

OPTIMIZATION OF ANTIOXIDANT ACTIVITY IN CORDYCEPS MILITARIS-ENRICHED FERMENTED RICE WATER

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ABSTRACT

This research investigated the chemical composition and biological activities of *Cordyceps militaris* extracts and their application in fermented rice water to enhance antioxidant properties for skincare development. HPLC analysis revealed that *C. militaris* fruiting body extract contained 6.96 mg/g cordycepin and 0.13 mg/g adenosine per DW extract, while mycelial extract contained cordycepin 0.81 mg/g DW extract without detectable adenosine. The aqueous mycelial extract demonstrated significant bioactivities: 69.11±0.25% DPPH radical scavenging and 652.42±0.60 mg/L Fe²⁺ chelating capacity. Water extraction yielded higher phenolic compounds, vitamin E, and polysaccharides, whereas ethanol extraction produced higher flavonoid content. Application studies incorporated *C. militaris* extracts into fermented rice water prepared from Jasmine rice (KDML 105) and glutinous rice using *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Saccharomycopsis fibuligera* (*S. fibuligera*). Late-stage supplementation significantly enhanced antioxidant capacity, phenolic content, flavonoids, and vitamin E levels, particularly with *S. fibuligera* fermentation. These findings demonstrate the potential of *C. militaris*-enriched fermented rice water formulations for developing high-efficacy skincare products with superior antioxidant protection.

Keywords: *Cordyceps militaris*; cordycepin; antioxidant activity; fermented rice water

1. Introduction

There has been a steady increase in the study of golden *Cordyceps* (*Cordyceps militaris*) extracts, including chemical and biological testing and their usefulness in creating nutritional supplements and medications. This is because golden *Cordyceps* contain a wide range of bioactive compounds with demonstrated health-promoting properties and include antitumor activity, promoting the immune system, and lowering the incidence of cancer [1] [2] [3]. Golden *Cordyceps* include a number of important active substances, including ergosterol, polysaccharides, proteins, and cordycepin. Furthermore, studies have been conducted on the antibacterial, anti-inflammatory, anti-photoaging, and antioxidant characteristics of golden *Cordyceps* [4] [5]. Beyond its established applications in regenerative medicine and nutraceuticals, including immunomodulation, sexual performance restoration, and physical vitality enhancement, *Cordyceps militaris* extracts have gained considerable commercial significance in the cosmeceutical sector, particularly in anti-aging and skin-conditioning formulations, owing to their potent antioxidant activity and melanogenesis-inhibitory properties [6] [7] [8] [9].

Mainly the stromata (fruiting bodies) of golden *Cordyceps* (a specialized reproductive structure that emerges from the mycelial network of fungi, functioning primarily to facilitate sexual reproduction and spore dispersal) are harvested for commercial usage because it has a high concentration of significant compounds. The culture media (the nutrient-rich foundation required for fungal growth) and mycelia (the vegetative part of a fungus, consisting of a complex, branching network of thread-like white filaments called hyphae) that make up the base are sold by farmers at a reduced cost. However, the fermented mycelium bases also possess antioxidant and antibacterial qualities, according to research by Dong et al. [10] and Torres-Martínez et al. [11]. As a consequence of the significantly higher concentration of bioactive constituents within the fruiting bodies of *Cordyceps*, this region commands a premium market valuation. In contrast, the residual culture media and mycelia, is frequently categorized as agricultural byproduct with negligible economic worth. Consequently, this research investigated the valorization of *Cordyceps* mycelium bases remnant as a primary substrate for the synthesis of novel value-added products, aiming to optimize the economic utility of this agricultural resource.

Rice constitutes a pivotal agricultural commodity in Thailand, with jasmine rice (*Oryza sativa* L.) enjoying widespread global recognition and commercial acceptance. Jasmine rice is a major export product, with Thailand being the second-largest rice exporter globally, holding a 16% market share [12] and The commercialization of jasmine rice has lifted many rural households out of poverty, particularly in the Northeast region, where its cultivation has been profitable since the 1980s [13]. The fermentation-based processing of Thai rice utilizing microbial agents has gained considerable industrial prominence, with yeasts representing one of the most extensively

employed microorganisms in large-scale fermentation processes. Yeasts function as catalytic agents in the bioconversion of sugar molecules to ethanol and carbon dioxide under aerobic conditions. Industrial fermentation primarily relies on diverse yeast strains, with *Saccharomyces cerevisiae* being the most widely used due to its high efficiency in converting glucose and fructose into ethanol [14] [15] [16]. However, some yeast species, such as *Pichia guilliermondii* and *Saccharomycopsis fibuligera*, exhibit limited starch utilization capabilities during fermentation, leading to the production of alternative metabolites such as vitamins, β -glucan, and bioactive peptides [17] [18]. Additionally, studies have shown that fermented rice water contains significantly higher levels of antioxidant compounds, particularly flavonoids and phenolic acids, compared to its non-fermented counterpart [19].

This study aims to investigate the potential of Cordyceps in enhancing yeast-based fermentation, with a focus on optimizing bioactive compound production. Specifically, to evaluate the feasibility of Cordyceps with conventional fermentation yeasts, assess its impact on antioxidant capacity (particularly flavonoid and phenolic acid content), and to investigate of augmenting antioxidant capacity in fermented rice water for cosmeceutical product development. Furthermore, the study focuses on developing cosmetic products from *Cordyceps militaris* through the utilization of mycelium substrate and cultivation medium—materials conventionally regarded as agricultural by-products from the cultivation process. This strategy generates value-added applications while supporting the progression of *Cordyceps militaris* cultivation toward Agriculture 4.0 via research initiatives aimed at achieving zero agricultural waste.

2. Materials and Methods

Raw material

A *Cordyceps militaris* sample was obtained from a farm in Samut Prakan Province, Thailand. Two kinds of rice, Jasmine brown rice RD 15 and Niao Kuang rice, procured from agricultural farms in Surin Province.

Extraction and preliminary analysis of bioactive properties of Cordyceps militaris extracts.

1. Extraction of Cordyceps militaris.

The 5.00 g samples of *Cordyceps militaris* fruiting body and mycelium were extracted with solvents at a 1:5 ratio, utilizing 95% ethanol and distilled water as extraction solvents. Extraction was performed using the maceration method at room temperature for 24 hours. The extract was subsequently filtered through filter paper, and the solvent was removed using a rotary evaporator. The crude extract was stored in amber bottles to preserve the bioactive compounds' integrity.

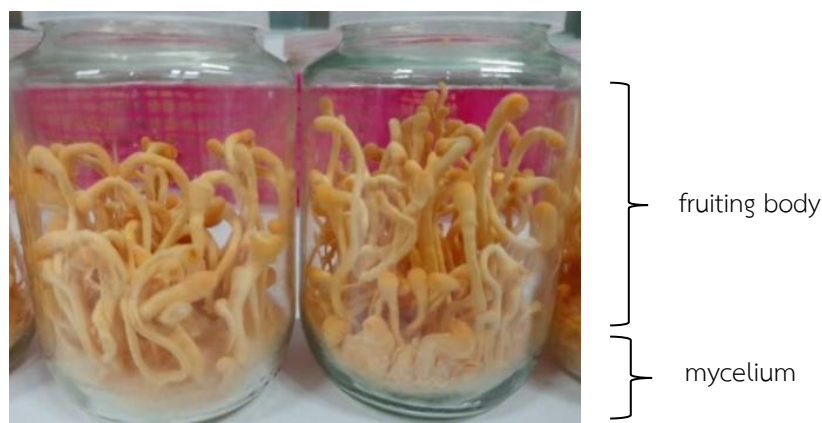


Figure 1. Constituents of *Cordyceps militaris* derived from culture medium cultivation.

In the cultivation of *Cordyceps militaris* (figure 1), the fungus presents two distinct structural forms that are the fruiting body, the orange, finger-like stalks reaching upward (often called "stromata") and the mycelium, the dense, pale, fuzzy layer located at the bottom of the jar.

2. HPLC-based quantification of cordycepin and adenosine concentrations.

Analysis of cordycepin and adenosine content using High-Performance Liquid Chromatography (HPLC) equipped with a Diode Array Detector (DAD) (Ultimate 3000, Thermo Scientific, USA) was performed according to a modified method of Huang et al. (2009) [20]. The stationary phase is Acclaim 120 C18 with a particle size of 5 microns. The mobile phase consisted of water and methanol in a 90:10 (v/v) ratio, with a flow rate of 1 mL/min. The injection volume was 10 μ L, and the column temperature was maintained at 32°C. Standard solutions of cordycepin and adenosine were prepared with concentration ranges of 0-100 ppm and 0-50 ppm, respectively.

3. Preparation of fermented rice water supplemented with *Cordyceps militaris* extract.

Clean samples of Jasmine brown rice RD 15 (RD 15) brown rice and Niao Kuang (NK) rice, an indigenous species of Surin Province, (50 g each) were combined with 100 mL of distilled water and sterilized by autoclaving at 121°C for 15 minutes. After cooling, an additional 100 mL of sterile distilled water was added. Fermentation was initiated using purified *Saccharomycopsis fibuligera* TISTR 5033 (*S. fibuligera*; SF) and *Saccharomyces cerevisiae* TISTR 5013 (*S. cerevisiae*; SC). The fermentation process was conducted at room temperature for 30 and 45 days, respectively. On the final day of fermentation, 10.00 g of *Cordyceps militaris* (C) mycelium extract was added, and the mixture was incubated for an additional 24 hours. The fermented product was subsequently filtered and pasteurized to obtain sample solutions for further analysis. Fermentation formula and corresponding codes are specified in Table 1.

Table 1. The fermentation formula and identification codes.

Identification codes	Fermentation formula
RD15-SF	Jasmine brown rice RD 15 + <i>Saccharomycopsis fibuligera</i>
RD15C-SF	Jasmine brown rice RD 15 + <i>Saccharomycopsis fibuligera</i> + <i>Cordyceps militaris</i> mycelium
NK-SF	Niao Kuang rice + <i>Saccharomycopsis fibuligera</i>
NKC-SF	Niao Kuang rice + <i>Saccharomycopsis fibuligera</i> + <i>Cordyceps militaris</i> mycelium
RD15-SC	Jasmine brown rice RD 15 + <i>Saccharomyces cerevisiae</i>
RD15C-SC	Jasmine brown rice RD 15 + <i>Saccharomyces cerevisiae</i> + <i>Cordyceps militaris</i> mycelium
NK-SC	Niao Kuang rice + <i>Saccharomyces cerevisiae</i>
NKC-SC	Niao Kuang rice + <i>Saccharomyces cerevisiae</i> + <i>Cordyceps militaris</i> mycelium

4. Determination of antioxidant capacity.

4.1 Determination of total phenolic content using the Folin-Ciocalteu method.

Determination of total polyphenolic content was performed using a modified method of Matthayom et al. [21] employing Folin-Ciocalteu reagent containing phosphomolybdate and phosphotungstate as active components. A standard curve was constructed using gallic acid concentrations ranging from 10-500 ppm. The assay procedure involved reacting samples with 1.00 mL of Folin-Ciocalteu reagent and 3.00 mL of 7.5% (w/v) sodium carbonate solution. The reaction mixture was incubated in darkness for 30 minutes at room temperature, resulting in the formation of a blue-coloured complex. Absorbance was measured at 745 nm using a spectrophotometer. Total polyphenolic content in sample solutions was analysed using the same methodology and calculated by comparison with the standard curve. Results were expressed as milligrams of gallic acid equivalents (mg GAE/g DW).

4.2 Determination of total flavonoid content using aluminium chloride colorimetric assay.

Determination of total flavonoid content was conducted using a modified method of Shraim et al. [22]. A standard curve was prepared using catechin at concentrations ranging from 10-50 ppm with sodium nitrite (NaNO_2) as the reagent. The assay procedure involved pipetting 1.00 mL of catechin standard solution and mixing with 0.20 mL of 0.5 M NaNO_2 and 0.20 mL of 0.3 M AlCl_3 . After incubation for 5 minutes, 1.00 mL of 1.0 M NaOH was added, and the mixture was allowed to stand for 5 minutes at room temperature. Absorbance was subsequently measured at 505 nm using a spectrophotometer. A standard curve was constructed using catechin

concentrations. Total flavonoid content in sample extracts was determined using the same methodology and expressed as milligrams of catechin equivalents (mg CAE/g DW).

4.3 Analysis of vitamin E content.

Vitamin E content analysis was performed using an alpha-tocopherol standard curve following a modified method of Bucci et al. [23]. Alpha-tocopherol standard solutions were prepared at concentrations ranging from 100-1000 ppm in DMSO solvent. Absorbance measurements were recorded at 298 nm wavelength using a spectrophotometer. For vitamin E analysis in extracts, 3.00 mL of concentrated extract (1 mg/mL in 95% ethanol) was pipetted and measured for absorbance at 298 nm wavelength. Vitamin E content was calculated from the standard curve and reported as vitamin E content per dry weight of sample.

4.4 Analysis of polysaccharide content using phenol-sulphuric colorimetric assay.

Total polysaccharide content analysis was performed according to the method of Masuko et al. [24]. A glucose standard curve was constructed using concentrations ranging from 100-500 ppm. The assay procedure involved pipetting 1.00 mL of standard solutions at various concentrations and mixing with 0.50 mL of 5% (w/v) phenol solution and 1.50 mL of concentrated sulphuric acid. The reaction mixture was allowed to stand for 20 minutes before measuring absorbance at 490 nm wavelength using a spectrophotometer. Polysaccharide content in the extracts was analysed by comparison with the standard curve and reported as polysaccharide content per dry weight of sample.

4.5 DPPH radical scavenging activity assay.

Antioxidant activity analysis using the DPPH method was performed following a modified procedure of Matthayom et al. [21]. The assay involved pipetting 1.00 mL of sample solution and mixing with 1.00 mL of 500 μ M DPPH solution. The mixture was adjusted to a final volume of 10 mL and incubated in darkness for 30 minutes to allow the reaction to proceed. Absorbance was measured at 505 nm wavelength, recorded as A_{sample} . The absorbance of DPPH solution without sample addition under identical conditions was measured as A_{control} . The percentage of DPPH radical scavenging activity was calculated using Equation (1).

$$\% \text{ DPPH free radical scavenging} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100 \quad (1)$$

where

A_{control} represents the absorbance of DPPH solution

A_{sample} represents the absorbance of DPPH solution with added extract

4.6 Antioxidant activity assay using 1,10-phenanthroline method.

Antioxidant capacity determination using the 1,10-phenanthroline method was performed following a modified procedure of Szydłowska-Czerniak et al. [25]. The assay involved pipetting 1.00 mL of extract sample, adding 1.00 mL of 0.2% (w/v) ferric chloride solution, and 0.50 mL of 0.5% (w/v) 1,10-phenanthroline solution. The reaction mixture was incubated in darkness for 30 minutes at room temperature, resulting in the formation of an orange-red coloured solution. Absorbance was subsequently measured at 512 nm wavelength using a spectrophotometer. Antioxidant activity was reported by calculating results in comparison with a ferrous sulphate standard curve at concentrations ranging from 100-500 ppm.

3. Results

1. HPLC analysis results for cordycepin and adenosine quantification.

HPLC analysis results for cordycepin and adenosine content in 95% ethanol extracts from *Cordyceps militaris* fruiting body (FB) and mycelium (MC) are presented in Table 2. The findings indicate that cordycepin and adenosine concentrations in fruiting body extracts were higher than those in mycelium extracts. This differential distribution can be attributed to metabolic regulation, as demonstrated by Thananusak et al. [26], who reported that light exposure enhances metabolic pathways in *Cordyceps militaris*, leading to increased transcription of genes involved in cordycepin and adenosine biosynthesis, particularly in the fruiting body compared to the mycelium. These analysis results are consistent with the study by Singpoonga et al. [27], which reported concentration ranges of 734.9–9336.0 and 255.8–670.8 mg kg⁻¹ for cordycepin and adenosine, respectively, from 39 *Cordyceps militaris* fruiting body extract samples analyzed using Near-Infrared Spectroscopy technique.

Table 2. HPLC analysis results of cordycepin and adenosine content in extracts from *Cordyceps militaris* fruiting body and mycelium.

Sample	Extracted colours	Cordycepin concentration (mg/g DW extract)	Adenosine concentration (mg/g DW extract)
<i>Cordyceps militaris</i> fruiting body	Dark yellow	6.96	0.13
Mycelium <i>Cordyceps militaris</i>	Light yellow	0.81	nd.

Note: nd. indicates not detected

Although the mycelium contains lower concentrations of cordycepin and adenosine than the fruiting body, its utilization in this study represents a value-added approach to post-harvest waste management in *Cordyceps militaris* cultivation. The mycelium, typically a residual of the harvesting process, was incorporated alongside fermented rice water due to its richness in vitamins and polysaccharides, particularly β -glucan, which are known for their skin-nourishing properties, including moisturization, antioxidant activity, and skin barrier support [28]. This approach therefore not only reduces agricultural waste but also enhances the functional value of the final formulation.

2. Antioxidant content and antioxidant activity analysis results.

The antioxidant compound analysis results are presented in Table 3, encompassing total phenolic contents, total flavonoid contents, vitamin E contents, and polysaccharide contents. Antioxidant compound concentrations in mycelium (MC) extracts obtained using distilled water as solvent were predominantly higher than those extracted with 95% ethanol. When considering antioxidant activity assessment using DPPH and 1,10-phenanthroline methods, crude extracts obtained using distilled water (WT) as solvent demonstrated superior antioxidant capacity compared to those extracted with 95% ethanol (ET). Antioxidant capacity represents the ability to accept electrons from free radicals, thereby converting free radicals into their oxidized forms. Consequently, extracts with high antioxidant compound concentrations typically exhibit superior antioxidant activity. In this study, antioxidant activity testing using DPPH and 1,10-phenanthroline methods yielded consistent results, with *Cordyceps* extracts prepared using distilled water demonstrating higher DPPH and 1,10-phenanthroline scavenging activities compared to extracts obtained with 95% ethanol solvent. These findings indicate that *Cordyceps* extracts exhibit excellent reducing agent efficiency, which is consistent with the research of Dong et al. (2024) [10], who investigated antioxidant compounds in *Cordyceps militaris* mycelium and found high reducing power values in the mycelium. Furthermore, the study of Ncama et al. (2025) [29] examines solvent extraction effectiveness for *Siraitia grosvenorii* (monk fruit) antioxidants, testing water, ethanol (50-95%), methanol, and acetone across phenolic/flavonoid yields via DPPH/ABTS assays. Key finding: 95% ethanol maximizes phenolics, while water excels for flavonoids and overall activity. This confirms that extraction efficiency depends on solvent type.

In this study, water was selected as the most appropriate solvent for extracting bioactive compounds from *Cordyceps militaris* mycelium for cosmetic skincare applications. This selection is supported by several key factors. First, fungal β -glucan possesses a water-soluble structure, enabling more efficient extraction compared to ethanol, and is well-documented for its roles as a humectant and skin barrier enhancer [28]. Second, MCWT demonstrated superior antioxidant activity, with DPPH radical scavenging activity of 69.11% and 1,10-phenanthroline value of 502.55

mg/L, both exceeding those of MCET. Finally, water is recognized as a green solvent that is non-irritating and safe for topical application, unlike ethanol, which may cause skin dryness or irritation, particularly in individuals with sensitive skin.

Table 3. Analysis results of antioxidant compound content and free radical scavenging activity of *Cordyceps militaris* mycelium extract.

Sample	Total Phenolic content (mg GAE/100g dry weight)	Total Flavonoid content (mg CAE/100g dry weight)	Vitamin E content (mg vit. E/100g dry weight)	Polysaccharide content (mg/100g dry weight)	DPPH (%radical scavenging activity)	1,10-phenanthroline (mg/L of Fe ²⁺)
MCWT	16.08±0.16	49.86±0.24	7,714.41±9.86	1,692.90±1.89	69.11±0.28	502.55±0.52
MCET	14.82±0.08	83.74±2.26	1,0974.62±4.93	1,157.81±8.25	62.08±0.26	186.86±0.20

Note: Values presented as antioxidant compound content ± standard deviation.

3. Preparation of fermented rice water supplemented with *Cordyceps militaris* extract.

Results of optimal fermentation time for this research were determined from the fermentation of two rice varieties: Jasmine brown rice RD 15 (RD15) and Niao Kuang rice (NK) for 30 and 45 days. The study evaluated antioxidant activity using DPPH and 1,10-phenanthroline methods derived from *S. fibuligera* yeast metabolism. The findings demonstrated that fermented rice water at 45 days exhibited higher antioxidant activity in both assays compared to 30-day fermentation, as shown in Figure 2 and 3. From the work of Razak et al. [30], it was found that fermentation with *Amylomyces rouxii* over 18 days increased the total phenolic content from 0.03 mg GAE/g to 3.94 mg GAE/g and flavonoid content from 0.04 to 1.71 mg QE/g in broken rice. The DPPH-radical scavenging activity improved from 9.03% to 94.22% by the end of the fermentation period. Consequently, the selected fermented rice water with a 45-day fermentation period was used for subsequent qualitative and quantitative analyses.

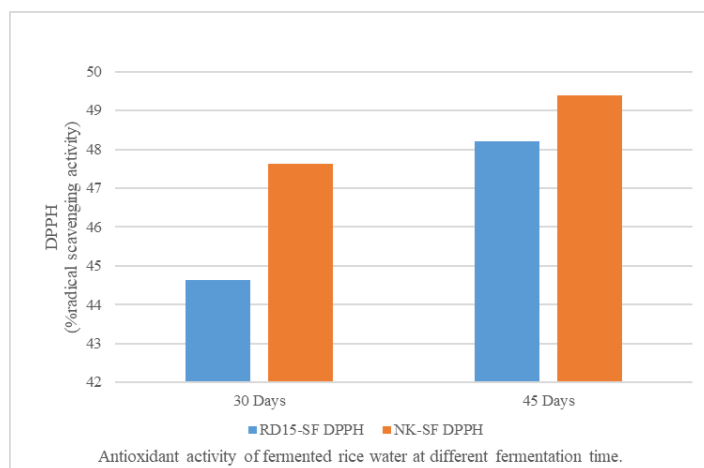


Figure 2. Antioxidant activity using DPPH method.

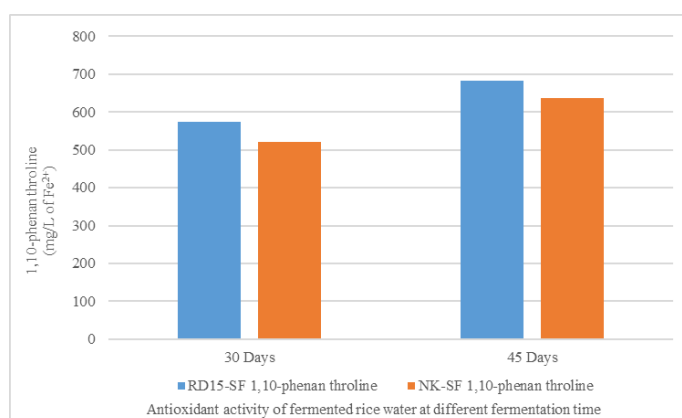


Figure 3. Antioxidant activity using 1,10-phenanthroline method.

Additionally, the acid-base characteristics of fermented rice water from Jasmine brown rice RD 15 and Niao Kuang rice using both types of yeast, and fermented rice water with added *Cordyceps militaris* mycelium extract at 45 days of fermentation, showed a pH range of 3.04 – 3.89. Color measurement results found that fermented rice water from both types of brown rice had lightness values (L*) in the range of 27 – 38, red color values (a*) in the range of -1.04 – 0.04, and yellow color values (b*) in the range of -2.90 – 2.92, indicating characteristics of a clear to light yellow solution.

4. The study of antioxidant capacity using the DPPH method and the 1,10-phenanthroline method of fermented rice water.

The study of antioxidant capacity using the DPPH method and the 1,10-phenanthroline method of fermented rice water obtained from Jasmine brown rice RD 15 and Niao Kuang rice using both types of yeast (*Saccharomyces fibuligera*, SF and *Saccharomyces cerevisiae*, SC) with the addition of *Cordyceps militaris* extract (C) and without extract addition, compared to the antioxidant capacity of *Cordyceps militaris* mycelium extracts, as shown in Figures 4 and 5.

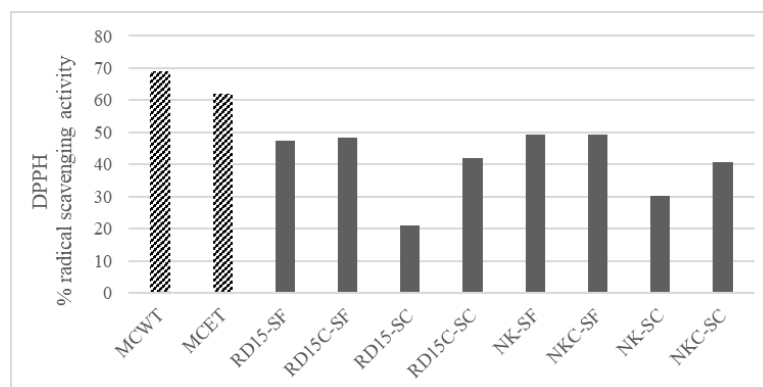


Figure 4. Percentage of antioxidant activity by DPPH radical scavenging method of fermented rice water under different conditions compared to *Cordyceps militaris* mycelium extracts.

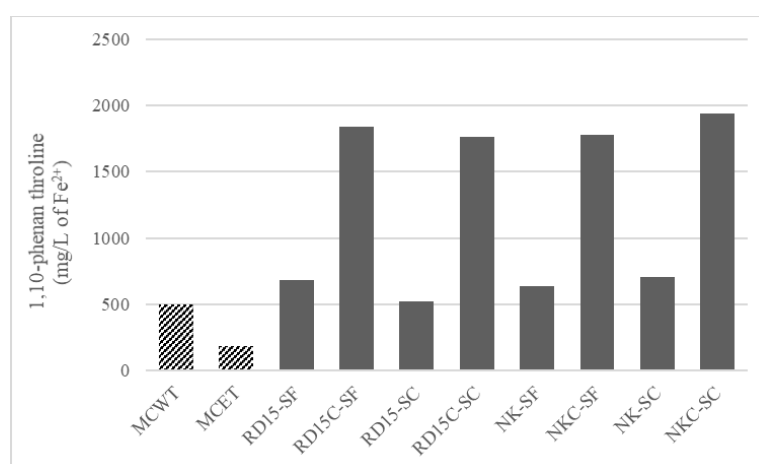


Figure 5. Percentage of antioxidant activity by 1,10-phenanthroline method of fermented rice water under different conditions compared to *Cordyceps militaris* mycelium extracts.

The study results of antioxidant capacity using the DPPH method, comparing between 2 types of fermented rice water with and without *Cordyceps militaris* mycelium extracts, found that fermented rice water obtained from *S. fibuligera* yeast showed higher DPPH antioxidant properties than fermented rice water using *S. cerevisiae* yeast. When considering rice types, it was found that Jasmine brown rice RD 15 had lower antioxidant percentage than Niao Kuang rice, with antioxidant percentages of 47.32% and 49.40% respectively. The addition of *Cordyceps militaris* mycelium extract was able to increase the antioxidant capacity. For the antioxidant study results using the 1,10-phenanthroline method, it was found that Jasmine brown rice RD 15 showed better reducing properties than Niao Kuang rice, with Fe²⁺ amounts of 682.88 and 636.04 mg/L respectively. The *Cordyceps militaris* mycelium extract showed good reducing efficiency, which is consistent with the research of Dong et al. (2014) [31], who studied the antioxidant properties of *Cordyceps militaris* mycelium and found that *Cordyceps militaris* mycelium extract had high

reducing power values, resulting in higher Fe^{2+} amounts when the extract was mixed in. In research by Hemagirri and Sasidharan [32], it was found that fermentation compounds using *Saccharomyces cerevisiae* BY611 yeast showed good trends in anti-aging and antioxidant properties.

4. Discussion and Research Recommendations

Fermented rice water obtained from fermentation by both yeast types yielded different results. Fermentation with *Saccharomycopsis fibuligera* TISTR 5033 yeast resulted in higher antioxidant efficiency of fermented rice water compared to fermentation with *Saccharomyces cerevisiae* TISTR 5013 yeast, which is due to the different metabolic pathways of both yeast types. Additionally, the filtrate from *S. fibuligera* fermentation contains peptide compounds called Galactomyces Ferment Filtrate, which has properties that stimulate skin cell function and inhibit melanin synthesis. The properties of fermented rice water depend on the type and strain of yeast used in the fermentation process. The addition of *Cordyceps militaris* mycelium extract to fermented rice water from both yeast strains resulted in increased antioxidant capacity, because The Cordyceps extract contains various antioxidant compounds such as phenolics, flavonoids, and vitamin E, etc. Fermentation outcomes in rice water vary significantly by yeast strain, with *Saccharomycopsis fibuligera* outperforming *Saccharomyces cerevisiae* in antioxidant capacity due to distinct metabolic pathways and peptide production like Galactomyces Ferment Filtrate for skin benefits.

Future studies might consider the appropriate amount of *Cordyceps militaris* mycelium extract for optimal antioxidant efficiency of *S. fibuligera*-fermented rice water. Measure antioxidant capacity (DPPH, ABTS, FRAP), peptide content, and skin-relevant bioactivity (melanin inhibition, cell proliferation) at 24, 48, and 72 hours post-mixing.

5. Acknowledgements

This research is part of a research project that received research funding support from the Office of the National Research Council of Thailand (NRCT) for research grant support. We would like to thank the Science Program, Faculty of Science and Technology, and the Research and Development Institute, Rajamangala University of Technology Suvarnabhumi, for their support and facilitation of instruments, equipment, and research facilities. We also thank the Department of Chemistry, Faculty of Science, Kasetsart University, for their other support.

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