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A THESIS FOR THE DEGREE OF

MASTER OF SCIENCE IN FOOD AND NUTRITION

Alcoholic Fermentation of Platycodi Radix and Bioconversion of Platycosides Using Co-culture of *Saccharomyces cerevisiae* KCTC 7928 and *Aspergillus awamori* FMB S900

Saccharomyces cerevisiae KCTC 7928 와

Aspergillus awamori FMB S900 를 이용한

도라지 알코올 발효와 도라지 사포닌의 생물전환

August, 2014

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Abstract

Alcoholic Fermentation of Platycodi Radix and Bioconversion of Platycosides Using Co–culture of *Saccharomyces cerevisiae* KCTC 7928 and *Aspergillus awamori* FMB S900

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The objectives of this study were to manufacture Platycodi radix (PR) wine, and assess the biotransformation pattern of PR platycosides using a co–culture of *Saccharomyces cerevisiae* KCTC 7928 with *Aspergillus awamori* FMB S900. The basal fermentation temperature was set to 15°C, and a high sucrose concentration (30% and above) was used to raise the level of ethanol production. The fermentation of PR by the co-culturing of *S. cerevisiae* with *A. awamori* was compared to that of a single culture of *S. cerevisiae* at concentrations of 30–50% (w/w) sucrose, and both types were analyzed at an interval of fifteen days during fermentation. The results showed that three major saponins (platycoside E, platycodin D_3, and platycodin D) were converted into 16–oxo–PD during the co-culturing of *S. cerevisiae* with *A. awamori* while producing up to 12.5% ethanol (with an initial sugar concentration of 40%). The potential use of such a co–culturing system suggested an opportunity to develop PR wine with bioconverted platycoside.

Keywords: PR, bioconversion, alcohol production, co–culture

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List of abbreviations

S. cerevisiae; *Saccharomyces cerevisiae* KCTC 7928

A. awamori; *Aspergillus awamori* FMB S900,

Platycodi radix; PR,

Platycoside E; PE

Platycodin D_3; PD_3

Platycodin D; PD

16–oxo–platycodin D; 16-oxo-PD

Platycodi radix saponins; PRS

1. Introduction

Platycodi radix (PR), the root of *Platycodon grandiflorum* A.DC. (Campanulaceae), is commonly known as Kilkyong in Korea, Jiegeng in China and Kikyo in Japan, and has been frequently used as a bioactive material in oriental medicine (1). PR has been used as an expectorant and applied to treat such adult diseases as bronchitis, asthma, pulmonary tuberculosis, and other respiratory ailments (2). In addition, PR has shown numerous biological activities for conditions such as hypercholesterolemia and hyperlipidemia (3) as well as diabetes (4).

Generally, the principal bioactive constituents of PR are a class of oleanane– type triterpenoid saponins, more commonly known as platycosides (5). Its extract and major components have been reviewed for their possession of various pharmacological actions, including anti-allergic reactions (6), antioxidant and anticancer activities (7), the inhibition of inducible nitric oxide synthase (8), anti–inflammation actions, and immunomodulation properties in mammalian systems (9).

Platycosides are composed of oleanane–type triterpenoid backbone structures with two side chains: a glucose unit attached through an ether linkage to the C–3 position of the triterpene and an ester linkage between C–28 and arabinose. In addition, its C–4 position has different substituents, such as methyl, carboxyl, or hydroxymethyl groups (1, 10). Previous studies of PR found that triterpenoid saponins were the main active chemical components, while the saponins referred to platycodins (A, D, D_2, and D_3), 2''– and 3''–O–acetyl polygalacin D_2, platyconic acid A, and platycosides (A, B, C, D, E, and F) were isolated in the extracts

of PR (8). To date, more than 30 platycosides have been found in PR. Platycoside E (PE), platycodin D_3 (PD_3) and platycodin D (PD), which is marker reference substance, are mainly reported in the published papers (11, 12).

Recently, a new platycoside, 3–O–β–D–glucopyranosyl 16–oxo–platycodigenin 28–O–β–D–apiofuranosyl–(1→3)–β–D–xylopyranosyl –(1→4)–α–L–rhamnopyranosyl–(1→2)–α–L–arabinopyra–nosyl ester, termed 16–oxo–platycodin D (16-oxo-PD), was isolated from PR. The same sugar constitution was shown to exist in both PD and 16–oxo–PD (13).

Most ingested glycosides are converted to non–glycoside components by the glycosidases produced by intestinal bacteria (14). Generally, the numbers and linkages of saccharides combined with the platycoside backbone structure play a role in their absorption and bioactive capacity in the human body. Physical, physicochemical, chemical, and biological pretreatment processes have been used to transform their structures in an effort to improve their bioactivity levels (15).

Microorganisms and their enzymes have been widely used as useful tools in structural modification and metabolism studies of both natural and synthetic organic compounds. They provide benefits related to their stereospecific reactions that take place under mild conditions as wells as good cost effectiveness (16, 17). Despite the advantage of bioconversion, very little research has yet been published on the fermentation of PR using microorganisms.

The aim of this study is to bioconvert platycosides and produce a new PR wine using a co–culture of *Saccharomyces cerevisiae* KCTC 7928

and *Aspergillus awamori* FMB S900. The effects of these two genera on alcoholic fermentation appear to be complementary, therefore, a mixed culture containing one representative of each genus was used. For the bioconversion of platycosides and ethanol production, these two different microorganisms could grow together successfully while at the same time yield better fermentation results.

2. Materials and Methods

2.1. Materials

2.1.1. The microorganism strains and culture conditions

S. cerevisiae KCTC 7928, used for fermentation, was purchased from the frozen stock culture collection of the Korean Culture Type Collection (Seoul, Korea). *S. cerevisiae* was activated aerobically at 25℃ for 24 h in a complex autoclaved medium containing 2% glucose, 0.6% yeast extract, 0.54% sodium sulfate, 0.32% ammonium chloride, 0.16% magnesium chloride hexahydrate, 0.15% monobasic potassium phosphate and 0.023% calcium chloride dihydrate in distilled water (18). All chemicals was purchased from Sigma Aldrich (Sigma, St, Louis, MO, USA).

A. awamori, used for co–culturing, was obtained from the Food Microbiology Lab. at the Department of Food and Nutrition in Seoul National University (Seoul, Korea). Culture was maintained on potato dextrose agar (PDA) (Difco, USA) at 30°C for two weeks.

2.1.2. Counting spores of *A. awamori*

Spores of *A. awamori* were harvested by washing the PDA slant cultures grown for two weeks with sterile saline containing Tween 80 0.05%. Sterile saline solution (20 ml) was added to each slope and a suspension of the spores made by lightly brushing the mycelium with a spreader. Spore numbers were estimated by counting an aliquot (20 $\mu\ell$) of the suspension using a haemocytometer. The suspension, calculated to give a final spore count of 4×10^7 spores per PR extracts (ml), was used as an inoculum (19, 20).

2.1.3. Chemicals and Reagents

PR, the roots of P. *grandiflorum*, were purchased from Kyung–dong market (Seoul, Korea). Acetonitrile and TFA used were of HPLC grade from J. T. Baker (Phillipsburg, NJ, USA) and Fisher Scientific (Pittsburg, PA, USA). Other chemicals for fermentation and separation were all of analytical grade from Sigma (USA).

2.2. Media and Fermentation Assays

2.2.1. Preparation of the extract of PR

Aqueous extracts of PR were prepared as follows: 1 kg of powdered dried plant material was refluxed with 10 L of distilled water at 80°C for 4 hr. After filtration through a double thickness of Whatman No. 5 filter paper (Whatman Ltd., Maidstone, United Kingdom), they were sterilized at 121℃ for 15 min (21).

2.2.2. Fermentation conditions

The activated *S. cerevisiae* in broth was inoculated into the PR extracts at 5% (v/v) level as a basal fermentation microorganism. The $4x10^7$ spores of *A. awamori* FMB S900, per one ml PR extracts were used as an inoculum for the co–culturing groups (Group D, E, F, G, H, and I). The levels of sucrose used as a carbohydrate source were as follows: 30% (w/w) (Group A, D, and G), 40% (w/w) (Group B, E, and H), 50% (w/w) (Group C, F, and I). Initial pH was adjusted to 5.00 by using 1 N citric acid. Batch fermentation was conducted in 1000–mL Erlenmeyer flasks sealed with a rubber cork and a one–way air valve to ensure anaerobic conditions. Anaerobic fermentation was carried out at 15°C for ninety days. Group D, E, F were performed under aerobic fermentation conditions (25°C, 120 rpm) during the initial five days before the anaerobic fermentation. During the fermentation of PR extracts, they were analyzed at an interval of fifteen days. The detail fermentation conditions are listed in Table I.

Table 1. Fermentation conditions of Group A–I

Group	Microorganism	Sugar level	Fermentation conditions
A	Only *S. cerevisiae*	30% (v/v)	Anaerobic fermentation, 15℃, 0 rpm for ninety days
B		40% (v/v)	
C		50% (v/v)	
D	Co–culturing of *S. cerevisiae* and *A. awamori*	30% (v/v)	Aerobic fermentation, 25℃, 120 rpm during the initial five days and then, Anaerobic fermentation, 15℃, 0 rpm for eighty five days
E		40% (v/v)	
F		50% (v/v)	
G	Co–culturing of *S. cerevisiae* and *A. awamori*	30% (v/v)	Anaerobic fermentation, 15℃, 0 rpm for ninety days
H		40% (v/v)	
I		50% (v/v)	

2.3. Chemical analysis of the samples

2.3.1. Determination of platycosides by high–performance liquid chromatography (HPLC)

Five ml of the fermented PR sample was dried by a freeze–dry process (Ilshin lab Co. Ltd., Korea). After drying, one ml of methanol was applied to extract the platycosides. The extracted solutions were filtered through a 0.2–μm PVDF membrane filter (PALL Acrodisc ® Syringe Filters, Ann Arbor, MI, USA) and analyzed via HPLC.

The HPLC analysis was carried out on a Dionex P680 HPLC pump instrument equipped with a UV–D170V detector and an ASI–100 automated sample injector. All separations were carried out on a ZORBAX SB C–18 column (250mm x 4.6mm id, particle size) from Agilent Technologies (Palo Alto, CA, USA) at 40°C. The flow rate was kept at 1.5 ml/min, and the absorbance was measured at a wavelength of 204 nm for the detection of platycosides. Mobile phases were distilled water (A mobile phase) containing 0.1% (v/v) trifluoroacetic acid (TFA) (Sigma, USA) and acetonitrile (B mobile phase). The multi–step gradient was performed as follows: 10–15% B (0–6 min), 15–25% B (6–24 min), 25–40% B (24–48 min), 40–100% B (48–50 min), 100–10% B (50–52 min), 10% B (52–65 min). Standard solutions of platycosides were prepared in methanol to calculate the concentration of platycosides in samples. HPLC assay conditions are shown in table 2.

Table 2. Operation conditions of HPLC analysis of platycosides of the fermented PR

Parameters	Conditions
Mobile phase A	Water (Containing 0.1% (v/v) of TFA
Mobile phase B	Acetonitrile
Composition	Multi–step gradient : 10–15% B (0–6 min), 15–25% B (6–24 min), 25–40% B (24–48 min), 40–100% B (48–50 min), 100–10% B (50–52 min), 10% B (52–65 min)
Column	ZORBAX SB C–18 column, 250mm x 4.6mm id, 5 mm
Flow rate	1.5 ml/min
Inj. volume	10 ㎕
Detection wavelength	204 nm
Column temperature	40°C
Instruments	Dionex P680 HPLC

2.3.2. Determination of alcohol concentration

Samples of PR extract were taken during the fermentation period and then distilled to obtain 70 mL distillate. Thirty mL deionized water was added and then alcohol degree was determinated by vinometer (22). The result were corrected using Gay Luccac Table (23). Each sample were analyzed in triplicate.

2.3.3. Measurements of pH and °Brix degree

Samples were capped and shaken for 3 min; then the pH was measured on a pH meter (pH-200L, ISTEK, Korea). °Brix degree was evaluated by refractometer (HI-96801, HANNA Company, Italy). Each sample were analyzed in triplicate.

2.3.4. Measurements of total sugar concentration

The total sugar concentrations in the samples were measured by the phenol–sulfuric acid method with glucose as a standard curve (24). One ml of the sample solution was pipetted into a 96–well plate, and 5% phenol (1 ml) added. After then, concentrated–sulfuric acid (5 ml) was added. After standing for 30 min at room temperature, the absorbance was measured at 470 nm using a spectrophotometer (25). The standards and the samples were analyzed in triplicate.

2.3.5. Measurements of antioxidative effect

1,1–Diphenyl–2–picrylhydrazyl (DPPH) radical scavenging activities of PR wines at the end of fermentation were measured by the method of Lee et al. (26). Five ml of the fermented PR sample was dried by a freeze–dry process. After drying, one ml of methanol was applied to concentrate the sample. Ascorbic acid (0.1mM) was used as a control. DPPH (150 μM) was prepared in methanol and 150 μl of this solution was mixed with 100 ul of sample solution and standard solution separately. These solution mixtures were kept in dark for 30 min at 37℃ and optical density was measured at 518 nm using spectrophotometer. Methanol (100 μl) with DPPH solution (150 μl) was used as blank. The optical density was recorded and electron donating ability (EDA, %) was calculated as shown below:

EDA (%) = (1 - ascorbic absorbance / blank absorbance) × 100

The standards and the samples were analyzed in triplicate.

3. Results and Discussion

3.1. Screening of alcohol–producing yeast strain

A preliminary experiment was carried out to select the yeast strain for alcoholic fermentation among the 18 different strains. The activated yeast in broth was inoculated into the PR extracts at a 5% (v/v) level for a basal fermentation. The level of sugar used as a carbohydrate source was 30% (v/v). Anaerobic fermentation was conducted at 15°C for 30 days. As a result, the ethanol production of *S. cerevisiae* KCTC 7928 was greater than the other experimental yeast strains (Table 3). Therefore, *S. cerevisiae* KCTC 7928 was used for alcohol fermentation.

Table 3. Screening of alcohol–producing yeast strain

Strain	alcohol production
Debaryomyces hansenii KCTC 7128	+++
Torulaspora delbrueckii KCTC 7680	+
T. delbrueckii KCTC 17764	++
Zygosaccharomyces rouxii KCTC 17700	++
Z. rouxii KCTC 17701	+++
Hanseniaspora vineae KCTC 17186	++
S. carlsbergensis KCTC 7233	++
S. chevalieri KCTC 7237	+++
S. chevalieri KCTC 7239	+++
S. bayanus KCTC 17223	+++
S. bayanus KCTC 17620	+++
S. cerevisiae KCTC 7904	+++
S. cerevisiae KCTC 7910	++
S. cerevisiae KCTC 7913	++
S. cerevisiae KCTC 7915	+
S. cerevisiae KCTC 7928	++++
S. cerevisiae KCTC 7238	+
S. cerevisiae KCTC 7919	+++

produces very well : ++++, produces well : +++, produces : ++,
Rarely produces : +

3.2. HPLC analysis of platycosides in fermented PR

A saponin fraction containing six major saponins (deapio–platycoside E, platycoside E, deapio–platycodin D_3, platycodin D_3, deapio–platycodin D, and platycodin D) was tested to produce a high level of platycodin D compound with seven glycosidases. As a result, cellulase, β–galactosidase, and β–glucosidase were found to be able to transform PE and PD_3 into PD (1). PD has one glucose molecule attached through an ester linkage at the C–3 position and shows inhibited pancreatic lipase activity in vitro (27). It has also shown an anti–obesitic effect in a mouse model (28). PD affected the values of $K_{m,\ app}$ and K_{cat}/K_m in a dose–dependent manner to mediate lipid metabolism in the intestinal tract (29). In addition, PD has shown anti–cancer effects (30, 31), anti–inflammatory activity (32), and anti–HCV activity (33).

Recently, a new triterpenoid saponin, entitled 16–oxo–PD, was found in the roots of *P. grandiflorum*. According to the basis of the spectroscopic data, the sugar constitutions of 16–oxo–PD and PD were identical (13).

In this study, different fermentation conditions using a PR extract containing three major saponins (PE, PD_3, and PD) and 16–oxo–PD (Fig. 1) were compared to assess the bioconversion of platycosides. They were analyzed at an interval of fifteen days during fermentation. The results are shown in Table 4.

As shown in Fig. 2, all of the co–culturing groups (–Groups D, E, F, G, H, and I) revealed high concentrations of 16–oxo–PD upon the end of the fermentation process. The concentrations of 16–oxo–PD in groups D,

E, and F, fermented under aerobic conditions during the initial five days, were particularly high compared to the other groups. In contrast, groups A, B, and C, using single *S. cerevisiae* for fermentation, showed low production levels.

Comparing the initial concentrations of PE, PD3, PD, and 16–oxo–PD (Fig. 3) with their final concentrations (Fig. 4), PE, PD_3 and PD were converted to 16–oxo–PD in the co–culturing groups (–Groups D, E, F, G, H, I). The PE, PD3, and PD concentrations in groups A, B, and C decreased during fermentation, whereas the concentrations of 16–oxo–PD did not increased. *Aspergillus* strains are effective when used to produce β−glucosidase (34) to cleave glycosidic bonds for transformation (1). Therefore, a co–culturing group using *A. awamori* may be more suitable for the bioconversion of platycosides. In particular, the conversion efficiency could be improved by using an aerobic environment for the activation of *A. awamori* during the initial five days. However, little is known about converted 16–oxo–PD, implying that more research is needed to determine its pharmacological effectiveness.

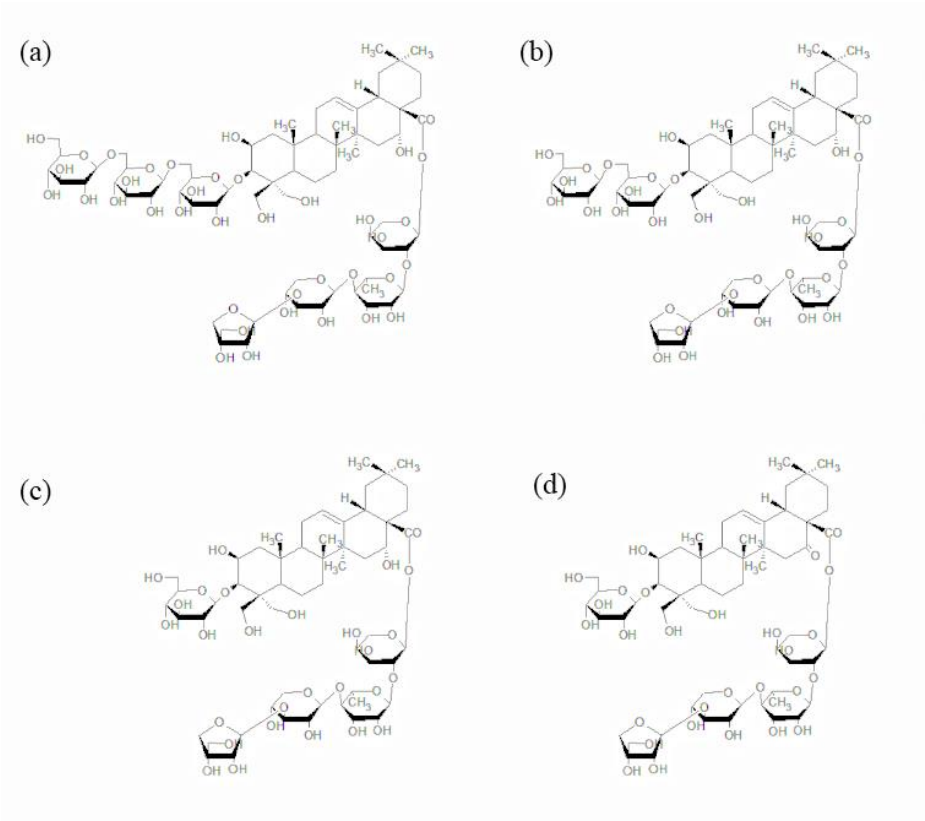

Figure 1. Chemical structures of four saponins from the roots of *P. grandiflorum*. (a) PE, (b) PD_3 (c) PD (d) 16-oxo-PD.

Table 4. Changes of PR saponins (PRS) concentration (μM) during fermentation

Group	PRS (μM)	Fermatation period (days)						
		0	15	30	45	60	75	90
A	PE	78.94	44.98	33.79	32.34	31.57	20.51	26.50
	PD3	42.50	15.59	20.18	20.80	19.45	25.78	13.89
	PD	167.47	55.92	45.33	46.52	40.65	53.16	38.62
	16-oxo-PD	9.15	22.34	18.99	18.83	22.27	10.86	17.21
B	PE	55.85	46.81	33.99	26.69	24.22	45.83	27.12
	PD3	33.92	24.70	13.00	17.17	9.24	23.62	15.36
	PD	106.80	85.12	54.56	65.57	46.73	66.23	48.48
	16-oxo-PD	16.79	26.39	16.57	26.43	7.83	18.76	20.96
C	PE	73.82	46.07	34.58	28.96	21.79	20.09	26.05
	PD3	36.61	15.97	13.71	11.66	6.53	11.39	9.89
	PD	141.69	29.34	46.61	51.18	57.68	33.39	57.61
	16-oxo-PD	17.65	47.91	12.48	11.48	15.50	7.19	16.50
D	PE	78.94	65.56	71.51	69.42	21.98	89.58	45.41
	PD3	42.50	33.05	36.46	20.87	49.02	59.92	61.47
	PD	167.47	62.64	88.90	88.45	109.93	80.31	19.96
	16-oxo-PD	9.15	32.30	30.18	42.28	127.75	229.37	213.89
E	PE	55.85	130.65	109.53	86.74	24.88	55.52	45.18
	PD3	33.92	40.64	25.37	38.13	50.09	50.60	18.46
	PD	106.80	135.75	90.60	105.09	114.65	45.13	23.10
	16-oxo-PD	16.79	32.58	32.81	32.02	166.43	146.23	246.93
F	PE	73.82	70.08	26.97	62.72	31.81	26.50	39.78
	PD3	36.61	27.07	23.44	4.34	46.63	53.61	47.86
	PD	141.69	101.49	77.62	76.04	53.07	30.12	32.49
	16-oxo-PD	17.65	27.36	17.93	35.35	24.08	63.17	205.57
G	PE	78.94	92.60	73.50	72.45	42.34	34.33	41.54
	PD3	42.50	23.98	40.13	22.71	28.19	28.08	41.47
	PD	167.47	128.14	98.20	94.55	90.17	25.88	47.99
	16-oxo-PD	9.15	22.50	32.95	15.57	46.79	62.59	90.97
H	PE	55.85	101.45	32.95	71.24	39.04	56.02	38.10
	PD3	33.92	42.08	11.99	13.83	32.60	23.74	34.01
	PD	106.80	122.80	65.40	80.17	49.67	67.23	56.66
	16-oxo-PD	16.79	39.53	19.18	24.72	16.10	62.27	104.69
I	PE	73.82	78.84	44.09	75.18	54.87	56.16	43.63
	PD3	36.61	25.69	23.10	19.47	27.17	29.29	35.14
	PD	141.69	113.69	72.01	88.43	80.36	65.69	57.69
	16-oxo-PD	17.65	28.52	14.74	28.19	18.41	47.69	103.19

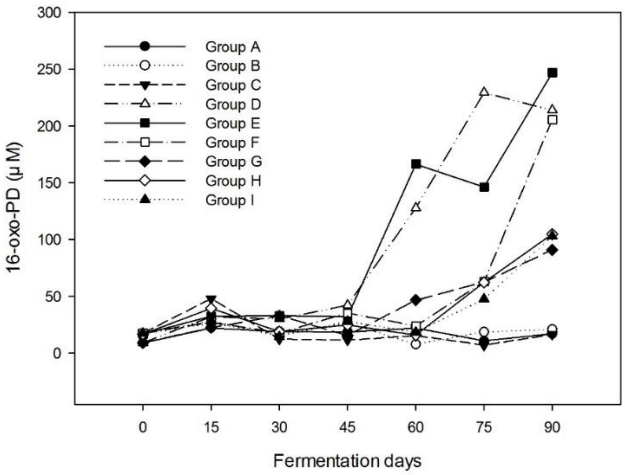

Figure 2. Changes of the 16–oxo–PD concentrations (μM) during fermentation.

Symbols: (●) Group A; (○) Group B; (▼) Group C; (△) Group D; (■) Group E; (□) Group F; (◆) Group G; (◇) Group H; and (▲) Group I (refer to Table 1 for details). The retention times of 16–oxo–PD are presented after 33.30 ± 0.41 min.

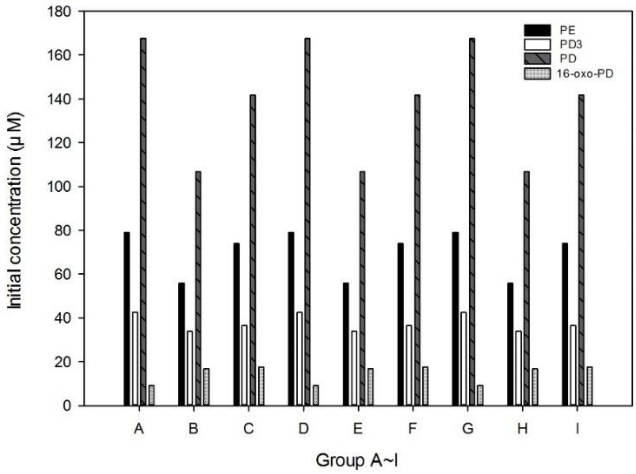

Figure 3. Initial concentrations of PE, PD_3, PD and 16–oxo–PD at the beginning of fermentation. The bars of PE (■), PD_3 (□), PD (▧) and 16–oxo–PD (▒) indicate the concentrations of the initial PR extracts, respectively (refer to Table 4 for details).

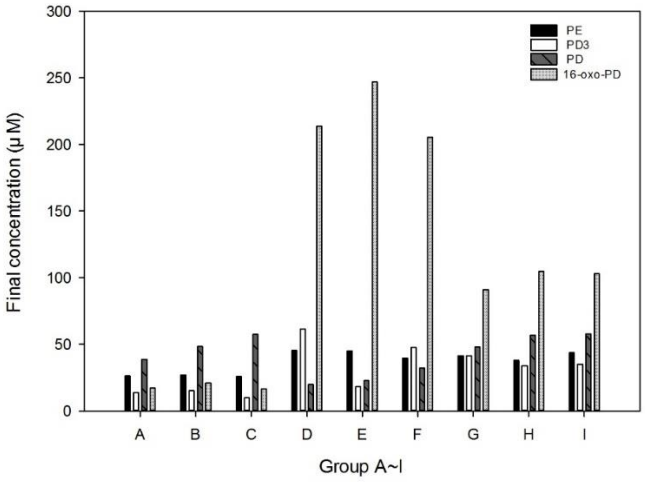

Figure 4. Final concentrations of PE, PD_3, PD and 16–oxo–PD at the end of fermentation. The bars of PE (■), PD_3 (□), PD (▧) and 16–oxo–PD (▒) indicate the concentrations of the fermented PR extracts, respectively (refer to Table 4 for details).

3.3. Ethanol Production from PR Extract

Natural foods containing saponins are increasingly recognized for their pharmaceutical effects and functional activities. The well-known medicinal herbs in Korea include the following: ginseng, gilkyung, sesame, jakyak, danggwi, and hwangki. Although medicinal herbs can be used as functional foods, consumption is not strongly advocated in Korea, due to their strong flavor. Enriching a good flavor by the process of fermentation has served to increase their value in the market place. Many of the active ingredients in medicinal herbs are glycosides, and they are converted into non–glycosides, which have greater bioavailability levels, through the fermentation process. For a variety of fermented foods, their development into alcoholic beverages has been carried out to help improve their taste. Traditional Korean wines that include medicinal herbs are usually made in one of two ways: fermenting rice wine with medicinal herbs or adding medicinal herbs to distilled liquor (35–39). In this study, unlike in existing methods, PR was used as the main ingredient for the fermentation of an alcoholic beverage.

The concentrations of ethanol produced during fermentation are presented in Fig. 5. The maximum ethanol yield was 12.5% (v/v) at 90 days in Group E. After 60 days, the ethanol production of Group E was greater than that of any of the other fermentation groups. From 0 to 15 days, the ethanol concentration under all fermentation conditions was less than 1% (v/v). All of the groups showed continuous increases in ethanol production up to 75 days during the fermentation period, with sharp increases starting at a time of 30 days after inoculation. For all of

the groups except Group I, the ethanol concentration appeared to increase only slightly after 75 days. The data shows that the end of the alcoholic fermentation period is 90 days after inoculation.

At the end of the fermentation period, the ethanol concentrations produced for each group were 3.9% for Group A, 4.3% for Group B, 4.8% for Group C, 10.3% for Group D, 12.5% for Group E, 9.6% for Group F, 5.9% for Group G, 6.5% for Group H and, 8.8% for Group I. Generally, the ethanol concentrations of Groups D, E, and F, which used aerobic fermentation conditions during the initial five days, were higher than those of the other groups. The ethanol concentrations of Groups A, B, and C, using single *S. cerevisiae* for fermentation, were less than 5% by the end of the fermentation period.

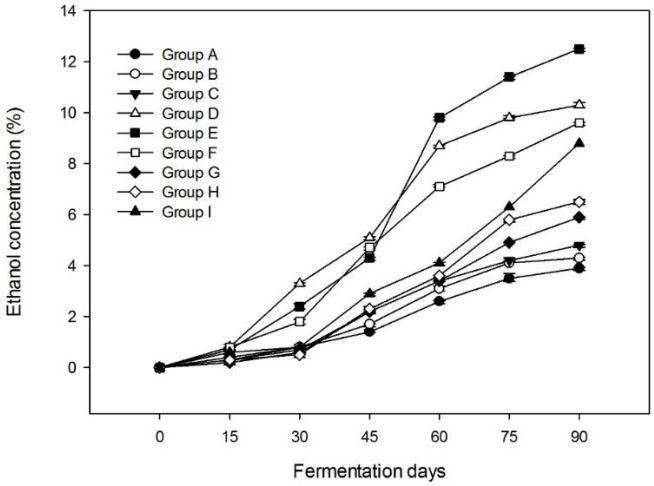

Figure 5. Changes of the ethanol concentration rate (%, v/v) during the alcoholic fermentation of PR extracts. Symbols: (●) Group A; (○) Group B; (▼) Group C; (△) Group D; (■) Group E; (□) Group F; (◆) Group G; (◇) Group H; and (▲) Group I (refer to Table 1 for details). The data are expressed as the averages of triplicate determinations. Bars of standard deviations are also represented.

3.4. Changes of pH in the fermented PR extract

A set of fermentation experiments were performed at same initial pH (5.0) to investigate the changes of pH in the fermented PR extracts, and they are presented in Fig. 6. The pH of all of the experimental groups gradually decreased during fermentation. By the end of the alcoholic fermentation period, the measured pH for each group was 3.34 for Group A; 3.41 for Group B; 3.38 for Group C; 3.95 for Group D; 4.08 for Group E; 4.35 for Group F; 4.34 for Group G; 4.27 for Group H; and 4.10 for Group I.

The pH of the fermentation process affects bacterial contamination in relation to the aging process (40) and the appropriate range of the pH is between 3.2 and 3.5 at the end of the alcoholic fermentation process (41).

With respect to storage, the pHs of Groups A, B, and C, using only *S. cerevisiae* for fermentation, were in the appropriate range. For most co–culturing groups (–Groups E, F, G, H, and I), except Group D, the pH of the PR extracts at the end were higher than 4.0. Therefore, they may need additional acid treatment after the end of batch fermentation to be suitable for long–term storage.

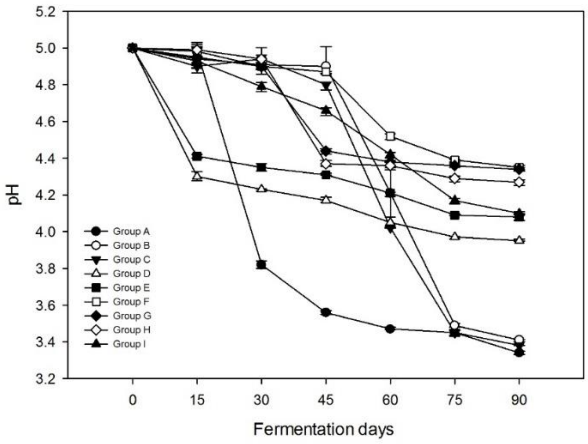

Figure 6. Changes of pH during the alcoholic fermentation of PR extracts. Symbols: (●) Group A; (○) Group B; (▼) Group C; (△) Group D; (■) Group E; (□) Group F; (◆) Group G; (◇) Group H; and (▲) Group I (refer to Table 1 for details). The data are expressed as average of triplicate determinations. Bars of standard deviations are also represented.

3.5. Changes of soluble solids (°Brix) during fermentation

The levels of the sugar used as a carbohydrate source were as follows: 30% (v/v) (Groups A, D, and G), 40% (v/v) (Groups B, E, and H), and 50% (v/v) (Groups C, F, and I). The initial soluble solids (°Brix) were measured as follows: 28.8° (Groups A, D, and G), 33.0° (Groups B, E, and H), and 37.4° (Groups C, F, and I). Fig. 7 shows the changes in the °Brix in the fermented PR extracts during fermentation. The °Brix in all of the experimental groups gradually decreased during fermentation, with a sharp decrease starting at 45 days after inoculation. At the end of the alcoholic fermentation period, the °Brix levels measured for each group were 24.1° for Group A, 26.3° for Group B, 30.2° for Group C, 11.5° for Group D, 13.4° for Group E, 21.1° for Group F, 18.3° for Group G, 23.7° for Group H, and 24.7° for Group I. The °Brix values of the samples at the end ranged from 11.5° to 30.2°. Under the same levels as the initial soluble solids (°Brix), Groups D, E, and F, which used aerobic fermentation conditions during the initial five days, were particularly lower than those of the other groups. In contrast, the °Brix levels of Groups A, B, C, which only used *S. cerevisiae* for fermentation, compared to the other groups, was decreased at a less degree than those of the other groups during the fermentation period.

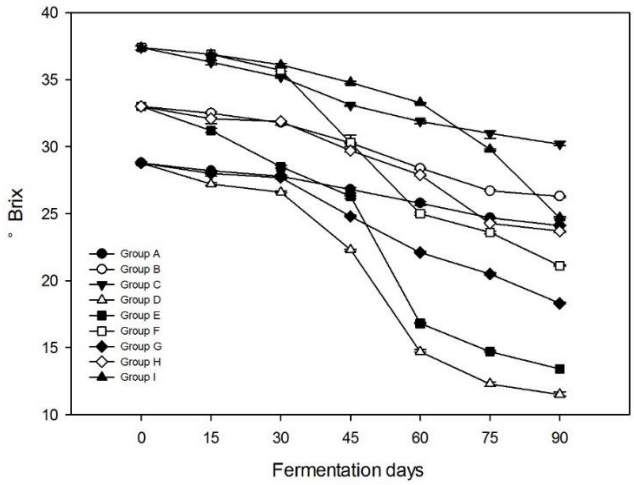

Figure 7. Changes of soluble solids (°Brix) during the alcoholic fermentation of PR extracts. Symbols: (●) Group A; (○) Group B; (▼) Group C; (△) Group D; (■) Group E; (□) Group F; (◆) Group G; (◇) Group H; and (▲) Group I (refer to Table 1 for details). The data are expressed as the averages of triplicate determinations. Bars of standard deviations are also represented.

3.6. Changes of total sugar during fermentation

The total sugar concentrations in the samples were measured by the phenol–sulfuric acid method, using glucose as a standard curve. The initial total sugar concentrations (g/L) were measured as follows, 286.6 g/L for Groups A, D, and G; 427.8 g/L for Groups B, E, and H; and 506.8 g/L for Groups C, F, and I. As shown in Fig. 8, the total sugar concentrations gradually decreased during fermentation. At the end of the alcoholic fermentation process, the total sugar concentrations measured for each group were 162.0 g/L for Group A, 245.4 g/L for Group B, 312.7 g/L for Group C, 50.0 g/L for Group D, 59.0 g/L for Group E, 143.5 g/L for Group F, 85.2 g/L for Group G, 155.9 g/L for Group H, and 191.9 g/L for Group I. The results showed that the decrement of groups D, E, and F, which used aerobic fermentation conditions over the initial five days, were noticeably higher than those of the other groups. In contrast, Groups A, B, and C, using only *S. cerevisiae* for fermentation, showed less of a reduction during fermentation compared to the other groups. To confirm the °Brix data, the sugar concentrations were also measured. These results showed that the concentration pattern was similar to the decreasing pattern of °Brix.

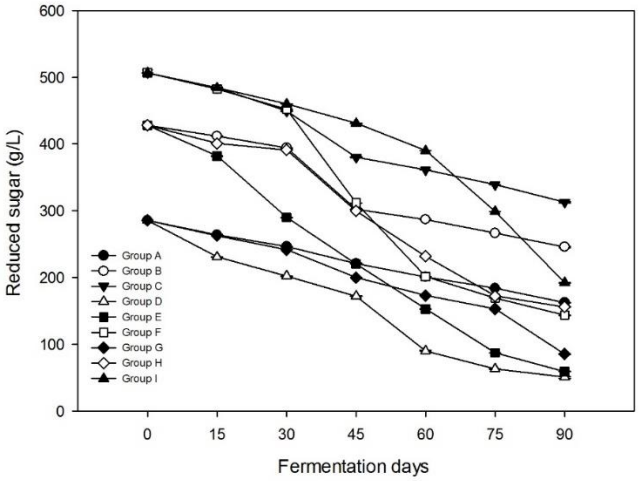

Figure 8. Changes in the total sugar (g/L) levels during the alcoholic fermentation of PR extracts. Symbols: (●) Group A; (○) Group B; (▼) Group C; (△) Group D; (■) Group E; (□) Group F; (◆) Group G; (◇) Group H; and (▲) Group I (refer to Table 1 for details). The data are expressed as averages of triplicate determinations. Bars of standard deviations are also represented.

3.7. DPPH radical scavenging activities

A damage by free radical is a major factor in toxicological processes, and DPPH has frequently been used to assess the free radical–scavenging activity of natural antioxidants (26). Fig. 9 shows the DPPH free radical scavenging activity of fermented PR extract at the end point of fermentation. Electron donating ability (EDA, %) determined for each groups were 47.25% for Group A; 48.17% for Group B; 48.47% for Group C; 58.17% for Group D; 69.48% for Group E; 77.45% for Group F; 60.33% for Group G; 65.11% for Group H; 65.76% for Group I; and 93.34% for Vitamin C (0.1mM). The EDA (%) values of using only *S. cerevisiae* group (group A, B, and C) were lower than that of co–culturing group (groups D, E, F, G, H, and I). So, co–culturing of *S. cerevisiae* with *A. awamori* might have increased antioxidant activities of PR wine.

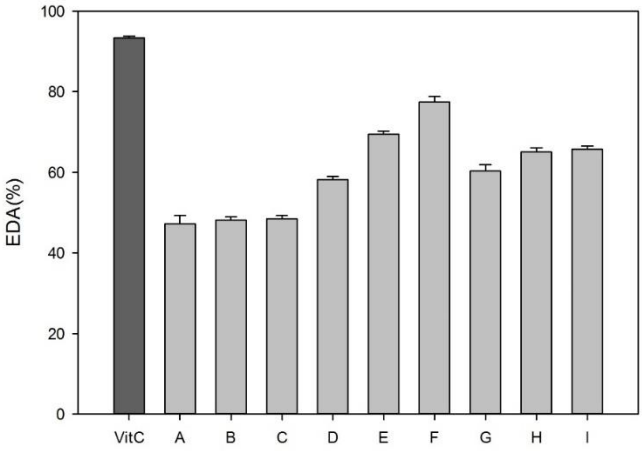

Figure 9. DPPH free radical scavenging activity of fermented PR extracts at the end of fermentation (refer to Table 1 for details).

3.8. Effect of co–culturing upon the production of fermented PR extract

3.8.1. Bioconversion of 16–oxo–PD and production of ethanol

The purpose of this study was to develop fermented PR products that contain high levels of 16–oxo–PD and ethanol. Figs. 1 and 4 show the 16–oxo–PD and ethanol content of the fermented PR extracts at the end of fermentation for each group. Interestingly, both the bioconversion of 16–oxo–PD and the production of ethanol were considerably enhanced by the co–culturing of *S. cerevisiae* with *A. awamori*. Above all, the production levels of 16-oxo-PD and ethanol in Groups D, E, and F, which used performed under aerobic fermentation conditions during the initial five days, were higher than those of the other groups. In contrast, Groups A, B, and C, using only *S. cerevisiae* for fermentation, were lower with regard to both their bioconversion of 16–oxo–PD and their production of ethanol. This shows that the aerobic growth of *A. awamori* during the initial fermentation period assisted with the bioconversion of platycoside and the production of ethanol. Among Groups D, E, and F, Group E showed particularly high efficiency.

3.8.2. Alcohol fermentation with different sugar concentration levels

Most alcoholic fermentation processes are usually run at a comparatively low carbohydrate concentration level to ensure speedy fermentation without inhibitory reactions caused by high sugar and alcohol levels. The four key factors of alcoholic fermentation are the sugar concentration, alcohol concentration, temperature, and duration. A high sugar concentration (30% and above) introduces a high level of osmolality stress, and a high concentration of alcohol inhibits the growth of the fermenting microorganisms (42). According to previous studies (43), a glucose concentration of either 40% or 50% w/w proved inhibitory to the growth of the yeast. At a glucose concentration of 30%, there is less inhibition of the fermentation process, while at 20% and 10%, alcoholic fermentation proceeds at the highest rate (42). These results suggest that it is important to find the optimum range of the initial sugar concentration during alcoholic fermentation. Looking at the relationship between temperature and duration, a higher temperature (23–28°C) generally causes a reduction of the fermentation period. However, fermenting at low temperatures (10−15°C) is a growing trend because it helps to retain volatile flavors and improve the wine's aroma, although it takes a longer fermentation time and produces a low concentration alcohol (44).

The aim of this study is to apply low–temperature fermentation and produce a high concentration of ethanol. Therefore, with the basal fermentation temperature set to 15°C, the three levels of sugar used as a carbohydrate source were as follows: 30% (w/w) (Groups A, D, and

G), 40% (w/w) (Groups B, E, and H), and 50% (w/w) (Groups C, F, and I). To assess the effect of the initial sugar concentration, the study compared the ethanol production levels in three different alcoholic fermentation systems: (A) using *S. cerevisiae* as the sole fermentation microorganism, (B) the co–culturing of *S. cerevisiae* with *A. awamori* performed under aerobic fermentation conditions to activate *A. awamori* during the initial five days, and (C) the co–culturing of *S. cerevisiae* with *A. awamori*. Comparing the alcohol production levels at the end of the fermentation process, system B was found to be the highest, followed by system C and then system A. With the same initial sugar concentrations, the co–culturing of *S. cerevisiae* with *A. awamori* produced more alcohol than the single culture of *S. cerevisiae*. Judging from the alcohol concentration produced, the co–culturing of *S. cerevisiae* with *A. awamori* was less inhibited by the concentration of sugar.

In addition, *S. cerevisiae* cannot tolerate alcohol beyond levels of around 12–15%. Therefore, natural wines generally produce 9–13% alcohol from fermentation (45). The alcohol concentration of system B was within this range (9–13%). In particular, Group E's initial sugar concentration at 40% (v/v) resulted in one of the highest alcohol concentration (12.5%). Contrary to the findings of a previous study (41), which showed that a sugar concentration of 30% gave the highest rate of alcoholic fermentation, the present result showed that it is possible to produce more alcohol using high sugar concentration levels (30% and above) by the co–culturing of *S. cerevisiae* with activated *A. awamori*. The potential use of such a co–culturing system for the production of

fermented PR wine with high levels of alcohol concentration would be of considerable economic interest. Further investigations are needed for a more detailed optimization of the initial sugar concentration and to determine aroma enhancing effects through low-temperature fermentation.

4. Conclusion

This study could establish that both bioconversion of 16–oxo–PD and production of ethanol through the fermentation process of PR extract by using co–culture of *Saccharomyces cerevisiae* KCTC 7928 and *Aspergillus awamori* FMB S900. The fermented PR product containing bioconverted platycoside was successfully produced by optimizing fermentation conditions and it contained high levels of bioconverted platycoside and ethanol. The process with optimized fermentation conditions described in the paper could be used for scaling up. Further investigations would be needed to know more detailed optimization of initial sugar concentration.

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국문 초록

도라지(*Platycodon grandiflorum* A. DC.)는 한국, 일본, 중국 동부 시베리아에서 자생하며 초롱꽃목 초롱꽃과(Campanulaceae)에 속하는 다년생 초본식물이다. 특히 도라지의 뿌리 부분인 길경은 전통적으로 폐질환, 가래, 기침, 천식, 진통, 해열 등의 증상에 많이 사용되어온 약초로, 주요 활성물질은 사포닌 계열의 화합물로 알려져 있다. 도라지 내 사포닌 함량은 2% 내외, 종류는 20 여 가지 이상으로 알려져 있다. 이 중에서 많이 연구되어온 물질은 platycoside 와 polygalacin 인데, 당 잔기, 작용기의 차이에 따라 다양한 형태로 존재한다. Platycoside 는 oleanane-type triterpenoid backbone structure 에 2 개의 side chain 이 연결된 구조인데 사포닌 화합물에서 구조, 작용기, 결합된 당의 종류와 수, 결합의 형태 및 다른 배당체와 상호작용 등은 이들 물질을 섭취했을 때 생체이용능 및 생리활성에 영향을 미치는 주요 인자이다. 한편, 대부분의 생리활성 배당체는 동물의 장 내에 존재하는 수많은 미생물들이 지니고 있는 효소에 의해 비배당체로 전환되어 흡수된다. 이때, 기질에 포함된 당의 종류, 결합된 형태 및 섭취자의 장내 미생물 환경에 따라 이들 배당체의 전환효율은 변화하기 때문에, 생리활성 역시 달라진다. 배당체의 비배당체로의 전환 혹은 구조, 작용기의 변환을 통한 이용효율의 향상은 고온, 고압 처리나 산, 알칼리 용액을 통한 가수분해 등과 같은 물리, 화학적 방법을 통해 이루어져 왔다. 이러한 처리를 통해 만들어진 비배당체 산물은 비특이적인 반응 특성으로 인해, 원하는 활성 물질의 생산을 분석하고 조절하는데 어려움이 있으며, 부가적으로 발생할 수 있는

독성물질의 제어에 제약이 있는 한계가 있다. 하지만 이들 배당체를 특이적으로 전환할 수 있는 효소 혹은 특정한 균과 곰팡이 등을 통해 전환 또는 발효한 경우, 목적으로 하는 비배당체를 선택적으로 생산하는 것이 용이하며, 비차별적인 화학결합의 파괴로 인한 독성물질의 생산으로 인해 식품의 안전성이 위협받는 것을 막을 수 있는 장점이 있다. 따라서 본 연구에서는 미생물을 이용하여 생물전환된 사포닌을 함유하는 도라지 발효주를 개발하여 제공하고 platycoside 전환능과 알코올 생산성이 높은 최적 조건을 알아보고자 하였다.

발효하는 동안 platycoside 전환능과 알코올 생산성이 우수한 조건은 다음과 같다: *S. cerevisiae* KCTC 7928 과 *A. awamori* FMB S900 의 공동발효 조건 하에 발효 초기 5 일 동안 25℃, 120 rpm 에서 호기 조건을 조성해 준 후 0℃, 0 rpm, 혐기 조건에서 85 일을 발효시켰을 때 수율이 가장 높았다. 그 중에서도 초기 설탕 농도가 40% (v/v) 조건에서 전환된 16-oxo-PD 의 양이 가장 많았고 알코올 생산량도 12.5%로 가장 높았다.

본 연구를 통해 생물 전환된 platycoside 를 함유한 도라지주가 발효 조건의 최적화에 의해 성공적으로 생산되었고, 이는 도라지를 이용한 발효식품의 개발에 활용될 수 있을 것이다.

주요어 : 도라지, 생물전환, 알코올 생산, 공동발효

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