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Enhancing nutritional and functional properties of yogurt with ergothioneine

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Abstract
The objective of the study is to extract ergothioneine from oyster plueteuros mushroom and encorporate in yoghurt to enhance the nutritional quality of the product, also now a days there are lot of diabetic patients, to reduce this disease sugar is replace with stevia which is 50 to 300 times sweeter than sugar. As ergothioneine is a bioactive compound which is native source of amino acid derived from oyster mushroom (pleurotus spp.) ergothioneine contains the antioxidant property which can help to protect cells from free radicals. Yogurt was prepared by using lactobacillus casein. The concentration of ergothioneine added in S1, S2 and S3 was 0.1,0.2 and 0.3 respectively, also stevia was added for sweetness. sensory evaluation was conducted on 9-point hedonic scale. The overall acceptability of product was good. Incorporation of ergothioneine in yoghurt increases the ash, moisture, fat, carbohydrate and protein. This suggest that ergothioneine has positive impact on health by addition of ergothioneine as bioactive compound in yoghurt.

Keywords: Ergothioneine, health benefits, fortified food, yoghurt, diabetic cure

Introduction

Ergothioneine (ERGO) is an amino acid having unique chemical structure found in the human diet which makes it a strong and stable antioxidant and anti-inflammatory compound (Allen T. Phillips 2021). Early work demonstrated that mushrooms contain the highest ERGO levels of any dietary source, other foods with high ERGO content include red beans, oat bran and liver (Dubost *et al*., 2006, Dubost *et al*., 2007, Dubost *et al*., 2007) [5]. The oyster mushroom, scientifically known as Pleurotus ostreatus is a versatile and highly valued edible fungus belonging to the family Pleurotaceae. Pleurotus ostreatus contains many chemical compounds that are beneficial to health, one of which is a rare amino acid compound, ergothioneine which was discovered by Charles Tanret in 1909 while investigating the ergot fungus *Claviceps purpurea* (Michael D. Kalaras, 2017). EGT exists as a tautomer between its thiol and thione forms, however at physiological pH it is primarily thione (J. Carlsson, 1974). EGT is a colorless, odorless compound. It has relative molecular mass of 229.30 and a relatively high solubility in aqueous solutions (aqueous solubility limit of 0.9 M at 25 °C) (E.B. Newton, 1927). Pleurotus has unique flavor and aromatic properties that are rich in carbohydrates, protein, vitamins, minerals and fiber (Naraian *et al*. 2016). Overall ET acts as an antioxidant by scavenging free radicals. It neutralizes them, and prevents oxidative stress, which protects the cells and tissues from damage. Due to these beneficial properties, ET has been described as a nutraceutical and longevity vitamin (Abhijith Rao and Sivaprasad Putluru, 2025). Ergothioneine (ET), exhibits potent antioxidant and cytoprotective properties, scavenging reactive oxygen and nitrogen species, and protecting against UV radiation damage, potentially offering benefits for neurological, cardiovascular, and anti-inflammatory disorders (Bindu D Paul, 2022). The rising expense of healthcare, longer life expectancy, and the desire to improve one's quality of life could all be contributing factors to the spike in demand for functional foods. Therefore, one of the key components of the research on consumer behavior with regard to functional foods is health (Emese Kápolna, Beáta Kápolna, 2008). Functional food are defined as foods that provide health benefits that extend beyond their fundamental nutritional value (Hammoudi Halat *et al*., 2023). Yogurt is considered one of the useful foods in people's diet due to its high digestibility and bioavailability of protein, calcium, potassium, and B vitamins (Zohreh Abdi-Moghadam, Majid Darroudi, 2023). Yogurt is a popular fermented dairy product produced by lactic acid bacteria, including Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus. In the process of production of yogurt, these bacteria (Lactobacillus delbrueckii subsp. Bulgaricus) produce lactic acid and decreases pH, causing milk protein to coagulate (Seiji Nagaoka, 2019). Yoghurt has a positive impact on human health due to their rich reservior of bioactive proteins, hydrolyzed carbohydrates, vitamins and minerals with improved bioavailability, it is consumed widely all over the world as indicated by production and per capita consumption (Gaurav Kr Deshwal and Swati Tiwari, 2021). It is prepared food that is high in nutrients and comparatively low in fat (Nelson, 2017) [12]. In the last years, consumption of dairy products has been growing faster in different parts of the world due to economic growth and income levels (Elena Hadjimbei, 2022). Probiotic yogurts having *Bifidobacterium* and *Lactobacillus* are among the most popular functional food items which promote digestion and intestinal health therefore sold worldwide (S Sarıtaş, 2024). One such bioactive that is getting attention is ergothionine, derived from the amino acid histidine and is only available in food; higher animals are unable to produce it. (Irina Borodina and Louise C. Kenny, 2020).

Materials and Methods

1. Materials

Yogurt was prepared using cow milk with a fat content of 3.5%. Stevia liquid sweetener (purity ≥ 95%) was obtained from the supplier SoSweet (sosweet.co.in). Fresh

mushrooms were sourced from Mundhwa Industrial Area, Mundhwa, Pune, Maharashtra 411036, India. All materials were stored under appropriate conditions to maintain their stability and quality before use. Cow milk was stored at refrigeration temperature ($4 \pm 1°C$) to prevent microbial growth and spoilage. Stevia liquid sweetener was kept in a cool, dry place away from direct sunlight to preserve its potency and avoid degradation. Fresh mushrooms were stored at $4 \pm 1°C$ in ventilated containers to maintain freshness and minimize moisture loss or fungal contamination.

2. Equipment Used

The study utilized various instruments and equipment to ensure precise sample preparation and analysis. Liquid Chromatography Tandem Mass Spectroscopy (LC-MS/MS) SHIMADZU 8045, Using Lab Solution Software for Integration, data was employed for the quantitative analysis of bioactive compounds. Micropipettes with volumes ranging from 10–200 µL and 100–1000 µL were used for accurate liquid handling during sample preparation. A vortex mixer and shakers facilitated thorough sample mixing and homogenization, while a sonicator was used to enhance extraction efficiency by applying ultrasonic energy. Centrifugation was performed to separate the supernatant from the precipitate at controlled speeds. Syringes and syringe filters were used to remove particulates and ensure sample clarity. A blender, operating at a minimum speed of 8000 rpm, was utilized for homogenizing yogurt and mushroom extracts. A vertical shaker, with adjustable settings and capable of holding a 500 mL Erlenmeyer flask, was employed to maintain consistent agitation. Erlenmeyer flasks of 500 mL capacity were used for sample storage, extraction, and mixing, while glass microfiber filters (5 cm diameter, 1.6 µm pore size) were used to filter out insoluble particles. Volumetric flasks with capacities of 10 mL, 20 mL, and 50 mL were used for accurate solution preparation and dilution.

3. Chemicals and Reagents

HPLC-grade acetonitrile and methanol were used for extraction and LC-MS/MS analysis. Milli-Q water was used for preparing aqueous solutions. LC-MS-grade formic acid improved ionization efficiency. Anhydrous magnesium sulfate and sodium chloride facilitated phase separation and drying. Primary secondary amine (PSA) was used for sample clean-up. All reagents were of analytical or LC-MS grade and handled following standard lab safety protocols.

4. Preparation of Yogurt from Milk

Yogurt was prepared using Amul milk (3.5% fat content). The milk was pasteurized at 85°C for 15 minutes and then cooled to 42°C. A commercial yogurt starter culture containing *Lactobacillus acidophilus* and *Lactobacillus casei* was inoculated into the milk. The inoculated milk was incubated at 42°C for 4–6 hours until the desired consistency and pH (4.5) were achieved. The prepared yogurt was then stored at 4°C until further analysis.

5. Sample Preparation for Ergothioneine

The extraction of ergothioneine from dried mushroom powder was carried out using a modified QuEChERS-based (Quick, Easy, Cheap, Effective, Rugged, and Safe) method. Initially, 5.0 ± 0.1 grams of finely ground dried mushroom powder was weighed and transferred into a 50 mL polypropylene centrifuge tube. To initiate the extraction process, 10 mL of Milli-Q water was added to the sample and allowed to stand for at least 5 minutes to ensure proper hydration and facilitate the release of water-soluble compounds. Following this, 10 mL of 1% formic acid prepared in HPLC-grade acetonitrile was introduced into the tube to enhance the extraction of ergothioneine and other polar compounds. The mixture was shaken thoroughly to ensure homogeneity. To promote phase separation and remove water content from the organic phase, 6 g of anhydrous magnesium sulfate and 1.5 g of sodium chloride were added to the tube. These salts also help in increasing the ionic strength of the solution, which aids in efficient extraction. The tube was then vigorously hand-shaken and vortexed for 3 minutes to ensure thorough mixing and interaction between solvents and sample components. The mixture was subsequently centrifuged at 5000 rpm for 5 minutes to separate the solid and liquid phases. After centrifugation, 1.5 mL of the clear supernatant (organic phase) was carefully transferred into a 2 mL microcentrifuge tube that had been preloaded with 150 mg of anhydrous magnesium sulfate and 25 mg of primary secondary amine (PSA) sorbent. This clean-up step using PSA helps to remove interfering matrix components such as organic acids, pigments, and sugars. The tube was again vortexed for 1 minute to ensure proper contact between the sorbents and the extract, followed by a second centrifugation at 5000 rpm for 5 minutes to obtain a purified extract. Finally, 1 mL of the resulting clear solution was filtered through a 0.22 µm nylon syringe filter into a 2 mL HPLC vial. This filtered extract was then subjected to analysis using the LC-MS/MS instrument to quantify the ergothioneine content accurately.

For instance, Tsiantas *et al*. (2021) [1] compared ultraviolet–visible spectrometry and LC–MS methods for evaluating ergothioneine content in various mushroom species. Similarly, Pesek *et al*. (2021) [6] developed a high-performance liquid chromatography method utilizing a silica hydride-based column to analyze ergothioneine in commercial mushrooms. Additionally, Dubost *et al*. (2006) [5] identified and quantified ergothioneine in cultivated mushrooms using liquid chromatography–mass spectrometry. Furthermore, a study by Liu *et al*. (2016) validated a hydrophilic interaction liquid chromatography method for analyzing ergothioneine in fermentation broth. These studies collectively underscore the efficacy of LC–MS techniques in the accurate determination of ergothioneine concentrations in mushroom samples.

6. Characterization of Ergothioneine

The extracted ergothioneine was characterized using multiple analytical techniques to confirm its identity, concentration, purity, structure, and stability. Identification of ergothioneine was performed using LC-MS/MS by comparing the retention time and mass-to-charge ratio (*m/z*) with a standard reference. Quantification of ergothioneine in the sample was carried out by constructing a standard calibration curve, and the detection and quantification limits were established accordingly. The purity of ergothioneine was assessed using HPLC, measuring its characteristic absorbance wavelength. Structural confirmation was achieved through mass spectrometry analysis, including fragmentation pattern evaluation. Furthermore, stability

studies were conducted to determine the solubility, thermal, and pH stability of ergothioneine under experimental conditions, ensuring its integrity throughout the analysis.

7. Flowchart of Yogurt Preparation

Pasteurization of Amul milk at 85°C for 15 minutes.

↓

Cooling to 42°C.

↓

Inoculation with *Lactobacillus acidophilus* and *Lactobacillus casei* starter culture.

↓

Incubation at 42°C for 4–6 hours until pH 4.5 and desired consistency.

↓

Storage at 4°C until further analysis.

8. Flowchart of Ergothioneine Extraction

Weigh 5 g dried mushroom powder
Add 10 mL water, extract for 5 minutes

↓

Add 10 mL 1% formic acid in acetonitrile, mix well. zx

↓

Add salts, centrifuge at 5000 rpm for 5 minutes.

↓

Filter through a 0.22 µm nylon syringe filter.

↓

Analyze sample using LC-MS/MS.
-09-090987

9. Yogurt Enriched Ergothioneine Extract

To develop a functional and palatable dairy product, yogurt was fortified with ergothioneine (EGT) extract derived from mushrooms. Initially, a plain yogurt prototype was prepared using standardized cow milk (3.5% fat), yogurt culture, stevia liquid sweetener and varying concentrations of ergothioneine extract. Multiple trials were conducted to finalize the optimal EGT-stevia ratio that balanced health benefits with acceptable sensory properties. This base formulation was then used for further product development.

Ergothioneine, known for its antioxidant and cytoprotective properties, can impart a slightly earthy or sulfur-like note. Alongside this, stevia, while being a diabetic-friendly natural sweetener, often leaves a lingering bitter or metallic aftertaste. To address this, the stevia concentration was carefully optimized (0.1–0.3 mL per 100 mL), and its addition was done post-fermentation to avoid degradation and preserve flavor quality. The yogurt's natural creaminess also helped round off stevia's sharp notes. Moreover, carefully selected flavoring agents were used to mask both EGT and stevia off-notes and improve overall acceptability.

After standardizing the base yogurt, ten distinct flavor variants were developed by incorporating natural or nature-identical flavoring agents: Vanilla, Pineapple, Cardamom, Rose, Butterscotch, Strawberry, Saffron, Pista, Mango, and Chocolate. These flavors were selected based on their compatibility with dairy matrices and their potential to harmonize with the subtle earthy notes of EGT.

10. Formulation

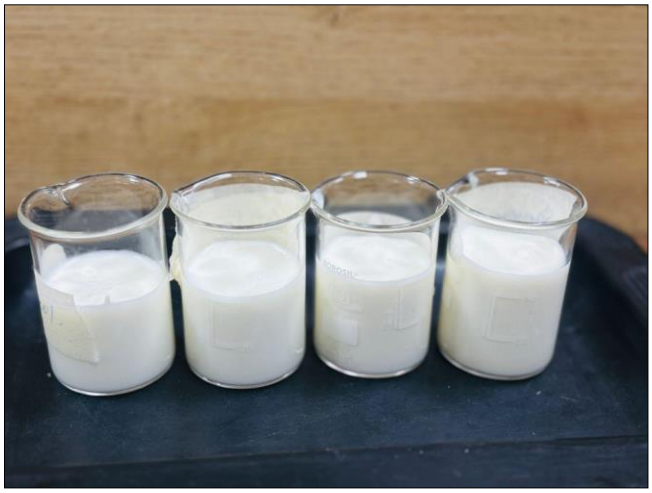
The yogurt formulation was standardized using 100 mL of cow milk with 3.5% fat as the base. To this, 2% yogurt starter culture was added for fermentation. The ergothioneine extract was incorporated in varying amounts ranging from 0.05 to 0.2 mL to evaluate optimal bioactivity and flavor balance. For sweetness, 0.1 to 0.3 mL of high-purity stevia liquid was used, carefully optimized to minimize its characteristic bitterness. Post-fermentation, each yogurt sample was enriched with 0.2 to 0.5 mL of natural flavoring agents, including vanilla, pineapple, cardamom, rose, butterscotch, strawberry, saffron, pista, mango, or chocolate. To improve consistency and mouthfeel, 0.1 to 0.2 g of stabilizer such as pectin or guar gum was optionally added. The fermentation was carried out at 42°C for 6 to 8 hours, followed by cooling and refrigeration. This formulation served as the base for all ten flavor variants of the ergothioneine-enriched yogurt.

All flavoring agents were added after fermentation to preserve their aromatic integrity and avoid thermal degradation. The samples were stored under refrigeration (4°C) until evaluation. A sensory analysis panel assessed each of the ten variants for flavor masking, texture, aftertaste, and overall consumer acceptability.

This development approach successfully yielded a series of antioxidant-rich, diabetic-friendly yogurt products with diversified flavor profiles, targeting both health-conscious and flavor-oriented consumers.

Result and Discussion

1. Product



2. Sensory Evaluation

Table 1: Sensory Evaluation of yogurt fortified product based on overall acceptability

Samples	Color	Flavor	Texture	Mouthfeel	Overall acceptability
S0	7.95±0.512	7.4±0.321	7.6±0.412	7.4±0.612	7.7±0.211
S1	7.9±0.621	7.8±0.423	7.7±0.413	7.8±0.418	7.7±0.210
S2	7.7±0.261	7.5±0.362	7.6±0.613	7.6±0.616	6.4±0.213
S3	8.1±0.421	7.8±0.623	8.2±0.203`	7.8±0.612	7.7±0.601

Sensory evaluation was carried out to assess the color, texture, aroma, and overall acceptability of the fortified yoghurt. A panel of 8 to 12 members participated in the evaluation, using a 9-point hedonic scale. Four different samples were tested: a control sample, and three fortified samples labeled S1, S2, and S3. The panelists were able to notice clear differences between the samples during the evaluation.

The sensory sessions were conducted at MIT ADT University, with faculty members serving as the panelists. In terms of flavor, the yoghurt samples contained components like aldehydes, esters, and terpenes, which contributed to their overall profile. The texture across samples was smooth and creamy, a result of the protein (casein) coagulation process typical of yoghurt formation.

When ergothioneine was added at concentrations of 0.1 ml, 0.2 ml, and 0.3 ml in S1, S2, and S3 respectively, a slight change in color was observed. Table 1 presents the average sensory scores for the fortified yoghurt products.

3. Proximate composition

Table 2: Proximate composition on wet basis of yogurt fortified product

Sample	Moisture	Ash	Fat	Protein	Carbohydrate
Control(S0)	75±0.601	0.8±0.13	1.61±0.01	1.39±0.04	12.05±0.21
S1	80.3±0.30	0.7±061	1.49±0.03	1.38±0.05	12.5±0.20
S2	85.32±0.41	0.6±0.22	1.55±0.04	2.23±0.08	15.68±0.88
S3	90±0.61	0.4±0.10	2.24±0.04	2.85±0.06	18.20±0.19

Based on the moisture content estimation (on a wet basis), the S2 sample was found to have the highest moisture content. This determination is important because moisture levels directly affect the shelf life and stability of yoghurt products.

As shown in Table 2, the control sample had a higher ash content compared to S3. Ash content provides an indirect measure of the mineral profile of yoghurt, giving insight into the concentration of essential minerals like calcium, magnesium, and phosphorus — all of which are crucial for healthy bone development and overall health.

The fat content was found to be higher in S3. Maintaining appropriate fat levels ensures good nutritional quality. Yoghurt fat is composed of saturated fats, unsaturated fats, and sometimes essential fatty acids. Interestingly, ergothioneine, a water-soluble antioxidant, may interact with lipids or other fat-soluble compounds present in the yoghurt.

S3 also showed the highest protein content among all the samples. This suggests that it offers greater health benefits, serving as a significant source of high-quality animal protein that contains all the essential amino acids needed by the body. Protein is vital for muscle repair, growth, and the proper functioning of the body.

Additionally, the carbohydrate content was higher in S3. Carbohydrates provide a primary source of energy in fortified yoghurt products, mainly derived from lactose — the natural sugar found in milk. Carbohydrates can also influence the absorption and bioavailability of ergothioneine and other bioactive compounds, further enhancing the nutritional value of the product.

Physicochemical Analysis

Table 3: Physicochemical Analysis of yogurt fortified product

Sample	pH	Titratable Acidity	TSS
S0	4.1±0.01	0.96±0.04	10.11±0.01°
S1	4.2±0.41	0.87±0.04	11.21±0.02°
S2	4.4±0.031	1.01±0.08	11.31±0.06°
S3	4.5±0.062	1.03±0.04	13.12±0.01°

The pH value of yoghurt should not drop below 4.0, as lower pH levels can encourage microbial growth. After 14 days of storage, a gradual increase in microbial count was observed, which corresponded with a decrease in pH. Maintaining a low pH is important because it helps ensure the yoghurt remains safe and free from microbial contamination. Among all the samples, S3 maintained a higher pH compared to the others, indicating better stability and microbial safety over the storage period. Titatable acidity is more in sample S3. It might be due to acid formation which is caused by fermentation process by microorganisms. Titatable acidity of 3 samples has different value against control sample.

The TSS (total soluble solids) should be range between 10 to 13 °brix. TSS increases as the concentration of Ergothioneine increases, more TSS is found in sample S3

Conclusion

Based on the findings of this study, it can be concluded that yogurt enriched with ergothioneine exhibits promising

functional properties that could serve as a nutritious alternative to regular yogurt. The enhanced nutritional profile suggests potential health benefits; however, further preclinical and clinical studies are necessary to validate its efficacy and safety. Additional research will help substantiate the use of this fortified yogurt as a functional food with potential applications in improving nutritional value and overall health.

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