Effect of Citrus Waste Substrate on the Production of Flavor Constituent of *Grifola Frondosa*

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Abstract: No studies have previously been published on the flavor content of *Grifola frondosa* cultivated on citrus wastes. The free sugars, non-volatile organic acids, and free amino acids components in *Grifola frondosa* mycelial extracts cultivated on citrus wastes such as citrus peel and premature Hallabong fruit drop, and their substrates were determined in the present study. The extracts of *Grifola frondosa* mycelium grown on citrus wastes contained fructose (11.0 - 168.4 mg/g), glucose (1.7 - 108.1 mg/g), sucrose (8.4 - 75.3 mg/g) and maltose (13.5 - 21.1 mg/g). The fructose and maltose contents were significantly higher, whereas glucose and maltose contents were declined in mushroom extracts using citrus peel and premature Hallabong as substrate than in their substrates. Non-volatile organic acids analysis showed that lactic acid (108.6 - 181.2 mg/g) is the major organic acid found in mushrooms cultivated on citrus wastes. Monosodium glutamate-like and sweet taste amino acids were 1.4- to 7-fold higher in *Grifola frondosa* mycelia cultivated on premature Hallabong drop than those of *Grifola frondosa* cultivated on sawdust and log substrates, which was reported in the previous findings. Taken together, citrus waste substrate may be responsible for the better taste of *Grifola frondosa* mycelia as compared with conventional substrate and it, therefore, could be utilized as a practical substrate to offer a viable alternative use for these abundant agricultural wastes with a double benefit: the cultivation of valuable mushrooms and a reduction in environmental impact.
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1. Introduction

Grifola frondosa (huishu hua; maitake), one traditional edible mushroom in Asia, has been used as food and food-flavoring material in soups and sauces for centuries, due to their unique and subtle flavor. Recently *Grifola frondosa* also has attracted as functional foods and as a source of physiologically beneficial medicine (Borchers et al., 2004; Inoue et al., 2002; Masuda et al., 2009). *Grifola frondosa* is generally produced in solid culture using composts or sawdust of broad leaf trees supplementing the source of nutrients such as rice or wheat bran, and this method makes it possible to cultivate on a large scale and to harvest all year around. Extensive research has been carried out to find the most efficient cultivation methods for the edible mushrooms by employing the optimal growing conditions exist within a limited range of temperature, moisture, humidity and other environmental factors (Garibay-Orijel et al., 2007; Sanchez, 2010; Chen st al., 2010). As one of the greatest challenges to mushroom cultivation, investigators have recently exerted their efforts to optimize existing cultural techniques for the mushrooms using cheap and locally sourced substrate materials such as agricultural and food wastes (Xiaoke and Shunxing, 2005; Chiu et al., 2000).

Citrus is the main fruit crop in the world, with a total production of 122 million tons in 2008 (Terol et al., 2010). In Korea, Jeju island is well known for its important production of citrus fruits (0.6 million tons in 2008/2009) (Department of Citrus Policy, Jeju Special Self-Governing Providence, Korea). Citrus fruits are utilized primary for juice recovery, where about half of the processed citrus including peels, segment membrane and seeds ends up as wastes. These solid residues are referred to as citrus wastes with estimated worldwide production of 15 million tons per year (Marı´n et al., 2007). Only a very small part of the wastes has been properly converted into useful or high-value products and most are disposed in landfills, constituting severe economic and environmental problems (Tripodo et al., 2004; Montgomery, 2004). In Jeju island, more than 50,000 tons of citrus wastes including citrus peels generated by juice processing facilities and canning industries, and premature fruit drops in orchards annually have been incinerated in disposal yards and dumped into the ocean (Yang et al., 2009). The by-products industry has a potential for growth since products have also been produced from citrus fruit residues. The peel of citrus fruits, the primary by-product, is a rich source of flavones as well as many polymethylated flavones, they could be which are very rare in other plants (Nogata et al., 2006). These beneficial actions suggest new value-added uses for these compounds as nutraceuticals and specialty

ingredients, and studies of the occurrence of the polymethoxylated flavones in byproducts generated from citrus juice processing or premature fruit drops caused by poor condition from climate change, some diseases and other stresses are currently of considerable interest to both pharmaceutical and food industries. Therefore, this study was performed to test the potentiality of using citrus wastes such as peels and premature fruit drops as nutrient supplements for growing *G. frondosa* mycelia. The present report analyzed the flavor components including free sugars, non-volatile organic acids, and free amino acids in the *Grifola frondosa* cultivated on citrus wastes and their substrates.

2. Material and Methods

2.1. Substrate

The peel waste of citrus fruit (Citrus *unshiu* Marc) after juice extraction was obtained from a local food processing company (Jeju Provincial Development Co., Jeju, Korea). The fruit peels were dissected, weighed, lyophilized and then ground into a fine powder using a blender. Premature Hallabong ([C. *unshiu* Marcov × C. *sinensis* Osbeck] × C. *reticulate* Blanco) fruit drops, kindly supplied by commercial orchards (Seogwipo-si, Jeju, Korea), were washed, dried in an oven with air circulation at 40 ºC, and ground with a mortal mile. The powered substrates were stored at -20 ºC prior to use.

2.2. Microorganism, inoculum and sample preparation

Grifola frondosa (KACC 50027), obtained from the RDA-Genebank Information Center, Suwon, Korea, was maintained on potato dextrose agar (PDA, Difco, Detroit, MI, USA) slant at 4 ºC. Fugal inoculum was prepared from mycelia grown on PDA for 5 days at 24 ºC in the dark and agar plugs taken from the periphery of the growing colony were used to inoculate media. An agar disk of the strain was inoculated (2%, v/v) in a 50 mL of a medium (pH 5) containing 50 mg of powdered substrate, 100 mg of $CaCO_3$ and 1 g of agar. After incubation for 10 days at 27 ºC, the 50 mL culture was added to 1 L media which consisted of powdered substrate (1 kg) and $CaCO_3$ (50 g). The fermentations were implemented in a 5-liter jar fermenter for 15 days at a 25 ºC. One gram of free-dried mycelia was ground into powder, extracted with 60 mL of 80% ethanol solution by ultrasonication at room temperature for 6 h, and then purified by using a Sep-Pak C_{18} cartridge and a 0.45 μm membrane filter (Waters, Milford, MA, US), which were used directly for analysis of their chemical components.

2.3. Analysis of free sugar

Free sugar analysis was performed by the high-performance liquid chromatography (HPLC) (Waters, Milford, MA, US) with an evaporative light scattering detector (ELSD 2000ES) using a Prevail carbohydrate ES column (5 μm, 4.6 mm × 250 mm; Alltech, Deerfield, IL, US) at 30 ºC and flow rate of 0.8 mL/min, with acetonitrile-water (70:30, v:v) used as the mobile phase. The acetonitrile was HPLC grade (Fisher Scientific) and the water was Milli-Q purified (Millipore, Bedford, MA, US). A calibration curve was obtained from six concentrations (0.16-5 mg mL^{-1}) of a mixture of standards containing D-glucose, D-fructose, sucrose and maltose (Fisher Scientific, Rockford, IL, US). Sugar content was expressed as mg sugar g^{-1} samples, on a lipid-free, dry weight basis.

2.4. Analysis of non-volatile organic acid

Organic acid analysis was performed by the same HPLC system as for sugar analysis. Separation of organic acids was by a Prevail organic acid column, 3 μm, 4.6 mm × 150 mm. The mobile phase was 25 mM KH_2PO_4, pH 2.5, and the flow rate was 1 mL/min. Sample injection volume was 20 μL. Tentative identification of organic acids was based on identical retention times of organic acid standards. Organic acid standards, oxalic acid dehydrate, DL-tartaric acid, DL-malic acid and lactic acid (Sigma Chemical Co., St. Louis, MO, US), were dissolved in 25 mM KH_2PO_4, pH 2.5 (pH adjusted with 1 N HCl). The profiles for separation of organic acids (Alltech) were used as references for organic acid identification. Three replicates were measured per sample class.

2.4. Analysis of free amino acid

The filtrate samples was mixed with *o*-phthalaldehyde reagent (Sigma) in an Eppendrof tube, shaken to facilitate derivastion and then immediately injected onto an X-Terra RP18 (5 μm, 4.6 mm × 150 mm) column of the Waters HPLC system equipped with a 626 pump, a fluorescence detector JASCO FP 1520 (Jasco, Tokyo, Japan) plus autosampler (Waters, Milford, MA) with the flow rate of 1.2 mL/min. The excitation and emission wavelengths were 442 and 480 nm, respectively. Each amino acid was quantified by the calibration curve of the authentic standards.

2.5. Statistical analysis

All analyses were replicated three times. Each data presented as means ± standard deviation. The data were statistically analyzed one-way analyses of variance followed by Duncan's multiple range tests (SPSS 12.0). Difference with *p* value less than 0.05 was considered statistically significant.

After multiple comparisons, the means in the following table and figures were followed with different small letter "a-d" based on their values and statistical differences. In the case that a mean was followed with "ab", this mean was not significantly different from a mean with "a", and was not significantly different from another mean with "b". However, means with different letters were significantly different at the level of 0.05.

3. Results and Discussion

Table 1 shows the free sugar contents in the *Grifola frondosa* mycelial extracts cultivated on citrus waste and their substrates. The free sugars found were fructose (11.0 - 168.4 mg/g), glucose (1.7 - 108.1 mg/g), sucrose (8.4 - 75.3 mg/g) and maltose (13.5 - 21.1 mg/g). Fructose (168.4 mg/g) had the highest concentration on a dry weight basis in the extract of *Grifola frondosa* mycelia cultivated on premature Hallabong fruit drop substrate (Table 1). The fructose and glucose contents were significantly higher, whereas maltose content was significantly lower in the extracts of *Grifola frondosa* mycelia cultivated on premature Hallabong fruit drop substrate than in the mushroom extracts cultivated on citrus peel substrate ($p < 0.05$) (Table 1).

Table 1. Free sugar composition of *Grifola frondosa* mycelium cultivated on citrus waste substrate

Free sugar (mg/g dry matter)	Citrus peel substrate	Premature Hallabong substrate	*Grifola frondosa* cultivated on citrus peel	*Grifola frondosa* cultivated on premature Hallabong
Fructose	11.0±0.71^{*,a}	111.5±5.19^{b}	13.1±0.22^{a}	168.4±5.30^{c}
Glucose	8.0±0.10^{a}	108.1±3.80^{b}	1.7±0.73^{c}	37.9±0.71^{d}
Sucrose	11.8±1.05^{a}	75.3±1.84^{b}	8.4±3.39^{a}	10.1±1.16^{a}
Maltose	16.4±0.36^{a}	13.5±0.29^{b}	21.1±1.33^{c}	17.7±0.93^{a}

*Mean ± S.D. for n=3; $^{a\sim d}$Values with different superscripts in a row are significantly different ($p < 0.05$).

Comparison between the results obtained using the two mushrooms grown on the citrus waste and their substrates showed that fructose and maltose were significantly higher in mushroom extracts than those in their substrates ($p < 0.05$) (Table 1). On the contrary, glucose and sucrose were declined in mushroom extracts using citrus peel (1.7 and 8.4 mg/g) and premature Hallabong (37.9 and 10.1 mg/g) as substrate than in their substrates (8.0 and 11.8 mg/g, and 108.1 and 75.3 mg/g, respectively). Sucrose was the only sugar which demonstrated no difference between mushrooms cultivated on citrus wastes (Table 1). Glucose has been associated with bitter flavor, and the taste attribute of sweetness decreased with increasing content of bitter glucose, influencing consumer acceptance (Bail et al., 2003). The type of sugar has an effect on flavor in addition to sweetness: Fructose is 5 times sweeter than maltose (Biester et al., 1925). Therefore, the high fructose and low glucose contents in mushrooms grown on citrus wastes would give rise to the sweet taste of *Grifola frondosa* (Table 1). This implied that citrus waste as substrate could possibly influence on flavor of *Grifola frondosa*.

Table 2. **N**on-volatile organic acid composition of *Grifola frondosa* mycelium cultivated on citrus waste substrate

Organic acid (mg/g dry matter)	Citrus peel substrate	Premature Hallabong substrate	*Grifola frondosa* cultivated on citrus peel	*Grifola frondosa* cultivated on premature Hallabong
Oxalic	0.02±0.004^{*,a}	0.32±0.026^{b}	0.02±0.008^{a}	1.7±0.22^{c}
Tartaric	1.4±0.03	2.4±0.06	1.7±0.14	2.8±1.46
Succinic	1.3±0.16^{a}	3.5±0.16^{b}	2.3±0.09^{ab}	6.5±0.96^{c}
Lactic	145.4±0.26^{a}	108.6±1.01^{b}	181.2±7.37^{c}	172.9±4.17^{c}

*Mean ± S.D. for n=3; $^{a\sim c}$ Values with different superscripts in a row are significantly different ($p < 0.05$).

Organic acids can be one of the compounds studies for its potential as flavor enhancers. Organic acids not only elicit sourness but also contribute to bitter and astringent taste quality (Thomas and Lawless, 1995; Kang et al., 2007). The composition and concentration of organic acids in mushroom are major factors in influencing their taste and flavor, and some organic acids contribute to antioxidant activity (Valentao et al., 2005). Hence, we investigated the effect of citrus waste substrates on the organic acids composition of *Grifola frondosa* in the following experiments. Four non-volatile organic acid contents of *Grifola frondosa* mycelia cultivated on citrus wastes and their substrates are shown in Table 2. These include lactic acid (108.6 - 181.2 mg/g), succinic acid (1.3 - 6.5 mg/g), tartaric acid (1.4 - 2.8 mg/g) and oxalic acid (0.02 - 1.7 mg/g). The results showed that lactic acid is the major organic acid found in mushrooms cultivated on citrus wastes and their substrates. The contents of lactic acid were significantly higher in the extracts of mushrooms grown on the citrus wastes than those in their substrates ($p < 0.05$), with the highest content found in the mushroom extracts cultivated on citrus peel (181.2 mg/g) (Table 2). In addition, mushroom extracts using premature Hallabong drop as a substrate show more succinic (6.5 mg/g) and oxalic (1.7 mg/g) acid contents than those of its substrate (3.5 and 0.32 mg/g), respectively ($p < 0.05$). However, no significant difference in tartaric content between mushrooms and their substrates was observed in this study (Table 2).

Table 3. Free amino acid composition of *Grifola frondosa* mycelium cultivated on citrus waste substrate

Amino acid (mg/g dry matter)	Citrus peel substrate	Premature Hallabong substrate	*Grifola frondosa* cultivated on citrus peel	*Grifola frondosa* cultivated on premature Hallabong
L-alanine	5.7±0.78[*,a]	11.3±1.24[b]	6.2±1.07[a]	14.8±2.36[b]
L-aspartic	3.1±0.66[a]	3.3±1.17[a]	2.0±0.36[b]	2.9±0.41[a]
L-glutamic	7.9±2.24[a]	11.7±3.13[b]	9.2±2.46[a]	13.4±3.01[b]
L-histidine	3.3±1.43[a]	3.6±1.04[a]	0.8±0.08[b]	0.7±0.03[b]
L-isoleucine	0.2±0.01	0.2±0.02	0.1±0.03	0.1±0.04
L-leucine	0.2±0.01	0.3±0.01	0.2±0.01	0.3±0.02
L-tyrosine	0.2±0.01[a]	0.8±0.04[c]	0.5±0.02[b]	0.6±0.02[b]
L-valine	0.5±0.02[a]	0.7±0.05[b]	0.7±0.04[b]	0.8±0.03[c]

[*]Mean ± S.D. for n=3; [a~c] Values with different superscripts in a row are significantly different ($p < 0.05$)

Free amino acids play an important role in the taste of mushroom (Mau et al., 1998). Thus, the quantitative analysis of free amino acids in *Grifola frondosa* grown on citrus wastes and their substrates is indispensable for the effective utilization of citrus wastes. Table 3 shows the free amino acid contents in the mushrooms and their substrates. Alanine (5.7 - 14.8 mg/g), aspartic acid (2.0 - 3.3 mg/g), glutamic acid (7.9 - 13.4 mg/g) and histidine (0.7 - 3.6 mg/g) were the major amino acids in the mushrooms and their substrates. Tabata et al. (2004) identified 15 amino acids in *Grifola frondosa* cultivated on sawdust and log substrates: the major one was alanine, aspartic acid, glutamic acid, histidine, and tyrosine (Tabata et al., 2004). However, our present results show that tyrosine is not major free amino acid.

Aspartic and glutamic acids are monosodium glutamate-like (MSG-like) components which give the most typical mushroom taste (Yamaguchi et al., 1971). MSG-like and sweet taste amino acids such as alanine would mainly be responsible for the attractive taste of *Grifola frondosa*. In the present study, the content of alanine, aspartic acid and glutamic acid were significantly higher for *Grifola frondosa* mycelial extract cultivated on premature Hallabong substrate (14.8, 2.9 and 13.4 mg/g) than those for *Grifola frondosa* mycelial extract cultivated on citrus peel substrate (6.2, 2.0 and 9.2 mg/g) ($p < 0.05$) (Table 3). These contents of mushroom cultivated on premature Hallabong drop were 1.4- to 7-fold higher than those of *Grifola frondosa* mycelia cultivated on sawdust (2.2, 1.6 and 8 mg/g) and log (3.1, 1.3 and 9.1 mg/g) substrates (Tabata et al., 2004). Moreover, the contents of bitter and tasteless amino acids histidine, tyrosine and valine in our study were appreciably lower in the mushrooms cultivated on citrus wastes than on sawdust (1.53, 1.77 and 0.96 mg/g) and log (0.94, 0.73 and 0.91 mg/g) substrates (Tabata et al., 2004). These findings indicate that citrus waste substrate may be responsible for the better taste of *Grifola frondosa* mycelia as compared with conventional substrate.

Together with these results, citrus waste could be utilized as a practical substrate for promotion of the cultivation and consumption of *Grifola frondosa*. Furthermore, the bioconversion of non-conventional substrates such as fruit peel and premature fruit drop by citrus offers a viable alternative use for these abundant agricultural wastes. This is not only useful in mushroom cultivation, but it will also keep the area green and environment good. A further sensory evaluation is in progress to confirm the findings of chemical compositions.

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