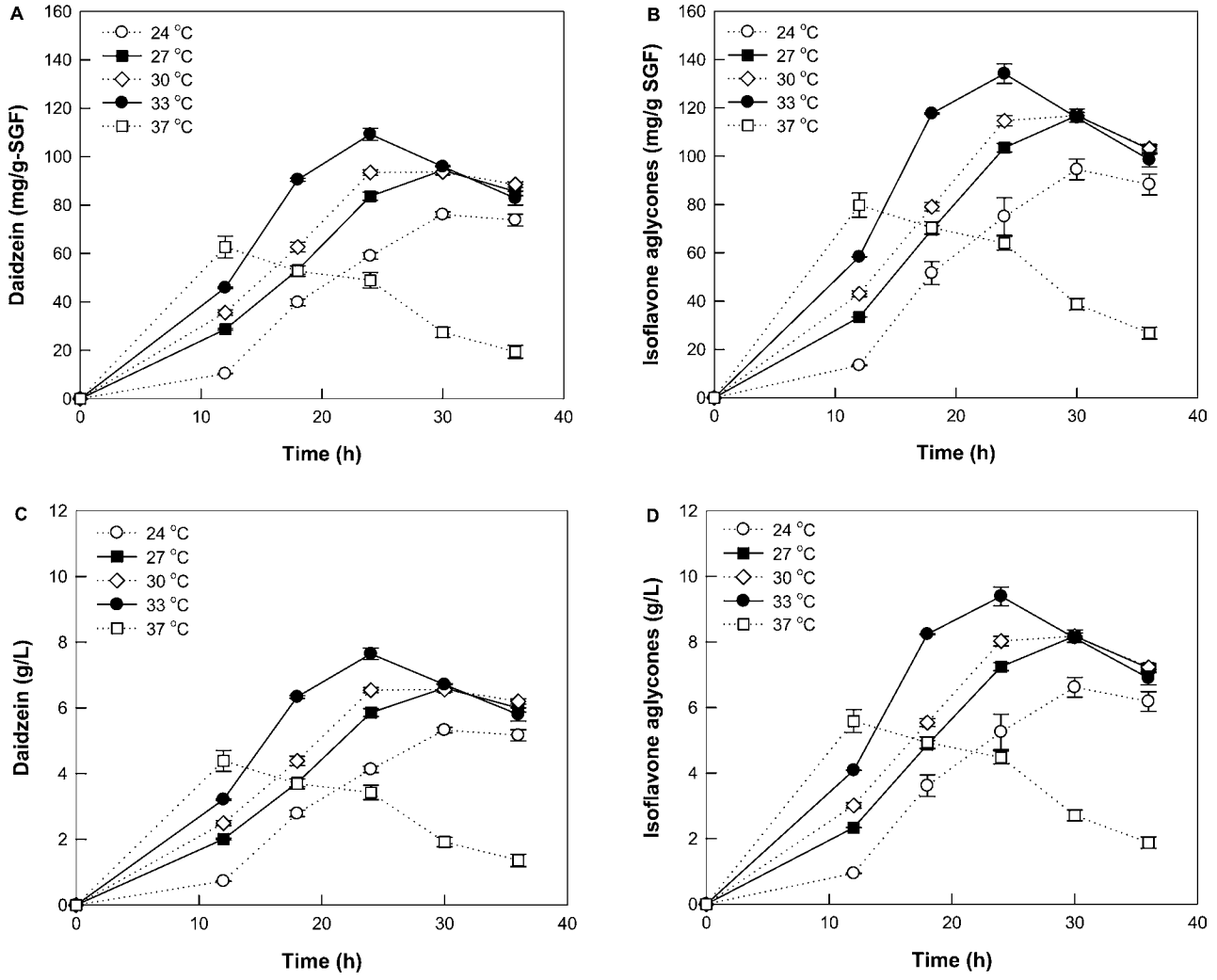

Figure 5. Effect of temperature on the production of isoflavone aglycons by *A. oryzae* KACC 40247. The reactions were performed with 7% SGF at an initial pH of 5.3 and 300 rpm in a 100 mL baffled flask for 42 h. Data represent the mean of three experiments, and error bars represent the standard deviation. (A) Daidzein content, (B) isoflavone aglycons content, (C) daidzein concentration, and (D) isoflavone aglycons concentration. Data are the mean of triplicate experiments, and error bars represent the standard deviations ($p < 0.05$).

isoflavone glycosides also resulted in the reduced content of isoflavone aglycons. The maximum concentrations of daidzein and isoflavone aglycons produced by *A. oryzae* KACC 40247 were 2.8 ± 0.1 g/L at 7% SGF after 30 h (Figure 3C) and 4.4 ± 0.2 g/L from 10% SGF after 36 h (Figure 3D), respectively. The contents of daidzein and isoflavone aglycons from 7% SGF after 30 h were 27 and 16% higher, respectively, than those at 10% SGF after 36 h. Therefore, 7% SGF was chosen as the substrate for the production of isoflavone aglycons.

The concentration of total isoflavone aglycons decreased with time after 30 h at 7% SGF and after 36 h at 10% SGF because total isoflavone aglycons might be degraded by other enzymes in cells. The degradation metabolism of total isoflavone aglycons to smaller metabolic products has not been fully investigated. The isoflavone aglycon daidzein was metabolized to hydroxy-*O*-desmethylangolensin by C-ring fission. The isoflavone aglycon genistein may be modified to dihydrogenistein or 6′-hydroxy-*O*-desmethylangolensin by other enzymes without hydroxylation.[53]

Effect of Agitation Speed on the Production of Isoflavones Aglycons by *A. oryzae* KACC 40247. *A. oryzae* KACC 40247 was incubated using 7% SGF by varying the agitation speed from 200 to 350 rpm in a 100 mL baffled flask for 42 h. Although the concentrations (contents) of daidzein and isoflavone aglycons at 350 rpm were higher than those at 300 rpm after 18 h, they were similar to those at 300 rpm after 24 h. At this time, the concentrations (contents) of daidzein and isoflavone aglycons at 300 rpm were 6.6 ± 0.04 g/L (93.7 ± 0.5 mg/g of SGF) and 8.0 ± 0.02 g/L (114 ± 0.3 mg/g of SGF), respectively (Figure 4). However, after 24 h, the production of daidzein and isoflavone aglycons at 350 rpm decreased faster than at 300 rpm. The higher oxygen transfer rate at the higher agitation speed of 350 rpm may stimulate not only the hydrolysis of isoflavone glycosides but also the degradation metabolism of isoflavone aglycons, resulting in more rapid hydrolysis and less stability of isoflavone aglycons. Thus, the optimal agitation speed for the production of isoflavone aglycons was determined to be 300 rpm.

Effects of Temperature and Initial pH on the Production of Isoflavones Aglycons by *A. oryzae* KACC 40247. *A. oryzae* KACC 40247 was incubated using 7% SGF at an initial pH of 5.3 and at 300 rpm for 42 h by varying the temperature from 24 to 37 °C. As the temperature increased, the initial production rates of daidzein and isoflavone aglycons increased but the time to reach the maximum concentration

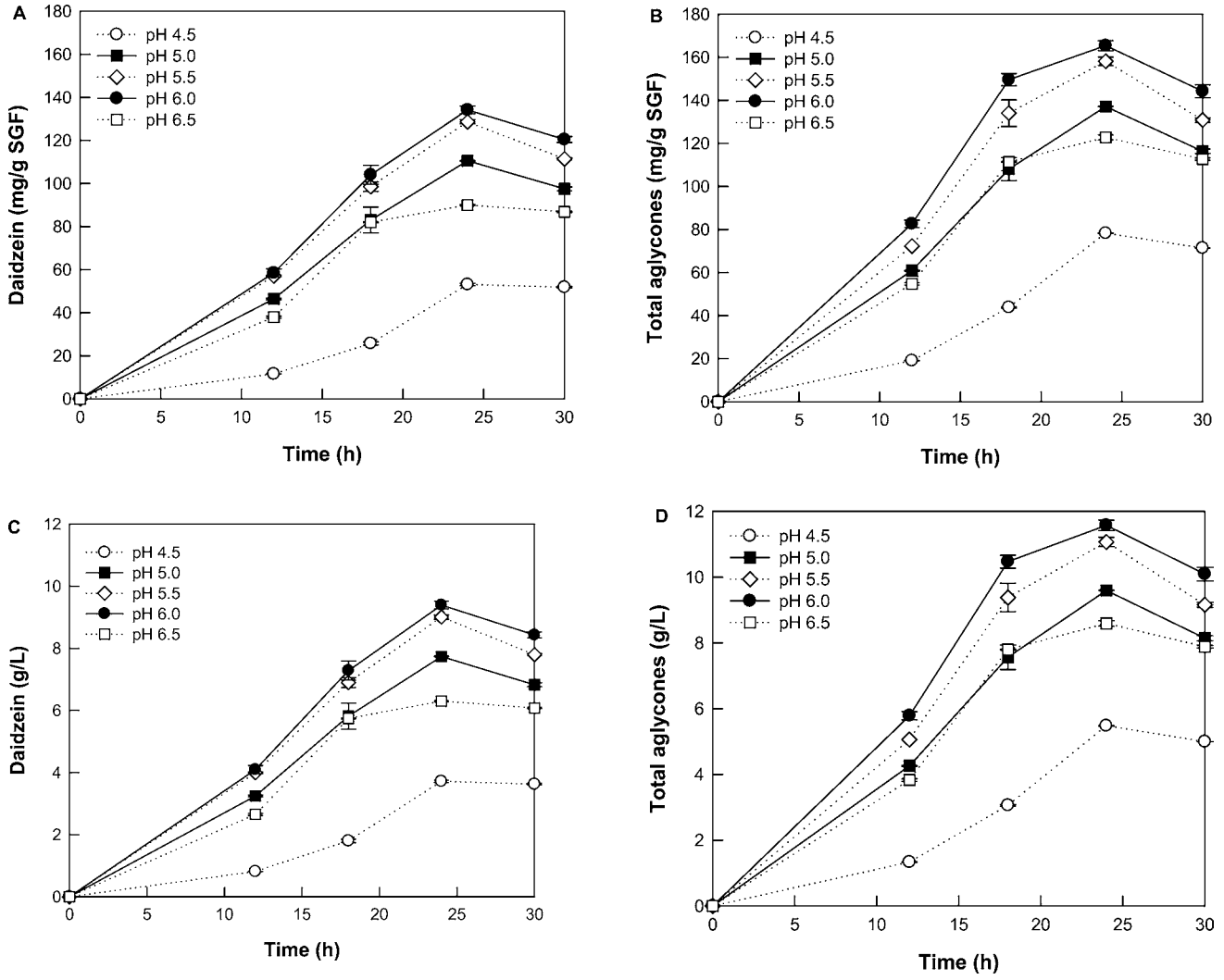

Figure 6. Effect of initial pH on the production of isoflavone aglycons by *A. oryzae* KACC 40247. The reactions were performed with 7% SGF at 300 rpm and 33 °C in a 100 mL baffled flask for 42 h. Data represent the mean of three experiments, and error bars represent the standard deviation. (A) Daidzein content, (B) isoflavone aglycons content, (C) daidzein concentration, and (D) isoflavone aglycons concentration. Data are the mean of triplicate experiments, and error bars represent the standard deviations ($p < 0.05$).

decreased. The production of daidzein and isoflavone aglycons was maximal at 33 °C. After 24 h, the concentrations (contents) of daidzein and isoflavone aglycons at 33 °C were 7.6 ± 0.2 g/L (109 ± 2.5 mg/g of SGF) and 9.4 ± 0.3 g/L (134 ± 4.1 mg/g of SGF), respectively (Figure 5). The conversion of isoflavone glycosides to isoflavone aglycons by *A. oryzae* CCT 4359 with whole soybean flour[41] and three *Aspregillus* strains using black bean[15] was performed at 30 °C. The optimum growth temperatures of the *Aspergillus* strains were in the range of 25−30 °C,[54] the optimum temperature for the production of *β*-D-glucosidase from *Aspergillus* sp. was 35 °C,[55] and the optimum temperature of *β*-D-glucosidase from *Aspergillus* strains was in the range of 50−60 °C.[43,55,56] The optimum temperature for the production of isoflavone aglycons by *A. oryzae* KACC 40247 was close to that for the production of *β*-D-glucosidase by *Aspergillus* sp.

The effect of pH on the production of daidzein and isoflavone aglycons by *A. oryzae* KACC 40247 was investigated using 7% SGF by varying the initial pH from 4.5 to 6.5 at 33 °C and 300 rpm for 42 h. The production of daidzein and isoflavone aglycons was highest at an initial pH of 6.0. After 24 h, the concentrations (contents) of daidzein and isoflavone aglycons were 9.4 ± 0.1 g/L (134 ± 0.85 mg/g of SGF) and 11.5 ± 0.1 g/L (165 ± 1.05 mg/g of SGF), respectively (Figure 6). The initial pH values of 4.5, 5.0, 5.5, 6.0, and 6.5 became 4.6, 5.1, 5.8, 6.0, and 6.2, respectively, at 12 h and 4.5, 4.8, 6.5, 6.7, and 7.3, respectively, at 24 h. The results indicate that the hydrolytic activity of *A. oryzae* KACC 40247 was optimal in the pH range of 6.0 to 6.7. The pH values for *A. oryzae* CCT 4359 and three other *Aspergillus* strains to produce isoflavone aglycons were not adjusted.[15,41] The optimum pH values for *β*-D-glucosidases from *Aspergillus* strains have been reported as 4−5.[43,48,55]

Production of Isoflavone Aglycons by *A. oryzae* KACC 40247 under Optimized Conditions. The nonoptimal and optimal conditions for *A. oryzae* KACC 40247 to produce isoflavone aglycons from isoflavone glycosides in SGF were as follows: 5% SGF, an initial pH of 5.3, 27 °C, and 200 rpm for 30 h, and 7% SGF, an initial pH of 6.0, 33 °C, and 300 rpm for 24 h in a 100 mL baffled flask, respectively. Under optimized conditions, the concentrations (contents) of daidzein and isoflavone aglycons were 9.4 ± 0.1 g/L (134 ± 0.85 mg/g of SGF) and 11.5 ± 0.1 g/L (165 ± 1.05 mg/g of SGF), respectively, with productivities of 391 ± 2.8 and 479 ± 4.1 mg/L/h, respectively. Optimization of culture conditions increased the content,

Table 2. Contents of Isoflavone Glycosides (mg/g) and Isoflavone aglycons (mg/g) in 7% (w/v) SGF by *A. oryzae* KACC 40247 under Optimized Conditions[a]

isoflavone	0 h	12 h	24 h
daidzin	188 ± 0.95 (58.9)	69 ± 0.59 (38.1)	2.8 ± 0.06 (1.7)
glycitin	88 ± 0.64 (27.6)	37 ± 0.35 (20.2)	1.5 ± 0.02 (0.9)
genistin	312 ± 0.06 (9.8)	3.6 ± 0.03 (2.0)	0.1 ± 0.01 (0.1)
isoflavone glycosides	308 ± 1.6 (96.3)	109 ± 1.2 (60.4)	4.4 ± 0.1 (2.6)
daidzein	8.0 ± 0.01 (2.5)	56 ± 1.00 (30.7)	134 ± 0.85 (79.5)
glycitein	2.5 ± 0.04 (0.8)	7.7 ± 0.26 (4.3)	11 ± 0.05 (6.4)
genistein	1.4 ± 0.01 (0.4)	8.4 ± 0.07 (4.6)	19 ± 0.15 (11.4)
isoflavone aglycons	11.9 ± 0.06 (3.7)	72 ± 1.33 (39.6)	165 ± 1.05 (97.3)
total isoflavones	320 ± 1.66 (100)	181 ± 2.53 (100)	169 ± 1.15 (100)

[a]The amounts of isoflavone glycosides and isoflavone aglycons were determined using 50% ethyl acetate at 25 °C for 2 min and with 70% methanol at 80 °C for 3 h, respectively. The percentage of each isoflavone from the total isoflavones is provided in parentheses. Data are the mean of triplicate experiments, and errors represent the standard deviations ($p < 0.05$).

Table 3. Production of Isoflavone Aglycons from Soy Products by Microorganisms

substrate	organism	temperature (°C)	time (h)	daidzein (mg/g)	aglycons (mg/g)	daidzein (g/L)	aglycons (g/L)	ref
defatted soy meal	*B. subtilis*	37	12	0.63	1.02[a]	0.031	0.051[a]	21
soy milk	*B. animalis*	37	24			0.035	0.078	22
soy milk	*L. paraplantarum*	37	12	1.31	3.91			24
soymilk	*S. thermophilus*	37	24			0.030	0.082	26
soybean milk	lactic acid bacteria mixture	37	90			0.013	0.052	40
soybean flour milk	lactic acid bacteria mixture	37	90	0.11	0.42	0.011	0.042	
black bean	*A. awamori*			0.14	0.25			15
	A. oryzae BCRC 30222			0.13	0.24			
	A. sojae BCRC 30103	30	72	0.27	0.56			
	R. azygosporus			0.25	0.49			
	Rhizopus sp.			0.40	0.82			
	R. oligosporous BCRC 31996	30	144	0.50	1.28[a]			25
soybean flour	*A. oryzae* CCT 4359	30	48	0.13	0.45			41
soy germ flour	***A. oryzae* KCTC 40247**	**33**	**24**	**134**	**165**	**9.40**	**11.5**	**this study**

[a]Only daidzein and genistein, without glycitein.

concentration, and productivity of isoflavone aglycons by 2.8-, 3.9-, and 4.8-fold, respectively, compared with those obtained under nonoptimized conditions.

The contents of isoflavones in black soybean,[57] black soybean koji,[57] soybean milk,[58] soy germ,[22] and soybean flour[34] were reported as 1.77, 1.81, 10.9, 12.1, 13.8, and 14.8 mg/g, respectively, and the proportions of isoflavone glycosides to total isoflavones were 94, 58, 87, 89, 94, and 80% (w/w), respectively, indicating that soybean flour had the highest previously reported content of isoflavones. Although the used solvent was different, the content of isoflavones in SGF (320 ± 1.66 mg/g of SGF with 96% isoflavone glycosides) was 22-fold higher than that in soybean flour (Table 2). Thus, SGF is the best source for the production of aglycons. The contents of isoflavone glycosides and isoflavone aglycons in 7% SGF were measured with *A. oryzae* KACC 40247 under optimized conditions. Isoflavones glycosides (96% of total isoflavones) in SGF, which consisted of 59% (w/w) daidzin, 27% (w/w) glycitin, and 10% (w/w) genistin, were converted to isoflavone aglycons (97% of total isoflavones), by *A. oryzae* KACC 40247 after 24 h, thereby providing 80% (w/w) daidzein, 6% (w/w) glycitein, and 11% (w/w) genistein.

The production of isoflavone aglycons from soy products by microorganisms is summarized in Table 3. The highest previously reported contents of isoflavone aglycons was 3.91 mg/g, which was produced by *Lactobacillus paraplantarum* from soymilk after 12 h.[24] The highest previously reported concentration and productivity of isoflavone aglycons were 0.082 mg/L and 3.4 μg/L/h, respectively, produced by *Streptococcus thermophilus*.[26] The content, concentration, and productivity of isoflavone aglycons achieved in the present study using *A. oryzae* KACC 40247 were 42-, 140-, and 140-fold higher, respectively, than the highest previously reported values.

The highest production of isoflavone aglycons among enzymatic methods has been reported in commercial β-glucosidase using 20% SGF.[42] The enzyme produced 1.3 g/L isoflavone aglycons after 5 h, with productivities of 260 mg/L/h. The concentration and productivity of isoflavone aglycons by the method used in the present study were 8.5- and 1.8-fold higher, respectively, than those by the enzymatic method. These results indicate that *A. oryzae* KACC 40247 produces the highest reported concentrations of isoflavone aglycons, considering both culture and enzymatic methods. Recently, 1.4 g/L isoflavone aglycons were produced from soy whey wastewater by acidic hydrolysis after 1.5 h, with a productivity of 933 mg/L/h.[20] The concentration of isoflavone aglycons in the present study was 8.2-fold higher than that by the acidic hydrolysis method, whereas the productivity of isoflavone aglycons in the present study was 1.9-fold lower.

In conclusion, *A. oryzae* KACC 40247 was selected as an efficient daidzein-producing fungus. The effective solvent systems for determining the amount of isoflavone glycosides and isoflavone aglycons were 70% methanol and 50% ethyl acetate, respectively. The culture conditions were optimized using SGF, an isoflavone-rich soy product, for increased

production of isoflavone aglycons. Under the optimized conditions, the highest level of production of isoflavone aglycons reported to date was obtained. These results should be useful in the soy product industry.

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Notes

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