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Production of Hydrolyzed Red Ginseng Residue and Its Application to Lactic Acid Bacteria Cultivation

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Enzymatic treatment conditions for red ginseng residue (RGR) were investigated to apply RGR as a microbial medium. Polysaccharide hydrolyase and protease were screened to obtain high solid and carbohydrate yields, and a good degree of carbohydrate hydrolysis. The optimal dosage and reaction time for Viscozyme, the chosen polysaccharide hydrolyase, were found to be 1.0% (w/w) and 3 h, respectively. Of the tested proteases, Flavourzyme, whose optimal dosage was 0.5% (w/w), was selected. Co-treatment with the optimal dosages of Flavourzyme and Viscozyme increased solid yield, carbohydrate yield, and degree of carbohydrate hydrolysis by 76%, 65%, and 1,865%, respectively, over levels in non-treated RGR. The culture characteristics of *Leuconostoc mesenteroides* strain KACC 91459P grown in enzymatically hydrolyzed red ginseng residue (ERGR) and RGR suspensions were compared. After cultivation for 6 h, the viable cell counts of both cell suspensions rapidly increased to 1.3×10^9 colony-forming units (CFU)/g. Moreover, while the viable cell population drastically decreased to 2.4×10^6 CFU/g for cells grown in RGR medium, it was maintained in cells fermented in ERGR medium for 24 h.

Keywords: Enzymatic hydrolysis, Lactic acid bacteria, Microbial medium, Red ginseng residue

INTRODUCTION

In Asia, ginseng (*Panax ginseng* C. A. Meyer) has long been used as a traditional herbal medicine for the prevention and cure of various diseases. Functional compounds in ginseng showing biological activity include ginsenosides (saponin), acidic polysaccharides, peptides, polyacetylenes, alkaloids, and phenolic compounds [1]. To extend its storage period and enhance it's efficacy, raw ginseng is often processed into white and red ginseng products in the Korean ginseng industry. Red ginseng is known to be more pharmaceutically active than white ginseng because of its higher content of specific ginsenosides such as Rg_3, Rh_2, and Rb_2 [2,3]. Among the processed products derived from red ginseng, the aqueous extract of red ginseng is the most important due to its higher applicability in the food industry. The water-soluble components, which mainly consist of ginsenosides and acidic polysaccharides, are recovered by direct extraction from red ginseng in hot water or a mixture of water and ethanol [4,5]. The water-insoluble part of red ginseng, red ginseng residue (RGR), is the by-product of this extraction process and is generally discarded as waste, even though it contains components with biological activities [6]. In previous studies, RGR has been utilized as a foodstuff and culture medium additive, and applied to the recovery of bioactive compounds such as acidic polysaccharides and lipid-soluble components [7-9].

Recently, microbial methods have been used in the

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transformation of medicinal herbs. In particular, a great deal of attention has focused on the fermentation of ginseng. Fermentation of red ginseng by intestinal microorganisms transforms ginsenosides such as Rb_1, Rb_2, Rc, and Rd into readily absorbable low-molecular-weight active products such as compound K, which is active against cancer and diabetes, and in immune stimulation [10-12]. Lactic acid bacteria, which are very safe but fastidious microorganisms with complicated nutritional requirements, have been highly applied in the production of fermented red ginseng. For efficient cultivation of lactic acid bacteria during ginseng fermentation, partial enzymatic degradation of ginseng constituents and subsequent use of the resulting hydrolysates as nutritional sources is preferable to supplementation with additional nutrients. Ceremix, a mixture of commercial polysaccharide hydrolases, was reported to be a suitable enzyme in the extraction of total saccharides from ginseng leaves, stems, and roots for use as a pretreatment for the production of fermented ginseng [13]. From an economical viewpoint, enzymatic treatment of ginseng residue may be preferable to similar treatment of whole ginseng roots for the supplementation of nutrients. However, to our knowledge, the enzymatic hydrolysis of RGR and use of the resulting hydrolysate have not before been studied, although a few reports have described the enzyme treatment of RGR for the purpose of extracting acidic polysaccharides [8].

In an attempt to apply RGR as a stand-alone microbial medium without any added nutrients, we explored the conditions for extracting total sugars from RGR through treatment with polysaccharide hydrolases and proteases, and compared the characteristics of lactic acid bacteria cultured in RGR and enzymatically hydrolyzed red ginseng residue (ERGR).

MATERIALS AND METHODS

Materials

RGR from ethanol extraction was provided by Greenbio Co. (Icheon, Korea) and ground to a particle size of less than 500 μm. Commercial polysaccharide hydrolases (Celluclast, Ceremix, Fructozyme, Pectinex, Ultraflo, and Viscozyme) and proteases (Alcalase, Flavourzyme, Kojizyme, Nutrase, and Protamex) were purchased from Novozyme (Bagsvaerd, Denmark). *Leuconostoc mesenteroides* (*L. mesenteroides*) KACC 91459P was obtained from the Korean Agricultural Culture Collection (Suwon, Korea) [14]. All other reagents were of analytical grade.

Enzyme treatments

RGR powder was suspended in distilled water at a concentration of 10% (w/v). After its pH was adjusted with 1 N NaOH or HCl such that it was optimal for each polysaccharide hydrolase or protease, the resulting RGR suspension was mixed with enzymes (1% [w/w] [based on the RGR powder weight]) and incubated at 50°C for 3 h in a shaking water bath. After enzymatic hydrolysis, the suspensions were boiled for 20 min and centrifuged at 2,000× g for 5 min. The resulting supernatant was analyzed for solid and carbohydrate contents.

Microbial cultivation

The RGR suspension was simultaneously incubated with Viscozyme and Flavourzyme for 3 h and then autoclaved at 121°C for 15 min. The resulting ERGR suspension (without any added nutrients) was used as a lactic acid bacteria culture medium. *L. mesenteroides* KACC 91459P was precultured in Lactobacilli MRS broth (Difco Laboratories, Detroit, MI, USA) at 30°C for 15 h on a rotary shaker with agitation (150 rpm). Cells were harvested from the preculture broth by centrifugation at 3,000× g for 15 min and washed twice with physiological saline. The washed pellets were suspended in a volume of physiological saline equivalent to that of the preculture broth. ERGR medium was then inoculated with washed *L. mesenteroides* KACC 91459P cell suspension (5% [v/v]). The cells were then cultured for 24 h under the same conditions used for the preculture. The pH of the fermented ERGR broth was measured using a 720P pH meter (Istek, Seoul, Korea). Titratable acidity was determined by titrating 5 g of the sample with 0.01 N NaOH using phenolphthalein as an indicator. Results were expressed as percentages of lactic acid. *L. mesenteroides* KACC 91459P growth was assessed by counting viable cells after plating the culture on MRS agar plates. The plates were incubated at 30°C for 36 h, and the culture density was estimated in terms of colony-forming units (CFU).

Analysis

RGR moisture, ash, crude lipid, and crude protein contents were determined according to the recommended protocols in the Korean Food Code (Korea Food and Drug Administration) [15]. The carbohydrate content was calculated by subtracting the moisture, ash, crude lipid, and crude protein weights from the total weight of the sample. Total solid, total carbohydrate, and reducing sugar contents in the polysaccharide hydrolase- and/or protein hydrolase-treated RGR were respectively deter-

mined using a PR-32α refractometer (Atago Co., Tokyo, Japan) using the phenol-sulfuric acid method [16] and by the DNS method [17] using glucose as a standard. Solid yield (SY), carbohydrate yield (CY), and degree of carbohydrate hydrolysis (DCH) were then calculated using the following equations [18-20]:

SY (%)=(total weight of solid in the ERGR supernatant/total RGR weight)×100

CY (%)=(total weight of carbohydrate in the ERGR supernatant/total RGR carbohydrate weight)×100

DCH (%)=(total weight of reducing sugar in the ERGR supernatant/total RGR carbohydrate weight)×100

The presented data for SY, CY, and DCH represent the means and standard deviation of values measured at least in triplicate.

RESULTS AND DISCUSSION

Enzyme screening

To screen enzymes for use in hydrolyzing RGR, the approximate chemical composition of RGR was determined. Its total carbohydrate and crude protein contents were 70.24% and 18.20%, respectively (Table 1). These results are very similar to those obtained for red ginseng tail and its extraction residues [7]. Composition data suggested that degradation of the major water-insoluble components (carbohydrate and protein) to readily usable low-molecular-weight products would be necessary for the growth of microorganisms on RGR. These are effective methods for generating carbon and nitrogen sources for use as growth nutrients. To choose the suitable polysaccharide-hydrolyzing enzyme, the RGR suspension was treated with commercially available carbohydratases (concentration 1% concentration on a solid weight basis). Of the tested polysaccharide hydrolases, Viscozyme (a mixture of β-glucanase, cellulase, hemicellulase, xylanase, and arabanase) and Ceremix (a mixture of β-glucanase, cellulase, pentosanase, proteinase, and α-amylase) gave the highest CY (46.9%) and SY (48.0%), respectively. The differences in yields between these two enzymes were not significant (Table 2).

Table 1. Approximate chemical composition of red ginseng residue

Components	Content (%)
Carbohydrate	70.24
Crude protein	18.20
Crude lipid	1.22
Ash	5.89
Water	4.45

Table 2. Effects of various enzyme treatments on the solubilization of red ginseng residue

Enzymes	Solid yield	Carbohydrate yield	DH for carbohydrate
Celluclast	38.7±3.1	36.1±6.1	7.5±1.5
Ceremix	48.0±1.0	43.3±5.7	17.0±3.2
Fructozyme	34.7±4.6	31.3±5.7	6.3±1.7
Pectinex	36.7±3.5	34.9±5.7	14.5±2.7
Ultraflo	36.0±2.0	32.6±7.4	3.6±1.1
Viscozyme	45.7±3.1	46.9±8.7	28.4±3.0
Alcalase	46.5±0.5	50.3±3.3	10.4±1.3
Flavourzyme	46.0±4.0	59.7±0.4	22.7±2.6
Kojizyme	41.0±1.0	47.2±1.6	20.0±2.6
Nutrase	46.5±3.5	54.5±2.5	6.6±1.1
Protamex	48.0±2.0	63.1±1.2	8.1±1.6
Control	31.0±1.7	32.6±7.4	2.3±0.4

Values are presented as percentage.
DH, degree of hydrolysis.

However, the value of DCH—the ratio of water-soluble reducing sugar to total carbohydrate content—increased from 2.3% (non-treated control) to 28.4% following treatment with Viscozyme, but to only 17.0% following treatment with Ceremix. Despite Ceremix having been reported to be effective for the extraction of total saccharides from ginseng leaves, stems, and roots [13], we concluded that Viscozyme was more suitable in our experiments using RGR as the substrate. These results indicate that the polysaccharide hydrolases degraded the RGR polysaccharides, which in turn increased the solubility of the polysaccharides, in accordance with the results of previous studies using vegetable materials as substrates [18,19]. A study also reported that a protease treatment improved the total solid and protein recovery from soymilk residue by hydrolyzing the protein part of peptidoglycan in plant cell walls and thereby increasing the solubility of both polysaccharides and proteins [19]. Notably, Flavourzyme, an exopeptidase, yielded the highest DCH (22.7%) of the tested proteases (Table 2). This enzyme had been also selected for the production of yeast extract [20] and heme-enriched peptide [21] via the enzymatic hydrolysis of yeast cells and hemoglobin, respectively.

Enzymatic hydrolysis of red ginseng residue

To optimize the enzyme treatment conditions, the RGR suspension was treated with different concentrations of Viscozyme (0% to 5.0% on a solid weight basis). Treatment for 3 h showed that SY, CY, and DCH strongly depended on a Viscozyme concentration in the range of 0% to 1% (Fig. 1A). Treatment with 1% Vis-

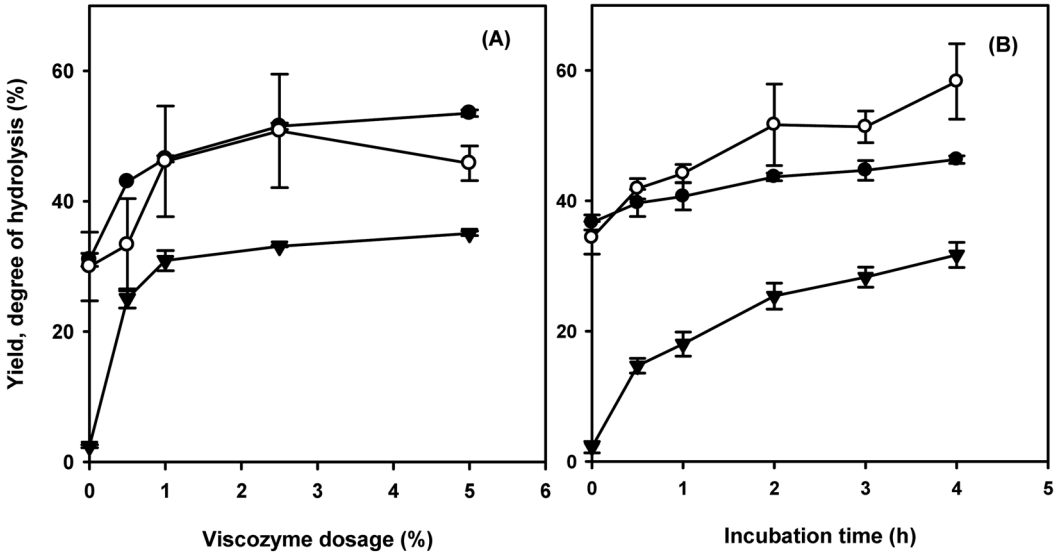

Fig. 1. Effects of Viscozyme dosage (A) and reaction time (B) on solid yield (●), carbohydrate yield (○), and degree of carbohydrate hydrolysis (▼). Reaction conditions (A): substrate, 10% red ginseng residue (RGR) suspension; temperature, 50°C; pH, 5.0; reaction time, 3 h. Reaction conditions (B): substrate, 10% RGR suspension; enzyme dosage, 1%; temperature, 50°C; pH, 5.0.

cozyme increased SY, CY, and DCH by 50%, 54%, and 1,187%, respectively (versus non-treated control). The optimal dosage of Viscozyme was found to be 1.0%. Time-course analysis of SY, CY, and DCH following treatment with 1.0% Viscozyme revealed that CY and DCH increased significantly with the increasing reaction time, while SY increased only slightly (Fig. 1B). CY, DCH, and SY increased by 50%, 1,186%, and 22%, respectively, after hydrolysis for 3 h. The marked enhancement of DCH was due to the enzymatic hydrolysis of glycoside bonds in polysaccharides and the release of soluble low-molecular-weight carbohydrates with reducing power, as occurs during the hydrolysis of soymilk residue [18].

As with Viscozyme, the effects of Flavourzyme dosage and reaction time on SY, CY, and DCH were determined. The effects of the Flavourzyme treatment followed a similar pattern to those of the Viscozyme treatment (data not shown). SY, CY, and DCH increased by 52%, 49%, and 731%, respectively, following treatment with 0.5% Flavourzyme (vs. non-treated control). Noticeably, DCH with Viscozyme was higher than with Flavourzyme. The optimal dosage and reaction time for Flavourzyme were found to be 0.5% and 1 h, respectively.

To identify the optimal enzyme combination, the RGR suspension was treated with different combinations of polysaccharide hydrolases and proteases. Hydrolysis was performed using the combination of Viscozyme and Flavourzyme. Values of SY, CY, and DCH following treatment with 0.5% Flavourzyme and various concentrations of Viscozyme, as well as with each enzyme alone, are shown in Table 3. Co-treatment with the optimized dosages of Flavourzyme (0.5%) and Viscozyme (1.0%) significantly increased the values of SY, CY, and DCH to 76% (26% → 46%), 65% (43.76% → 72.27%), and 1,865% (1.93% → 37.93%), respectively (vs. non-treated control). This increase in hydrolysis achieved through treatment with two different enzymes was very similar to those reported in studies on the hydrolysis of hemoglobin [21] and chlorella [22], and the extraction of astaxanthin from *Haematococcus pluvialis* [23]. As shown in both the present study and previous investigations, the use of multiple enzymes is a helpful strategy

Table 3. Effects of enzymes co-treatments on the solubilization of red ginseng residue[1)]

Enzyme dosage		Solid yield	Carbohydrate yield	DH for carbohydrate
Flavourzyme	Viscozyme			
0	0	26	43.76	1.93
0	1.0	40	66.23	28.16
0.5	0	38	69.76	17.85
0.5	1.0	46	72.27	37.93
0.5	2.0	48	72.72	39.55
0.5	5.0	50	74.35	43.62

Values are presented as percentage.
DH, degree of hydrolysis.
[1)]Enzymatic hydrolysis was carried out for 3 h by two enzymes co-treatment. Reaction conditions: 0.5% Flavourzyme, different concentration of Viscozyme, 50°C and pH 5.5. Enzyme dosage was based on solid content.

for maximizing substrate degradation.

The cultivation of lactic acid bacteria on enzymatically hydrolyzed RGR

To verify the utility of ERGR as a microbial culture medium, changes in cell growth and the pH and titratable acidity of ERGR and RGR suspensions fermented with *L. mesenteroides* KACC 91459P were monitored (Fig. 2). For the RGR medium, while the pH of the fermented broth decreased from 6.0 to 4.08 after 6 h of fermentation, its titratable acidity increased from 0.05% to 0.414% and was thereafter maintained at around 0.43% for a further 18 h. For the ERGR medium, the pH similarly decreased (from 6.0 to 3.91 after 6 h of fermentation, and then more gradually to 3.69 at 24 h), while its titratable acidity increased hyperbolically from 0.05% to 0.846% at 24 h. The ERGR and RGR media were initially inoculated with approximately 6–8×10^7 CFU/g of *L. mesenteroides* KACC 91459P. After cultivation for 6 h, the viable cell counts for both cultures had increased to 1.3×10^9 CFU/g. However, while the viable cell population decreased dramatically to 2.4×10^6 CFU/g for the RGR culture, it was maintained during 24 h of fermentation in ERGR medium. These results are superior to those obtained in previous studies involving lactic acid bacteria cultivated in a mixture of milk and cultured ginseng extract [24] or MRS medium containing ginseng powder [25]. When *Lactobacillus plantarum* (*L. plantarum*) MG208 was cultivated with MRS medium containing 5% red ginseng powder, the viable cell counts, pH, and titratable acidity of the broth changed to 4.7×10^8 CFU/mL, 3.73, and 0.59%, respectively, after 24 h of fermentation [25]. Although *L. plantarum* MG208 differs from *L. mesenteroides* KACC 91459P, the fermentation characteristics of lactic acid bacteria were superior in ERGR medium than in MRS medium containing 5% red ginseng powder. These data suggest that the ERGR medium on its own contains sufficient nutrients to support bacterial growth and can be utilized as a microbial growth medium without the addition of nutritional supplements. Previous studies have investigated the potential of adding RGR to existing fermentation media as a nutritional supplement [7,26]. To the best of knowledge, the present study is the first to explore the possibility of using RGR as a non-supplemented microbial cultivation medium.

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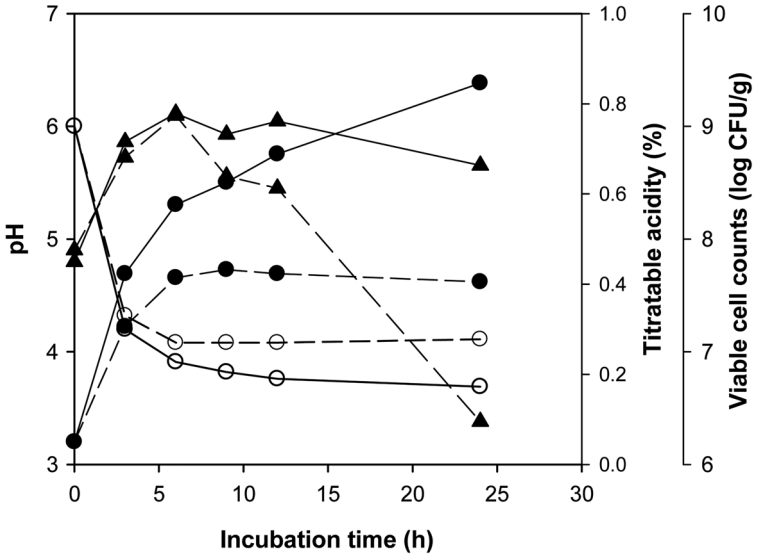

Fig. 2. Changes in the growth (▲) of *Leuconostoc mesenteroides* KACC 91459P and in the titratable acidity (●) and pH (○) of the red ginseng residue medium (broken line) and enzymatically hydrolyzed red ginseng residue medium (solid line) during lactic acid fermentation at 30°C.

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