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Impact of Organic Additives on Non-Enzymatic Antioxidant Activity in *Lentinus edodes* (Berkley)

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ABSTRACT

Shiitake mushroom (Lentinus edodes) is a globally recognized cultivated species, yet it remains underrepresented in Indian markets. This is primarily due to the lack of cultivation technologies using locally available substrates and the unavailability of suitable high-temperature tolerant strains. The present study investigates the effect of various organic additives, including rice flour, wheat flour, corn flour, horse gram flour, sorghum flour, black gram flour, green gram flour, and tapioca flour, on biomass production and non-enzymatic antioxidant activity in Lentinus edodes. The results demonstrated that among the tested additives, sorghum flour significantly enhanced the antioxidant content compared to the other organic additives. These findings suggest that sorghum flour could be a promising substrate for improving the antioxidant properties of Lentinus edodes, with potential applications in mushroom cultivation and food industries.

Keywords: *Lentinus edodes, Shiitake mushroom, organic additives, non-enzymatic antioxidants*

Introduction

Mushrooms, particularly edible species such as *Lentinus edodes* (shiitake), are gaining significant attention worldwide due to their remarkable nutritional and medicinal properties. They are naturally rich in dietary fiber, vitamins, minerals, and bioactive compounds, making them a valuable addition to the human diet. Studies have shown that mushrooms provide an array of health benefits, including antioxidant, anti-inflammatory, antimicrobial, and anticancer properties, contributing to their increasing popularity in functional food and medicinal markets [1]. Their ability to provide essential nutrients while being low in fat and calories further enhances their appeal, especially in the context of modern diets focused on health and wellness. The nutritional composition of mushrooms is unique compared to other plant-based foods. Cultivated mushrooms, such as shiitake, contain high levels of crude protein (ranging from 20% to 44% of dry matter) with minimal fat content (3% to 7% of dry matter) [2]. This makes them an excellent source of protein for vegetarians and individuals seeking plant-based alternatives to animal proteins. Moreover, mushrooms are abundant in essential amino acids, making them a complete protein source, which is crucial for body function and growth. They are also rich in minerals such as potassium, phosphorus, iron, and zinc, along with various vitamins, including B-vitamins (B1, B2, B3, and B5), vitamin D, and other micronutrients that contribute to their functional properties.

Beyond their nutritional value, mushrooms have garnered attention for their medicinal potential. Medicinal mushrooms, including shiitake, reishi (*Ganoderma lucidum*), and maitake (*Grifola frondosa*), have been utilized for thousands of years in traditional medicine to treat a wide range of health conditions. Modern research has confirmed the efficacy of certain compounds found in mushrooms for preventing and treating diseases, further elevating their status as both functional foods and natural therapeutics.

For example, shiitake mushrooms are known to contain bioactive compounds such as polysaccharides (e.g., lentinans), sterols, and other triterpenes, which have been shown to exhibit immunomodulatory, anti-cancer, and antioxidant properties [3-4]. One of the most significant bioactive properties of mushrooms, including shiitake, is their antioxidant capacity. Antioxidants are compounds that help neutralize free radicals, which are highly reactive molecules that can cause oxidative stress, leading to cellular damage and contributing to various chronic diseases, including cancer, heart disease, and neurodegenerative conditions. Non-enzymatic antioxidants, such as phenolic compounds, flavonoids, and ascorbic acid, are particularly important because they scavenge free radicals and help protect the body from oxidative damage [5]. Shiitake mushrooms, in particular, have been found to have high antioxidant activity, which is largely attributed to their rich content of polyphenolic compounds, such as flavonoids and phenolic acids, the production and cultivation of shiitake mushrooms have not yet reached their full potential in many regions, including India. This is due to several factors, such as the lack of suitable cultivation technologies using locally available substrates and the unavailability of high-temperature tolerant strains. In India, the absence of advanced cultivation techniques and strains adapted to the local environment has limited the widespread adoption of shiitake mushrooms [6]. Cultivation of shiitake typically requires specific conditions, such as controlled temperature and humidity, which can be challenging to replicate in areas with high ambient temperatures or limited resources.

These challenges, recent studies have explored the use of organic additives to enhance mushroom cultivation and improve the nutritional quality of the mushrooms produced. Organic additives, such as rice flour, wheat flour, sorghum flour, and others, have been shown to influence the growth, yield, and nutritional composition of mushrooms.

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The addition of these organic materials to the substrate used for mushroom cultivation provides essential nutrients that support better growth and increased production of bioactive compounds, including antioxidants [7]. Moreover, organic additives can help optimize the cultivation process by providing an enriched substrate that supports the growth of mycelium, thereby enhancing biomass production and increasing the antioxidant potential of the harvested mushrooms.

Among the various organic additives, sorghum flour has shown particular promise due to its unique composition and nutrient profile. Sorghum is a rich source of carbohydrates, proteins, fiber, and minerals, which can serve as an excellent substrate for mushroom growth. Studies have suggested that sorghum flour can improve the antioxidative properties of mushrooms, potentially making it a valuable tool for enhancing the health benefits of shiitake mushrooms. By optimizing the use of organic additives like sorghum flour, it may be possible to not only increase mushroom yield but also improve the nutritional quality, particularly the antioxidant content, of mushrooms cultivated under Indian conditions.

The present study aims to evaluate the effect of various organic additives on the biomass production and non-enzymatic antioxidant activity of *Lentinus edodes*. The specific additives under investigation include rice flour, wheat flour, corn flour, horse gram flour, sorghum flour, black gram flour, green gram flour, and tapioca flour. By assessing the influence of these additives on the antioxidant properties of shiitake mushrooms, this study seeks to identify the most effective additive for improving the nutritional profile of mushrooms and contributing to the development of sustainable cultivation practices in India [8]. This research is part of a broader effort to promote shiitake mushroom cultivation in India and enhance its market potential by improving the nutritional and health benefits of the product. By leveraging locally available organic additives, this study aims to support the development of cost-effective, high-yield mushroom cultivation systems that can provide both economic and health benefits to local communities.

Materials and Methods

Effect of Organic Additives on Non-Enzymatic Antioxidants in *Lentinus edodes* (Shiitake)

The effect of various organic additives on non-enzymatic antioxidants in *Lentinus edodes* was investigated by incorporating different organic substrates into Czapek's broth. The organic additives tested included rice flour, wheat flour, horse gram flour, sorghum flour, and tapioca flour, each added at concentrations of 1% and 2% (w/v) [9]. The amended media were prepared by dissolving the respective additives in Czapek's broth to obtain the desired concentrations. A total volume of 50 mL of the amended media was dispensed into 100 mL Erlenmeyer flasks.

The flasks containing the amended media were sterilized by autoclaving at 15 psi for 1 hour to eliminate any contaminants. After cooling to room temperature, each flask was inoculated with a 9 mm diameter disc obtained from the peripheral growth of an 11-day-old culture of *Lentinus edodes* (strain used in the study). The inoculated flasks were incubated at 25±2°C for a period of 15 days.

Each treatment was replicated three times, with a flask containing only Czapek's broth (without any organic additives) serving as the control. After the incubation period, the mycelium growth was evaluated.

The supernatant from each flask was carefully extracted, and the mycelial growth retained on Whatman No. 1 filter paper was harvested and weighed to determine the fresh weight of the mycelium [10]. To determine the biomass, the fresh mycelium was subsequently dried in an oven at 80°C for 6 hours. The dry weight of the mycelium (biomass) was then recorded for each treatment.

Preparation and Extraction of the Sample

For the analysis of non-enzymatic antioxidants in *Lentinus edodes*, the mycelial mat from each treatment was collected from the Whatman No. 1 filter paper after filtration of the culture filtrate. A 5g portion of the mycelial mat from each sample was transferred into a beaker, and methanol was added at a ratio of 1:10 (w/v). The mixture was stirred for one hour and left to stand overnight for extraction. The extract was then separated from the residue by filtration through Whatman No. 1 filter paper. To ensure complete extraction, the residue was re-extracted twice with fresh methanol, and the two extracts were combined. The combined extract was lyophilized, and the residual solvent was removed under reduced pressure at 40°C using a rotary evaporator. The resulting extracts were produced in triplicates for each treatment and were used for the assay of antioxidant activity [11].

Biochemical Analysis

The mushroom samples were prepared in a 0.1 M phosphate buffer and used for the analysis of non-enzymatic antioxidants, including total glutathione, vitamin A, vitamin C, vitamin E, and total carotenoids.

Estimation of Total Glutathione

For the estimation of total glutathione, 0.5 mL of the enzyme extract was mixed with 0.5 mL of 5% trichloroacetic acid (TCA). The mixture was centrifuged at 1000 rpm for 10 minutes to precipitate the proteins. A 0.1 mL portion of the supernatant was made up to 1.0 mL with 1.0 mL sodium phosphate buffer (pH 8.0), and 2.0 mL of freshly prepared 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reagent was added. The absorbance was measured after 10 minutes at 412 nm against a reagent blank. A standard curve was also prepared by treating a set of glutathione standards in the same manner. The amount of glutathione in the sample was expressed as μg of glutathione per mg of protein.

Estimation of Vitamin A

For the estimation of vitamin A, 1.0 mL of a 10% homogenate was pipetted into a test tube, and 1.0 mL of saponification mixture (2 N KOH in 90% alcohol) was added. The mixture was heated at 60°C for 20 minutes. After cooling, 25 mL of distilled water was added, and the solution was transferred to a separating funnel. The mixture was then extracted three times with 10, 15, and 25 mL portions of petroleum ether (40-60°C). The ether extracts were pooled, washed repeatedly with 50-100 mL of distilled water until the wash was free of alkali, and then dried using anhydrous sodium sulfate. The volume of the final extract was noted. For spectrophotometric measurement, 3 mL of the petroleum ether extract was transferred into a cuvette, and absorbance was measured at 420 nm against a petroleum ether blank immediately to prevent evaporation of the solvent and degradation of carotenoids by light. This reading was recorded as A1. A β-carotene working standard was measured at 450 nm to generate a standard curve.

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The petroleum ether extract was evaporated to dryness at 60°C in a water bath, and the residue was resuspended in 2 mL of trifluoroacetic acid (TFA) reagent. The mixture was quickly transferred to a cuvette, and absorbance was measured at 620 nm exactly after the addition of TFA. This reading was recorded as A2. A vitamin A working standard was read at 620 nm for comparison.

Calculation of Vitamin A Content

To accurately calculate the vitamin A content in the mushroom samples, it was necessary to correct for the absorbance contributed by carotene at 620 nm. The following formula was used:

$$A_3 = A_2 - A_1$$

Where:

- A_1 = Absorbance at 450 nm (absorbance contributed by carotene)
- A_2 = Absorbance at 620 nm (absorbance contributed by both carotene and vitamin A)
- A_3 = Absorbance at 620 nm (corrected absorbance attributed to vitamin A alone)

The final result for the vitamin A content in the mushroom sample was expressed as μg of vitamin A per gram of tissue.

Estimation of vitamin C (Sumathi, 1998)

The estimation of vitamin C (ascorbic acid) in *Lentinus edodes* was performed using the dinitrophenylhydrazine method, which involves the formation of an orange-red azazone compound. Initially, 1.0 mL of enzyme extract was pipetted into a test tube, and the volume was adjusted to 3.0 mL by adding distilled water. To this, 1.0 mL of dinitrophenylhydrazine (DNPH) reagent was added along with 1-2 drops of thiourea. The thiourea enhances the reaction between ascorbic acid and DNPH. A blank was prepared in a similar manner but with distilled water instead of the enzyme extract. The test tubes were mixed thoroughly and incubated at 37°C for three hours to allow the reaction to proceed. After incubation, the tubes were placed in an ice bath to halt the reaction. Following this, 7.0 mL of 80% sulfuric acid was added drop by drop to dissolve the orange-red azazone crystals formed. The tubes were then allowed to stand at room temperature for 30 minutes. After the standing period, the absorbance was measured at 540 nm using a spectrophotometer, with the blank used for calibration. The concentration of vitamin C in the sample was determined by comparing the absorbance to a standard curve generated from known concentrations of ascorbic acid. The results were expressed as micrograms of vitamin C per gram of tissue. This method provides a reliable measure of the vitamin C content, which is an important non-enzymatic antioxidant in the mushrooms.

Estimation of Vitamin E

To estimate the vitamin E content in *Lentinus edodes*, 1.5 mL of tissue extract from each sample, along with 1.5 mL of the standard solution and 1.5 mL of water (for the blank), were pipetted into three separate stoppered centrifuge tubes (test, standard, and blank). To the test and blank tubes, 1.5 mL of ethanol was added, while to the standard tube, 1.5 mL of water was added. Then, 1.5 mL of xylene was added to all the tubes. The tubes were stoppered, mixed well, and centrifuged. After centrifugation, 1 mL of the xylene layer was transferred into another clean stoppered tube, making sure not to include any ethanol or protein.

To each tube, 1 mL of 2,2-dipyridyl reagent was added, the tubes were stoppered, and the mixture was mixed thoroughly. Next, 1.5 mL of the mixtures from each tube was transferred into spectrophotometer cuvettes, and the absorbance of the test and standard tubes was measured against the blank at 460 nm. Following this, beginning with the blank, 0.33 mL of ferric chloride solution was added to each tube. The tubes were mixed well and after exactly 1.5 minutes, the absorbance of the test and standard tubes was read at 520 nm against the blank. The results were expressed as micrograms of vitamin E per gram of tissue. The amount of vitamin E in the sample can be calculated using the formula:

$$\text{Vitamin E } (\mu g / g) = \frac{(\Delta A_{520nm} - \Delta A_{450nm} \times \text{conc [S]} \times 0.29 \times \text{Total volume})}{\Delta A_{520nm} \times \text{Volume for experiment} \times \text{Weight of sample}}$$

Estimation of Total Carotenoids

To estimate the total carotenoids in *Lentinus edodes*, a sample weighing 5 to 10 g was first saponified for approximately 30 minutes in a shaking water bath at 37°C after extracting the alcoholic potassium hydroxide (KOH) solution. The saponified extract was then transferred into a separating funnel, packed with glass wool and calcium carbonate, and treated with 10 to 15 mL of petroleum ether. The carotenoid pigments were extracted into the petroleum ether layer, while the lower aqueous phase was discarded. The extraction process was repeated with fresh petroleum ether until the aqueous phase was colorless, ensuring complete extraction of the carotenoids. The petroleum ether extract containing the carotenoid pigments was collected into an amber-colored bottle. A small quantity of sodium sulfate was added to the petroleum ether extract to remove any turbidity. The final volume of the petroleum ether extract was noted and adjusted if necessary, using a known dilution factor. The absorbance of the extract was measured at 450 and 503 nm in a spectrophotometer, with petroleum ether serving as the blank. The carotenoid concentration was then calculated and expressed as micrograms of carotenoids per gram of tissue.

Calculation

$$\text{Carotenoids } (\mu g) = \frac{P \times 4 \times V \times 100}{W}$$

P = Optical density of the sample

V= Volume of the sample

W= Weight of the sample

Result and Discussion

The results of the experiment (Tables 1 and 2) clearly indicate that among the various organic additives, sorghum flour significantly enhanced the levels of antioxidative substances in *Lentinus edodes* compared to the other additives. The non-enzymatic antioxidative substances, such as total glutathione (14.84 μg/g), vitamin A (1.90 μg/g), vitamin C (0.41 μg/g), vitamin E (0.13 μg/g), and total carotenoids (16.00 μg/g), were found to be at their maximum concentrations in the medium amended with sorghum flour. These findings are consistent with the results of [12] who reported a similar increase in enzymatic and non-enzymatic antioxidative substances in *Volvariella volvacea* when sorghum flour was added to the growing medium. In addition, several studies have reported similar findings for the antioxidative properties of shiitake mushrooms, including those by [13-16].

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Therefore, the higher levels of both enzymatic and non-enzymatic antioxidative substances observed in *Lentinus edodes* in this study suggest that it could serve as an excellent food supplement, contributing to improved health and well-being.

Conclusion

The present study demonstrates the positive influence of organic additives on the production of non-enzymatic antioxidative substances in *Lentinus edodes*. Among the various organic additives tested, sorghum flour was found to significantly enhance the levels of antioxidative compounds, including total glutathione, vitamin A, vitamin C, vitamin E, and total carotenoids. These findings align with previous studies that have highlighted the role of organic supplements in boosting the antioxidative capacity of edible mushrooms. Given the observed increase in antioxidative substances, particularly with sorghum flour, *Lentinus edodes* holds considerable potential as a functional food supplement that could contribute to human health by providing essential antioxidants. The results suggest that optimizing the cultivation of shiitake mushrooms with the right organic additives can further enhance its nutritional value, making it a valuable addition to diets aimed at improving overall health and preventing oxidative stress-related diseases.

Table 1: Effect of Organic Additives on Biomass Production of Lentinus edodes

Tr. No.	Additives	Mycelial Fresh Weight (g)	Mycelial Dry Weight (g)
		1%	2%
1	Rice flour	3.31 ± 0.10	5.25 ± 0.12
2	Wheat flour	4.93 ± 0.11	6.75 ± 0.09
3	Tapioca flour	5.65 ± 0.12	7.13 ± 0.10
4	Horse gram flour	6.21 ± 0.13	7.27 ± 0.08
5	Sorghum flour	7.72 ± 0.10	8.95 ± 0.11
6	Control	2.51 ± 0.09	—

Values are expressed as mean ± standard deviation. Means followed by the same letter in each column are not significantly different (P ≤ 0.05).

Table 2: Effect of organic additives on non- enzymatic antioxidative substances in L.edodes

Tr. No.	Additives	Total Glutathione (µg/mg)	Vitamin A (µg/g)	Vitamin C (µg/g)	Vitamin E (µg/g)	Total Carotenoids (µg/g)
		1%	2%	1%	2%	1%
1	Rice flour	8.71 ± 0.16	10.62 ± 0.14	1.36 ± 0.08	1.56 ± 0.06	0.29 ± 0.03
2	Wheat flour	10.16 ± 0.18	12.09 ± 0.13	1.51 ± 0.07	1.71 ± 0.04	0.35 ± 0.02
3	Tapioca flour	11.82 ± 0.17	13.41 ± 0.14	1.70 ± 0.06	1.88 ± 0.05	0.38 ± 0.02
4	Horse gram flour	12.19 ± 0.14	14.06 ± 0.11	1.76 ± 0.07	2.03 ± 0.04	0.41 ± 0.03
5	Sorghum flour	14.84 ± 0.12	16.00 ± 0.10	1.90 ± 0.06	2.18 ± 0.03	0.41 ± 0.02
6	Control	7.30 ± 0.11	—	1.08 ± 0.05	—	0.25 ± 0.01

Values are expressed as mean ± standard deviation. Means followed by the same letter in each column are not significantly different (P ≤ 0.05).

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