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ORIGINAL RESEARCH PAPER

Evaluation of Antibacterial Activity of Tempeh and Optimization of Supplementary Ingredients for Enhanced Production

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Abstract

Tempeh, a traditional Indonesian fermented soybean product, derives its characteristic texture, flavor, and enhanced nutritional properties from the metabolic activity of the filamentous fungus Rhizopus oligosporus. This study aimed to isolate and identify R. oligosporus from fresh Hibiscus leaves collected in Dharan, Nepal, for application in tempeh production and evaluation of antibacterial activity. Fungal isolation from leaf samples was carried out using Potato Dextrose Agar (PDA). Identification was based on macroscopic colony morphology and microscopic characteristics. The isolate was further evaluated for growth at different temperatures (4℃, 20℃, 30℃, and 37℃) to determine optimal conditions. Tempeh was prepared by inoculating cooked soybeans with a spore suspension (10^6 spores/ml) of R. oligosporus and incubating at 30 ± 2℃ for 36-48 h. Fermentation efficiency was enhanced by supplementing soybeans with flours from potato, maize, wheat, and rice at concentrations ranging from 0.5% to 2%. Antibacterial activity of fresh tempeh extract was assessed against Staphylococcus aureus and Escherichia coli using a membrane-sterilized (0.45 µm) filtrate. Out of 30 samples, 4 isolates (13%) were identified as R. oligosporus based on morphological and microscopic features. The selected strain exhibited optimal growth at 37℃ and effectively fermented soybeans into compact tempeh. Supplementation studies indicated that 1% potato starch and 0.5% rice flour produced superior tempeh quality. The tempeh extract demonstrated inhibitory activity against S. aureus but not E. coli. These findings suggest that Hibiscus leaves may serve as a sustainable local source of R. oligosporus, supporting cost-effective, regionally adapted starter culture development and functional food production in Nepal.

Keywords:
Tempeh
Rhizopus oligosporus
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Antibacterial activity
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Introduction

Tempeh is a traditional fermented soybean product that originated in Indonesia, particularly on the island of Java, where it has been consumed for centuries as a primary source of dietary protein. Its development is closely associated with the Javanese people of Central Java, reflecting indigenous fermentation practices adapted to tropical environmental conditions. Although the earliest documented reference to tempeh appears in the early nineteenth-century Javanese literary work *Serat Centhini* (1815), historical evidence suggests that its production and consumption likely predate this record by several centuries, possibly as early as the seventeenth century (Shurtleff & Aoyagi, 2020).

Tempeh is produced by fermenting dehulled, soaked, and cooked soybeans with filamentous fungi of the genus *Rhizopus*, particularly *R. oligosporus*, whose mycelium binds the beans into a compact cake. Over time, tempeh spread beyond Indonesia to other regions of Asia, Europe, and the Americas, especially during the twentieth century alongside the expansion of vegetarian and plant-based diets. Contemporary studies recognize tempeh as both a culturally significant Indonesian food and a globally valued sustainable protein source (Ratnaningsih et al., 2025). Moreover, its fermentation-derived bioactive compounds have attracted increasing attention in functional food research due to their potential health-promoting properties (Duniaji et al., 2019; Rizzo, 2024).

Tempeh is widely recognized as a nutritionally valuable fermented soybean product due to its high protein content, balanced amino acid composition, dietary fiber, and essential micronutrients, including iron, calcium, magnesium, and B-complex vitamins (Ratnaningsih et al., 2025). Originating in Indonesia, tempeh has been consumed for over three centuries as an affordable and sustainable plant-based protein source (Shurtleff & Aoyagi, 2020). The fermentation process, primarily mediated by *R. oligosporus*, enhances nutrient bioavailability by degrading antinutritional factors such as phytic acid and oligosaccharides. This process improves protein digestibility and promotes the formation of bioactive compounds, including isoflavone aglycones, antioxidant peptides, and other health-promoting metabolites (Rizzo, 2024; Wang et al., 2023). Fermentation may also increase the levels of certain micronutrients, including vitamin B12, while reducing allergenic components (Ahnan-Winarno et al., 2021). Additionally, tempeh contains probiotic and prebiotic components that support gut health and metabolic function. Traditionally prepared by frying, steaming, boiling, or incorporating into soups and curries, tempeh has also been adapted into modern products such as burgers, chips, and meat substitutes, contributing to its growing global acceptance as a functional and environmentally sustainable food.

R. oligosporus occurs naturally in diverse ecological niches, particularly on plant surfaces and decomposing organic matter, making it an accessible and sustainable source for starter culture development (Sparringa et al., 2002). Recent research has emphasized the value of isolating beneficial fungal strains from unconventional habitats, such as leaves, roots, and other tissues of medicinal and ornamental plants, which harbor diverse microbial communities (Verma et al., 2009). *Hibiscus rosa-sinensis*, a widely distributed ornamental and medicinal plant, provides a favorable microenvironment for microbial colonization due to nutrient-rich leaf surfaces and diverse phytochemicals (Zulkurnain et al., 2023). However, it has also been isolated from commercial tempeh, starter cultures, and other plant leaves such as teak (*Tectona* spp.) (Duniaji et al., 2019). Although considered a domesticated strain, similar *Rhizopus* species commonly occur in soil and decaying organic substrates.

Identifying and utilizing native strains of *R. oligosporus* has implications for local tempeh production, microbial ecology, and food biotechnology. Indigenous isolates may exhibit distinctive physiological or biochemical traits, including enhanced sporulation, environmental stress tolerance, or superior fermentative efficiency, which could improve product quality (Wang et al., 2023; Wijaya et al., 2024). This study, therefore, aimed to isolate and identify *R. oligosporus* from the phylloplane of *Hibiscus* leaves collected in Dharan, Nepal, and to evaluate its soybean fermentation capacity under controlled laboratory conditions, thereby supporting the development of locally adapted starter cultures.

Materials and Methods

Sample collection

Fresh and mature *Hibiscus* leaves were collected from multiple locations within Dharan Sub-Metropolitan City, Nepal. The research was carried out between April and December 2021 in the Microbiology Laboratory of the Central Campus of Technology, Tribhuvan University, Dharan. Leaves from different *Hibiscus* species were used as sources for the isolation of *R. oligosporus*, a filamentous fungus widely employed in tempeh fermentation. Collected samples were placed in sterile polyethylene bags, transported to the laboratory under ambient conditions, and processed within 24 h of collection to minimize contamination and physiological deterioration (Jennessen et al., 2008).

In the laboratory, the leaves were rinsed thoroughly under running tap water to remove adhering debris, cut into small segments under aseptic conditions, and directly inoculated onto Potato Dextrose Agar (PDA) plates. The inoculated plates were incubated at 28 ± 2°C for 2-5 days to facilitate fungal growth (Klich, 2002; Pitt & Hocking, 2009). Emerging colonies were observed daily, and morphologically distinct fungal growth was subcultured repeatedly to obtain pure cultures. Isolates exhibiting fast-growing, cottony white-to-greyish mycelia, characteristic of *Rhizopus* species, were transferred to fresh PDA plates until culture purity was confirmed by consistent colony morphology.

Morphological identification of *R. oligosporus*

Macroscopic characteristics of the fungal isolates, including colony color, texture, surface appearance, and growth rate, were carefully recorded following incubation. Microscopic examination was performed to observe reproductive and vegetative structures. A small portion of fungal mycelium was mounted on a clean glass slide using lactophenol cotton blue (LPCB) stain, covered with a coverslip, and examined under a compound microscope at 40× magnification. Microscopic features such as sporangiophores, sporangia, columella, rhizoids, and sporangiospores were documented (Jennessen et al., 2008). The observed morphological characteristics were compared with standard fungal identification keys and taxonomic descriptions to confirm that the identity of *R. oligosporus,* which was differentiated from closely related *Rhizopus* species based on a combination of macroscopic and microscopic features (colony morphology, rhizoids and stolons, sporangia and columella, sporangiophores)(Díaz et al., 2010).

Tempeh production

Soybeans were soaked in water for 12-16 h to ensure adequate hydration, followed by manual dehulling and cooking until softened while maintaining structural integrity. The cooked beans were drained and cooled to room temperature under aseptic conditions. A spore suspension of the isolated *R. oligosporus* was prepared from a sporulated culture grown on potato dextrose agar. Sterile distilled water containing 0.1% (v/v) Tween-80 was added to the culture surface, and spores were gently dislodged using a sterile loop. The suspension was filtered through sterile muslin cloth to remove mycelial fragments. Spore concentration was determined using a Neubauer hemocytometer under light microscopy, and the density was calculated using the standard hemocytometer equation. The suspension was adjusted to 1 × 10^6 spores/mL by dilution with sterile Tween-80 solution or by concentration through centrifugation and resuspension.

The cooled soybeans were inoculated with the prepared spore suspension (10^6 spores/mL) and mixed thoroughly for uniform distribution. The inoculated beans were packed into perforated polyethylene bags (1-2 mm perforations) to allow aeration and incubated at 30 ± 2°C for 36-48 h to facilitate fungal growth and tempeh formation (Nout & Kiers, 2005; Wang et al., 2023).

Optimization of temperature of *R. oligosporus* in Tempeh preparation for 24 h

To determine the optimal temperature for fungal growth in tempeh preparation, the isolated *R. oligosporus* was inoculated onto soybean and incubated at different temperatures (4°C, 20°C, 30°C, and 37°C) for 24 h. Colony growth was monitored to identify the temperature conditions that supported maximum mycelial development (Raimbault, 1998).

Optimization of tempeh production with different ingredients and their proportions

The effects of different supplemental substrates on tempeh production were evaluated by incorporating potato starch, wheat flour, maize flour, and rice flour at concentrations of 0.5%, 1%, 1.5%, and 2% (w/w) into 100 g of soybeans. The mixtures were inoculated with *R. oligosporus* and incubated at 37°C for 24 h. Tempeh quality was assessed based on the extent of *Rhizopus* mycelial development and the resulting compactness of the fermented soybean matrix. Observations of fungal growth patterns, mycelial coverage, and textural attributes were conducted to determine the influence of the added substrates on fermentation performance. Finally, the products were categorized as excellent, very good, good, and poor cake formation (Nout & Kiers, 2005; Duniaji et al., 2019).

Antimicrobial test against *S. aureus* and *E. coli*

The tempeh sample was homogenized by adding 100 mL of sterile distilled water to obtain a uniform slurry. The homogenate was first filtered through sterile fine mesh cloth to remove coarse particles and debris. The filtrate was subsequently sterilized by passing it through a 0.45 µm membrane filter to ensure the removal of microbial contaminants while retaining soluble bioactive compounds (Yudiono et al., 2021).

The antibacterial activity of the prepared tempeh extract was evaluated against *S. aureus* and *E. coli* using the agar well diffusion method. Sterile Mueller-Hinton agar (MHA) plates were inoculated with standardized bacterial suspensions adjusted to the 0.5 McFarland turbidity standard. Wells of uniform diameter were aseptically bored into the agar, and measured volumes of the tempeh extract were dispensed into each well. The plates were incubated at 37°C for 24 h. Following incubation, the zones of inhibition surrounding the wells were measured in millimeters, and the antibacterial activity of the tempeh extract was determined based on the diameter of the clear zones.

Results

Isolation and identification of *R. oligosporus*

Thirty samples of fresh *Hibiscus* leaves were collected from the Dharan sub-metropolitan area. The *R. oligosporus* was isolated from only 4 (13%) of the 30 leaf samples (Figure 1).

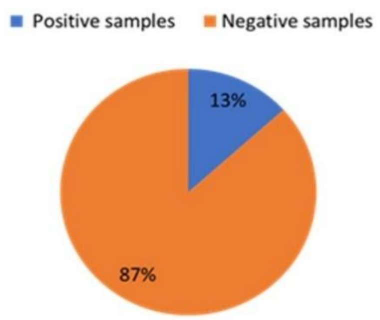

Figure 1
Percentage of positive isolates among the total collected samples

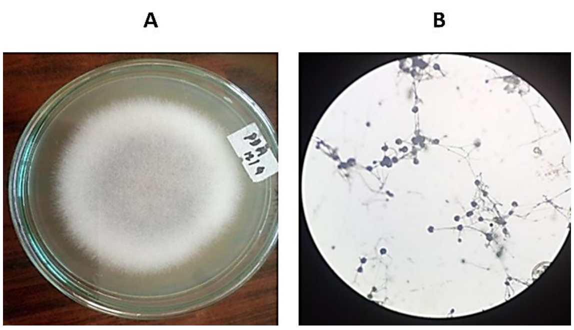

Figure 2
Morphological identification for *R. oligosporus*
A: Macroscopic observation
B: Microscopic observation (Lacto-phenol cotton blue)

R. oligosporus, a filamentous fungus belonging to the family Mucoraceae (phylum Mucoromycota), was observed in the present study. This species is characterized by a rapidly growing, branching hyphal network that forms a dense, cottony mycelium. The isolate recovered from *Hibiscus* leaves exhibited morphological features typical of *R. oligosporus*. The vegetative mycelia appeared white, indicating active growth during the early stages of culture. With maturation, reproductive structures developed, including brownish sporangia borne on sporangiophores. The sporangiospores were oval to ellipsoidal in shape, smooth-walled, and produced abundantly within the sporangium (Figure 2). These macroscopic and microscopic characteristics are consistent with the standard morphological description of *R. oligosporus* (Table 1).

Table 1
Morphological observations of *R. oligosporus* isolate

Parameter	Observation
Colony morphology	Dense, cottony, rapidly spreading growth
Colony color (initial)	White
Colony color (mature)	Grayish to brownish due to sporangial development
Colony texture	Fluffy, aerial mycelium
Growth rate	Rapid growth within the incubation period
Hyphal structure	Broad, branched, coenocytic (aseptate) hyphae
Mycelium	Well-developed, filamentous network
Sporangiosphores	Erect, unbranched, arising from mycelium
Sporangia	Spherical, brownish, terminally borne
Sporangiospores	Oval to ellipsoidal, smooth-walled
Rhizoids	Present at the base of sporangiophores

Optimization of growth temperature of *R. oligosporus* during Tempeh fermentation (24 h)

The effect of temperature on the growth of *R. oligosporus* during soybean fermentation was evaluated after 24 h of incubation. Minimal mycelial growth was observed at 4°C, indicating that low temperature significantly limited fungal metabolic activity. Moderate mycelial development was recorded at 20°C, while a clear increase in mycelial density and spread was evident at 30°C, suggesting favorable growth conditions.

Table 2
Growth of *R. oligosporus* during Tempeh preparation at different temperatures after 24 h

Temperature (°C)	Observation after 24 h
4	No visible mycelial growth
20	Slight mycelial growth
30	Moderate mycelium growth
37	Dense and compact mycelial growth

The highest and most compact mycelial growth was observed at 37°C, where the soybean substrate was uniformly covered with dense white mycelium. This indicates that 37°C provides optimal conditions for fungal metabolism, enzyme activity, and substrate colonization during tempeh fermentation. These findings demonstrate that *R. oligosporus* exhibits temperature-dependent growth, with 37°C being the optimum among the tested conditions for effective tempeh production (Table 2).

Tempeh production using *R. oligosporus*

Successful fermentation of soybeans with *R. oligosporus* resulted in the formation of compact tempeh cakes within 24-48 hours of incubation at 37°C. Visible fungal growth was observed as a dense white mycelial network that uniformly covered and bound the soybean substrate. Progressive mycelial proliferation led to the consolidation of individual soybeans into a firm, cohesive structure characteristic of well-fermented tempeh (Figure 3). No contamination or abnormal discoloration was observed during the fermentation period. The final product exhibited a stable cake-like form, indicating effective fungal colonization and substrate binding.

Figure 3
Prepared Tempeh at 37°C in 48 h by using *R. oligosporus*

Effect of added ingredients on Tempeh cake formation

The addition of various ingredients at different concentrations influenced the quality of tempeh cakes based on the extent of *Rhizopus* mycelial development and the resulting compactness of the fermented soybean matrix (Table 3).

Table 3
Evaluation of added ingredients on Tempeh cake formation

Added ingredient	*Tempeh cake quality with ingredient percentage*			
	0.5%	*1%*	*1.5%*	*2.0%*
Potato starch	Good	Excellent	Very good	Poor
Wheat flour	Poor	Excellent	Very good	Very good
Maize flour	Good	Very good	Very good	Very good
Rice flour	Excellent	Very good	Very good	Good

Potato starch produced excellent cake formation at 1%, but higher concentrations (2%) reduced quality. Wheat flour resulted in excellent binding at 1%, with slightly reduced quality at other concentrations. Maize flour consistently yielded very good cakes at concentrations from 1% to 2%. Rice flour produced the best cake formation at the lowest concentration (0.5%), with a slight decrease in quality at higher percentages. These findings indicate that both the type and proportion of added ingredients significantly affect tempeh texture and structural integrity.

Antibacterial activity of Tempeh extract

Tempeh extract (crude) showed a 9.8 mm zone of inhibition against *S. aureus,* but could not inhibit *E. coli* in agar well assay performed on MHA medium (Figure 4).

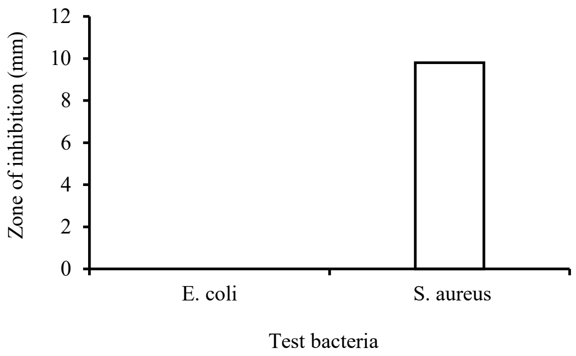

Figure 4
Antibacterial activity of Tempeh extract

Discussion

The present study demonstrates that *R. oligosporus* can be isolated from natural plant surfaces such as *Hibiscus* leaves, although the recovery rate was relatively low (13%). The occurrence of *R. oligosporus* in environmental niches outside traditional fermentation settings supports the view that this fungus is not exclusively domesticated but can persist as a saprophyte on plant materials rich in carbohydrates and organic debris (Pawlowska et al., 2018; Samson et al., 2019). Members of the genus *Rhizopus* are known to inhabit soil, decaying vegetation, and leaf surfaces, where they contribute to organic matter decomposition through the secretion of hydrolytic enzymes (Pitt & Hocking, 2009). The relatively low isolation frequency in this study may be attributed to ecological competition with other phylloplane microorganisms and environmental conditions influencing spore deposition and survival.

Morphological characterization of the isolate revealed classical features of *R. oligosporus*, including rapid cottony growth, coenocytic hyphae, terminal brown sporangia, and oval to ellipsoidal smooth-walled sporangiospores. The presence of rhizoids at the base of sporangiophores further confirmed taxonomic placement within *Rhizopus* (Samson et al., 2019). These observations align with standard descriptions used for the identification of *R. oligosporus* in food fermentation and mycological studies (Pitt & Hocking, 2022; Tamang et al., 2020). The progressive change from white vegetative mycelium to grayish-brown mature colonies reflects active sporulation and is a typical developmental pattern of the species under aerobic culture conditions.

Temperature optimization results indicated that fungal growth was strongly temperature dependent, with maximum mycelial density observed at 37°C after 24 h. This finding is consistent with previous reports that *R. oligosporus* exhibits optimal growth between 30°C and 37°C, conditions that enhance enzymatic activities such as protease, lipase, and amylase production essential for soybean fermentation (Górska et al., 2025; Hazarika & Hazarika, 2025). Limited growth at 4°C reflects metabolic suppression at low temperatures, while moderate growth at 20-30°C suggests suboptimal but permissive conditions. The superior performance at 37°C supports its suitability for rapid tempeh fermentation, where uniform mycelial coverage is necessary for structural binding and product quality.

Successful tempeh formation within 24-48 h further confirms the technological competence of the isolated strain. The dense mycelial network binding soybeans into a compact cake is a hallmark of effective *R. oligosporus* fermentation (Shurtleff & Aoyagi, 2020). The absence of contamination and abnormal discoloration indicates that the strain maintained competitive dominance and produced conditions unfavorable for spoilage organisms, possibly through rapid substrate colonization and metabolite production.

The incorporation of additional ingredients significantly influenced cake formation and texture. Starch-rich supplements such as potato starch and rice flour enhanced structural integrity at lower concentrations, likely due to increased carbohydrate availability that supports fungal growth and extracellular polysaccharide production (Górska et al., 2025). However, excessive supplementation (e.g., 2% potato starch) reduced cake quality, potentially due to altered moisture balance or substrate compaction limiting aeration. Wheat and maize flours provided consistent improvements, suggesting that moderate levels of complex carbohydrates and proteins optimize substrate structure and fungal metabolism. These findings highlight the importance of balancing nutrient enrichment and physical substrate properties in fermentation systems.

The antibacterial assay showed that crude tempeh extracts inhibited *S. aureus* but not *E. coli*. This selective activity aligns with previous studies demonstrating that tempeh fermentation can generate antimicrobial peptides, organic acids, and phenolic compounds, effective mainly against Gram-positive bacteria due to their simpler cell wall structure (Górska et al., 2025; Ketnawa & Ogawa, 2021). Gram-negative bacteria such as *E. coli* possess an outer membrane that limits penetration of many antimicrobial metabolites, which may explain the absence of inhibition. The observed zone of inhibition (9.8 mm) indicates moderate antibacterial activity and supports the functional food value of tempeh beyond its nutritional value.

Overall, the study confirms that naturally occurring *R. oligosporus* strains can be isolated from plant sources and effectively applied in soybean fermentation under optimized temperature conditions. Ingredient supplementation further modulates product quality, while the resulting tempeh exhibits selective antibacterial properties. These findings reinforce the microbiological, technological, and functional significance of *R. oligosporus* in traditional and improved tempeh production systems.

Conclusions

The isolation of *R. oligosporus* confirms that *Hibiscus* leaves serve as a natural habitat for tempeh-producing fungi. The strain obtained from Dharan, possessing specific enzymatic and metabolic capabilities, enabled successful tempeh production. The optimum temperature for fermentation was determined to be 37°C, supporting maximal mycelial growth and substrate binding. The addition of substrates such as 0.5% rice flour, 1% potato starch, and 1% wheat flour significantly enhanced tempeh cake formation compared to various concentrations of maize flour. Tempeh extracts exhibited inhibitory activity against *S. aureus*, suggesting the presence of antibiotic peptides produced by *R. oligosporus*. In contrast, no activity was observed against Gram-negative bacteria such as *E. coli*. Overall, the identification of *R. oligosporus* from *Hibiscus* leaves and its successful application in tempeh fermentation provides a basis for exploring alternative, plant-derived sources of fermentation microbes. This approach contributes to sustainable food systems and offers opportunities for indigenous bioprospecting of microbial diversity for food and functional applications.

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Conflict of Interest

The authors declare no conflict of interest.

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