Scientific Research Publishing

Advances in Microbiology, 2017, 7, 719-728
http://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Cultivation of *Flammulina velutipes* on Modified Substrate Using Fermented Apple Pomace

Chikao Hiramori[1,2], Katsuki Koh[3], Sayaka Kurata[4], Yutaka Ueno[3], Sanath Gamage[5], Poyu Huang[5], Shoji Ohga[5]

[1]Kyozen Syoji Co., Ltd., Nagano, Japan
[2]Interdisciplinary Graduate School of Science and Technology, Shinshu University, Nagano, Japan
[3]Faculty of Agriculture, Shinshu University, Nagano, Japan
[4]Graduate School of Agriculture, Shinshu University, Nagano, Japan
[5]Faculty of Agriculture, Kyushu University, Fukuoka, Japan
Email: kkkohss@shinshu-u.ac.jp, ohga@forest.kyushu-u.ac.jp, gamage.wgsb@gmail.com

How to cite this paper: Hiramori, C., Koh, K., Kurata, S., Ueno, Y., Gamage, S., Huang, P. and Ohga, S. (2017) Cultivation of *Flammulina velutipes* on Modified Substrate Using Fermented Apple Pomace. *Advances in Microbiology*, **7**, 719-728.
https://doi.org/10.4236/aim.2017.711057

Received: October 20, 2017
Accepted: November 10, 2017
Published: November 13, 2017

Abstract

In present study, a cultivation experiment was performed on modified substrates for the *Flammulina velutipes,* which contains 0% (control group), 4.8% (S group), 9.6% (M group) and 14.4% (L group) of fermented apple pomace (FAP) substituting corncob on a dry matter basis. The pH of all substrates was maintained at the required level, although the pH of FAP was low (3.9). Addition of FAP affected the mycelial growth and full colonizing in test groups to a minor degree, but extended the period by one day in L group. The initiations of fruit bodies were extended in all FAP groups by one day, but there was a little difference in terms of total cultivation time duration among groups due to FAP groups showing raise of growth rates in later phase. The effect of FAP on fruit bodies yield was observed clearly: the yield has the positive correlation with increasing levels of FAP. Fruit bodies showed little difference concerning Brix degree, proximate composition, and organic acid profile among groups. In conclusion, it is suggested that FAP can be used as an alternative to corncobs as a *F. velutipes* substrates raw material.

Keywords

Flammulina velutipes, Mushroom Cultivation, Fermented Apple Pomace

1. Introduction

In Japan, Nagano Prefecture has the highest production for *Hypsizygus tessellatus* and *Flammulina velutipes* and second highest production for apple [1]. However, securing low-cost raw materials to use as substrates and the disposal

of apple pomace have been two mains straggles in the local agriculture industry. In light of these issues, we focus on the reutilization of apple pomace biomass as a raw material for substrates to grow mushrooms in Nagano Prefecture. Although attempts had already been made to use apple pomace as substrates material [2] [3] [4], such studies used apple pomace that was preserved using the freezing method. However, the freezing method is considered costly and impractically. In this paper, we prepared and used dried apple pomace as substrate material for growing *F. velutipes* and compared the fruit body yield to the corncobs substrates [5]. While the cost of drying the apple pomace was cheaper than freezing it, however, to reduce the moisture content where the apple pomace is suitable as a substrate material can be difficult. Therefore, we developed a low-cost apple pomace preservation technique [6], which using lactic acid fermentation to preserve apple pomace in a cost-effective manner. This fermented apple pomace (FAP) that was used as substrates for growing *H. tessellatus* was at a concentration up to 9% dried matter [6]. However, in the *H. tessellatus* experiment, when FAP was added, there was no benefit in either promoting growth of the mushrooms or increasing the yield.

In this study, we demonstrated a trial of cultivation on *F. velutipes*, which is the main type of mushroom grown in Nagano Prefecture, by progressively replacing the corncob component of the substrate with FAP and surveying the quality of the fruit bodies in order to evaluate whether FAP were feasible replacement for corncobs.

2. Materials and Methods

2.1. Test Mycelial Strain and Status of FAP

The test strain in this study used the commercial G-6 *F. velutipes* strain from the Agricultural Technology Institute of Nagano Farmers' Federation. The FAP was obtained from the same lot as used in the previous report [6]. FAP was made by adding *Lactobacillus plantarum* to apple juice pomace obtained from a food company in Nagano Prefecture and allowing the apple pomace to ferment at room temperature (moisture content 78%) at a pH of 3.9. **Table 1** shows the proximate composition of FAP.

Table 1. Proximate composition of corncob and (FAP).

	Corncob	FAP
Moisture	6.8%	84.2%
Crude protein[1]	2.8%	10.8%
Ether extract[1]	0.5%	5.7%
Nitrogen-free extract[1]	36.7%	46.8%
Crude fiber[1]	53.2%	33.5%
Crude ash[1]	6.8%	3.2%

[1]Dry matter basis.

2.2. Test Substrates Media Preparation

A control substrate mix was made from corncobs and nutritionally enrichments using rice bran, cottonseed hull, wheat bran, sugar beet powder, and soy pulp, while oyster shells and lime were used as pH adjustors (Table 2). It is known from previous experience that FAP is highly viscous, which provides resistance and reduces workability when mixed with raw materials as substrates. Thus, a certain volume of tap water was added beforehand to ensure final substrates' moisture content of approximately 60%. The mixture was completely mixed and tap water was again added to adjust the final moisture content of the substrate mix to 64%. Various test substrate mixes were prepared in this manner by progressively replacing the corncob component in the control substrate mix with FAP: 4.8% (S substrate), 9.6% (M substrate), and 14.4% (L substrate) of the total mix on a dry matter basis. Samples (580 g) of each substrate mix were placed in individual 850 ml culture bottles and sterilization was conducted using autoclaving for 45 minutes at 120˚C at 0.12 Mpa.

2.3. Culture Conditions and Incubation

There were 22 sample bottles for each test substrate mix; six of the bottles were used for measuring pH as described below and 16 were used in the cultivation test. After inoculating each of the sterilized culture bottles with approximately 10 grams of the test strain, the bottles were incubated at a temperature of 17˚C and relative humidity of 70%. Once spawn running had been completed in 10 of the 16 bottles, all 16 bottles were scratched. After scratching, pinning was performed in all bottles (temperature 14˚C, relative humidity 95%) for 10 days followed by flushing (temperature 4.5˚C, relative humidity 95%) for three days, after which they were kept in an environment to promote fruiting (temperature 5˚C, relative humidity 90%). Light exposure was limited to 0.5 hours per day at approximately 300 lx only during the flushing period; the rest of the cultivation was completed in complete darkness.

2.4. Measurement and Observations

The following items were used as samples for measuring substrate pH; immediately

Table 2. Dry weights and compositions of the test substrates.

	Control	S	M	L
Corncob (g)	70.0	60.2	50.4	40.6
FAP (g)	0	9.8	19.6	29.4
Rice bran (g)	57.0	57.0	57.0	57.0
Cottonseed hull (g)	22.5	22.5	22.5	22.5
Wheat bran (g)	21.0	21.0	21.0	21.0
Sugar beet powder (g)	15.0	15.0	15.0	15.0
Soy pulp (g)	15.0	15.0	15.0	15.0
Other (g)	3.4	3.4	3.4	3.4

after mixing and sterilization, at scratching, and after harvesting fruit bodies. First, substrate mixes were tested immediately after mixing and prior to being placed in culture bottles. Substrate mixes from culture bottles were tested after removal from the autoclave. Lastly, substrate mixes from the bottles were tested on the day of scratching, and after fruit bodies were harvested. Although the substrate mixes after mixing were obtained directly from the mixer, other substrate mix samples were obtained from three culture bottles for each test substrate mix type. The pH of the samples was measured using a pH meter (AS-211, Horiba, Ltd., Kyoto). The number of days for spawn running was starting from the day of inoculation to the day on which spawn running was complete. As previously mentioned above, last day of spawn running was measured for each test substrate mix; the average value is shown below. Pinning period was starting from the day of scratching to the day when the first pins were verified. Furthermore, cultivation period was begun from the day of scratching to the day of harvest. The growing period was the total number of days in the spawn running period plus the cultivation period. Harvesting was conducted in accordance with the mushroom growing index [7] at a stem length of approximately 14 cm and a cap diameter of 10 to 12 mm. After the fruit bodies have been weighted, they were stored at −20˚C.

2.5. Composition Analysis of Fruit Bodies

The preserved fruit bodies were later thawed and used for Brix degree, proximate composition, and organic acid analyses. Each substrate mix was randomly divided into nine parts, which all the parts were used for Brix. Next to organic acid analysis was performed on five parts, which were randomly selected. Three samples then were randomly selected from the five parts that were selected in the previous analysis and sent for proximate composition analysis. As part of the proximate composition analysis, moisture content was measured using a heat drying method at normal atmospheric pressure (105˚C). Crude protein was measured using the Kjeldahl method; crude fat was measured using the Soxhlet extraction method; crude fiber was measured using the improved Henneberg-Stohmann method; and crude ash was measured using the dry ash method. Sugars were separately calculated from moisture content, crude protein, crude fat, crude fiber, and crude ash. In order to analysis sugars, fruit bodies were crushed into a mortar and a refractometer (PAL-S, Atago Co., Ltd., Tokyo) was used to measure the Brix value of the resulting extract. The remaining extract was spun in a centrifuge for 10 minutes after being diluted with the required amount of distilled water. Organic acids were measured using high-performance liquid chromatography (LC-2000 System, JASCO Corporation, Tokyo) and separated using 0.02% perchloric acid and 10% acetonitrile at 40˚C with two Inertsil ODS-3 4.6 × 250 mm columns (GL Sciences, Inc.). Organic acids were detected using ultraviolet absorption spectrometry at 210 nm.

2.6. Statistical Analysis

Measurements obtained were analyzed using a one-way analysis of variance. Tukey-Kramer test was performed on results that were deemed to be significant to further examine the significant difference. The level of significant difference was set at $p < 0.05$.

3. Results

3.1. pH of the Growing Media

Immediately after mixing, the pH of the control substrate mix was 7.0, whereas the pH of the test substrate mixes had dropped by approximately 1. Although the pH of the control substrate mix had dropped by 0.7 and S substrate mix had dropped by 0.6 after being placed in the culture bottles and undergoing heat sterilization, the pH of the M and L substrate mixes had not dropped. At the time of scratching, the pH in all substrate mixes had increased and the pH values were 7.0 - 7.4. At the time of harvest, the pH values were lower by 1 than the time of scratching and were the same right after sterilization. **Table 3** shows pH of test substrates.

3.2. Mycelial Growth and Fruit Bodies Formation

The spawn running period was 24 days for the control, S substrate mix, and M substrate mix which was one day longer than L substrate mix. The pinning period was 11 days for all the bottles those containing the control substrate mixture. However, the pinning period for the other three substrate mixes was an average of one day longer and showed some slight variations among mixes. The growing period was 26 days for the control, S substrate mixes, and M substrate mixes; and L substrate mix was one day shorter during the growing period. There was no variation in the length of the growing period among samples of the same substrate mix. We can see adding FAP to the substrate mixes resulted in longer spawn running period and the pinning period was shorter. However, the overall length of the cultivation period for all the substrate mixes was the same at 50 days. **Figure 1** shows that the weight of the fruit bodies significantly increased as the added FAP volume increased ($p < 0.05$). With the L substrate mix, it was 1.24 times higher than the control substrate mix. **Table 4** shows Effects of FAP on cultivation period and fruiting body yield of *F. velutipes.*

Table 3. The pH of test substrates.

	Control	S	M	L
Immediately after mixing[1]	7.0	6.3	5.9	5.9
Immediately after autoclaving[2]	6.3 ± 0^{a}	5.7 ± 0^{b}	5.9 ± 0^{b}	5.9 ± 0.1^{b}
On the day of flushing[2]	7.4 ± 0.1^{a}	7.1 ± 0.1^{b}	7.1 ± 0.1^{ab}	7.0 ± 0.1^{b}
After harvesting[2]	6.3 ± 0.1^{a}	5.9 ± 0^{b}	5.9 ± 0^{b}	6.0 ± 0^{ab}

[1]The SE was not obtained because they were not bottled. [2]Average of three observations ± SE. [a,b]Values with different superscripts differ significantly ($p < 0.05$).

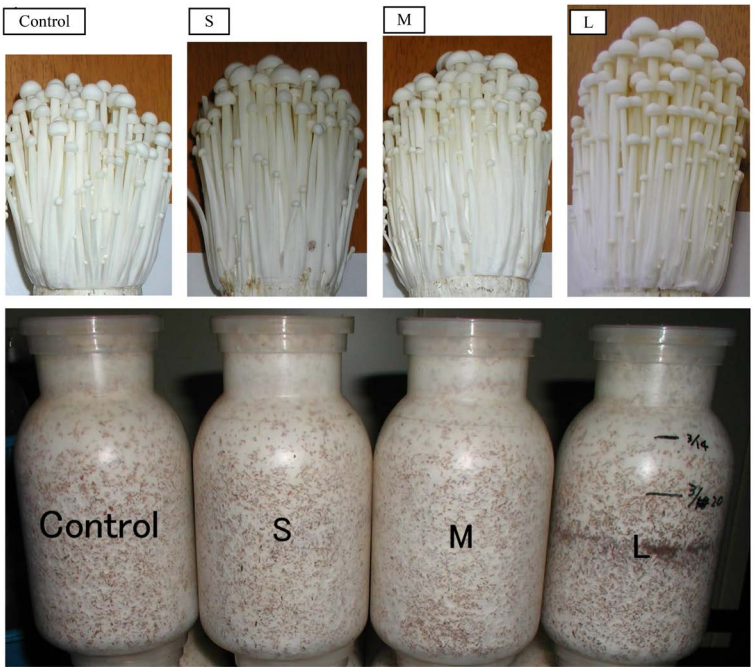

Figure 1. Size of the fruit bodies and state of fully colonization of L, M, S and control substrates.

Table 4. Effects of FAP on cultivation period and fruiting body yield of *F. velutipes*.[1]

	Control	S	M	L
Period (day)				
Spawn running[2]	24	24	24	25
Incubation[3]	11.0 ± 0.0[a]	12.0 ± 0.17[b]	12.0 ± 0.2[b]	12.1 ± 0.12[b]
Fruiting body formation[4]	26.0 ± 0	26.0 ± 0	26.0 ± 0	25.0 ± 0
Total cultivation[5]	50.0 ± 0	50.0 ± 0	50.0 ± 0	50.0 ± 0
Yield of fresh fruiting bodies (g)	189.3 ± 5.8[a]	201.3 ± 6.6[ab]	212.3 ± 3.2[b]	233.9 ± 2.7[c]

[1]Values indicated are based on 16 observations ± SE, but periods of mycelium running were based on 16 observations as they were evaluated according to group. [2]Period required for spreading of mycelium. [3]Period from spawn running to flushing. [4]Period from flushing to harvest. [5]Period from inoculation to harvest. [a,b,c]Values with different superscripts differ significantly ($p < 0.05$).

3.3. Brix and Organic Acid Content of Fruit Bodies

Fruit bodies Brix ranged from 8.5% to 9% for all substrate mixes and no influence from adding FAP was observed. In terms of the proximate composition of fruit bodies, components influenced by adding FAP were moisture content, crude protein, crude fiber, and carbohydrates in comparison there was nearly no influence on crude fats and crude ash. When adding FAP, there was a significant decline in moisture and carbohydrates, on the other hand crude protein and crude fiber increased. With regard to the organic acid content of fruit bodies, oxalic acid, tartaric acid, malic acid, citric acid, acetic acid, and fumaric acid were detected. Although levels of tartaric acid, malic acid, acetic acid, and fumaric acid were higher in the S substrate mix compared to the control

Table 5. Brix, proximate composition, and organic acid profile of *F. velutipes.*[1]

	Control	S	M	L
Brix (%)	8.7 ± 0.2	9.0 ± 0.3	8.5 ± 0.2	8.9 ± 0.4
Proximate composition (%)				
Moisture	82.1 ± 0.2[a]	81.5 ± 0.0[abc]	80.7 ± 0.2[c]	80.7 ± 0.4[bc]
Crude protein	19.2 ± 2.0	22.4 ± 1.9	21.0 ± 0.9	25.7 ± 1.8
Ether extract	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.0
Crude fiber	7.4 ± 1.2	7.4 ± 0.4	7.9 ± 0.1	8.7 ± 0.7
Crude ash	3.7 ± 0.4	3.7 ± 0.1	3.5 ± 0.0	3.5 ± 0.0
Carbohydrate	80.4 ± 2.1	77.1 ± 1.9	78.7 ± 0.9	74.0 ± 1.8
Organic acids (mg/ml)				
Acetic	11.4 ± 0.4[a]	18.8 ± 0.8[b]	11.6 ± 0.5[a]	12.2 ± 1.1[a]
Oxalic	9.3 ± 0.7	10.8 ± 0.6	10.2 ± 0.4	10.3 ± 0.7
Succinic	Trace	Trace	Trace	Trace

[1]Values indicated are based on five observations ± SE. [a,b,c]Values with different superscripts differ significantly ($p < 0.05$).

substrate mix, yet no such tendency was observed in the M and L substrate mixes. Therefore, there was no correlation between the concentration of these substances and the volume of added FAP. Table 5 shows Brix, proximate composition, and organic acid profile of *F. velutipes.*

4. Discussion

There are few studies on the use of fermented apple pomace (FAP) in mushroom substrates and mainly focusing on *Pleurotus pulmonarius* and *H. tessellatus* [8] [6]. When FAP was added to the substrate mixes instead of corncobs and soy pulp, the cultivation period of *P. pulmonarius* was extended by 2 days as FAP was added at a rate of 11.7% on a dry matter basis. There was also a major boost of weights of the fruit bodies, approximately 1.6 times heavier; also spawn running was inhibited when FAP was added at a rate of 17.6%. Furthermore, as FAP was added to the substrate mixes as a nutritional supplement with substances such as wheat bran, the cultivation yield of *H. tessellatus* was basically the same as the control substrate mix with an FAP addition rate of 4.6% and 9.1% on a dry matter basis. Furthermore, spawn running was inhibited when FAP was added at a rate of 13.2%.

The present study is the first to consider the effect of adding FAP to the substrate mixes on *F. velutipes*. While spawn running and pinning were slightly delayed as FAP was substituted for corncobs in the substrate mix, the fact that spawn running was not inhibited even as FAP was added at a rate of 14.4% (L substrate mix) and a faster growth rate after spawn running showed no increase in the overall length of the cultivation period even with the addition of FAP. In light of this, with *F. velutipes*, it was extremely noticeable that there was nearly no negative effect adding FAP to the substrate mixes, which was different from

the previous studies on *H. tessellatus* [6]. Substances contained FAP could have a negative effect on the growth of microorganisms included organic acids, such as lactic acid and acetic acid [9]. If the antimicrobial action of the organic acids contained in FAP was the cause of suppressing mushroom growth, it demonstrated that *F. velutipes* have a higher resistance to organic acids than *H. tessellatus*. Furthermore, it is conceivable that organic acids could have been the cause of the reduction in the substrate mixes' pH. The pH of substrate mixes was within the acceptable range in this study that it was not measured in previous studies.

A distinct influence from adding FAP was observed in terms of *F. velutipes* yield of the fruit bodies, such as the yield increased as the FAP volume increased. The L substrate mix has been increased approximately 1.24 times compared to the control substrate mix. Though it was not as much as the 1.6 times increase in yield observed from *P. pulmonarius* [8], this shows that FAP also has a yield-increasing effect on *F. velutipes*. The present study, FAP substituted only corncobs, which can be speculated that yield-increasing effect on fruit bodies is caused by unique substances of the FAP. A comparison of the proximate composition of corncobs and FAP (**Table 1**) revealed that there were more crude proteins, crude fats, and sugars in FAP and less crude fibers and crude ash. Consequently, it is plausible that the increase in easily decomposable nutrients is one of the factors that increased fruit bodies yield [10]. FAP can be better than corncobs as a raw material for *F. velutipes* substrate mixes due to its ability to increase fruit bodies yield. In this study, we only considered FAP components of up to 14.4% (L substrate mix) and further investigations are required to identify the maximum FAP in substrate mixes, which will increase yield without causing deterioration in cultivation performance.

The *Flammulina velutipes* has been grown in substrates containing sweet bean paste wastes [11], there was a drop in crude proteins and crude fats and a growth in organic acid concentration, especially citric acid. Same species, *F. velutipes* was grown in substrates where the sawdust component was replaced by corncobs and reported a rise in the crude fats and Brix of fruiting bodies as well as a decline in crude proteins [12]. It is known that changes occur in the substances found in fruit bodies is according to the makeup of the substrates in which they are grown, and changes in tastes are expected to occur if such substances are changed. However, in this study, the effects of adding FAP to substrate mixes in terms of the proximate composition of *F. velutipes* fruit bodies was a significant decrease in moisture content but no significant differences in Brix or organic acid concentration among the different substrate mixes were observed. Therefore, there is the possibility that a reduction in fruit bodies' moisture content could result in a better shelf life with little changes in taste.

5. Conclusion

In conclusion, adding fermented apple pomace (FAP) to *F. velutipes* substrate

mix as a substitute for corncobs at a rate of up to 14.4% of the total mix on a dry matter basis increases the fruit bodies yield in a dose-response manner without prolonging the cultivation period. Moreover, it was clear that FAP had no negative influence on fruit body proximate composition, Brix, or organic acid content. It suggests that FAP is a promising local biomass, which could be used as a replacement for corncobs in *F. velutipes* substrate mixes.

Acknowledgements

This study was supported by the Project to Support the Advancement of Strategic Core Technologies (Title: *Using Fermented Apple Pomace in Mushroom Substrates and Using Post-Use Substrates as Highly Functional Animal Feed*), which was run by the Ministry of Economy, Trade and Industry's Kanto Bureau of Economy, Trade and Industry. The authors would like to acknowledge this and express their gratitude. The funding source had no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

References

[1] Ministry of Internal Affairs and Communications (2014) FY2014 Income from Forestry Production Statistics Report. Statistics Bureau, February 2016. http://www.e-stat.go.jp/SG1/estat/List.do?lid=000001147435.

[2] Worrall, J.J. and Yang, C.S. (1992) Shiitake and Oyster Mushroom Production on Apple Pomace and Sawdust. *HortScience*, **27**, 1131-1133. http://hortsci.ashspublications.org/content/27/10/1131.full.pdf+html

[3] Nakazato, Y. (1998) Trial Regarding Adding Nutritional Supplements to Mushroom Substrates. Bulletin of the Forestry and Forest Products Research Institute. *Aomori Prefectural Industrial Technology Research Center*, **48**, 15-21.

[4] Nakazato, Y. (2001) Research Regarding the Use of Apple Juice Dregs in Mushroom Substrates, Bulletin of the Forestry and Forest Products Research Institute. *Aomori Prefectural Industrial Technology Research Center*, **51**, 12-21.

[5] Hiramori, C., Takeuchi, K., Yamada, S. and Koh, K. (2012) Adding of Apple Pomace to Enokitake Mushroom (*Flammulina velutipes*) Substrate Improves Nutritive Value and *In Vitro* Digestibility of the Spent Mushroom Substrate without Decreasing Mushroom Yield. *Proceedings of the* 15th *AAAP Animal Science Congress,* Thailand, 26-30 November 2012, 1977-1980.

[6] Hiramori, C., Ueno, Y. and Koh, K. (2015) Effects of Lactic Acid Bacteria on Fermented Apple Pomace (FAP) Production and Inclusion of FAP in Substrates for Brown Beech Mushroom (*Hypsizygus marmoreus*) Cultivation. *Mushroom Science and Biotechnology*, **23**, 120-124.

[7] JA Nagano Central Union of Agricultural Cooperatives (2010) Mushroom Growing Index. Edited by Nagano Prefecture, Nagano Chapter of the National Federation of Agricultural Co-operative Associations, J-Forest Nagano, **69**, Nagano.

[8] Hiramori, C. and Koh, K. (2014) Cultivation of Oyster Mushrooms (*Pleurotus pulmonarius*) in Beds Using Apple Pomace. *Collection of Summaries of Research Papers Presented at the* 64th *Annual Meeting of the Japan Wood Research Society*, Matsuyama, March 15 2014

[9] Matsuda, T., Yano, T., Maruyama, A., Kumagai, H. (1994) Antimicrobial activities of Organic Acids Determined by Minimum Inhibitory Concentrations at Different pH Ranging from 4.0 to 7.0. *Japanese Society for Food Science and Technology,* **41**, 687-701.

[10] Park, Y.J., Park, H.R., Kim, S.R., Yoon, D.E., Son, E.S., Kwon, O.C., Han, W. and Lee, C.S. (2012) Apple Pomace Increases Mycelial Growth of *Pleurotus ostreatus*. *African Journal of Microbiology Research*, **6**, 1075-1078 http://www.academicjournals.org/article/article1380268962_Park%20et%20al.pdf

[11] Takabatake, K., Nabejima, Y. and Katoh, T. (2003) Effect of using Azuki Bean Paste Refuse as a Substrate on the Yield and Constitution of Fruiting Bodies in Sawdust Based Cultivation of *Flammulina velutipes*. *Mushroom Science and Biotechnology*, **11**, 71-78.

[12] Shiroishi, M., Nishizawa, K., Nozaki, K., Amano, Y. and Kanda, T. (2005) Effect of Culture Medium Components on Cultivation of *Flammulina velutipes* and Fruiting Body Characteristics. *Journal of Applied Glycoscience*, **52**, 133-136. https://www.jstage.jst.go.jp/article/jag/52/2/52_2_133/_pdf