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Optimization of Enzymatic Pretreatment for the Production of Fermented Ginseng using Leaves, Stems and Roots of Ginseng

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Abstract : This study sought to optimize the extraction and enzymatic treatment conditions of *Panax ginseng* leaves, stems, and roots for the production of fermented ginseng. The optimization enhanced the extraction of total saccharide, a nutrient and growth-activating factor for *Lactobacillus* bacteria. The hydrolysis of ginseng leaves, stems, and roots was tested with eight enzymes (Pentopan, Promozyme, Celluclast, Ultraflo, Pectinex, Ceremix, Viscozyme, and Tunicase). The enzymatic hydrolysis conditions were statistically optimized by the experimental design. Optimal particle size of ginseng raw material was <0.15 mm, and optimal hydrolysis occurred at a pH of 5.0–5.5, a reaction temperature of 55–60°C, a Ceremix concentration of 1%, and a reaction time of 2 hr. Ceremix produced the highest dry matter yield and total saccharide extraction. Ginseng leaves were found to be the most suitable raw material for the production of fermented ginseng because they have higher carbohydrate and crude saponin contents than ginseng roots

Key words : enzyme treatment, ginseng, leaves, stems, roots

INTRODUCTION

Korean ginseng belongs to the *Panax* genus in the family Araliaceae. It grows primarily in northern China, Korea, and eastern Siberia and is a popular herbal remedy that has been used in Asia for several thousand years. Studies have demonstrated the beneficial effects of ginseng in treatment of diabetes [1], the central nervous system [2], cancer [3], the immune system [4], blood pressure [5], inflammation [6], and allergies [7, 8]. Saponins (ginsenosides) are considered to be the main active polysaccharide component of ginseng, and more than 40 ginsenosides have been identified [9-11]. Non-saponin physiological components of ginseng include polyacetylene, phenolic compounds, acidic polysaccharides, peptides, and alkaloids [9-11]. Human health is increasingly threatened by factors related to industrialized civilization, environmental pollution, increased income, rising standards of living, overnutrition, and increased fat intake. Health foods and herbal medicines have therefore attracted increasing attention.

Recent studies have employed new technologies to maximize the herbal benefits of ginseng, and have focused on the development of new ginseng health products [12-14]. Fermented ginseng has received particular attention because it contains structurally modified ginsenosides that enhance the herb's beneficial effects. Structural modifications can be achieved with treatments that employ heat [15,16], mild acids [17,18], alkalines [3,19], and micro-organic enzymes. For example, ginsenoside Rb1 has been transformed to Rd in *Rhizopus sp.* with enzyme treatment [20], ginsenoside Rb_1 has been hydrolyzed by β-glucosidases from human intestinal bacteria [21, 22], gypenoside-5 has been hydrolyzed into Rd by gypenoside-α-L-rhamnosidase [23], and the ginsenosides Re and Rg1 have been hydrolyzed by lactase from *Penicillium sp.* into 20(S)-ginsenoside Rh_1 [6-O-beta-D-glucopyranosyl-20(S)-protopanaxatriol] [24]. Dammarane-type triterpenoid skeletons in ginseng saponins can be modified by saccharides such as glucose, arabinose, and xylose, producing various saponins depending on the method of

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modification.

The active compounds in orally administered ginseng must be degraded in the digestive system to yield the desired pharmacological effects. Polysaccharides obtained from the plant can be metabolized by the enzymes of human intestinal bacteria such as *Lactobacillus* [25], but not by human digestive enzymes. Furthermore, the active compounds in ginseng are not simply saponins, but saponin metabolites hydrolyzed by intestinal bacteria [25]. The metabolic pathway and absorption rate of ginseng saponins therefore depend on various factors of the intestinal bacteria [26]. Bacterial or enzymatic transformation of saponin can be induced by a process in which bacterial â-glucosidase hydrolyzes the saccharides of saponin. Each bacterial hydrolytic enzyme is characterized by a specific saponin substrate and preferred cleavage site [27].

Unfortunately, most studies of ginseng leaves and stems, the by-products of ginseng processing, have been limited to the simple analysis of ginsenoside content and nutritional factors [28, 29]. Some recent studies, however, have focused on the pharmacological effects of compounds in ginseng leaves and stems. This research has revealed that ginseng leaves have a higher saponin content than that of the roots (~4 to 5 times) and stems (>9 times) [30-33]. The efficient use of these ginseng by-products has therefore become a matter of interest. Fermented ginseng production has traditionally used only the roots, but we expect that the leaves and stems may also be very useful. Treatment of enzymes before fermentation may also improve the efficiency of fermented ginseng production.

The production of fermented ginseng has primarily employed *Lactobacillus*, which has important nutritional requirements. The components in ginseng must be hydrolyzed to allow their efficient uptake as a nutritional source. The present study thus aimed to optimize hydrolysis conditions to maximize the extraction of total saccharide in the leaves, stems, and roots of ginseng.

MATERIALS AND METHODS

Ginseng and reagents

The leaves, stems, and roots of 6-year-old *Panax ginseng* plants were provided by the Gimpo Agricultural Technology Center (Gimpo-si, Gyeonggi-do, Korea). Pentopan, Promozyme, Celluclast, Ultraflo, Pectinex, Ceremix, and Viscozyme carbohydrate hydrolases were purchased from Nordisk A/S (Copenhagen, Denmark), and Tunicase of *Athrobacter sp.* ATCC 21712 was obtained from Diawa Kasei (Okazaki, Japan). All other reagents used in this study were of analytical grade.

Determination of the contents of proximate compositions and crude saponin

The contents of proximate compositions (i.e., moisture, ash, crude lipid, crude protein, and crude fiber) were determined according to recommended protocols in the 'Food Code' [34], using the air-oven method, dry ashing Soxhlet extraction, the semimicro-Kjeldahl method with a 6.25 nitrogen coefficient [35], and the Henneberg-Stohmann method, respectively. Carbohydrate content was then calculated by subtracting the sum of crude protein, total fat, moisture, and ash from the total weight of the sample. Crude saponin content was measured using the analytical protocol for red ginseng compounds described in the 'Food Code' [34]. Each 5 g sample of ginseng leaves, stems, and roots was suspended in 50 ml of water-saturated butanol. Extraction was performed three times at 85°C for 1 hr in a water bath (SWB-10, Jeio Tech, Korea). All extracts were combined and centrifuged at 3,200 rpm (1,969 x g) for 10 min using a Union 55R centrifuge (Hanil, Korea). Distilled water (50 ml) was added to the supernatant, and the mixture was incubated at room temperature for 6 hr. After removing the water layer, extracts in the water-saturated butanol were concentrated with a vacuum evaporator at 80°C. Ether (50 ml) was added to the extracts and they were incubated at 46°C for 30 min. The extracts were then dried at 105°C for 20 min, and dry weight was measured to calculate the crude saponin content.

Enzymatic treatment

Ginseng leaves, stems and roots were pulverized using a KT-34 machine (Korea Medi Co., Ltd., Korea), and separated by particle size (<0.15 mm, 0.15–0.18 mm, and 0.18–0.28 mm). Distilled water containing 6% (w/v) pulverized ginseng was mixed with 1% (w/w) polysaccharide hydrolase, and incubated at 40–50°C for 2 hr in a water bath (SWB-10). After enzymatic hydrolysis, the mixture was centrifuged at 3,200 rpm (1,969 x g) for 15 min using a Union 55R centrifuge, and the dry matter yield and total saccharide in the supernatant were determined. The characteristics of the eight carbohydrate hydrolases used in this study are summarized in Table 1.

Experimental design to optimize extraction

To optimize the enzymatic hydrolysis conditions for extracting total saccharide from ginseng leaves, stems,

Table 1. Characteristics of the enzymes used for the hydrolysis of ginseng leaves, stems, and roots

Enzyme	Type	Enzyme characterization	Optimum conditions	
			pH	Temp (°C)
Pentopan	Mono	Xylanase	5.5	55
Promozyme	Mono	Pullulanase	5.5	55
Cellulclast	Mono	Cellulase	5.0	55
Ultraflo	Complex	β-glucanase, cellulase xylanase, pentosanase, arabanase	6.5	60
Pectimex	Complex	pectin transeliminase, cellulase hemicellulase, polygalacturonase	5.0	55
Ceremix	Complex	β-glucanase, cellulase, pentosanase proteinase, α-amylase	5.0	60
Viscozyme	Complex	β-glucanase, cellulase hemicellulase, zylanase, arabanase	5.0	50
Tunicase	Mono	β-glucanase	7.0	50

and roots, we formulated an experimental design based on the central composite design of the response surface method (RSM) using Design Expert 6.0 software (Stat-ease Inc., MN, USA).

Determination of dry matter yield and total saccharide

Dry matter yields in the hydrolase-treated extracts of ginseng leaves, stems, and roots were determined using a refractometer (Model N.O.W. 507-1, Nippon Optical Works, Tokyo, Japan). Total saccharide was determined with the phenol-sulfuric acid method [36]. Briefly, 0.6 ml of ginseng extract was mixed with 0.3 ml of 5% phenol and 1.5 ml of concentrated sulfuric acid, and incubated at 85°C for 30 min in a water bath (SWB-10). After cooling to room temperature for 5 min, 0.2 ml of the mixture was transferred to a 96-well plate, and absorbance was measured at 490 nm using a Versa Max microplate reader (Molecular Devices**,** CA, USA). Standard curves were obtained by using D-glucose as a reference standard for colorimetric analysis.

RESULTS AND DISCUSSION

Determination of the contents of proximate compositions and crude saponin

Proximate composition and crude saponin contents were determined for the leaves, stems, and roots of 6-year-old ginseng plants cultivated in Gimpo, Korea. The total carbohydrate content of ginseng leaves was 69.0%, that of stems was 82.6%, and that of roots was 80.2% (Table 2). Kim *et al.* [37] analyzed the leaves, stems and roots of 6-year-old ginseng plants cultivated in the Jeung Pyeong open test field of the KT & G Central Research Institute, finding total carbohydrate contents of 55.4%, 36.1%, and 65.9%, respectively. Our results show that each ginseng element may contain much higher carbohydrate levels than those found by Kim *et al.* [37]. We found a much higher crude saponin content in ginseng leaves (24.8%) than in stems (4.6%) and roots (5.3%), also inconsistent with the results of Kim *et al.* [37] (Table 3). Furthermore, Chang *et al.* [33] reported that dry ginseng-leaf tea contained high levels of crude saponin, ranging from 6.86% to 7.5% depending on the manufacturing pro-

Table 2. Approximate compositions of ginseng leaves, stems, and roots (dry basis, %)

Components	Leaves	Stems	Roots
Carbohydrate	69.0±0.04	82.6±0.02	80.2±0.05
Crude protein	15.5±0.06	8.1±0.06	12.5±0.04
Crude lipid	5.4±0.13	1.2±0.04	2.0±0.01
Crude ash	10.0±0.05	8.0±0.06	5.5±0.05

Table 3. Crude saponin content (total oligosaccharide) of ginseng leaves, stems, and roots (dry basis, %)

Components	Leaves	Stems	Roots
Crude saponin	24.8	4.6	5.3
Total oligosaccharide	0.9	2.0	3.2

cess. Our findings clearly show that ginseng leaves contain high amounts of carbohydrates and crude saponin, and also suggest that the leaves may be a better source for fermented ginseng produced by enzymatic treatment and *Lactobacillus*. We therefore investigated the use of ginseng leaves in the production of fermented ginseng.

Effects of enzyme treatment on total saccharide extraction

To optimize the concentration of a ginseng source for enzyme treatment, we determined the crude polysaccharide and total carbohydrate contents obtained from various concentrations of ginseng leaves, stems, and roots. Tark *et al.* [38] extracted high amounts of saccharide from a concentrated ginseng source, although the yield of this extraction was remarkably decreased. Our study also confirmed that the extraction of crude saccharide varied in direct relation to the concentration of ginseng leaves, stems, and roots (data not shown). Total saccharide extraction was maximized with a 6% concentration, but did not increase with concentrations >6% (data not shown).

To optimize conditions for total saccharide extraction from ginseng leaves, stems, and roots, pulverized ginseng was incubated at 50°C for 2 hr with eight different carbohydrate hydrolases [final conc. 1% (v/v)]. After the hydrolytic process, microwave treatment was performed for 4 min, and dry matter yield and total saccharide extraction were determined (Fig. 1). The highest dry matter yield was obtained with Ceremix (β-glucanase, cellulase, pentosanase, protensase, and α-amylase), although this difference was not statistically significant. Ceremix increased the total saccharide yield more than 1.4-, 1.7-, and 2.2-fold (*vs.* control group) in ginseng leaves, stems, and roots, respectively.

To investigate whether particle size affected dry matter yield and total saccharide extraction, ginseng root samples with different particle sizes were divided into two groups. One set of samples was treated with Ceremix and the other was left untreated as a control. Ceremix treatment increased the total saccharide yield about two-fold in ginseng roots. Particle size did not significantly affect yield in either the Ceremix or control group (Fig. 2). The dry

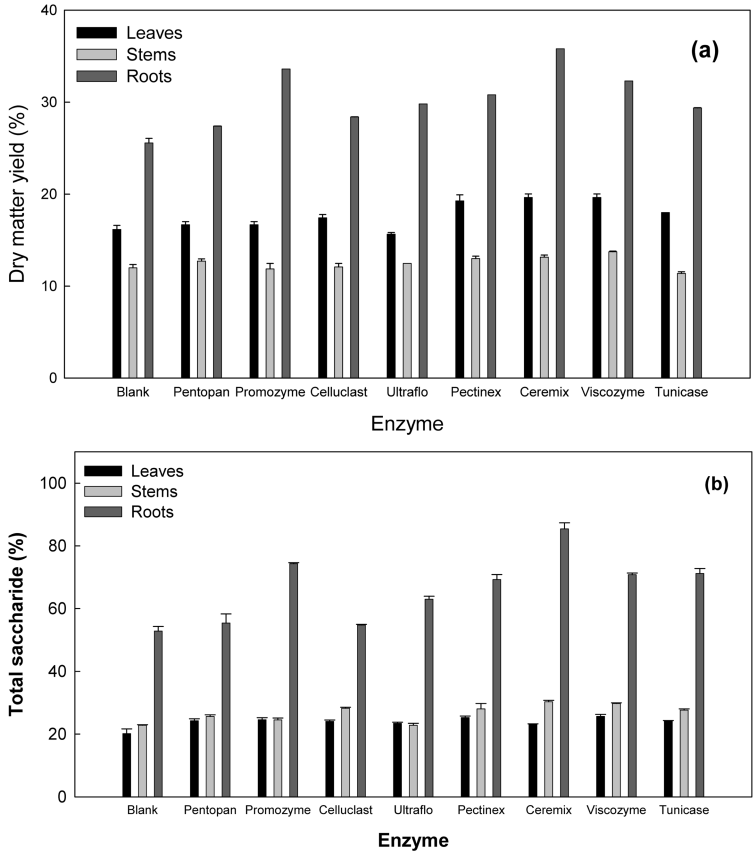

Fig. 1. The effects of enzyme type on dry matter yield (a) and total saccharide extraction (b) from ginseng leaves, stems, and roots.

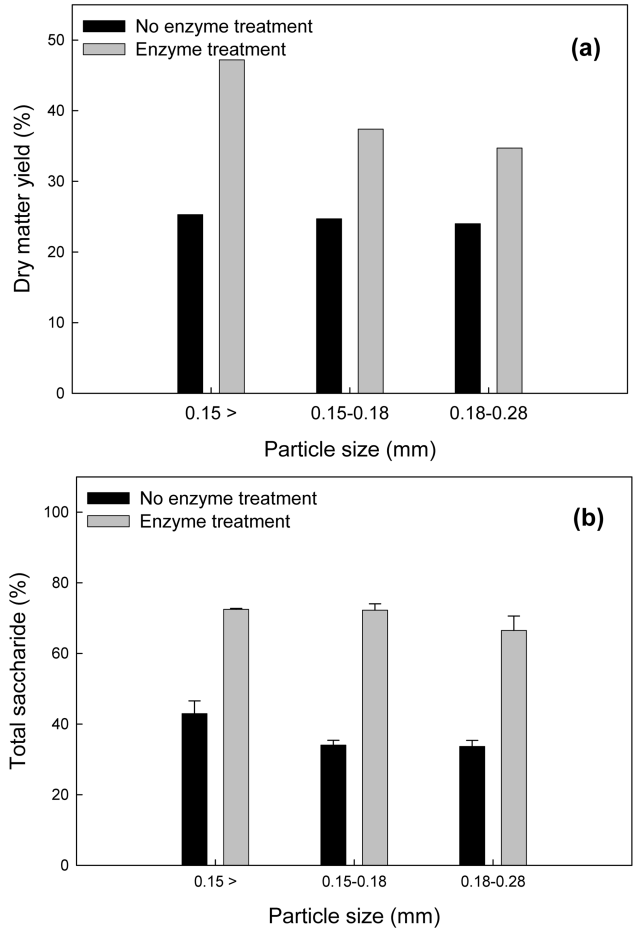

Fig. 2. The effects of ginseng particle size on dry matter yield (a) and total saccharide extraction (b) from ginseng roots.

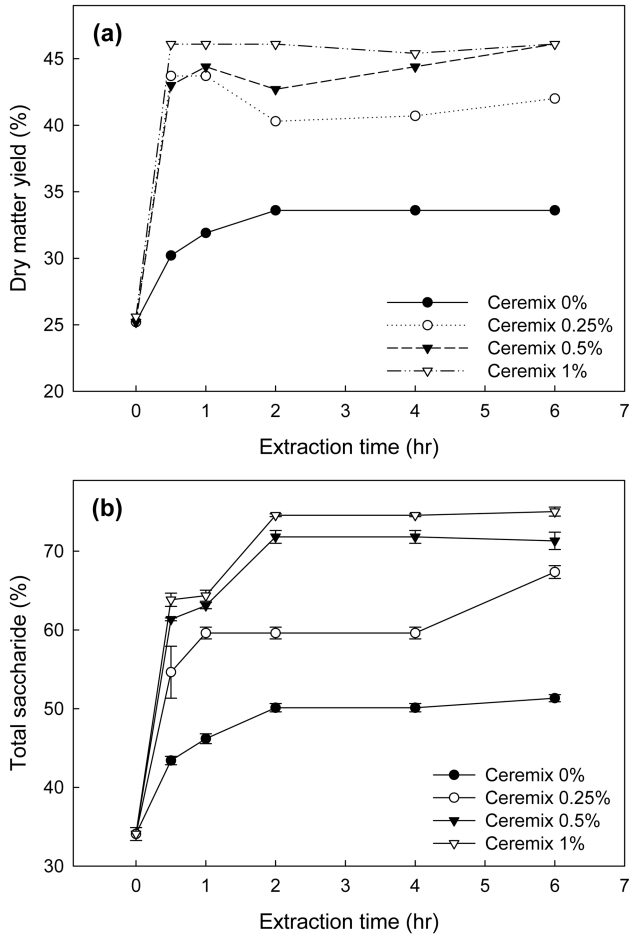

Fig. 3. The effects of extraction time and Ceremix concentration on dry matter yield (a) and total saccharide extraction (b) from ginseng leaves, stems, and roots.

matter yields of Ceremix-treated ginseng roots with particle sizes of 0.18–0.28 mm, 0.15–0.18 mm, and <0.15 mm were 34.7%, 37.4%, and 47.2%, respectively (Fig. 2). Control samples with corresponding particle sizes had dry matter yields of 24.0%, 24.7%, and 25.3%, respectively (Fig. 2). Small particle size (<0.15 mm) may thus increase dry matter yields. These results are consistent with those of Cho *et al.* [39], who showed that the use of red ginseng powder reduced extraction time and increased extraction yield.

We also investigated the effects of extraction time and Ceremix concentration on dry matter yield and total saccharide extraction. Pulverized ginseng leaf, root, and stem samples with small particle sizes (<0.15 mm) were suspended in distilled water [final conc. 6% (w/v)] and incubated with different concentrations of Ceremix (0%, 0.25%, 0.5%, and 1%) at 50°C for up to 6 hr. The ginseng mixture was centrifuged every hour after enzyme treatment, and the supernatant was used to calculate dry matter yield and total saccharide extraction. At an extraction time of 1 hr, the sample treated with 1% (w/w) Ceremix produced a 25% dry matter yield and a 35% total saccharide extraction. An additional 1 hr of extraction time significantly increased both dry matter yield (46%) and total saccharide extraction (64%) (Fig. 3), but further extraction time did not produce additional increases. While overall yield may initially be related to extraction time, longer extraction time does not necessarily increase the efficiency of the process. In fact, Lee *et al.* [40] showed that two to three repetitions of a 1–2 hr extraction time may produce the most efficient yield. Based upon our results, we set a single 2-hr extraction time for the following experiments.

Experimental design to optimize extraction

We used an RSM-generated model to investigate the

Table 4. Response surface central composite design for the enzymatic hydrolysis of ginseng leaves, stems, and roots

Factor	Parameter	Level	Low Level	High Level	Standard Deviation
A	Temperature	45	30	60	0
B	pH	5	4	6	0
C	Enzyme concentration	1	0.5	1.5	0

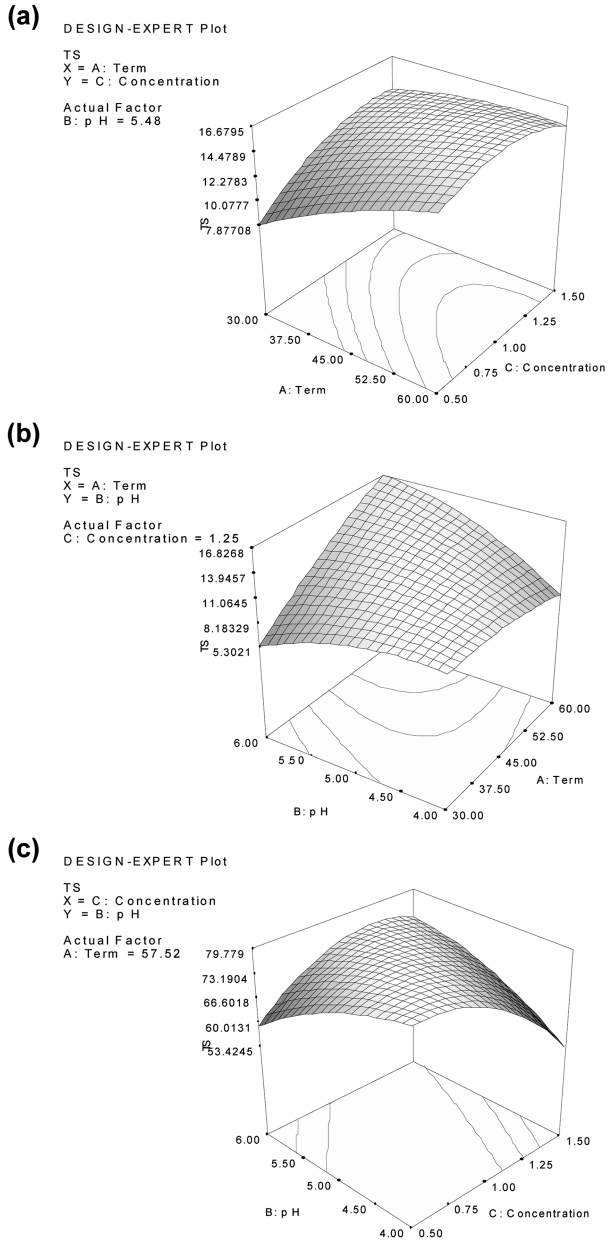

Fig. 4. Response surface for total saccharide recovery as a function of extraction temperature (X_1), enzyme concentration (X_2), and pH (X_3) using ginseng leaves (a), stems (b), and roots (c).

optimal conditions for extracting total saccharide from ginseng leaves, stems, and roots. Extraction temperature (X_1, 30–60°C), Ceremix concentration (X_2, 0.5–1.5%), and pH of the extracting solution (X_3, 4–6) were treated as dependent variables, while total saccharide was the independent variable (Table 4). Analysis of variance (ANOVA) was used to examine the interaction between dependent and independent variables (Fig. 4). The total saccharide extracted from ginseng leaves was 11.10–16.78%, that extracted from stems was 6.04–13.58%, and that extracted from roots was 48.44–75.73%. A Ceremix concentration of 1% (w/w), extracting solution pH of 5.0–5.5, and extracting temperature of 55–60°C were identified as the optimum conditions of enzymatic pretreatment.

In summary, this study showed that Ceremix was the most efficient of the eight carbohydrate hydrolases we tested for the extraction of total saccharide from ginseng leaves, stems, and roots. We optimized the Ceremix treatment conditions for the production of fermented ginseng. However, the effects of this optimized pre-treatment process on *Lactobacillus* fermentation and the overall efficiency of the fermented ginseng production process should be further evaluated in future studies.

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