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Optimization of Carbon Sources to Improve Antioxidant Activity in Solid State Fermentation of Persimmon peel Using Lactic Acid Bacteria

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ABSTRACT

The present study was conducted to develop persimmon peel, a by-product of dried persimmon manufacturing, as a feed additive via lactic acid bacteria fermentation. *Pediococcus pentosaceus*, *Lactobacillus plantarum*, and three strains of *Leuconostoc mesenteroides* were used as a starter culture in the solid state fermentation of persimmon peel, and antioxidant activity and total polyphenol content were assessed. *Leuconostoc mesenteroides* KCTC 3100 showed high antioxidant activity ($p<0.05$), whereas *Pediococcus pentosaceus* showed high total polyphenol content ($p<0.05$). These two strains were thus selected as starter culture strains. Glucose, sucrose and molasses were used as variables for optimization and a total 15 experimental runs were produced according to Box-Behnken design. Regarding significant effects of variables, molasses showed linear and square effects on antioxidant activity of persimmon peel fermentation ($p<0.05$). In conclusion, optimum concentrations of glucose, sucrose, and molasses were determined to be 4.2, 3.9 and 5.3 g/L, respectively, using a response surface model. Antioxidant activity was also improved 2.5 fold after optimization.

(**Key words** : Persimmon peel, Solid state fermentation, Antioxidant activity, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*)

Ⅰ. INTRODUCTION

Persimmon (*Diospyros kaki*), a deciduous fruit, is broadly cultivated in Korea, Japan, China, Brazil, and Italy. Persimmon contains several bioactive compounds such as polyphenols, flavonoids, terpenoids, steroids, carotenoids, dietary fiber, starch, and minerals (Yokozawa et al., 2007). Bioactive compounds extracted from the leaves, peel, and flesh of persimmon are reported to possess anti-tumor, anti-hyperlipidemia, anti-diabetes, antioxidant, and neuroprotective activities (Itoh et al., 2011). Either astringency removal using carbon dioxide or ripening is required for direct consumption of persimmon fruit since it contains 1~5% of tannins after maturation (Kim, 2005). Hence, some persimmons are consumed in dried form. During manufacturing of dried persimmon, a considerable amount of peel is wasted. Actually, antioxidant-rich phenolic compounds are more abundant in persimmon peel than in persimmon flesh (Gorinstein et al., 2001). However, antioxidant-rich compounds in persimmon peel are not stable, and are spontaneously degraded (Yokozawa et al., 2007). Therefore, an appropriate

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treatment may be necessary.

Fermentation using lactic acid bacteria (LAB) is frequently used to increase the shelf-life of foods or vegetables. *Lactobacillus plantarum, Lactobacillus brevis, Leuconostoc mesenteroides, Pediococcus pentosaceus* and *Pediococcus acidilactici* are reported as representative strains for the fermentation of vegetables (Chang and Chang, 2010; Gardner et al., 2001). One of the representative benefits of lactic acid fermentation is the improvement of the bioactivity of vegetables or their components. In a study by Hong (2011), fermentation of fruit using lactic acid bacteria increased the water-soluble polyphenol concentration and antioxidant activity. The performance of fermentation, including cell yield and metabolite production, depends on the medium ingredients used and fermentation conditions (Cho et al., 2010). Therefore, the optimization of medium or fermentation conditions is important to achieving effective, economical fermentation.

One factor at a time method (OFAT), which investigates the effect of a single factor while maintaining all others at constant levels, has been traditionally used in microbiological studies (Coman and Bahrim, 2011). However, OFAT does not examine all possible interactions among the factors, and this is particularly true when there are several factors investigated within an experiment (Amdoun et al., 2010). Response surface methodology (RSM) was developed in order to overcome the inherent problems of OFAT, which can simulate the behavior of a system responding to factors and eventually calculate the optimum conditions related to target performance of the system (Box and Behnken, 1960; Coman and Bahrim, 2011).

Therefore, the present study was designed to develop persimmon peel as an antioxidant-rich feed additive through lactic acid fermentation, and the optimum formulation for medium ingredients was investigated using statistical methods.

Ⅱ. MATERIALS AND METHODS

1. Experimental materials

All chemicals and medium ingredients were purchased from Sigma Chemical Co. (St. Louis, Mo. USA) and Becton, Dickinson and Company (BD, Le Pont de Claix France), respectively.

Leuconostoc mesenteroides KCCM 11324 and KCCM 35046 were purchased from the Korean Culture Center of Microorganisms (Seoul, Korea). *Leuconostoc mesenteroides* KCTC 3100 was purchased from the Korean Collection for Type Culture (Daejeon, Korea). *Pediococcus pentosaceus* and *Lactobacillus plantarum* were wild type strains. They were isolated from Kim-Chi and identified using 16S rRNA gene sequence analysis. All strains were cultivated in MRS broth at 30°C in an incubator with agitation (150 rpm).

Persimmon peel was obtained from a dried persimmon farm located in Sangju city, Korea and was dried at 60°C for 48 h, after which it was finely grinded using a cutter miller (Philips HR2860, Netherlands) prior to the experiment.

2. Solid state fermentation conditions of persimmon peel

Solid state fermentation (SSF) method was employed to ferment persimmon peel. To screen for effective starter cultures, the SSF medium was prepared with the following ingredients; 600

g of dried persimmon peel, 3 g of yeast extract, 1 g of sodium acetate, 1 g of sodium chloride, 0.1 g of dipotassium phosphate, 0.01 g of magnesium sulfate and 400 ml of distilled water. In the optimization, basal medium was prepared with 600 g of dried persimmon peel, 3 g of yeast extract, 1 g of sodium acetate, 1 g of sodium chloride, 0.1 g of dipotassium phosphate, 0.01 g of magnesium sulfate and 400 ml of distilled water, after which three carbon sources were added according to the experimental design. The prepared medium was autoclaved at 121°C for 15 min. For the fermentation conditions, 50 g of medium was placed in an Erlenmeyer flask capped with a cotton plug, after which the flask was incubated at 30°C in an incubator without agitation for 3 days.

3. Experimental design for optimization

Three carbon sources, glucose, sucrose and molasses, were used as variables. Each variable was present at three concentration levels (labeled as −1, 0 and +1). Thus, a total of 15 experimental runs were comprised of three variables according to Box-Behnken design (1960). The configuration of the experiment and the actual concentration of variables are summarized in Table 1.

4. Analysis

After fermentation, 50 mL of cold sterilized 0.8% NaCl solution was added to the flask, after which it was immediately used for viable cell count assay. The remaining persimmon peel

Table 1. Box-Behnken design configuration for the effects of carbon sources on persimmon peel fermentation and their responses

Run	Variables, g/L						Responses		
	Glucose		Sucrose		Molasses		Viable cell count, $\log_{10}$ (CFU/mL)	Antioxidant activity, ascorbic acid mM equivalent	Total polyphenol content, gallic acid mg/L equivalent
	C	U	C	U	C	U			
1	−1	1.0	−1	1.0	0	5.5	8.97±0.05[2)]	7.60±0.15	4.13±0.02
2	1	10.0	−1	1.0	0	5.5	8.97±0.10	7.47±0.11	4.31±0.02
3	−1	1.0	1	10.0	0	5.5	9.00±0.04	7.36±0.13	4.03±0.04
4	1	10.0	1	10.0	0	5.5	8.92±0.05	7.41±0.16	4.03±0.05
5	−1	1.0	0	5.5	−1	1.0	8.82±0.06	7.44±0.02	4.01±0.04
6	1	10.0	0	5.5	−1	1.0	8.98±0.07	6.60±0.11	3.74±0.04
7	−1	1.0	0	5.5	1	10.0	8.99±0.07	7.10±0.04	3.95±0.02
8	1	10.0	0	5.5	1	10.0	8.77±0.06	7.21±0.09	4.00±0.01
9	0	5.5	−1	1.0	−1	1.0	8.80±0.03	7.51±0.14	3.96±0.03
10	0	5.5	1	10.0	−1	1.0	8.65±0.13	7.10±0.06	3.97±0.02
11	0	5.5	−1	1.0	1	10.0	8.98±0.02	7.31±0.13	3.99±0.03
12	0	5.5	1	10.0	1	10.0	8.86±0.07	6.45±0.09	3.80±0.01
13	0	5.5	0	5.5	0	5.5	8.86±0.06	7.93±0.17	3.81±0.04
14	0	5.5	0	5.5	0	5.5	8.58±0.22	8.18±0.06	4.02±0.03
15	0	5.5	0	5.5	0	5.5	8.91±0.10	7.69±0.08	3.88±0.05

[1)] C and U mean coded value (experimental point) and uncoded value (actual amount of variables), respectively.

[2)] Mean ± standard deviation (n=3).

was dried at 60°C overnight and then it was finely grinded using a cutter miller and used for analysis of antioxidant activity and total polyphenol assay. Viable cell count and anti-oxidant activity were determined according to the method of Chang et al. (2010), and antioxidant activity was represented as ascorbic acid mM equivalent (Chang and Chang, 2010). Total polyphenol content was analyzed using Folin-Ciocalteu's phenol according to Juan and Chou (2010) and represented as gallic acid mg/L equivalent (Juan and Chou, 2010).

5. Statistical analysis

In screening for starter cultures, the effects of different LAB stains were determined in a general linear model with Duncan's post-hoc test using statistical program R. In the optimization study, experimental design, analysis of variance and calculation of optimum point of variables were performed using Minitab, version 14 (Cho et al., 2010; Minitab Inc., 2010).

Ⅲ. RESULTS AND DISCUSSION

1. Bacterial strains as starter cultures for fermentation of persimmon peel

Three type strains, *Leuconostoc mesenteroides* KCCM 11324 (LM1), KCCM 35046 (LM2), KCTC 3100 (LM3), *Pediococcus pentosaceus* (PP) and *Lactobacillus plantarum* (LP) were investigated as starter cultures for the fermentation of persimmon peel. Following fermentation, anti-oxidant activities and total polyphenol contents of fermented persimmon peel using different starter cultures were determined (Fig. 1). For antioxidant activity, LM3 showed higher ($p<0.05$)

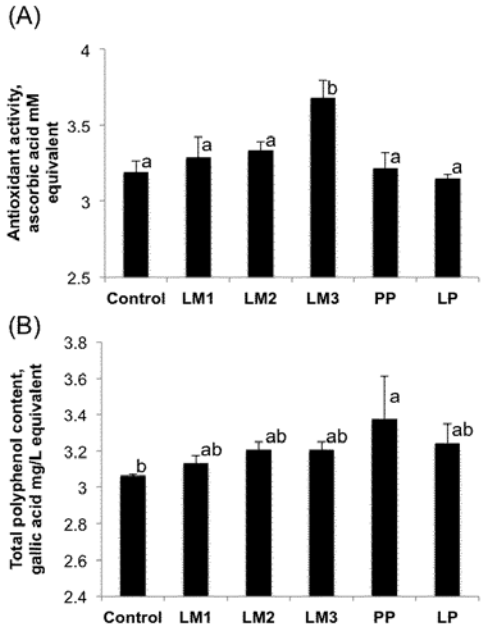

Fig. 1. Effects of different bacterial strains on antioxidant activity (A) and total polyphenol content (B) of fermented persimmon peel. Control, non-fermented; LM1, *Leuconostoc mesenteroides* KCCM 11324; LM2, *Leuconostoc mesenteroides* KCCM 35046; LM3, *Leuconostoc mesenteroides* KCTC 3100; PP, *Pediococcus pentosaceus*; LP, *Lactobacillus plantarum*.

activity compared to the control and other strains (Fig. 1A). For total polyphenol content, higher ($p<0.05$) content was observed upon PP treatment (Fig. 1B). Therefore, *Leuconostoc mesenteroides* and *Pediococcus pentosaceus* were selected as starter cultures for the next step of the experiment. Lactic acid bacteria are known to be heavily involved in vegetable fermentation and interestingly the characteristics of fermented vegetables depend upon the LAB strain used. Commonly used LAB belong to the genera Leuconostoc, Lactobacillus, and Pediococcus (Gardner et al., 2001). Leuconostoc strain, a hetero-fermentative LAB, is reported to be

dominant in fermented vegetables compared to Lactobacillus and Pediococcus strains (Gardner et al., 2001). *Pediococcus pentosaceus* is known to be involved in both vegetable and dairy product fermentation, representing antibacterial activity through the production of organic acids, hydrogen peroxide and bacteriocin (Abrams et al., 2011). *Lactobacillus plantarum* is a frequently used LAB in agricultural applications, including silage making, dairy products, and direct-fed micro-organisms (de Vries et al., 2006; Lee et al., 2008).

2. Carbon sources for fermentation of persimmon peel using *L. mesenteroides* and *P. pentosaceus*

Carbon sources are important nutrients for the growth and production of metabolites in LAB fermentation. In this study, three carbon sources, glucose, sucrose, and molasses, were used as variables in the medium, and three concentration levels of each variable were assigned to 15 experimental runs according to Box-Behnken

Table 2. Analysis of variance for the effects of carbon sources on persimmon peel fermentation

Viable cell count					
Source	df	SS	MS	F-value	P-value
Regression	9	0.1354	0.0150	0.81	0.631
Linear	3	0.0299	0.0152	0.82	0.537
Quadratic	3	0.0676	0.0226	1.22	0.395
Interaction	3	0.0379	0.0126	0.68	0.600
Residual error	5	0.0393	0.0185		
Lack-of-Fit	3	0.0301	0.0100	0.32	0.815
Pure error	2	0.0626	0.0313		
Total	14	0.2281			
Antioxidant activity					
Source	df	SS	MS	F-value	P-value
Regression	9	2.3331	0.2592	2.96	0.122
Linear	3	0.4329	0.2214	2.53	0.171
Quadratic	3	1.6145	0.5382	6.15	0.039
Interaction	3	0.2857	0.0952	1.09	0.434
Residual error	5	0.4375	0.0875		
Lack-of-Fit	3	0.3169	0.1057	1.75	0.383
Pure error	2	0.1206	0.0603		
Total	14	2.7706			
Total polyphenol content					
Source	df	SS	MS	F-value	P-value
Regression	9	0.2067	0.0230	1.76	0.276
Linear	3	0.0397	0.0273	2.09	0.220
Quadratic	3	0.1239	0.0413	3.17	0.123
Interaction	3	0.0431	0.0144	1.10	0.430
Residual error	5	0.0652	0.0130		
Lack-of-Fit	3	0.0409	0.0136	1.12	0.504
Pure error	2	0.0243	0.0122		
Total	14	0.2719			

df, degree of freedom; SS, sum of square; MS, mean square.

design as summarized in Table 1. Cell growth represented in logarithmic value with base 10, antioxidant activity and total polyphenol content as responses are shown in Table 1. Cell growth responses in the experimental runs differed from 8.5 to 9.0 $\log_{10}$ (CFU/mL), and antioxidant activities ranged from 6.4 to 8.1 ascorbic acid mM equivalent. Total polyphenol contents in the experimental runs ranged from 3.8 to 4.3 (gallic acid mg/L equivalent). Analysis of variance of each response was performed, and a significant effect was found in the quadratic regression of antioxidant activity (Table 2). Thus, although it is marginal, it could be concluded that the three carbon sources used in this study could influence antioxidant activity of fermented persimmon peel. Glucose and sucrose are known to be efficient carbon sources for both metabolite production and LAB growth, although their effects on cell growth and / or production of metabolite such as enzymes or bacteriocin can vary according to their concentrations or combinations (Powell et al., 2007; Todorov et al., 2004). Molasses is a frequently used carbon source for the fermentation of various LAB with industrial potential due to its cheap price (Rodrigues et al., 2006).

3. Optimization of carbon sources

Based on analysis of variance, optimum concentrations of the three variables for maximization of antioxidant activity were determined using response surface methodology. Calculated regression coefficients and their probabilities are summarized in Table 3. Significant differences were found in the linear ($p = 0.043$) and in the quadratic effects ($p = 0.011$) of molasses. Although these values were generated by a mathematical approach, nonetheless, it could be concluded that molasses was the most important carbon source affecting the antioxidant activity of persimmon peel fermentation. Although significant interaction among the three carbon sources was not detected, contour plot analysis was performed to investigate the possible interactions of variables, and the results are summarized in Fig. 2. The highest antioxidant activity was convergent to the center of the plot in all plots. Optimum concentrations of the three carbon sources were calculated using the optimization tool of Minitab program as shown

Table 3. Regression coefficients and their probabilities for the effects of carbon sources on antioxidant activity of persimmon peel fermentation using *L. mesenteroides* and *P. pentosaceus*

Term	Coefficient	Standard error of coefficient	T-value	P-value
Constant	7.0285	0.52630	13.355	<0.001
Glucose	0.0296	0.10374	0.285	0.787
Sucrose	0.1024	0.10374	0.987	0.369
Molasses	0.2796	0.10374	2.695	0.043
Glucose × Glucose	−0.0117	0.00760	−1.544	0.183
Sucrose × Sucrose	−0.0116	0.00760	−1.529	0.187
Molasses × Molasses	−0.0300	0.00760	−3.949	0.011
Glucose × Sucrose	0.0022	0.00730	0.302	0.775
Glucose × Molasses	0.0118	0.00730	1.614	0.167
Sucrose × Molasses	−0.0055	0.00730	−0.754	0.485

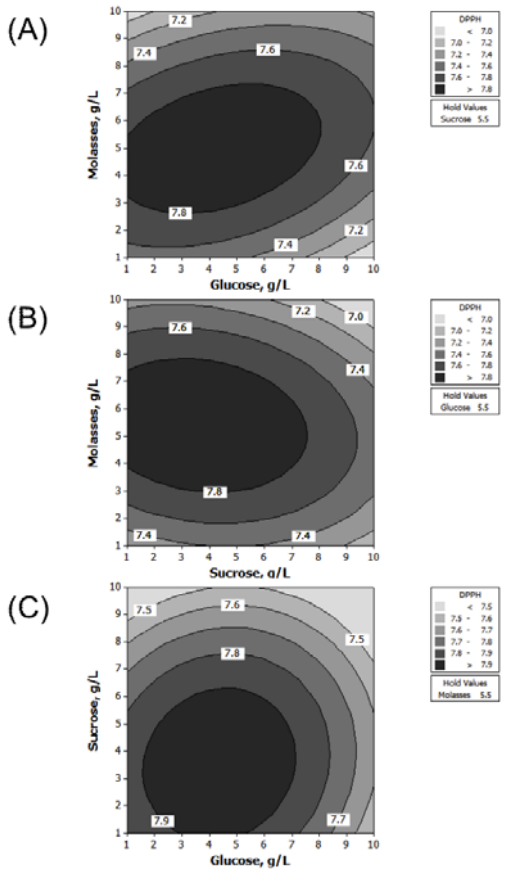

Fig. 2. Contour plots of the interaction of various carbon sources on antioxidant activity of fermented persimmon peel using *Leuconostoc mesenteroides* and *Pediococcus pentosaceus*. (A), Interaction between molasses and glucose; (B), interaction between molasses and sucrose; (C), interaction between sucrose and glucose.

in Fig. 3. Finally, 4.2 g/L of glucose, 3.9 g/L of sucrose, and 5.3 g/L of molasses were determined to be the optimum concentrations for improvement of the antioxidant activity of persimmon peel fermentation using *L. mesenteroides* and *P. pentosaceus* as starter cultures. The simulated antioxidant as a result of optimization was 8.0 ascorbic acid mM equivalent. The antioxidant activity after optimization was 2.5 times higher

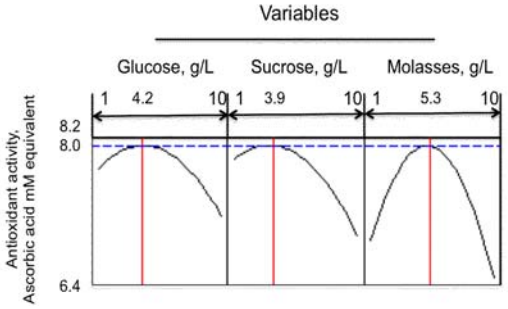

Fig. 3. Optimization plot of carbon sources for maximizing antioxidant activity in persimmon peel fermentation.

than that before optimization (see Fig. 3 in relation to the control in Fig. 1).

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