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Comparison of bioactive compounds contained in discard solid state culture and *Cordyceps militaris* fruiting body wines

Sasirindara Labua[1*] and Tanatya Kenkhunthot[2]

[1]Faculty of Food Technology, College of Agricultural Innovation and Food Technology, Rangsit University, Pathum Thani 12000, Thailand
[2]Faculty of Agricultural Innovation, College of Agricultural Innovation and Food Technology, Rangsit University, Pathum Thani 12000, Thailand

*Corresponding author; E-mail: sasirin.l@rsu.ac.th

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Abstract

For centuries, bioactive compounds such as cordycepin and phenolic compounds, found in fruiting bodies and solid-state cultures of *Cordyceps millitaris*, have shown antioxidant activities which promote positive immune modulating effects. Wine is one of the most popular alcoholic beverages consumed worldwide which contains bioactive compounds and phytochemical components such as resveratrol, flavonoids, polysaccharides and phenolic compounds. Daily moderate wine consumption promotes longevity and prevents disease. Based on this, the aim of this research is to compare the bioactive compounds and antioxidant activity in discard solid state culture and *C. millitaris* fruiting body wines which benefits health and promotes positive immune modulating effects. Each wine was collected to assess alcohol content, total phenolic compounds, antioxidant activity and cordycepin content. In this study, alcohol content, antioxidant activity by DPPH (1, 1diphenyl-2-picryl hydrazyl) radical scavenging method and total phenolic compounds in discard solid state culture and *C. millitaris* fruiting body wines increased during fermentation compared to initial amounts. The maximum ethanol content of both wines was quite similar which was about 11.20-11.70% (v/v). The total phenolic compounds corresponding to the absorbance of gallic acid and antioxidant activity of discard solid culture medium wine was significantly higher than that of *C. millitaris* fruiting body wine. The highest measured antioxidant activity and total phenolic compounds in discard solid state culture wine for 30 days were 118.18 ± 2.82 mg AAE/L and 489.88 ± 8.52 mg GAE/L, respectively. Cordycepin, a nucleic acid antibiotic found in discard solid state culture and *C. millitaris* fruiting body wines fermented for 30 days was 7.96 ± 0.45 mg/kg and 66.78 ± 0.08 mg/kg, respectively. This study concludes that bioactive compounds and antioxidant activity in discard solid state culture wine were significantly higher which may benefit health in preventing damages and encourage disease prevention.

Keywords: *antioxidant activity; bioactive compound; Cordyceps millitaris fruiting body wine; discard solid state culture wine.*

1. Introduction

For centuries, *Cordyceps militaris* (*Ascomycota or Hypocreales*) has been globally recognized as a great medicinal fungus highly utilized in functional foods, pharmacology and modern medicine (Nxumalo, Elateeq, & Sun, 2020). *C. militaris* consists of the grass part (stalk or fruiting body) and the insect part (sclerotium) (Zhang, Wen,

Duan, Zhang, & Ma, 2019). *C. militaris* plays an important role in the ability to infect and parasitize lepidopteran during development until finally killing the host (Kim et al., 2010). Bioactive compounds such as cordycepin, polyphenol, amino acids, and trace elements found in this variety have many positive effects on the human immune and respiratory systems. Cordycepin (3-deoxyadenosine) is a nucleic acid antibiotic which has anti-bacterial, anti-viral, anti-tumor, anti-inflammatory, anti-restenosis and immunomodulatory activity (Tang, Qian, & Zhu, 2015). Cordycepin is used in the treatment of several diseases such as diabetes, cancer, hypocholesterolemia, hepatic dysfunctions and also shows antimicrobial activities (Bawadekji, Al Ali, & Al Ali, 2016; Ghosh et al., 2014; Lert-Amornpat, Maketon, & Fungfuang, 2017). Cordycepin has growth- inhibiting properties for several bacteria and associated bacterial diseases, such as *Clostridium perfringens, Clostridium paraputrificum, Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium adolescentis, Lactobacillus casei*, and *Lactobacillus acidophilus* (Ahn, Park, Lee, Shin, & Choi, 2000). Recently, numerous studies have found that *C. militaris* has the potential to resolve male reproductive organ issues such as infertility and reproductive function (Bawadekji et al., 2016). Further, high antioxidant activities provide health benefits in preventing damages due to free radicals and oxidative stress, cancer prophylaxis and therapy, and longevity (Sharma, 2015). Cordycepin has been synthesized both by chemical and biological fermentation. However, cordycepin obtained from chemical synthesis is hard to purify and is more expensive than microbial fermentation (Masuda, Urabe, Honda, Sakurai, & Sakakibara, 2014).

Currently, artificial cultivation by solid state culture (SSC) and liquid state culture of *C. militaris* is much easier. Rice is widely used for the main carbon source in SSC due to its low cost (Cui, 2015). During cultivation, the mycelium and fruiting bodies of *C. militaris* secrete a large amount of cordycepin into the SSC (Masuda et al, 2007). After cultivation, SSC is discarded as food waste. The use of discarded SSC not only lowers food waste and is environmentally friendly but is also profitable to human health and the manufacturer. The fermented *C. militaris* is probiotic and rich in bioactive compounds such as polyphenols, polysaccharides, glucans, and triterpenes which play an important role in immune system regulation, mitigating the effects of oxidation and inflammation and exhibiting anti-obesity properties (Chuang, Hsieh, & Lee, 2020).

Wine is one of the most popular alcoholic beverages consumed worldwide which contains bioactive and phytochemical components, such as resveratrol, flavonoids, polysaccharides and total phenolic compounds (Artero, Artero, Tarín, & Cano, 2015). Importantly, red wine has a high total phenolic compound and antioxidant activity (Cravero, 2019). The total phenolic compounds, such as anthocyanins, tannins and flavonoids influence the astringency, bitterness and color of the wine (Hosu, Cristea, & Cimpoiu, 2014). The most effective antioxidants in wine which are active in cardiovascular diseases are anthocyanins, catechins and resveratrol (Snopek et al., 2018). Wine is also rich in organic acids such as formic acid, lactic acid, amino acids and vitamins (Chen et al., 2017). Alcohol drinking which results in cardiovascular risk associated with mortality depends on frequency, pattern and consumed amount (Chiva-Blanch, & Badimon, 2020). Daily moderate alcohol consumption is 125 mL for women and 250 mL for men. Many researchers have considered that moderate wine consumption promotes longevity and prevents chronic diseases (Pavlidou et al., 2018; Castaldo et al., 2019). Based on this background, this research aimed to investigate bioactive compounds in discard solid state culture compared with *C. millitaris* fruiting body wines. This study could elucidate which bioactive compounds in discard solid culture medium and fruiting body wine have benefits on health promotion and disease prevention.

2. Objectives

The objective of this study was to investigate bioactive compounds contained in discard solid state culture compared with *C. millitaris* fruiting body wines.

3. Materials and methods

3.1 Culture medium and cultivation

C. militaris was purchased from a Thai mushroom farm located at Bangna, Bangkok, Thailand. The suspension was pipetted and spread using a Drigalsky spatula onto petri dishes containing potato dextrose agar (PDA 15 g/L, agar, 15 g/L, dextrose 200 g/L, potato tubers in 1 L of dH_2O). After

incubation at 25°C for 15 days in a total darkness, the fungal colony was cut to about 1 cm in diameter using cork borer. *Saccharomyces cerevisiae* was purchased from Thailand Institute of Scientific and Technological Research. YMA medium (yeast extract 3 g/L, peptone 5 g/L, glucose 10 g/L, agar 20 g/L) was used to cultivate *S. cerevisiae.*

3.2 Liquid state culture for *C. militaris*

MPDA medium (potato broth 200 g/L, brown rice broth 200 g/L, yeast 5 g/L, dextrose 15 g/L, molasses 30 g/L, KH_2PO_4 0.36 g/L, $MgSO_4.7H_2O$ 0.6 g/L, KCl 0.1 g/L, $NaNO_3$ 1.58 g/L, distilled water 1 L) was used for the cultivation of *C. militaris*. MPDA medium, 100 mL, was added into each bottle, then autoclaved at 121°C, pressure 15 lb_f/in^2 for 15 min. The 2 fungal colonies' discs, each about 1 cm diameter, were inoculated in each bottle then incubated for 21 days in total darkness.

3.3 Solid state culture for *C. militaris*

Brown rice and modified culture medium as mention above were prepared for solid state culture. The 40 g brown rice and 40 ml liquid medium were added in each bottle. The 4% (v/v) of inoculum was transferred in each bottle then incubated for 35 days in total darkness. After fermentation, the mycelium and fruiting bodies were harvested.

3.4 Chemicals and materials

1,1-dipheyl-2-pierylhydrazyl (DPPH), acetonitrile, methanol, potassium di-hydrogen-phosphate, and all standard samples were analytical grade supplied by Sigma-Aldrich (St. Louis, U.S.A.), Merck (Darmstadt, Germany) and HiMedia Laboratories Pvt. Ltd (Mumbai, India). All glassware and analysis equipment were provided by the Chemistry and Microbiology Laboratory, College of Agricultural Innovation and Food Technology, Rangsit University and the Central Laboratory (Thailand) Co., Ltd.

3.5 Fermentation of discard solid state culture and *C. militaris* wines

The fresh discard solid state culture and fruiting body of *C. militaris* were harvested, cut and weighed. Water was added at 1:5 and 1:10 (w/v), respectively then heated to 80°C for 5 min. After cooling to 25°C, the pH was adjusted between 3.0-3.5 by citric acid and total soluble solid was adjusted to 22°Brix by sucrose. The inoculum of 10% (v/v) *S. cerevisiae* was added in each sample. The samples of discard solid state culture and *C. militaris* fruiting body wine were fermented at 25°C for 30 days in 3 replications. Each individual wine was collected to assess alcohol, total phenolic, antioxidant activity and cordycepin content. Results are reported as mean ± standard deviation.

3.6 Determination of ethanol content

The ethanol content was determined by Gas Chromatography (Shimadzu, Japan) with spitless mode. The samples of discard solid state culture and *C. militaris* fruiting body wine were collected and filtered through a membrane (0.45 µm, Millipore) before injection. The analysis conditions were carried out with capillary columns; CBP Series: a CBP-20 (250 x 0.33 mm) at 100°C injector and 260°C FID detector. The ethanol content was expressed as % (v/v).

3.7 Determination and analysis of total phenolic compounds

The most abundant phenolic compound in wine was gallic acid. The redox action of gallic acid which resulted in potential increase was used to determine the phenolic content in wine. The quantitative analysis of total phenolic content relied on the Folin–Ciocalteu colorimetric method corresponding to the absorbance of standard gallic acid at 765 nm. 1.5 mL of Folin–Ciocalteu solution (0.2 mol/L) was added to 0.3 mL of wine appropriately diluted with distilled water. The mixture was allowed to react for 5 min then 1.2 mL of 0.7 mol/L $Na_2CO_3$was added. Samples were incubated at room temperature for 120 min in the dark place. A calibration curve was obtained using 0–100 µg gallic acid/mL. The potential change was based on permanganate ion as an indicator. The absorbance of samples was in the range of 0.20–0.80. The total phenolic content was expressed as the amount of gallic acid equivalent (GAE) per liters of wine (mg GAE/L) (Sun, Zhang, Xu, Wang, & Kou, 2019)

3.8 Determination and analysis of total antioxidant activity

The total antioxidant activity was determined by DPPH radical scavenging according to the method of Marques et al., (2012). 0.25 mL of wine was diluted with distilled water to ensure that the absorbance was in the range of 0.20–0.80 then 3.0 mL of 0.09 mg/mL methanolic solution of DPPH was added. Samples were mixed and kept at 20°C for 20 min in the dark. Calibration was performed using ascorbic acid as standard, in the concentration range of 0.150–0.275 mg/mL. The absorption rate of A_{sample} was measured at 517 nm (Agilent 8453UV). A_{blank} was measured without the wine samples. The total antioxidant activity was calculated as milligrams of ascorbic acid equivalents (AAE) per liters of wine (mg AAE/L) (Eq. (1))

$$\% \text{ inhibition} = 100 [A_{blank} - A_{sample}]/ A_{blank} \quad (1)$$

3.9 Determination and analysis of cordycepin

The wine solution was filtered through a 0.45 μm filter membrane. Following this, the filtrate was analyzed by High Performance Liquid Chromatography (Waters, Milford, MA, U.S.A.), using a reverse phase column (RP 18/4.6 × 150 mm, 5 μm). The mobile phase was 10 mmol/L $KH_2 PO_4$, dissolved in methanol/distilled water (6:94). The isocratic elution rate was performed at 1 mL/min with 45°C column temperature and 259 nm UV wavelength (Kang et al., 2014). To identify the suitable cordycepin content, standard cordycepin at various concentrations was dissolved in distilled water and consecutively injected for calibration. The injection volume was 10 μL.

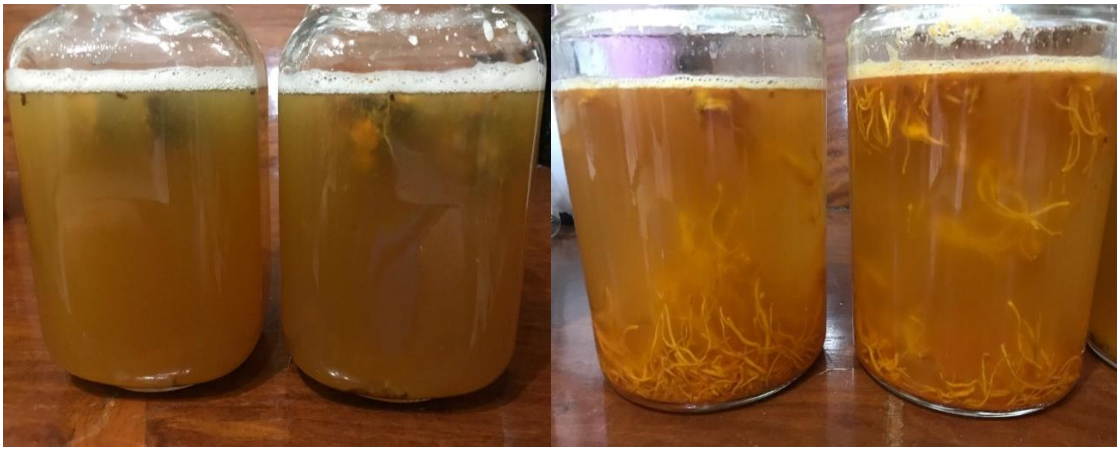
3.10 Statistical Analysis

All experiments were performed in triplicate. The data were expressed as mean ± SD. An analysis of variance (ANOVA) for multiple comparisons was applied at $p<0.05$. The statistical data was analyzed and performed in Statistical Package for the Social Sciences (SPSS) program version 20.0 (SPSS Inc., Chicago, U.S.A.).

4. Results

4.1 Fermentation of discard solid state culture and *C. militaris* fruiting body wines

After fresh discard solid state culture and *C. militaris* fruiting body were harvested, cut and weighed, water was added at 1:5 and 1:10 (w/v), then the total soluble solid and pH were adjusted, respectively. The 10% (v/v) *S. cerevisiae* was added as an inoculum. The fermentation as shown with carbon dioxide or bubble production of discard solid state culture and *C. militaris* fruiting body wines increased rapidly during the first 20 days as shown in Figure 1. The lower fermentation, the decrease, or no bubble (CO_2) occur in the vessels. Carbon dioxide production was nearly depleted before day 20. The discard solid state culture and *C. militaris* fruiting body wines were fermented at 25°C for 30 days



(A) (B)

Figure 1 Discard solid state culture wine (A) and *C. militaris* fruiting body wine (B) during fermentation

4.2 Ethanol content

The ethanol production during discard solid state culture and *C. militaris* fruiting body wine fermentation increased rapidly with the maximum ethanol values of 11.70 ± 0.10% (v/v) and 11.20 ± 0.20% (v/v), respectively. The maximum ethanol content of both wines was quite similar and showed the same pattern as shown in Figure 2. The trend of ethanol production in this experiment is same as other wines. According to this result, it can be concluded that the metabolisms of yeasts in fermentation treatments for ethanol production were the same.

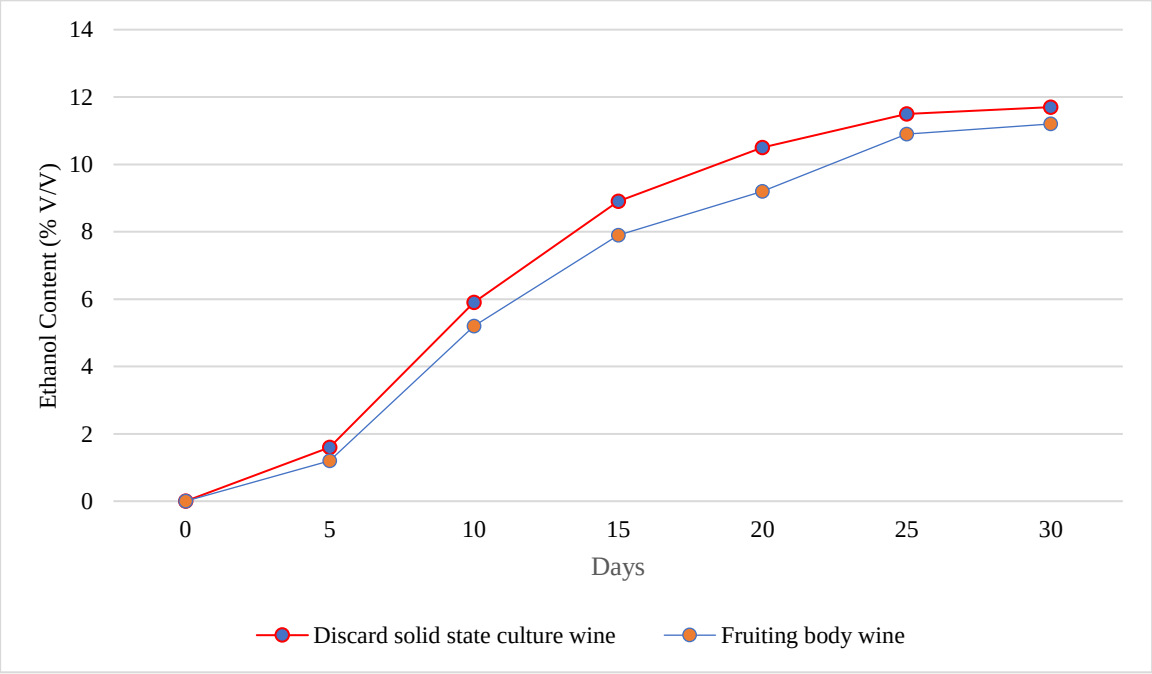


Figure 2 Ethanol production during discard solid state culture and *C. militaris* fruiting body wines fermentation

4.3 The antioxidant activity of discard solid state culture and *C. militaris* fruiting body wines

Many diseases, such as cancer, diabetes and arteriosclerosis occur due to oxidative stress which is associated with an abnormal immune system. Antioxidant activity is protecting the body against oxidation (Zhang et al., 2019). The antioxidant activity determined by DPPH radical scavenging expressed in terms of ascorbic acid was calculated with correlation coefficients of R^2 = 0.9985. The DPPH scavenging results showed that during fermentation the antioxidant activity of discard solid state culture wine changed faster than fruiting body wine (Table 1). This result demonstrated that discard solid state culture wine contained more substances that could react with free radicals. Free radical formation occurs in this fermentation both enzymatic involved in the respiratory and nonenzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing reactions (Lobo, Patil, Phatak, & Chandra, 2010). The highest amount of antioxidant activity (118.18 mg AAE/L) was derived from discard solid state culture wine with a significant difference ($p<0.05$) compared to *C. millitaris* fruiting body wine (108.80 mg AAE/L).

Table 1 Antioxidant activity during fermentation of discard solid state culture and *C. militaris* fruiting body wines

Days	Antioxidant activity (mg AAE/L)	
	Discard solid state culture wine	*C. millitaris* fruiting body wine
0	93.69 [a] ± 0.64	37.90 [b] ± 1.41
5	94.08 [a] ± 0.51	48.66 [b] ± 0.26
10	103.76 [a] ± 1.60	50.46 [b] ± 2.56
15	105.04 [a] ± 3.14	52.52 [b] ± 1.03
20	107.47 [a] ± 10.06	75.84 [b] ± 0.51
25	108.56 [a] ± 2.05	95.46 [b] ± 1.47
30	118.18 [a] ± 2.82	108.80 [b] ± 2.88

Different letters in superscript within column indicate a significant difference at $p<0.05$ according to SPSS (SPSS Inc., Chicago,U.S.A.) version 20.0

4.4 The total phenolic compounds of discard solid state culture and *C. militaris* fruiting body wines

The total phenolic compounds present in wine have a biological effect in some cancers and decrease the risk of cardiovascular diseases (Nunes, Freitas, Almeida, & Laranjinha, 2019). The total phenolic compounds such as gallic acid in wine also contribute to sensorial characteristics and indicates state of the art of wine (Sun et al., 2019). The classic Folin–Ciocalteu colorimetric method was used to calculate, with the high correlation coefficients of $R^2 = 0.9995$, the total phenolic compounds which are considered to have antioxidant activity. *C. militaris* is naturally rich in polyphenol, antioxidant activity and cordycepin content (Bawadekji et al., 2016). Our results showed that at 30 days the amount of total phenolic compounds in discard solid state culture (273.35 ± 8.81) was higher than that of *C. millitaris* fruiting body wine (244.94 ± 8.52) (Table 2). Liu et al. (2018) also demonstrated that the total phenolic compounds of discard solid state culture vinegar was 2.08 times higher than that of rice vinegar. The results are in linear correlation with antioxidant activity (Table 1).

Table 2 Total phenolic compounds during fermentation of discard solid state culture and *C. militaris* fruiting body wines

Days	Total phenolic compounds (mg GAE/L)	
	Discard solid state culture wine	*C. millitaris* fruiting body wine
0	134.43 [a] ± 0.57	107.15 [b] ± 1.70
5	213.13 [a] ± 13.35	138.13 [b] ± 6.54
10	217.39 [a] ± 3.41	148.07 [b] ± 1.71
15	221.65 [a] ± 1.99	154.60 [b] ± 1.99
20	246.65 [a] ± 5.40	159.72 [b] ± 8.24
25	253.18 [a] ± 1.14	212.84 [b] ± 0.02
30	273.35 [a] ± 8.81	244.94 [b] ± 8.52

Different letters in superscript within column indicate a significant difference at $p<0.05$ according to SPSS (SPSS Inc., Chicago,U.S.A.) version 20.0

4.5 The cordycepin content of discard solid state culture and *C. militaris* fruiting body wine

Cordycepin is a unique bioactive compound from *C. militaris* which is used in modern medicine (Bawadekji et al., 2016). Cordycepin plays an important role in anticancer effects, antimicrobial and immune modulation (Zhang et al., 2019). The temperature and pH values for good cordycepin production were 25°C and a pH range of 5.0–6.0 (Lee et al., 2019). The cordycepin content was significantly affected by the increase of pH and temperature (Adnan, Ashraf, Khan, Alshammari, & Awadelkareem, 2017). Results in this study found that cordycepin decreased from the initial amount due to the variation of pH and temperature during fermentation. Moreover, the cordycepin content of discard solid state culture wine fermented for 30 days (66.78 mg/kg) was 8.4 times more than *C. militaris* fruiting body wine (7.96 mg/kg). Liu et al. (2018) also found that cordycepin of discard solid state culture vinegar was 31 times higher than rice vinegar. The results obtained clearly demonstrate that bioactive compounds such as cordycepin, antioxidants and total phenolic content derived from discard solid state culture wine can be used as functional foods for disease prevention and health protection.

Table 3 Cordycepin content during fermentation of discard solid state culture wine and *C. militaris* fruiting body wines

Days	Cordycepin content (mg/kg)	
	Discard solid state culture wine	*C. millitaris* fruiting body wine
0	$97.25^{a} \pm 0.12$	$33.30^{b} \pm 0.20$
15	$69.24^{a} \pm 0.15$	$12.16^{b} \pm 0.03$
30	$66.78^{a} \pm 0.08$	$7.96^{b} \pm 0.45$

Different letters in superscript within column indicate a significant difference at $p<0.05$ according to SPSS (SPSS Inc., Chicago,U.S.A.) version 20.0

5. Discussion

Many researchers have investigated the bioactive compounds and antioxidant activity in *C. militaris* fungus and wine. Consequently, scientists have developed many methods to enhance the cultivation of *C. militaris* with shorter time, lower risk of contamination but higher quantity of fruiting bodies. Currently, artificial cultivation of *C. militaris* by SSC uses rice because of its low cost. However, the large amount of food waste discard from solid state culture after finished cultivation causes environmental problems. The use of discard SSC to ferment wine is one of the methods to lower food waste. Furthermore, the fermentation of *C. militaris* and discard solid state culture wines are also rich in bioactive compounds and phytochemicals such as cordycepin, polyphenols and other antioxidants which play an important role in immune system regulation, anti-inflammation and exhibiting other medicinal effects. The characteristic of discard solid state culture wine in this present investigation showed that the bioactive compounds and antioxidant activity are valuable sources of medicinal compounds. This research has demonstrated that nutrition plays a crucial role in the prevention of chronic diseases.

6. Conclusion

The results of this research may be further used to establish the bioactive compounds in discard solid state as food waste in order to improve the production of medicinal compounds and facilitate a better health promotion and disease prevention. Various cultivation parameters and their optimization are needed for the scale up and automation process.

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