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Research article

Optimization of White Popinac Substrate Composition for Milky Mushroom Cultivation

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Abstract

This study was aimed at optimizing the substrate composition of white popinac wood chips for milky mushroom cultivation. Response surface methodology and central composite design were used to evaluate the effects of rice bran, dolomite, and phosphate rock concentrations. The optimized substrate composition, which was determined to maximize biological efficiency, consisted of 5% rice bran, 2.5% dolomite, and 2.5% phosphate rock, which yielded a biological efficiency of 89.72%. Proximate analysis of the cultivated mushrooms revealed 82.15% moisture, 20.14% protein, 1.60% fat, 64.87% carbohydrate, and 8.29% ash content. The glutathione concentration was 58.46±6.14 μM. The DPPH and ABTS radical scavenging assays showed IC50 values of 93.20±8.90 and 67.08±9.61 μg/mL, respectively. The results of this study underscore the potential of organic milky mushroom cultivation and highlight the suitability of white popinac wood chips as a sustainable substrate.

Keywords: milky mushroom; white popinac; response surface methodology; mushroom cultivation; biological efficiency

1. Introduction

Calocybe indica P&C, commonly known as milky mushroom, is a prized species due to its impressive size, excellent taste, and exceptional nutritional value. It is a rich source of protein, fiber, carbohydrates, vitamins, and minerals, making it a valuable component of a balanced diet (Chelladurai et al., 2021). Furthermore, it contains some medicinal properties due to the presence of bioactive compounds, which exhibit various bioactivities, including anti-cancer, antioxidant, anti-obesity, anti-aging, anti-inflammatory, antimicrobial, hepatoprotective, and immune-boosting effects (Shashikant et al., 2022).

The cultivation of milky mushrooms is widespread, particularly in Asia, where the mushrooms can be grown on various substrates. Although rice straw is commonly used, other cellulosic substrates such as corn cob, rice husks, sugarcane bagasse, plant leaves,

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and stalks may be used as alternatives. The mushroom cultivated on rice straw results in a biological efficiency of 95%-132.4% (Vijaykumar et al., 2014; Kerketta et al., 2018). In Thailand, the average market price of milky mushrooms ranges from 100 to 150 THB per kilogram, depending on factors such as quality and location of sale.

White popinac (*Leucaena leucocephala* (Lamk.) de Wit) is a fast-growing tree known for its nitrogen-fixing ability. It is used for various purposes, including animal feed, green manure, fuel, soil stabilization, and soil fertility improvement. The wood of the white popinac is rich in cellulose, hemicellulose, and lignin, which are essential nutrients for mushroom growth. This substrate is suitable for the cultivation of certain edible mushroom species, such as the oyster mushroom, *Pleurotus ostreatus* (Abeer et al., 2013); however, not all mushroom species can grow on white popinac, and studies should be conducted to evaluate its suitability as a substrate before commercial cultivation.

In this study, response surface methodology (RSM) was employed to investigate the suitability of substrate concentration when using white popinac wood chips as the basic substrate for milky mushroom production. The results of this study could provide valuable insights into the optimal conditions for cultivating the mushrooms on this substrate, contributing to the development of sustainable and efficient mushroom cultivation practices.

2. Materials and Methods

2.1 Mushroom strain and culture condition

The milky mushroom was obtained from the Kru Payong-Anan Mushroom Farm (Ang Thong, Thailand). A pure culture of the mushroom was maintained on potato dextrose agar (potato infusion 200 g/L, dextrose 20 g/L, and agar 15 g/L) at 30°C and sub-cultured monthly to ensure viability and productivity. For short-term storage, the culture was stored at 4°C, which minimized metabolic activity and reduced the risk of genotypic and phenotypic changes.

2.2 Optimization of mushroom substrate concentration using response surface methodology (RSM)

The optimal concentration of the mushroom substrate was analyzed by RSM using a central composite design. The experiment was conducted with three variables, which included rice bran (0-10%), dolomite (0-5%), and phosphate rock (0-5%) to determine the optimal substrate concentration for biological efficiency when white popinac wood chips were used as a basic substrate. Each factor was determined using five scoring levels (-2, -1, 0, 1, 2) as shown in Table 1. A set of 20 experiments with six central points, six axial points, and eight factorial points was designed and the experiments were performed with two replicates.

The data obtained for biological efficiency were subjected to analysis of variance (ANOVA). The relationship between the response (biological efficiency) and the three independent variables (concentration of rice bran, dolomite, and phosphate rock) was approximated by quadratic equation 1.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{23} BC + \beta_{13} AC + \beta_{123} ABC \quad (1)$$

where Y is the dependent response and A, B, and C represent the independent variables.

2.3 Mushroom cultivation

For spawn preparation, sorghum grains were submerged overnight and boiled until they were soft. They were drained to remove the excess water. After transfering to a bottle, they were sterilized in an autoclave at 15 lb pressure (121°C) for 20 min. A 0.5 cm disc from a seven-day-old mycelium culture was inoculated into the sorghum medium and incubated at 30°C for 14 days. For mushroom cultivation, 1.2 kg of approximately 0.5 × 0.5 × 0.5 cm white popinac wood chip were submerged overnight and drained. They were then mixed with rice bran, dolomite, and phosphate rock and sterilized in an autoclave at 15 lb pressure (121°C) for 20 min. After cooling down, 60 g of spawn was added and mixed well. The substrate was then transferred to a 10-inch-diameter × 7.5-inch-tall plastic pod, loosely wrapped with a plastic bag, and incubated at 30±2°C for 30 days. For casing, 600 g of soil was moistened to approximately 70%, sterilized in an autoclave at 15 lb pressure (121°C) for 20 min, and overlaid onto the substrate after cooling down. The pod was then transferred to a chamber with a temperature range of 28 to 32°C and the humidity was maintained at 80-90% using mist spray. The first and second mushroom flushes were harvested and measured as the total yield of mushrooms. The biological efficiency was calculated according to formula 2.

$$\text{biological efficiency (\%)} = \frac{\text{weight of mushroom fresh weight}}{\text{weight of dried substrate}} \times 100 \quad (2)$$

2.4 Proximate analysis

The moisture, protein, fat, total carbohydrate, and ash content were determined based on the AOAC method (Latimer, 2016).

2.5 Determination of glutathione content

The total glutathione (GSH + GSSG) of the milky mushroom was measured by the EnzyChrom™ GSH/GSSG Assay Kit (Cat. No. EGTT-100, BioAssay Systems, USA) according to the manufacturer's instruction.

2.6 Determination of antioxidant activities by DPPH and ABTS radical scavenging assays

The mushroom was dried, and ground into powder. The mushroom powder (50 g) was then extracted using the maceration technique by soaking the powder in 250 mL ethanol for 24 h. The resulting extract was filtered through Whatman filter paper no. 1 and the solvent was evaporated using a rotary evaporator. The dried extracts were resuspended in dimethyl sulfoxide. The DPPH and ABTS radical scavenging assays were performed according to Romruen and Bangyeekhun (2024).

3. Results and Discussion

White popinac wood chips were used for the cultivation of milky mushrooms using a tray culture technique (Figure 1). After inoculation, 25-28 days were required for the spawn run. After casing with soil, a pinhead was observed 35-40 days following inoculation. The first and the second crop were harvested on days 48-52 days and 70-80 days after inoculation,

respectively. By supplementing the wood chip substrate with rice bran, dolomite, and phosphate rock enhanced production of a mushroom fruiting body was observed. Each supplement increased mushroom's biological efficiency by 4-9% (Figure 2).

Thus, the substrate composition for milky mushroom production was optimized by varying the concentration of rice bran, dolomite, and phosphate rock using a central composite design. The range and the levels of the variables examined are shown in Table 1. The central composite design with three variables in 20 experimental trials was conducted and the resulting biological efficiencies are given in Table 2. The lowest and highest biological efficiency was 80.94% and 91.89%, respectively. The relationship between the variables and biological efficacy is shown by equation 3.

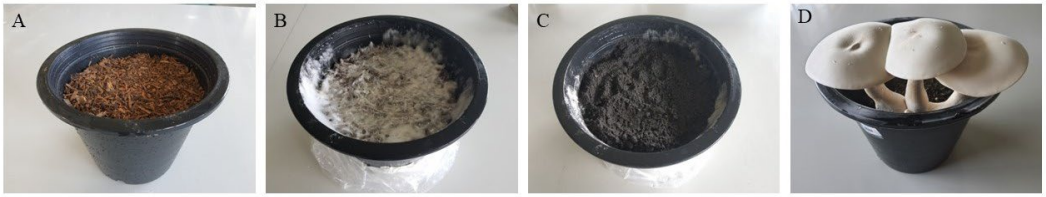

Figure 1. Cultivation of milky mushroom using white popinac wood chip substrate by a tray culture technique. A: Substrate preparation and inoculation. B: Fully colonized mycelium on substrate. C: Soil casing. D: Fruitification.

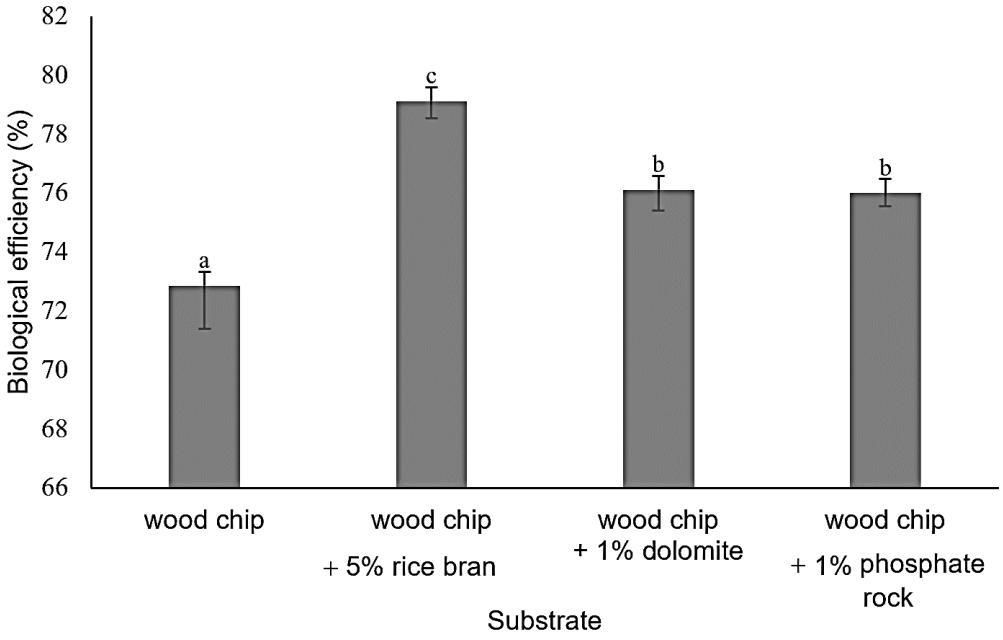

Figure 2. Biological efficiency of milky mushroom cultivated on white popinac wood chip with different supplements. The values are the means of triplicate experiments. The error bars indicate the standard deviations of the means. There are no significant differences between the points marked with the same letter ($P<0.05$).

$$\text{Biological efficiency (\%)} = 89.61 + 1.142A + 0.502B + 0.138C - 1.175A^2 - 1.621B^2 - 1.390C^2 + 0.149AB - 0.534AC - 0.761BC + 1.936ABC \quad (3)$$

where A, B, and C correspond to rice bran, dolomite, and phosphate rock, respectively.

Table 1. Range and levels of variables used in central composite design

Variables	Range and Levels				
	-2	-1	0	1	2
Rice bran (%)	0	2.5	5	7.5	10
Dolomite (%)	0	1.25	2.5	3.75	5
Phosphate rock (%)	0	1.25	2.5	3.75	5

Table 2. Experimental design using central composite design and biological efficiency

Run No.	Coded Variable			Natural Variable			Biological Efficiency (%)	
	Rice Bran	Dolomite	Phosphate Rock	Rice Bran	Dolomite	Phosphate Rock	Observed	Predicted
1	-1	-1	-1	2.5	1.25	1.25	81.62	80.57
2	1	-1	-1	7.5	1.25	1.25	86.53	87.49
3	-1	1	-1	2.5	3.75	1.25	87.88	86.67
4	1	1	-1	7.5	3.75	1.25	85.64	86.44
5	-1	-1	1	2.5	1.25	3.75	87.46	87.31
6	1	-1	1	7.5	1.25	3.75	82.49	84.35
7	-1	1	1	2.5	3.75	3.75	82.93	82.62
8	1	1	1	7.5	3.75	3.75	86.30	88.00
9	-2	0	0	0	2.5	2.5	80.94	82.63
10	2	0	0	10	2.5	2.5	89.54	87.20
11	0	-2	0	5	0	2.5	82.61	82.13
12	0	2	0	5	5	2.5	84.30	84.13
13	0	0	-2	5	2.5	0	83.22	83.79
14	0	0	2	5	2.5	5	85.57	84.35
15	0	0	0	5	2.5	2.5	91.89	89.61
16	0	0	0	5	2.5	2.5	89.35	89.61
17	0	0	0	5	2.5	2.5	87.25	89.61
18	0	0	0	5	2.5	2.5	89.98	89.61
19	0	0	0	5	2.5	2.5	89.28	89.61
20	0	0	0	5	2.5	2.5	90.58	89.61

A quadratic regression analysis using ANOVA was conducted to determine the significance of the coefficients and *p* values (Table 3). The analysis revealed that the *P* value obtained for the model was <0.001, indicating that the relationship between the predictor variables and the response variable was sufficient to ensure confidence. The positive coefficient for rice bran^2, dolomite^2, and phosphate rock^2 indicated a quadratic effect that increased biological efficiency. An F-value of 4.570, which is greater than the significant F-value of 0.016, suggests that the between-group variation is significantly larger than the within-group variation. This indicates that the model provides a good fit to

Table 3. Analysis of variance for the experimental results of the central composite design quadratic model for biological efficiency

Source	Coefficients	Standard Error	t Stat	*P*-value
Intercept	89.613	0.765	117.165	<0.001*
Rice bran	1.142	0.479	2.382	0.041*
Dolomite	0.502	0.479	1.047	0.322
Phosphate rock	0.138	0.479	0.288	0.780
Rice bran*Rice bran	-1.175	0.382	-3.072	0.013*
Dolomite*Dolomite	-1.621	0.382	-4.239	0.002*
Phosphate rock*Phosphate rock	-1.386	0.382	-3.624	0.006*
Rice bran*Dolomite	0.149	0.678	0.219	0.831
Rice bran*Phosphate rock	-0.534	0.678	-0.787	0.451
Dolomite*Phosphate rock	-0.761	0.678	-1.123	0.291
Rice bran*Dolomite*Phosphate rock	1.936	0.678	2.856	0.019*

R-Sq = 83.55% R-Sq (adj) = 65.27%

* a significance level of 0.05 ($p < 0.05$)

the data, as the observed differences between groups are statistically significant. The value of the determination coefficient (R^2) was 0.8355, which suggests that the equation can explain up to 83.55% of the variability in biological efficiency.

The contour and response surface curves revealed that the highest biological efficiency occurred with 5% rice bran, 2.5% dolomite and 2.5% phosphate rock (Figure 3). Under optimal conditions, the biological efficiency was 89.72% (mean of run No. 15-20 in Table 2) compared with the predicted biological efficiency of 89.61%. Different substrates used for milky mushroom cultivation may affect biological efficiency. Cultivating the mushrooms on wheat straw, paddy straw, soybean straw, coconut coir pith, cotton waste and sugarcane bagasse yielded biological efficiencies of 146.3%, 132.4%, 126.1%, 108.7%, 92.07% and 51.57%, respectively (Vijaykumar et al., 2014). Moreover, the size of the substrate could affect mushroom production. In the present study, white popinac wood chips with a size of approximately 0.5 × 0.5 × 0.5 cm were used for mushroom culture by the tray culture technique. A small space between the wood chips was observed, even though they were tightly packed in a pod. The aeration within the small space may have affected mycelial growth. The rice bran was provided as a nitrogen source for mushroom cultivation and its concentration must be optimized (Moonmoon et al., 2011; Larounga et al., 2022). Supplementing the substrate with rice bran increases its C:N ratio. In *Agaricus brasiliensis*, the highest, intermediate, and lowest mycelial growth was observed on a substrate with a C:N ratio of 11:1, ranging from 15:1 to 50:1, and 100:1, respectively (Junior et al., 2010). In the present study, supplementing wood chips substrate with rice bran resulted in a biphasic response for biological efficiency, which was likely due to the C:N ratio. Dolomite ($CaMg(CO_3)_2$) is commonly used as a supplement for mushroom cultivation substrates. In the oyster mushroom, *Pleurotus ostreatus*, adding dolomite to the mushroom growing media (baglog) yielded the best results for mycelium growth and fruit body weight (Sianturi et al., 2021). Dolomite provides both calcium and magnesium, which are important nutrients for mushrooms. Calcium is essential for enhanced mycelial growth and fruiting body development of the oyster mushroom (Suzuki et al., 2020). Magnesium is also important for the growth and development of the pink oyster mushroom, *Pleurotus djamor* (Zięba et al., 2021). Phosphate rock is a non-detrital sedimentary rock that contains high

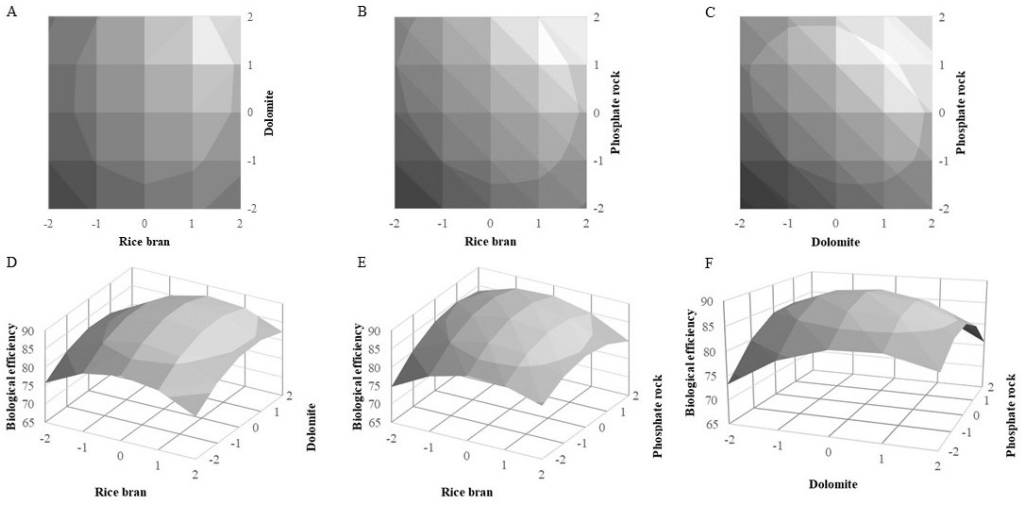

Figure 3. Contour (A, B and C) and response surface (D, E and F) plots showing the interaction of rice bran and dolomite (A and D), rice bran and phosphate rock (B and E), and dolomite and phosphate rock (C and F) on biological efficiency (BE).

amounts of phosphate minerals, primarily fluorapatite ($Ca_5(PO_4)_3F$) and hydroxyapatite ($Ca_5(PO_4)_3OH$ or $Ca_{10}(PO_4)_6(OH)_2$). It is useful for increasing the phosphate content during cultivation. Phosphate rock in combination with other supplements had an important role in the mycelial growth rate of the white button mushroom, *Agaricus bisporus* (Rashid et al., 2018).

The nutritional composition and bioactive compounds of milky mushrooms can be influenced by the substrates used for cultivation. Lakshmipathy et al. (2012) found that the cultivation of mushrooms on different substrates including coconut coir pith, maize straw, rice straw, sugarcane bagasse, sugarcane leaves and vettivera leaves yielded 78-85% moisture, 27-31% protein, 0.7-0.9% fat, 53-58% carbohydrate, 35-38% fiber and about 8% ash. In this study, fresh weight of milky mushroom grown on white popinac wood chip contained 82.15% moisture, 20.14% protein, 1.60% fat, 64.87% carbohydrate, and 8.29% ash. Glutathione is an essential antioxidant that plays an important role in maintaining cellular health. We found that the mushrooms grown on wood chips contained 58.46±6.14 µM reduced glutathione. Selvi et al. (2007) found that fresh and powdered milky mushrooms contained 0.156±0.025 nmol/g (approximately 0.05 µM) and 0.123±0.015 nmol/g (approximately 0.04 µM) of reduced glutathione, respectively. The antioxidant properties of the mushroom were also evaluated using DPPH and ABTS assays. The ethanol extract of the mushroom exhibited radical scavenging IC50 values of 93.20±8.90 µg/mL and 67.08±9.61 µg/mL for DPPH and ABTS, respectively. It is important to note that the tested antioxidant activity may be attributed not only to glutathione, but to other compounds present in the mushroom. Mirunalini et al. (2012) found that the ethanol extract of the milky mushroom showed radical scavenging IC50 values for DPPH and ABTS of 33.4 µg/mL and 26.3 µg/mL, respectively. These results suggest that the white popinac wood chip substrate may affect but does not negatively impact the nutritional composition and bioactive compounds of the mushroom.

Organic agriculture for mushroom cultivation emphasizes the use of natural and sustainable practices to promote ecological balance and minimize the environmental impact. This study demonstrated the potential of organic milky mushroom cultivation using organic substrates. White popinac is a fast-growing tree that can produce a large amount of biomass, making it a sustainable source of substrate material for mushroom cultivation. Rice bran, a byproduct of rice milling, is also a nutrient-rich substrate that can be used as a cost-effective and sustainable material in organic mushroom cultivation. To obtain organic rice bran, it is essential to cultivate organic rice using sustainable farming practices. Dolomite and phosphate rock are also part of an organic system that slowly releases essential nutrients such as calcium, magnesium, and phosphorus. These natural materials create a more resilient and self-sustaining ecosystem for agriculture.

4. Conclusions

In this study, we demonstrated the feasibility and efficacy of cultivating milky mushrooms on white popinac wood chips supplemented with rice bran, dolomite, and phosphate rock. Through response surface methodology, the optimal substrate composition that yielded maximum biological efficiency was identified. The cultivated mushrooms exhibited comparable nutritional composition and significant levels of bioactive compounds. These findings highlight the suitability of white popinac wood chips as a sustainable substrate material for organic milky mushroom cultivation. Future studies are needed to identify additional factors that affect mushroom cultivation to further enhance production and quality.

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6. Authors' Contributions

EB conceived the study, designed and performed the experiments, interpreted the results, wrote and edited the manuscript, supervised the project; KS performed the experiments, collected and analyzed the data; UR performed the experiments, collected and analyzed the data.

7. Conflicts of Interest

The authors declare no conflicts of interest.

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