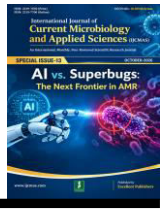
*Int.J.Curr.Microbiol.App.Sci* (2026) *Special Issue-13: 84-93*

*International Journal of Current Microbiology and Applied Sciences*
***ISSN: 2319-7706*** **Special Issue-13 [October-2026]**
Journal homepage: http://www.ijcmas.com




**Original Research Article** https://doi.org/10.20546/ijcmas.2026.sp13.011

# *Invitro* Bioactive and Nutritional Properties of Fermented Black Rice (*Oryza sativa* L. indica) Liquid Produced using *Aspergillus oryzae* and *Bacillus coagulans*

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**Keywords**

Karung Kavuni; black rice; *Aspergillus oryzae; Bacillus coagulans*; DPPH; α-amylase; Pancreatic lipase; GC-MS; fermented rice liquid

**Article Info**

***Received:***
18 August 2026
***Accepted:***
20 September 2026
***Available Online:***
10 October 2026

**A B S T R A C T**

Fermented Karung Kavuni black rice (*Oryza sativa L. indica*) liquid was produced by sequential fermentation with *Aspergillus oryzae* MTCC 1847 followed by *Bacillus coagulans* MTCC 5856 and evaluated for total acid content, antimicrobial activity, DPPH radical-scavenging activity, pancreatic lipase inhibition, α-amylase inhibition, proximate composition, and GC-MS profile. Ground rice mixed with distilled water (1:2.5, w/v) was autoclaved, inoculated with A. oryzae, and fermented for approximately 72 h. The broth was heated at 75°C for 15 min to inactivate fungal spores, cooled, inoculated with 2 mL *B. coagulans* culture, and fermented for approximately 72 h. The supernatant obtained after filtration and centrifugation was used for analysis. Total acid content was 4.288 g/100 mL. Inhibition zones against Escherichia coli were 19, 21, 24, and 25 mm at 25, 50, 75, and 100 µL per well, respectively; against *Staphylococcus aureus* the zones were unreported at 25 µL and 10, 13, and 15 mm at the higher volumes. No antifungal activity was observed against *Candida albicans* or *Aspergillus niger.* DPPH inhibition increased from 11.27% to 35.42%, pancreatic lipase inhibition from 1.10% to 36.01%, and α-amylase inhibition from 36.718% to 73.437% across the tested range of 10–50 µL/mL. Proximate composition was 1.85 g protein, 0.40 g fat, and 23.45 g carbohydrate per 100 mL, corresponding to 105 kcal/100 mL. GC-MS analysis detected 47 peaks; the major peak-area assignments (fucosterol 31.95%, cholest-5-enol 24-propylidene-(3β) -16.89 %, and ergosta-5, 22-dien-3-ol (3β, 22E) 8.60%) are reported as tentative library matches. The results are limited to the in vitro conditions described and do not establish clinical efficacy or product safety.

## Introduction

Rice is a staple food for more than half of the world's population. Pigmented varieties such as black rice (*Oryza sativa L. indica*) contain anthocyanins in the pericarp that contribute measurable antioxidant activity in chemical assays (Hu *et al.,* 2003; Deng *et al.,* 2013). Karung Kavuni (also called Kavuni rice) is a traditional black rice landrace of Tamil Nadu, India, reported to possess elevated levels of phenolics, carotenoids, and other phytochemicals relative to non-pigmented rice (Hemamalini *et al.,* 2018; Valarmathi *et al.,* 2014; Kalavani *et al.,* 2018).

Fermentation with food-grade microorganisms can modify the extractable constituents of cereal substrates. *Aspergillus oryzae* is widely used in East Asian fermentations because it secretes amylases, proteases, and other hydrolytic enzymes; *Bacillus coagulans* is a spore-forming lactic-acid bacterium employed in food and probiotic research. Sequential or mixed-culture fermentation of rice may therefore alter acidity, antimicrobial activity, radical-scavenging capacity, and enzyme-inhibitory properties (Murooka and Yamashita, 2008; Sharma *et al.,* 2020).

The present study prepared a liquid product from Karung Kavuni black rice by sequential fermentation with *A. oryzae* MTCC 1847 and *B. coagulans* MTCC 5856. The liquid was examined for total acidity, antibacterial and antifungal activity, DPPH radical scavenging, pancreatic lipase and α-amylase inhibition, proximate composition, and GC-MS profile.

## Materials and Methods

### Rice samples and microbial cultures

Karung Kavuni (*Oryza sativa L. indica*) black rice was purchased from a local market in Tamil Nadu, washed, and air-dried. Approximately 50 g of grain was ground and mixed with distilled water at a ratio of 1:2.5 (w/v). The slurry was autoclaved and cooled before inoculation. *Aspergillus oryzae* MTCC 1847 and *Bacillus coagulans* MTCC 5856 were obtained from the Microbial Type Culture Collection and Gene Bank (IMTECH, Chandigarh, India). Cultures were identified by lactophenol cotton blue staining (*A. oryzae*) and Gram staining *(B. coagulans*). *A. oryzae* was maintained on potato dextrose agar/broth and *B. coagulans* on MRS medium.

### Sequential fermentation

The cooled rice slurry was inoculated with *A. oryzae* and incubated on a shaker under atmospheric (aerobic) conditions for approximately 72 h. After the fungal stage the broth was heated at 75°C for 15 min to reduce viable fungal spores, cooled, and inoculated with approximately 2 mL of *B. coagulans* culture. Incubation continued for a further approximately 72 h. Solids were removed by filtration through cotton mesh; the filtrate was centrifuged at 5000 rpm for 10 min and the supernatant stored at −20°C until analysis.

### Total acid content

Ten millilitres of fermented liquid was diluted with 40 mL distilled water, four drops of phenolphthalein were added, and the mixture was titrated with 0.1 N NaOH to a pale-pink endpoint. Results are expressed as g/100 mL. The acid equivalent used for calculation, number of replicates, and blank correction were not stated in the source records and remain to be confirmed.

### Antibacterial and antifungal assays

Antibacterial activity was evaluated by the agar-well diffusion method (Holder and Boyce, 1994) against *Escherichia col*i MTCC 443 and *Staphylococcus aureus* MTCC 96 obtained from the Microbial Type Culture Collection and Gene Bank (IMTECH, Chandigarh, India). Inocula were prepared by the direct colony suspension procedure (CLSI) and standardised to 0.5 McFarland turbidity (absorbance 0.08–0.13 at 625 nm). One hundred microlitres of an 18-h culture (OD adjusted to 0.6) was spread on Mueller–Hinton agar. Wells of 6 mm diameter were made with a sterile cork-borer and loaded with 25, 50, 75 or 100 µL of fermented liquid. Sterile water served as the negative control and azithromycin (20 µg/mL) as the positive control. Plates were incubated at 37°C for 24 h and the diameter of the zone of inhibition was measured with an antibiotic zone scale (HiMedia, Mumbai, India). Antifungal activity was tested against *Aspergillus niger* MTCC 404 and *Candida albicans* MTCC 227 (IMTECH, Chandigarh) by the agar-well diffusion method (Holder and Boyce, 1994). Potato dextrose agar plates were inoculated either with a 5-mm mycelial disc *(A. niger)* or by swabbing an 18-h yeast culture *(C. albicans).* Wells were loaded with 25, 50, 75 or 100 µL of fermented liquid; clotrimazole served as the positive control. Plates were incubated for 72 h and zones of inhibition were measured.

## DPPH radical-scavenging assay

DPPH radical-scavenging activity was determined according to Von Gadow *et al.,* (1997). Two millilitres of $6 \times 10^{-5}$ M methanolic DPPH solution were mixed with 50 µL of sample. The decrease in absorbance at 515 nm was recorded continuously for 16 min at room temperature. Percentage inhibition was calculated by the formula of Yen and Duh (1994): IP = [(A_C(0) − A_A(t))/A_C(0)] × 100, where A_C(0) is the absorbance of the control at t = 0 min and A_A(t) is the absorbance of the sample at t = 16 min. Samples were tested at a nominal concentration of 1 mg/mL; tabulated results are expressed as µL/mL.

## Pancreatic lipase inhibition

A substrate emulsion of 1% (v/v) triolein and 1% (v/v) Tween 40 in 0.1 M phosphate buffer (pH 8) was prepared. The reaction mixture contained 1600 µL emulsion, 200 µL porcine pancreatic lipase preparation (0.5 g pancreatin in 15 mL buffer), and 200 µL sample. After 30 min at 37°C, absorbance was read at 450 nm. Inhibition (%) = [(A_control − A_sample)/A_control] × 100 (modified from Etoundi *et al.,* 2010).

## α-Amylase inhibition

α-Amylase inhibitory activity was assayed according to Jung *et al.,* (2006). The reaction mixture containing 200 µL of 0.02 M sodium phosphate buffer, 20 µL enzyme and sample (20–100 µg/mL) was incubated for 10 min at room temperature. After addition of 200 µL starch solution the reaction was terminated with 400 µL DNS reagent, placed in a boiling water bath for 5 min, cooled, diluted with 15 mL distilled water and the absorbance measured at 540 nm. Percentage inhibition was calculated as [(A_control − A_sample)/A_control] × 100. Tabulated results are expressed as µL/mL.

## Proximate Composition

Protein content was estimated by the Bradford dye-binding method (Bradford, 1976) at 595 nm using bovine serum albumin as standard. Carbohydrate was determined by the phenol–sulphuric acid method of Dubois *et al.,* (1956) at 488 nm with glucose as standard. Fat was extracted with chloroform: methanol (2:1, v/v); the dried extract was treated with concentrated sulphuric acid and vanillin reagent and quantified at 490 nm. Energy was calculated using Atwater factors (4 kcal/g protein, 4 kcal/g carbohydrate, 9 kcal/g fat).

## GC-MS analysis

GC-MS analysis was performed on a Shimadzu GC-2010 Plus gas chromatograph equipped with a 15 m Alltech EC-5 column (250 µm i.d., 0.25 µm film thickness). Injection was split (10:1), and the carrier gas was helium at a constant flow of 2 mL/min. The oven temperature program started at 35°C (hold 2 min) and was ramped at 20°C/min (instrument method to be verified from the original file). Mass spectra were acquired in electron-ionisation mode with GCMS Solution software (version 2.6). Compounds were identified by matching spectra against the NIST library (version 11) and a GC/MS metabolomics database using retention index. All identifications remain tentative library matches.

## Results and Discussion

## Total acid content

The fermented Karung Kavuni black-rice liquid contained 4.288 g total acid per 100mL. The acid equivalent used for calculation was not specified.

## Antibacterial activity

Inhibition zones increased with sample volume (Table 1). The 25 µL value for S. aureus was not recorded in the source table and is therefore shown as unreported. No antifungal activity was observed against *C. albicans* or *A. niger* under the conditions tested.

## GC-MS profile

Forty-seven peaks were detected. The three largest peak-area percentages were assigned by library matching to fucosterol (31.95%), cholest-5-enol, 24-propylidene-(3β)- (16.89%), and ergosta-5,22-dien-3-ol (3β,22E) (8.60%). Additional sterol-like assignments included stigmasta-5,24(28)-dien-3-ol (4.58%) and 26,27-dinorergosta-5,23-dien-3β-ol (4.51%).

These identifications remain tentative pending verification with authentic standards, retention indices, and corrected instrument parameters. Peak-area percentage is not equivalent to absolute concentration or biological activity.

**Table.1** Antibacterial activity of fermented Karung Kavuni black-rice liquid (zone of inhibition in mm).

| Test material | Volume | *S. aureus* (mm) | *E. coli* (mm) |
|---|---|---|---|
| Fermented liquid | 25 µL | — | 19 |
| | 50 µL | 10 | 21 |
| | 75 µL | 13 | 24 |
| | 100 µL | 15 | 25 |
| Azithromycin | 20 µg/mL | 20 | 30 |

**Table.2** DPPH radical-scavenging activity of fermented black-rice liquid.

| Sample level (µL/mL) | Inhibition (%) |
|---|---|
| **10** | 11.27 |
| **20** | 15.61 |
| **30** | 18.67 |
| **40** | 25.76 |
| **50** | 35.42 |

**Table.3** Enzyme inhibition by fermented black-rice liquid.

| Sample level (µL/mL) | Lipase inhibition (%) | α-Amylase inhibition (%) |
|---|---|---|
| **0** | 0 | 0 |
| **10** | 1.10 | 36.718 |
| **20** | 19.28 | 54.687 |
| **30** | 26.51 | 60.156 |
| **40** | 30.01 | 64.843 |
| **50** | 36.01 | 73.437 |

**Table.4** Proximate composition of fermented black-rice liquid.

| Parameter | Value |
|---|---|
| **Protein** | 1.85 g/100 mL |
| **Fat** | 0.40 g/100 mL |
| **Carbohydrate** | 23.45 g/100 mL |
| **Energy** | 105 kcal/100 mL |

**Table.5** Selected GC-MS peaks from fermented Karung Kavuni black-rice liquid

| Peak | RT (min) | Compound (library match) | M.W. | Peak area (%) |
|---|---|---|---|---|
| **27** | 32.271 | Cholest-5-enol, 24-propylidene-(3β)- | 426.7 | 16.89 |
| **28** | 32.301 | Stigmasta-5,24(28)-dien-3-ol, (3β)- | 412.69 | 4.58 |
| **29** | 32.446 | Fucosterol | 412.7 | 31.95 |
| **35** | 34.197 | β-Sitosterol | 414.71 | 0.77 |
| **37** | 36.139 | Squalene | 410.73 | 0.36 |
| **38** | 36.300 | Stigmasta-4,24(28)-dien-3-one | 410.7 | 2.88 |
| **42** | 37.407 | 26,27-Dinorergosta-5,23-dien-3β-ol | 370.6 | 4.51 |
| **43** | 37.451 | Ergosta-5,22-dien-3-ol, (3β,22E) | 398.7 | 8.60 |
| **44** | 37.610 | 22,23-Dibromostigmasterol acetate | 454.7 | 1.92 |

*Note: RT = retention time; M.W. = molecular weight. Peak area % is relative.*

**Figure.1** Karung Kavuni black rice grains used as the study substrate.

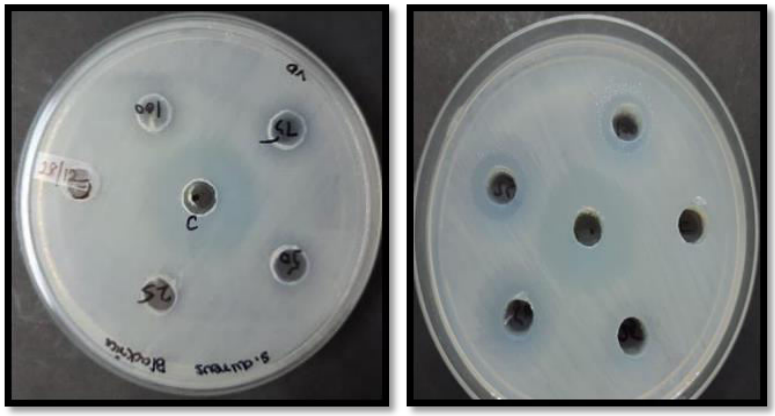


**Figure.2** Starter-culture broths of *Aspergillus oryzae* and *Bacillus coagulans.*

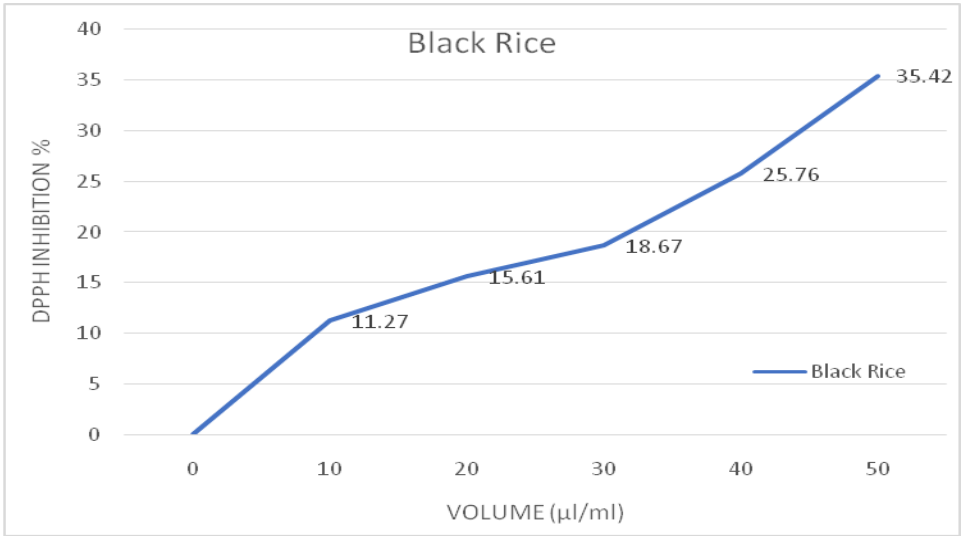



**Figure.3** Agar well-diffusion plates showing antibacterial activity of fermented black-rice liquid against *Staphylococcus aureus* and *Escherichia coli.*

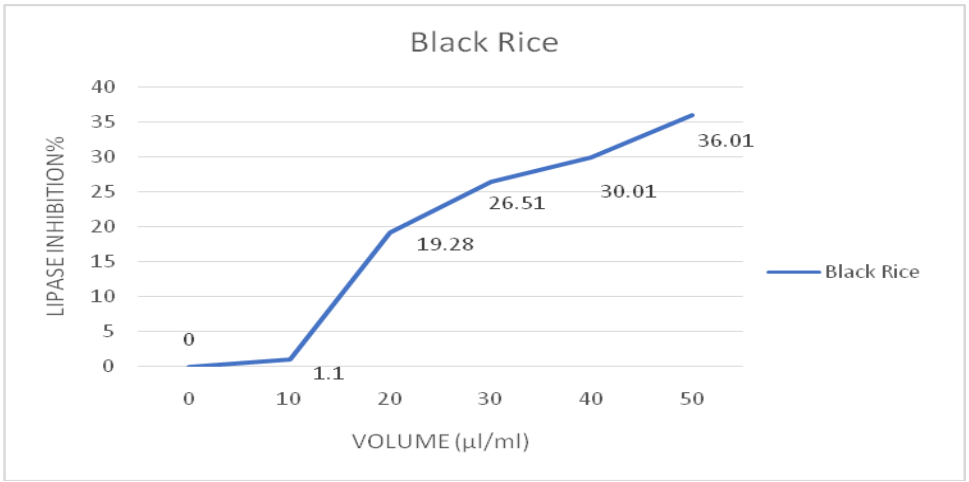




**Graph.1** DPPH radical-scavenging inhibition of fermented black-rice liquid across the tested sample levels.




**Graph.2** Pancreatic lipase inhibition, and α-amylase inhibition of fermented black-rice liquid across the tested sample levels.



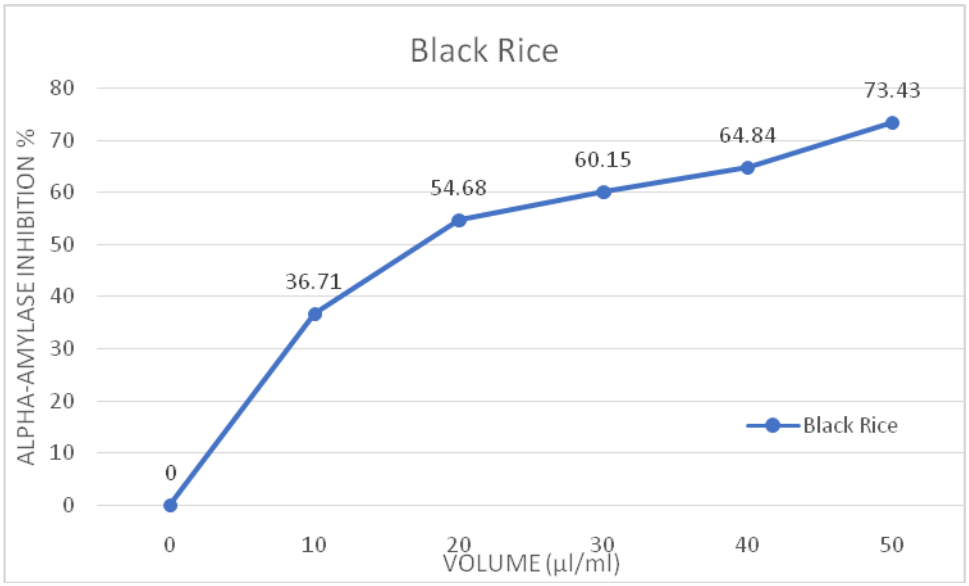
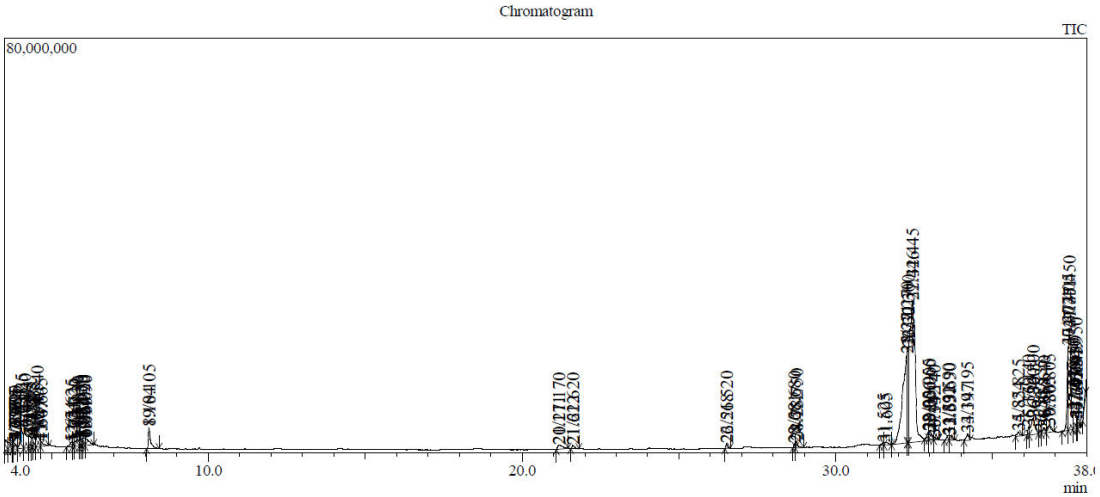



**Figure.6** Total ionchromatogram (TIC) of fermented Karung Kavuni black-rice liquid obtained by GC-MS analysis.




Fermented foods have attracted global attention because controlled microbial growth and enzymatic conversion of food components can improve nutritional value, organoleptic properties and bioactive potential (Sharma *et al.,* 2020). Submerged fermentation of pigmented rice with food-grade fungi and bacteria remains comparatively less explored. In the present study, sequential fermentation of Karung Kavuni black rice with *Aspergillus oryzae* followed by *Bacillus coagulans* yielded a liquid that displayed measurable antibacterial, antioxidant and enzyme-inhibitory activities under the laboratory conditions tested. The fermented black-rice liquid produced clear zones of inhibition against both Staphylococcus aureus and Escherichia coli, with zone diameters increasing with sample volume (Table 1). No antifungal activity was recorded against Candida albicans or *Aspergillus niger*. The absence of antifungal activity in the present assay indicates that the active principles, if present, are more effective against the bacterial strains examined than against the fungal and yeast strains tested. Agar-well diffusion remains a screening method; determination of minimum inhibitory concentrations and

identification of the responsible compounds are still required. DPPH radical-scavenging activity increased from 11.27% to 35.42% across the tested concentration range (Table 2). Pigmented rice bran is known to be a rich source of phenolic antioxidants (Sompong *et al.,* 2011), and fermentation can release phenolic aglycones that possess higher radical-scavenging capacity than their glycoside counterparts (Heim *et al.,* 2002; Dey and Kuhad, 2014). The progressive increase observed here is therefore consistent with the liberation or formation of antioxidant metabolites during fermentation, although the absolute values remain modest compared with some longer solid-state fermentations reported elsewhere. Pancreatic lipase and α-amylase were inhibited in a concentration-dependent manner, reaching 36.01% and 73.437%, respectively, at the highest sample level tested (Table 3). Inhibition of pancreatic lipase is a recognised strategy for reducing dietary fat absorption, while α-amylase inhibition can delay starch digestion and post-prandial glucose rise (Jung *et al.,* 2006). The α-amylase inhibition value is comparable to that reported for red-koji fermented rice bran. These enzyme assays are, however, strictly in vitro and do not demonstrate anti-obesity or anti-diabetic efficacy in vivo. Proximate analysis showed 1.85 g protein, 0.40 g fat and 23.45 g carbohydrate per 100 mL, corresponding to an energy value of 105 kcal/100 mL (Table 4). The relatively high carbohydrate figure for a filtered liquid warrants verification against calibration and dilution records. GC-MS analysis detected 47 peaks (Table 5). The largest peak-area assignments were fucosterol (31.95%), cholest-5-enol 24-propylidene-(3β)- (16.89%) and ergosta-5,22-dien-3-ol (3β,22E) (8.60%). These sterol-like library matches remain tentative; confirmation with authentic standards, retention indices and a verified instrument method is essential before any biological attribution can be made (Bae *et al.,* 2020). The principal limitations of the present data set are the incomplete documentation of fermentation parameters (especially inoculum density and precise temperature), the absence of replicate statistics, inconsistent concentration units between method descriptions and result tables, and the unverified status of the GC-MS identifications. Resolution of these points from laboratory notebooks will strengthen the reliability of the findings and facilitate future development of the fermented liquid as a functional-food ingredient.

In conclusion, Sequential fermentation of Karung Kavuni black rice (*Oryza sativa L. indica*) with *Aspergillus oryzae* MTCC 1847 followed by *Bacillus coagulans* MTCC 5856 produced a liquid that exhibited measurable in vitro bioactivity. Under the conditions tested, the fermented liquid showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, concentration-dependent DPPH radical-scavenging activity (up to 35.42%), and progressive inhibition of pancreatic lipase (up to 36.01%) and α-amylase (up to 73.437%). Proximate analysis indicated 1.85 g protein, 0.40 g fat and 23.45 g carbohydrate per 100 mL, corresponding to an energy value of 105 kcal/100 mL. GC-MS analysis detected 47 peaks; the major library assignments were sterol-like compounds, although these identifications remain tentative pending confirmation with authentic standards. The present findings are limited to the laboratory assays described and do not demonstrate clinical efficacy, product safety, or suitability as a commercial functional beverage. Standardisation of fermentation parameters (particularly oxygen availability, inoculum density and temperature), inclusion of replicate measurements with statistical analysis, correction of the GC-MS temperature program from the original instrument method, and verification of selected compound identities are recommended before the fermented liquid can be considered for further development as a functional food ingredient.

## Declarations

**Funding:** No external funding was received for this study.

**Conflict of interest:** The authors declare that they have no competing interests.

**Ethics approval:** Not applicable; the study involved only laboratory in vitro assays.

## Author Contributions

Vinayakapriya. V – Conceptualization, Methodology, Investigation, Formal Analysis, and Writing – Original Draft. Gomathi. N. – Supervision, research guidance, critical evaluation of the work, validation of the results, and writing – review and editing

### Data availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

## Acknowledgements

The authors thank the Department of Microbiology, Valliammal College for Women, for laboratory facilities.

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**How to cite this article:**

Vinayakapriya V. and Gomathi Natrajan. 2026. *Invitro* Bioactive and Nutritional Properties of Fermented Black Rice (*Oryza sativa L. indica*) Liquid Produced Using *Aspergillus oryzae* and *Bacillus coagulans*. *Int.J.Curr.Microbiol.App.Sci.* Special Issue-13: 84-93. **doi:** https://doi.org/10.20546/ijcmas.2026.sp13.011