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Optimization of Submerged Fermentation of Cordyceps militaris Mycelium with Coconut Broth

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ABSTRACT

Cordyceps militaris is a well-known medicinal mushroom celebrated for its immunomodulatory, antioxidant, and anti-inflammatory properties. This study explores the optimization of submerged fermentation for *C. militaris* mycelium using coconut broth as a sustainable nutrient medium. Coconut broth, consisting of coconut sugar, white sugar, and skim milk, was investigated for its effectiveness in promoting biomass yield and bioactive compound production. Using a 2-Level Full Factorial Design and Box-Behnken Design, the study examined varying concentrations of coconut sugar at 9 °Brix (100-200 ml/L), white sugar (10-30 g/L), and skim milk (5-10 g/L) to identify the most influential factors affecting the dry weight of *C. militaris* mycelium (DWCM). Statistical analysis revealed that skim milk, white sugar, and coconut sugar significantly contributed to increased DWCM, with contribution rates of 97.09%, 88.38%, and 74.67%, respectively. The steepest ascent method determined the direction of optimization, and the final regression model identified the ideal conditions for maximum DWCM (27.172 g/L) as 128.38 ml/L of coconut sugar at 9 °Brix, 25.476 g/L of white sugar, and 11.466 g/L of skim milk. These results indicate that coconut broth is an eco-friendly and suitable for scaling up production of bioactive compounds and developing innovative functional health products.

Keywords: *Cordyceps militaris*; Coconut sugar; Optimization; Submerged fermentation

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1. Introduction

Thailand is one of the world's largest producers of coconuts, with numerous plantations located in the southern and central regions. This allows the country to produce millions of coconuts annually. In 2023, the central region alone produced an estimated 387.8 million metric tons of coconuts [1, 2]. The coconut industry supports numerous small-scale farmers and contributes to the production of various coconut products, including coconut milk, coconut oil, and dried coconut. Therefore, the coconut industry is important to the Thai economy, generating income for farmers and raw materials for the food and beverage industry. However, the price of coconuts fluctuates according to supply and demand. During the peak production season, prices tend to decrease, while during the off-season, prices increase, leaving farmers without income throughout the year [3].

Producing coconut sugar for cooking directly from the farm provides farmers with a way to earn income throughout the year. Coconut sugar, also known as coconut palm sugar, is a natural sweetener derived from the nectar of the coconut tree. Its production process is both traditional and sustainable, supporting local farming communities [4, 5]. A sweet liquid rich in natural sugars and nutrients is gently heated over low heat to evaporate the water content. As the water evaporates, the liquid thickens to a syrup-like consistency. Once the syrup reaches the desired thickness, it is poured into molds or left to cool and harden, inducing coagulation. The hardened syrup is then broken down into granules or powder to produce the final coconut sugar product [6, 7].

Fermentation of coconut water, which contains natural sugars such as glucose, fructose, and sucrose [8], using *Saccharomyces cerevisiae* yeast to produce alcoholic beverages such as wine and liquor [9], followed by aeration to convert the alcohol into vinegar [10], which gives the beverage a coconut flavor and aroma, is another approach to processing. However, the fermentation of alcoholic beverages in Thailand has traditionally been dominated by large, long-established companies. Recent legislative changes aim to liberalize the industry, allowing small-scale producers to enter the market. Despite these changes, funding and government support for alcohol production remains limited [11, 12]. There are research studies that utilize coconut sugar as a carbon source for kombucha fermentation [13] and yeast fermentation [14], as well as studies that use coconut husk as a carbon source for ethanol production [15]. Additionally, coconut oil has been used to enhance the fermentation efficiency of *C. militaris* [16]. However, there is currently no research found on the use of coconut sugar specifically as a carbon source for the fermentation of *C. militaris*.

Fermentation of edible microorganisms with edible raw materials results in edible products, thus this research focuses on developing creative and health-promoting beverages, leading to the exploration of fermentation techniques using a variety of natural ingredients, leading to the idea of producing a beverage from fermenting *C. militaris* in coconut sugar. *C. militaris* is a medicinal fungus known to contain bioactive compounds that have received widespread research interest in nutrition and medicine [17], especially for its many medicinal properties, such as immunostimulant, anti-inflammatory [18], anti-cancer [19], antiviral, anti-tumor, and anti-hyperglycemic properties [20]. Coconut sugar, a natural sweetener obtained from the sap of coconut palm inflorescences, offers a delightful flavor and is notably rich in potassium, magnesium, and amino acids. These nutrients make it an ideal substrate for supporting microbial growth during fermentation. When used as a carbon source, coconut sugar not only boosts the nutritional profile of fermented beverages but also enhances the distinct qualities of coconut water, a refreshing and nutrient-dense liquid derived from the same tree, resulting in products with improved health benefits and sensory appeal [21].

This study aimed to investigate the optimum conditions for the submerged fermentation of *C. militaris* mycelium using coconut sugar, a natural and underexplored carbon source in fungal fermentation. Despite the growing interest in functional beverages, limited research has examined the use of coconut sugar to enhance the taste, nutritional profile, and bioactive compound yield in *C. militaris* fermentation. By integrating traditional fermentation practices with modern optimization techniques, this study seeks to address this gap and contribute to the development of sustainable and nutritious functional beverage products that align with consumer demand for natural, health-promoting foods.

2. Materials and Methods

2.1 Preparation of *C. militaris mycelium*

The *C. militaris* strain on solid media was obtained from the Maejo Mushroom Learning Base, Faculty of Agro-Industry, Maejo University. The culture medium was sterilized in an autoclave at 121 °C for 20 min. The culture medium, 100 ml containing 24 g/L potato dextrose broth (PDB), was autoclaved at 121 °C for 20 min. The *C. militaris mycelium* on solid media grown at 24 °C was cut into 1 cm2 sections for inoculation into culture medium in a lamina flow incubator. The *C. militaris mycelium* was grown in a shaking incubator at 150 rpm at 24 °C for 7 days.

2.2 Preparation of coconut sugar

Red rose brand of coconut palm sugar (liquid) is a pure liquid coconut sugar made from natural coconut nectar. It has a sweet taste and a distinctive aroma, making it ideal for preparing desserts. It was purchased from Friend Kitchen Chiangmai Bakermart. Coconut sugar at 9 °Brix is prepared by 250 g of to boiling in 1 L of boiling water for about 20 minutes, then filtered through a cloth to separate the molasses from the water. Distilled water is used to adjust the volume to obtain the sweetness of coconut sugar at 9 °Brix. The preparation of coconut broth was adapted from the method used to prepare longan juice for use as a culture medium [22] and preliminary experiments.

2.3 Submerged fermentation of *C. militaris mycelium* mycelium

The *C. militaris mycelium* mycelium grown in liquid media at 24°C was centrifuged using a sterile apparatus. Five milliliters of the fine *C. militaris mycelium* mycelium were inoculated into 100 ml of various coconut broth in a laminar flow incubator. The *C. militaris mycelium* mycelium was grown in a shaking incubator at 150 rpm at 24 °C for 7 days. The experimental method was adapted from research on the submerged fermentation of *C. militaris mycelium* [23].

2.4 Determination of DWCM

After the submerged fermentation process was completed, samples of *C. militaris mycelium* mycelium in coconut broth were obtained. The samples were measured for growth values based on the DWCM.

Incompletely fermented samples contain sugar in the liquid medium, so they need to be washed out by sucking out 10 ml with a pipette, mixing with 40 ml of distilled water, and pouring into a 50 ml tube. The samples in the test tubes were mixed using a vortex to minimize the amount of residual sugar. The samples in the test tubes were centrifuged at 5000 rpm to separate the mycelium and supernatant. The *C. militaris mycelium* mycelium was dried in a Binder hot air oven model 9010-0305 at 70 °C for 1-2 days until the dry weight of the *C. militaris mycelium* mycelium was constant. The DWCM at 10 ml was converted to g/l.

2.5 Selection of factors using the 2-level full factorial design method

Factors affecting the growth of *C. militaris mycelium* mycelium include carbon sources from coconut sugar and white sugar, and nitrogen sources from skim milk. The optimal conditions were determined by selecting variables and setting each factor at two levels of submerged fermentation: high (+1) and low (-1). The experiment was designed using the 2-level full factorial design method with 3 factors (2^3), as shown in Tables 1.

Table 1. Factors and levels for selection of factors for submerged fermentation of *C. militaris mycelium* mycelium in coconut broth.

Factors	Unit	Level	
		-1	1
Coconut sugar at 9 °Brix	ml/L	100	200
White sugar	g/L	10	30
Skim milk	g/L	5	10

The selection of various factors in the experiment to influence the growth of the *C. militaris mycelium* mycelium was based on the coefficients and P-values from the statistical analysis. Factors with low P-values were identified as significant, resulting in high coefficients, which were then considered within the appropriate range.

2.6 Steepest ascent method to find the optimum range

The coefficients from the statistical analysis influence the level of the factor. If a coefficient is negative (-), the amount of the independent variable should be decreased. Conversely, if a coefficient is positive (+), the amount of the independent variable should be increased. The steepest ascent method was tested under various conditions, as shown in Table 5. The value of the dependent variable gradually increased until it reached its maximum, after which it began to decrease. The optimum range was identified between the maximum value and the point where the optimum range was obtained. This range was then used to determine the next optimum condition.

2.7 Development of mathematical models using response surface method

The condition that yielded the highest value of the dependent variable using the steepest ascent method was selected as the appropriate range for designing the response surface method experiment. The factors were divided into three levels: minimum (-1), middle (0), and maximum (1), as shown in Table 2.

Table 2. Factors and levels for the response surface for submerged fermentation of*C. militaris mycelium* mycelium in coconut broth.

Factors		Unit	Level		
			-1	0	1
Coconut sugar at 9 °Brix	x_1	ml/L	100	130	160
White sugar	x_2	g/L	20	25	30
Skim milk	x_3	g/L	5	10	15

The Box-Behnken Design was employed to design the experiments under various conditions, as shown in Table 6. Quadratic polynomial equations were used to predict the relationship between the factors and responses, as shown in Eq. (2.1).

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_{12} + \beta_{22} x_{22} + \beta_{33} x_{32}, \quad (2.1)$$

where Y is the response value, β_0 is a constant, x_1, x_2, x_3 are the experimental factors, $\beta_1, \beta_2, \beta_3$ are the linear coefficients, $\beta_{12}, \beta_{13}, \beta_{23}$ are the interaction coefficients between the variables, $\beta_{11}, \beta_{22}, \beta_{33}$ are the quadratic coefficients.

2.8 Optimization by matrix method

Finding the optimal condition involves determining the maximum or minimum value of the equation. When Equation 1 was used to develop a 3D model to study the effect of submerged fermentation factors on achieving maximum dry weight, the model showed a peak at a slope of 0. Therefore, to determine the optimum condition, the predictive model can be used by differentiating with respect to the three independent variables: $\frac{dy}{dx_1}$, $\frac{dy}{dx_2}$ and $\frac{dy}{dx_3}$. These derivatives must be equal to 0, as shown in Eqs. (2.2)-(2.4).

$$\frac{dy}{dx_1} = \beta_1 + \beta_{12} x_2 + \beta_{13} x_3 + 2\beta_{11} x_1 = 0$$
$$\text{or } 2\beta_{11} x_1 + \beta_{12} x_2 + +\beta_{13} x_3 = -\beta_1 \quad (2.2)$$
$$\frac{dy}{dx_2} = \beta_2 + \beta_{12} x_1 + \beta_{23} x_3 + 2\beta_{22} x_2 = 0$$
$$\text{or } \beta_{12} x_1 + \beta_{22} x_2 + +\beta_{23} x_3 = -\beta_2 \quad (2.3)$$
$$\frac{dy}{dx_3} = \beta_3 + \beta_{13} x_1 + \beta_{23} x_2 + 2\beta_{33} x_3 = 0$$
$$\text{or } \beta_{13} x_1 + \beta_{23} x_2 + +\beta_{33} x_3 = -\beta_3 \quad (2.4)$$

(2.5)

Eqs. (2.2)-(2.4) can be combined into a matrix form, as shown in Eqs. (2.5)-(2.6).

$$\begin{bmatrix} 2\beta_{11} & \beta_{12} & \beta_{13} \\ \beta_{12} & 2\beta_{22} & \beta_{23} \\ \beta_{13} & \beta_{23} & 2\beta_{33} \end{bmatrix}_{3\times3} \times \begin{bmatrix} x_1 \\ x_2 \\ x_3 \end{bmatrix}_{3\times1} = \begin{bmatrix} -\beta_1 \\ -\beta_2 \\ -\beta_3 \end{bmatrix}_{3\times1}, \quad (2.6)$$

$$\beta_{3\times3} X_{3\times1} = Y_{3\times1} \quad (2.7)$$

Therefore, the optimum condition is predicted from Eq. (2.7).

$$X_{3\times1} = \beta^{-1}_{3\times3} Y_{3\times1} \quad (2.8)$$

2.9 Statistical analysis

One-way ANOVA was performed using SPSS version 29, with each experiment conducted in triplicate to ensure reliability, and statistical significance set at the 0.05 level. Additional statistical analyses were carried out in Microsoft Excel 365. The accuracy of the prediction equation was assessed based on Multiple R, R Square, and Adjusted R Square values; metrics approaching 1 indicate high predictive precision and the ability to estimate maximum yield effectively. The ANOVA results were further evaluated using the Significance F value, coefficients, and P-values to elucidate the relationships between independent and dependent variables. Furthermore, STATISTICA 64 (version 12) was utilized to construct 3D graphs that visually illustrate the influence of independent variables on the dependent outcome.

3. Results and Discussion

To develop a healthy beverage from coconut broth, food-grade ingredients available in the market and baking supply stores were selected. The key components considered include coconut sugar with a sweetness level of 9°Brix as a source of carbon and various compounds, white sugar as a carbon source, and skim milk as a source of both carbon and nitrogen. The response variable measured was DWCM. Determining the optimum conditions involves four steps: selecting variables, finding the appropriate range, developing a mathematical model, and identifying the optimum conditions [24].

3.1 Selection of factors for submerged fermentation of *C. militaris mycelium* mycelium in coconut broth using the 2-level full factorial design method

A 2-Level Full Factorial Design was used for variable selection. Three submerged fermentation factors at two levels were used: coconut sugar at 9 °Brix with volumes of 100 and 200 ml/L, white sugar at 10 and 30 g/L, and skim milk at 5 and 10 g/L. These were designed into a 2^3 factorial experiment, resulting in 8 conditions, as shown in Table 3. The experimental results of the DWCM under each condition were analyzed using statistical regression, as shown in Table 4. Table 4 shows the results of the correlation and regression analysis between the three factors and the response variable of the DWCM. Considering the *P*-value which affects the confidence level, it was found that skim milk, white sugar, and coconut sugar influence the DWCM, respectively.

Table 3. 2-level full factorial design (2^3) for selecting factors in the submerged fermentation of *C. militaris mycelium* mycelium in coconut broth.

Coconut sugar at 9 °Brix (ml/L)	White sugar (g/L)	Skim milk (g/L)	DWCM (g/L)
100	30	5	15.833 ± 3.763
100	10	5	15.333 ± 3.076
100	10	10	18.500 ± 2.588
200	10	5	14.000 ± 2.756
200	10	10	16.166 ± 2.639
200	30	10	17.500 ± 1.870
100	30	10	19.166 ± 0.983
200	30	5	17.333 ± 3.723

Based on the coefficients, it was found that the factors of white sugar and skim milk have positive values, indicating that increasing the amounts of white sugar and skim milk will positively affect the DWCM. Conversely, coconut sugar at 9 °Brix has a negative coefficient, suggesting that reducing the amount of coconut sugar at 9 °Brix will result in an increase in the DWCM. White sugar, or sucrose, is commonly used as a carbon source in fermentation processes. It provides the necessary energy for growth, significantly impacting the biomass production of mycelium [25] and effectively supporting yeast fermentation [26]. Skim milk is rich in proteins and other nutrients, making it an important nitrogen source beneficial for mycelium growth [27]. Research has shown that skim milk improves fermentation characteristics and increases nutrient absorption [28]. Although coconut sugar is a carbon source that microorganisms can utilize, excessive amounts exhibit antimicrobial, antioxidant, and anti-inflammatory properties. This can inhibit the growth of fungi and other microbial activities due to mechanisms related to high sugar concentrations, which create an environment with strong osmotic pressure. Water from microbial cells is drawn out, causing cell shrinkage and disruption

Table 4. ANOVA of 2-level full factorial design (2^3) for selecting factors in the submerged fermentation of *C. militaris* mycelium in coconut broth.

Factors	Coefficients	*P*-value	Confidence level
	16.75	1.5E-06	99.99
Coconut sugar at 9 °Brix, x_1 (ml/L)	-0.5	0.253	74.67
White sugar, x_2 (g/L)	0.75	0.116	88.38
Skim milk, x_3 (g/L)	1.25	0.029	97.09

Multiple R = 0.899, R Square = 0.808, Adjusted R Square = 0.664, Significance F = 0.064

of metabolic functions. Moreover, coconut sugar contains phytochemicals with antimicrobial effects [29, 30].

3.2 Steepest ascent method to find the optimum range for submerged fermentation of *C. militaris* mycelium in coconut broth

The coefficients of the three factors were used to design the steepest ascent experiment for submerged fermentation of C. militaris mycelium in coconut broth to determine the optimal range. Based on Table 4, the coefficients were utilized to determine the step values for the steepest ascent, ranging from run 0 to run 7, as shown in Table 5. It was found that the ascent consisted of the intermediate value of coconut sugar at 9 °Brix 150 ml/L, decreasing by 10 ml/L each time; the intermediate value of white sugar at 20 g/L, increasing by 3 g/L each time; and the intermediate value of skim milk at 7.5 g/L, increasing by 1.25 g/L each time, where x_1 is the coded value and X_1 is the actual value of coconut sugar at 9 °Brix, x_2 is the coded value and X_2 is the actual value of white sugar, and x_3 is the coded value and X_3 is the actual value of skim milk.

From Table 5, the results of the steepest ascent method indicated that at step 2 and step 6, the DWCM was among the highest, at 27 g/L and 28.33 g/L, respectively. This is attributed to coconut sugar at 9 °Brix and white sugar being crucial carbon sources for microbial growth [22]. Therefore, decreasing coconut sugar while increasing white sugar creates the optimal conditions for the carbon source influencing DWCM. Specifically, the first condition has the highest coconut sugar content (step 2), while the other condition has the highest white sugar content (step 6). Excessive coconut sugar may contain certain components that inhibit microbial growth [21]. However, since this research aims to utilize coconut water, Step 2 was selected to determine the optimal range. Therefore, the optimal range is coconut sugar at 9 °Brix 100-160 ml/L, white sugar 20-30 g/L, and skim milk 5-15 g/L.

3.3 Development of mathematical model for submerged fermentation of *C. militaris* mycelium in coconut broth using response surface method

The optimal range for submerged fermentation of C. militaris mycelium in coconut broth was utilized to design the experiment using the Box-Behnken Design with 15 conditions. Each factor included low (-1), medium (0), and high (+1) levels, as shown in Table 6.

When the experimental results of the submerged fermentation factors on the DWCM, the response surface variance was analyzed. The analysis revealed statistically significant differences (Significance F<0.05), with Multiple R, R Square, and Adjusted R Square values exceeding 0.95, indicating the coefficients as shown in Table 7.

From Table 7, Eq. (2.1) can be formulated to predict the amount of coconut sugar at 9 °Brix (x_1), the amount of white sugar (x_2), and the amount of skim milk (x_3) on the DWCM (y), as shown in Equation 8. By considering the P-value < 0.05 and the coefficients, it was found that a decrease in the amount of coconut sugar at 9 °Brix (x_1), along with an increase in the amounts of white sugar (x_2) and skim milk (x_3), results in an increase in the DWCM.

$$\begin{aligned} Y =& 27 - 1.042x_1 + 0.958x_2 + 0.667x_3 \\ &+ 0.75x_1x_2 + 1.667x_1x_3 - 0.667x_2x_3 \\ &- 4.458x_{12} - 3.792x_{22} - 0.875x_{32}. \end{aligned} \tag{3.1}$$

Table 5. Steepest ascent method for submerged fermentation of *C. militaris* mycelium in coconut broth.

Run	Steps	x_1	x_2	x_3	X_1 (ml/L)	X_2 (g/L)	X_3 (g/mL)	DWCM (g/L)
	Δ	**-0.2**	**0.3**	**0.5**	**-10**	**3**	**1.25**	
0	Intermediate value + 0Δ	0	0	0	150	20	7.5	21.17±0.75 ^{g}
1	Intermediate value + 1Δ	-0.2	0.3	0.5	140	23	9	22.00±0.89 ^{f,g}
2	Intermediate value + 2Δ	-0.4	0.6	1	130	26	10	27.00±0.00 ^{a,b}
3	Intermediate value + 3Δ	-0.6	0.9	1.5	120	29	11	23.83±0.75 ^{d,e}
4	Intermediate value + 4Δ	-0.8	1.2	2	110	32	13	22.83±0.41 ^{e,f}
5	Intermediate value + 5Δ	-1	1.5	2.5	100	35	14	25.83±2.14 ^{b,c}
6	Intermediate value + 6Δ	-1.2	1.8	3	90	38	15	28.33±0.52 ^{a}
7	Intermediate value + 7Δ	-1.4	2.1	3.5	80	41	16	24.83±0.41 ^{c,d}

Remark: Data are expressed as means ± SD (n = 3). Means followed by the same letter are not significantly different according to Waller-Duncan's test (P >0.05)

Table 6. Box-Behnken Design for submerged fermentation of *C. militaris* mycelium in coconut broth.

Run	Coconut sugar at 9 °Brix (ml/L)	White sugar (g/L)	Skim milk (g/L)	DWCM (g/L)	
	X_1	X_2	X_3	Y experimental	Y predicted
1	100 (-1)	20 (-1)	10 (0)	19.333	19.583
2	160 (1)	20	10	15.000	16.000
3	100	30 (1)	10	21.000	20.000
4	160	30	10	20.000	19.417
5	100	25 (0)	5 (-1)	24.333	23.708
6	160	25	5	19.333	18.292
7	100	25	15 (1)	21.333	21.708
8	160	25	15	22.667	22.958
9	130 (0)	20	5	20.333	20.042
10	130	30	5	22.000	23.292
11	130	20	15	24.333	22.708
12	130	30	15	23.000	23.292
13	130	25	10	27.333	27.000
14	130	25	10	27.000	27.000
15	130	25	10	26.667	27.000

Table 7. ANOVA of RSM for submerged fermentation of *C. militaris* mycelium in coconut broth.

	Coefficients	Standard Error	P-value	Confident level
Intercept	27.000	0.711	0.000	100.000
x_1	-1.042	0.435	0.062	93.780
x_2	0.958	0.435	0.079	92.100
x_3	0.667	0.435	0.186	81.370
x_1x_2	0.750	0.616	0.278	72.244
x_1x_3	1.667	0.616	0.042	95.755
x_2x_3	-0.667	0.616	0.328	67.162
x_{12}	-4.458	0.641	0.001	99.906
x_{22}	-3.792	0.641	0.002	99.803
x_{32}	-0.875	0.641	0.230	76.961

Multiple R = 0.976, R Square = 0.953, Adjusted R Square = 0.867, Significance F = 0.008

Eq. (3.1) was utilized to generate 3D images illustrating the response surfaces of the amount of coconut sugar at 9 °Brix, the amount of white sugar, and the amount of skim milk in relation to the DWCM, as shown in Figs. 1-3.

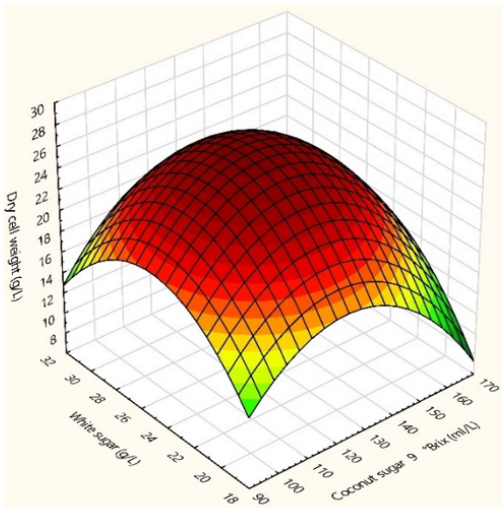

Fig. 1. The response of coconut sugar at 9 °Brix and white sugar on the DWCM at a skim milk concentration of 10 g/L.

3.4 Optimization for submerged fermentation of *C. militaris* mycelium in coconut broth by matrix method

The optimal conditions for submerged fermentation of *C. militaris*

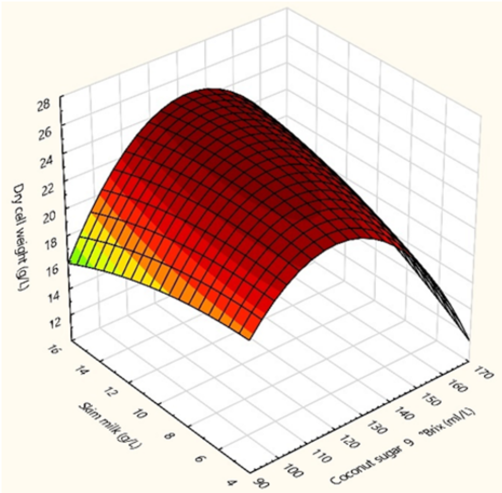

Fig. 2. The response of coconut sugar at 9 °Brix and skim milk on the DWCM at a white sugar concentration of 25 g/L.

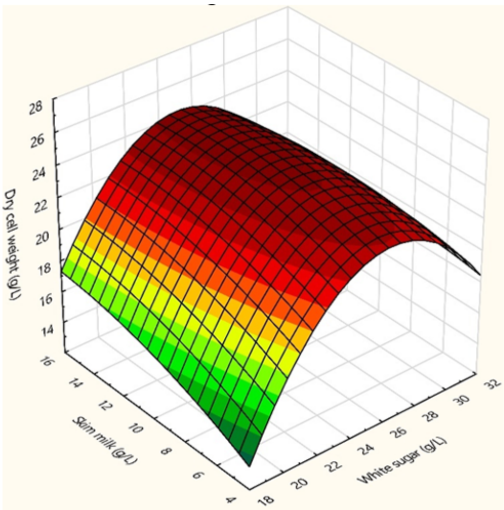

Fig. 3. The response of white sugar and skim milk on the DWCM at a coconut sugar at 9 °Brix concentration of 130 ml/L.

mycelium in coconut broth were determined by substituting the coefficients from Table 7 and Eq. (3.1) into Eq. (2.5), as shown in Eq. (3.2), with the inverse of β matrix (β^{-1}) presented in Eq. (3.3).

$$\begin{bmatrix} 2(-4.458) & 0.75 & 1.667 \\ 0.75 & 2(-3.792) & 0.050 \\ 1.667 & 0.050 & 2(-0.875) \end{bmatrix}_{3\times3} \times \begin{bmatrix} x_1 \\ x_2 \\ x_3 \end{bmatrix}_{3\times1} = \begin{bmatrix} 1.042 \\ -0.958 \\ -0.667 \end{bmatrix}_{3\times1}, \quad (3.2)$$

$$\beta^{-1}_{3\times3} = \begin{bmatrix} -0.136 & -0.002 & -0.129 \\ -0.002 & -0.136 & 0.050 \\ -0.129 & 0.050 & -0.713 \end{bmatrix}_{3\times3} \quad (3.3)$$

Therefore, the optimal condition was calculated from Eq. (2.7) to obtain the coded values of the factors, as shown in Eq. (3.4).

$$\begin{bmatrix} x_1 \\ x_2 \\ x_3 \end{bmatrix}_{3\times1} \begin{bmatrix} -0.054 \\ 0.095 \\ 0.293 \end{bmatrix}_{3\times1}. \quad (3.4)$$

From Table 8, converting the coded values of the optimal conditions for submerged fermentation of C. militaris mycelium in coconut broth to actual values, it was found that coconut sugar at 9 °Brix 128.38 ml/L, white sugar 25.476 g/L, and skim milk 11.466 g/L can produce the highest DWCM at 27.172 g/L, which are the optimal conditions.

Table 8. Converting code values to actual values of factors for submerged fermentation of *C. militaris* mycelium in coconut broth.

Factors	Unit	Optimization		
		Coded	Actual	
Coconut sugar at 9 °Brix	ml/L	-0.054	130+(-0.054*30)	128.38
White sugar	g/L	0.095	25+(0.095*5)	25.476
Skim milk	g/L	0.293	10+(0.293*5)	11.466

Fermentation was conducted in a 10-liter bioreactor with a working volume of 7 liters under optimized conditions, comprising coconut sugar at 9° Brix (130 mL/L), granulated sugar at 25 g/L, and whey at 11.5 g/L. These conditions yielded DWCM at a concentration of 27.333□0.707 g/L. Greater biomass results in increased production of bioactive compounds due to a higher number of active cells and the presence of necessary enzymes and biosynthetic pathways. These cells are capable of producing bioactive substances such as cordycepin, adenosine, and polysaccharides. Moreover, medium optimization not only supports cellular growth but also supplies precursors essential for metabolite synthesis [32, 33]. Therefore, the results of this research can be applied to the production of bioactive compounds from *C. militaris* mycelium with medicinal properties at an industrial level [34]. These optimal conditions result in higher yields and improved quality of mycelial biomass and bioactive compounds [35]. However, if the objective is to increase the amount of bioactive compounds, pH control should be intensified [36]. This, however, results in a salty taste due to the accumulation of acidic and basic ions, making the product unsuitable for use as a health food. Although using coconut sugar as a carbon source is more expensive than using fresh coconut water, because of its labor-intensive production process and the lack of large-scale industrial manufacturing, it still supports local communities or small-scale farmers. This approach introduces an innovative method for transforming coconut into a health-promoting beverage by fermenting medicinal mycelium. Coconut sugar was selected as the primary ingredient for its nutritional value and was used as a substrate for fermenting *C. militaris*, a medicinal fungus. Research findings have demonstrated that a functional beverage developed through submerged fermentation of *C. militaris* holds promise as an immune-enhancing dietary supplement, without inducing toxicity in the liver, kidneys, or blood components [37]. Although Thailand is rich in various sources of carbon and nitrogen that medicinal fungal mycelium can utilize, mycelium-based food products are still relatively new to the country. As a result, there is currently no official information or regulatory approval from the Food and Drug Administration allowing mycelium to be used as an ingredient in dietary supplements or pharmaceuticals, which poses a significant obstacle to the advancement of this research. Nevertheless, in Taiwan and many other countries around the world, fungal mycelium is gaining considerable attention for its potential in developing biobased materials and health-related products, particularly within the framework of the Bio-Circular-Green (BCG) economy model.

4. Conclusion

This study successfully identified the optimal conditions for submerged fermentation of Cordyceps militaris mycelium using coconut broth as a nutrient medium. By applying factorial design, steepest ascent analysis, and response surface methodology, the ideal concentrations of coconut sugar at 9 °Brix (128.38 ml/L), white sugar (25.476 g/L), and skim milk (11.466 g/L) were determined, yielding a maximum dry weight of *C. militaris* mycelium (DWCM) at 27.172 g/L. These findings underscore the efficiency of coconut-based fermentation systems for promoting fungal growth and metabolite production. Furthermore, the optimized medium offers a sustainable and locally adaptable solution that can be scaled for industrial applications. This approach enhances the utilization of agricultural resources and supports the development of value-added functional health products derived from medicinal fungi. However, the gap in this research lies in its focus on health beverages. Therefore, the optimal conditions emphasized biomass yield rather than the analysis of bioactive compounds. It is thus necessary to conduct a detailed analysis of these bioactive substances. If the goal is to develop a beverage product, sensory evaluation tests should also be included. Moreover, to develop bioactive compounds from fermentation processes at an industrial scale, it is essential to design and build large, complex bioreactors. This includes designing the fermentation system, sterilizing the bioreactor using a high-pressure steam boiler, implementing air and temperature control systems, and equipping tools for measuring bioactive compounds. Such development requires expertise in chemistry, biotechnology, food technology, and mechanical engineering. Consequently, industrial-scale development demands significant investment in equipment and human resources. It is necessary to seek funding from the private sector to establish a prototype factory.

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