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ORIGINAL ARTICLE

Effect of Fungal Fermentation on Enhancement of Nutritional Value and Antioxidant Activity of Defatted Oilseed Meals

Ruma Dutta[1] · Saheli Ghosal[1] · Dipak K. Bhattacharyya[1] · Jayati Bhowal[1]

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Abstract

Agro-industrial residues contain high nutritive value. Nowadays, various advanced researches have been done for the production of various value-added products, using these wastes as substrates in the fermentation media. Flaxseed, mustard, and rice bran meal, residues of oil industry, were used as substrates for fermentation. Submerged fermentation with soil-isolated fungal species of the genus *Aspergillus* sp. was done for oil production by using these substrates in the fermentation media. Effect of fermentation by the oleaginous species of *Aspergillus* on the nutritive value and functional properties of flaxseed, mustard, and rice bran meal has been discussed for the first time in the present study. After fermentation, the seed meals showed substantial increase in the protein and ash content. The fungal strains utilized the carbohydrate present in the seed meals for the production of highly nutritional metabolites, which decrease the sugar contents of the meals. The fungi also showed extracellular amylase and cellulase activities which helped to hydrolyze the carbohydrates present in these meals, to utilize them for their metabolism. The enhancement was also observed in terms of antioxidant activity of the meals. Increase in the total phenolic and flavonoid contents was observed after fermentation along with radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl and 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid reagents and ferric reduction potential. These effects of fermentation modify these cheap waste materials into nutrient dense substrates, which could be further used in the formulation of value-added products.

Keywords Oilseed meals · Antioxidant activity · Nutritive value · Enzyme activity · *Aspergillus* sp

Introduction

Oil seed meals are by product of oil manufacturing industry and they contained high nutritional value; mainly, they are rich in various macro- and micronutrients along with important bioactive compounds which are beneficial for health [1]. Flaxseed meal (FSM), the byproduct

✉ Jayati Bhowal
jayatibhowal@gmail.com

[1] School of Community Science and Technology, Indian Institute of Engineering Science and Technology, Shibpur, Howrah, West Bengal 711103, India

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of flaxseed oil industry, is used in poultry diets due to its high protein content (30 to 34.9%) [2]. In addition, FSM contains high levels of α-linolenic acid (ALA) which reduce serum cholesterol level [3]. Mustard oil and rice bran oil are the two most widely used edible oils in India, resulting in large amount of wastes from these oil industries. India has been ranked three for the production of mustard meal (MM) as more than 60% of the seed becomes residuals after oil extraction [4]. Mustard and rice bran meal (RBM) both contain high protein, vitamin, minerals, and indigestible carbohydrate [5]. Mustard meal contained significant antioxidant property in the term of potential free radical scavenging activity [6]. RBM is an important agro-industrial residue as fermented rice bran produces biologically active metabolites like protein and lipid and enhances the production vitamin B, and essential amino acids along with phenolic compounds like ferulic acid, vanillic acid, protocatechuic acid, ɣ-oryzanol, phytic acid, and inositol [7]. These lignocellulosic oil industrial waste products are mainly used for the formulation of low-cost, high nutritious poultry feed.

Fungi are a group of microorganisms capable of producing a variety of important metabolites. Single cell oil (SCO) is the oil produced by microorganisms intracellularly by converting the carbohydrate present in the fermentation media into lipids, having similar composition as vegetable oils [8]. Although very few literatures are found, but these oil industry waste products could serve as nutrient supplements in the fermentation media for improving the production of SCO by fungi [9].

The product qualities of the seed meals, such as nutritional value and digestibility, were improved by fermentation. The process might also increase the bioactivity of these wastes by producing value-added products like antioxidants [10]. Microorganisms improve protein quality of seed meals and nutrient accessibility; reduce tannins and phytates; and destroy many anti-nutritional components and inhibitors by their endogenous enzymes [11]. Previous studies have reported that *Aspergillus niger* produce enzyme β-glucosidase, which degrades cyanogenic glycosides present in FSM [12–14]. Besides these features of enhancing food quality, shelf life [15], nutritional value, and organoleptic properties, microbial fermentation also increases the production and extraction yields of antioxidants in the food used for pharmaceutical industries [16]. There are various studies reported showing the enhancing effect of fungal fermentation on different oil seed meals [17–19], but no literature is found studying the effect of various oleaginous *Aspergillus* strains on both the nutritional and functional properties of flaxseed, mustard, and rice bran meal.

Filamentous ascomycete fungi, *Aspergillus* spp., are known to produce various hydrolytic and oxidative enzymes that could degrade the lignocellulosic materials present in these meals and utilize them for the production of value-added products such as single cell protein (SCP), SCO, and organic acids [20]. Almost all fungi of genus *Aspergillus* synthesize saccharolytic enzymes [21].

The present study was aimed for the evaluation of the effect of fungal fermentation on the nutritive values in terms of total protein and ash contents along with the antioxidant activity for flaxseed, mustard, and rice bran meal. The study also evaluated the activity of extracellular cellulase and amylase enzymes in the oil-producing media fermented with soil-isolated *Aspergillus spp.* In this study, submerged fermentation was done for the production of single cell oil using oil seed meals as supplementation in the culture media.

Materials and Methods

Materials

Potatoes, rice bran, flaxseed, and black mustard seeds were bought from local market of Shibpur, West Bengal, India. All the media components used for fermentation purpose were purchased from Hi-media Laboratories Pvt. Ltd., India (Mumbai, India). All chemicals and solvents used were analytical grade, purchased from MERCK. Chemicals used for antioxidant assay, (2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS)) were purchased from Sigma (St. Louis, MO).

Collection of Soil

Soil sample was collected from drained waste of rice bran oil industry situated in Saktigarh, Burdwan, West Bengal, India, and used for isolation of oleaginous fungi. Sample was collected from a depth of 5–10 cm below the surface using sterile spatula and kept in sterile plastic container and stored at 4 °C under refrigeration immediately until use.

Screening and Isolation of Fungi

Fungi were isolated from the collected soil sample by serial dilution and plating method. One gram of soil after diluting with 9 ml of 0.9% (w/v) sterile saline water was added in 100-ml conical flask and shaken vigorously for 5 min. Serial tenfold dilutions of the original suspension ranging from 10^{-1} to 10^{-6} were prepared. From each test tube, 0.1 ml of solution was spread individually into Czapek-Dox Agar (CYA) plate, containing 3% sucrose, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, $7H_2O$, 0.05% KCl, 0.01% $FeSO_4$, $7H_2O$, and 2% agar. Streptomycin was added to the medium (at a concentration of 10,000 U/ml) to prevent bacterial growth. Each dilution was plated in triplicate. The screening plates were incubated at 28 °C for 7 days to form fungal colonies.

The isolated colonies of different fungal strains were further screened for their lipid-producing abilities by qualitative analysis with the Sudan Black B staining technique [22] and observed under microscope on oil immersion for the presence of blue or grayish colored fat globules within the cell.

Microscopic examination of the isolated colonies was done for confirmation of the pure cultures. Similarity in the septate hyphae and tree-like branching within individual colonies confirmed the pure cultures [23].

The potential lipid-producing pure fungal isolates were maintained at 30 °C on CYA slants and subcultured once every month.

Identification of Fungi

Fungal DNAs were extracted from isolated pure cultures by using Biopure kits (Bio Axis DNA Research Centre). After extraction of total DNA, polymerized chain reaction (PCR) amplification of the ribosomal DNA in internal transcribed spacer (ITS) region was performed by using a pair of primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4

(TCC TCC GCT TAT TGA TAT GC). The amplification conditions were as the following: one cycle represents initial amplification at 94 °C for 5 min followed by 35 cycles of denaturation (94 °C for 30 s), annealing (53 °C for 1 min), and extension (72 °C for 1 min), and finally, the process ends by final extension at 72 °C for 7 min. The amplified (PCR) product was subjected to electrophoresis using agarose gel 1% in 1 × Tris–acetate-EDTA (TAE) buffer and visualized by staining with ethidium bromide. The PCR product was purified by washing with sodium acetate and 70% of ethanol and eluted from gel. The forward and reverse sequencing reactions of PCR amplicon were carried out on ABI 3730XL sequencer to obtain the sequence. The assembled DNA sequence was used to carry out the basic local alignment search tool (BLAST) with the database of the National Center for Biotechnology Information (NCBI).

Preparation of Oil Seed Meals

Twenty-five grams each of the flaxseed and mustard seeds was dried to reduce the moisture content. Dried seeds were crushed in a grinder and the powders were put into individual thimbles and subjected to oil extraction separately by Soxhlet by using hexane. After 7 cycles, oil was removed from the seeds and the thimbles were taken out to obtain the seed meals.

Oil extraction from the rice bran was done in the similar process by Soxhlet as described above. After completion of the oil extraction process, all the thimbles were subjected to solvent evaporation individually, in a hot air oven (Labin, Dombivli, Maharashtra, India). The dried defatted flaxseed, mustard, and rice bran meals were taken out of the individual thimble and kept in different air tight containers labeled as flaxseed meal (FSM), mustard meal (MM), and rice bran meal (RBM) respectively. The meals were stored at 4 °C in refrigerator for further use.

Proximate Composition of the Meals

Proximate composition of the defatted flaxseed, mustard, and rice bran meals, i.e., moisture, carbohydrate, protein, and ash and fiber contents, was determined by standard AOAC methods [24].

Fungal Inoculums Preparation

A part of each isolated pure culture was individually transferred into Czapek-Dox media by aseptically cutting 5 mm each of the CYA plate culture. Flask cultures were then prepared in 250-ml Erlenmeyer flasks, each containing 50 ml sterilized Czapek-Dox medium. The flasks were then inoculated with 10^7 spores per milliliter of individual fungal cultures and incubated at 30 °C for 72 h to develop the inoculums for fermentation.

Preparation of Fermentation Media and Submerged Fermentation

The submerged fermentation by individual isolated lipid-producing fungi was carried out separately in 250-ml Erlenmeyer flasks, for oil production by each fungal culture, at static condition. Each fermentation media contained 100 ml potato dextrose broth (PDB) composed of potato extract (200 g/l) and glucose (20 g/l), designated as control. Another set of

fermentation was carried out by adding 1 g each of FSM, MM, and RBM separately into the individual control media. All the flasks containing media were sterilized at 121 °C for 15 min and inoculated with 10% (v/v) of the isolated pure culture inoculums separately. After inoculation, the flasks were incubated at 30 °C for 7 days. All experiments were run in triplicate.

Fungal Dry Mass Determination

The biomasses from each fermentation flask were harvested by filtering and washed twice with distilled water. The harvested biomasses were then freeze dried (Eyela FDU-1110, Japan) to constant mass and weighted dry cell weight.

The residual culture filtrate was centrifuged (R-24 Research centrifuge REMI, Mumbai, Maharashtra) at 10,000 RPM for 5 min, to obtain cell free culture medium. The culture filtrate obtained from each fungal fermentation flask was concentrated by lyophilization (Eyela FDU-1110, Japan). The concentrated culture media were stored at − 30 °C for determination of extracellular enzyme activity.

Extraction of Lipid Compounds from Biomasses

The dried fungal biomasses, filtered from each fermentation broth, were subjected to lipid extraction according to the method described by Bligh and Dyer, with methanol, water, and chloroform in a ratio of 2:1:1 [25]. The lower chloroform layer containing lipid was collected individually and lipid contents of different fungal biomasses were measured by evaporating the solvent.

The lipid content in biomass, i.e., SCO productivity ($Y_{L/X}$), was calculated using Eq. 1.

$$Y_{L/X} = \frac{L_{max}}{X} \tag{1}$$

where L_{max} is the maximum lipid yield, g/l, and X is the biomass yield corresponding to the maximum lipid yield, g/l.

Collection of Seed Meals After Fermentation

After fermentation was completed, each culture medium was centrifuged (R-24 Research centrifuge REMI, Mumbai, Maharashtra) separately for 10 min at 5000 rpm and then freeze dried (Eyela FDU-1110, Japan) to concentrate. The pellet was then collected and washed twice with distilled water to obtain culture-free seed meals. The fermented flaxseed meal (FFSM), mustard meal (FMM), and rice bran meal (FRBM) were then freeze dried to constant mass. All the dried meals and the freeze-dried culture filtrates were stored separately in air tight containers at − 30 °C with proper labeling for further analysis.

Determination of Enzyme Activity of the Fungal Isolates

Determination of Amylase Activity of Fungi

Activity of the extracellular amylase enzyme was measured by taking 0.05 ml concentrated culture filtrate of individual fungus in separate tubes and mixing 0.05 ml of 1% starch

solution, dissolved in 0.1 (M) sodium phosphate buffer (pH 7.4), to each tube. All the test tubes were incubated at 37 °C for 10 min. Then 3 ml of DNS solution was added to each tube to stop the reaction and the mixtures were heated in a boiling water bath until color change occurred. The color intensity was read after cooling the tubes, at the wavelength of 540 nm using the spectrophotometer (Jasco V-630 UV–VIS Spectrophotometer, Meryland, USA) [26]. One tube without any culture filtrate was used for the measurement of blank. One unit of amylase activity (IU/ml) was defined as the number of milligrams of glucose liberated by 1 ml of enzyme solution per minute using glucose as standard.

Determination of Cellulase Activity of Fungi

Enzymatic activity of each fungus to hydrolyze cellulose was determined by analyzing the individual freeze-dried culture media filtrates according to the method described by Ghosh, 1987 [27]. The activity was measured by mixing 0.1 ml each of the freeze-dried culture media filtrate with 0.1 ml of 1% carboxy methyl cellulose (CMC) solution, dissolved in 0.1 (M) citrate buffer (pH 5.5), in individual tubes, followed by incubation at 50 °C for 30 min. The reaction was stopped by adding 3 ml of DNS solution to all the tubes and the mixtures were heated in a boiling water bath until the color changed. The color intensity was read after cooling the tubes, at the wavelength of 540 nm using the spectrophotometer (Jasco V-630 UV–VIS Spectrophotometer, Meryland, USA) [21]. One tube without any culture filtrate was used for the measurement of blank. One unit of cellulase activity (IU/ml) was defined as the amount of enzyme which releases 1 μmol of reducing sugars per minute with glucose as standard by 1 ml of enzyme solution per minute.

Determination of the Effect of Fermentation on the Nutrient Composition of the Meals

The effect of submerged fermentation on the nutrient composition of FSM, MM, and RBM was determined individually by measuring soluble carbohydrate, total protein, and ash content of the fermented meals. Carbohydrate, protein, and ash contents of fermented meals were determined by standard AOAC methods [24], to compare with the contents of raw meals.

Determination of the Effect of Fermentation on the Antioxidant Activity of the Meals

Preparation of Extract of Metabolites from the Raw and Fermented Meals

The effect of fermentation on antioxidant activity was determined by extract in the secondary metabolites from all the raw and fermented freeze-dried meals separately. The extraction of the antioxidant compounds was done by mixing 1 g each of the raw meals individually with hexane (1:10) by shaking overnight (18–24 h) at room temperature. The extracts were then filtered separately through Whatman paper No. 1 by vacuum filtration. The process was repeated three times. The combined hexane filtrates were collected and concentrated under reduced pressure. Solvent was evaporated and the residues were dried in rotary vacuum evaporator to yield the crude metabolites from the raw meals.

Similar procedure was followed for the extraction of secondary metabolites from the fermented seed meals.

Quantitative Analysis of Antioxidants Present in the Extracts of Raw and Fermented Meals

Determination of Total Phenolic Content (TPC) of Raw and Fermented Meal Extracts TPC of all the raw and fermented seed meal extracts were estimated individually using Folin–Ciocalteau reagent–based assay using gallic acid as standard described by Singleton et al. with some modification [28]. Of each raw meal extract, 0.2 ml was taken in separate test tubes; with the extracts, 0.8 ml of solvent (hexane), 2.5 ml of (11%) Folin–Ciocalteau reagent, and 2 ml of (7.5%) Na_2CO_3 were added to each test tube. The mixtures were incubated at room temperature for 30 min in the dark and the absorbance of the color developed in each tube was recorded at 765 nm using UV–vis spectrophotometer (Jasco V 630, UV–VIS Spectrophotometer, Meryland, USA). Determination of TPC of the extracts of fermented meals was done using the similar method described above. The same procedure was repeated to 1 ml aliquots of 20, 40, 60, 80, and 100 μg/ml methanolic-gallic acid solutions used as standard for calibration curve. Total phenolic content was obtained from the regression equation $y = 1.16x - 0.102$ with $R^2 = 0.9998$ and expressed as mg/g gallic acid equivalent using the formula $C = cV/M$, where C is the total content of phenolic compounds in mg GAE/g; c is the concentration of gallic acid established from the calibration curve; V is the volume of seed meal extracts; and M is the weight of seed meal extract taken.

Determination of Total Flavonoid Content (TFC) of Raw and Fermented Seed Meal Extracts The total flavonoid contents of the seed meal extracts, before and after fermentation, were measured according to the method described by Jagadish et al., with slight modification [29]. One hundred microliters of each raw meal extract was taken in separate test tubes and the volume made up to 1 ml with solvent (hexane). To each test tube, 5 ml of distilled water and 0.3 ml of 5% $NaNO_2$ solution were added and incubated for 5 min. After that, 0.6 ml of 10% $AlCl_3$ solution was added to each tube and the mixtures were allowed to stand for 6 min. Then, 1 ml of 1 (N) sodium hydroxide solution was added to all the mixtures individually and the final volume of the mixtures was made up to 10 ml with distilled water. Another set of reaction mixtures was prepared similarly, for the determination of TFC of the fermented meal extracts. All the solutions were mixed well and the absorbance of each tube was measured one by one at 510 nm against blank. One milliliter of aliquots of 20, 40, 60, 80, and 100 μg/ml catechin solutions was used as standard for calibration curve. Total flavonoid content was obtained from the regression equation $y = 0.0955x + 0.001$ with $R^2 = 0.9939$ and expressed as milligrams of catechin equivalents per gram of sample (mg/g) using the formula $C = cV/M$, where C is the total content of flavonoid compounds in mg/g catechin; c is the concentration of catechin established from the calibration curve; V is the volume of seed meal extracts; and M is the weight of seed meal extract taken.

Antioxidant Activity Assays of the Raw and Fermented Meal Extracts

Three different activity assays were done for analyzing the antioxidant activity of the raw and fermented seed meal extracts individually. Antioxidant activities were assayed by using free radical scavenging DPPH assay, ABTS assay, and FRAP assay. Scavenging

activities were expressed in terms of IC_{50} value (μg/ml), that is, the concentration of the extracts required for 50% inhibition. All experiments were done in triplicate and mean values were taken.

1,1-Diphenyl-2-picrylhydrazyl Assay of Raw and Fermented Meal Extracts DPPH is a stable, nitrogen-centered free radical which produces violet color in methanol solution. When a substrate that can donate a hydrogen atom added in DPPH solution, it reduced the color of diphenylpicryl hydrazine [30]. The free radical scavenging activity of each sample was determined using the DPPH method according to Adhikari et al. with little modification [31]. DPPH solution (0.05 g/l) was prepared in 95% methanol. Each raw meal extract (0.2 ml) was diluted separately with solvent (hexane) to make the total volume of 3 ml. One milliliter of DPPH solution was added in all the test samples separately and incubated for 30 min at room temperature in darkness. Similar procedure was followed for the assessment of DPPH activity using fermented meal extracts. One test tube was run as control (without the sample), which contained only the solvent mixed with DPPH solution. After 30 min, the absorbance was measured at 517 nm. Free radical scavenging activity was expressed as a percentage.

The percentage of the DPPH radical scavenging was measured by comparing with the scavenging activity of ascorbic acid and calculated as Eq. 2.

$$\text{Inhibition of DPPH radical (\%)} = \frac{(\text{Control absorbance} - \text{sample absorbance})}{(\text{Control absorbance})} \times 100 \tag{2}$$

2,20-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid Assay of Raw and Fermented Meal Extracts Radical scavenging activity of all the raw and fermented seed meal extracts was determined according to the method described by Miller et al. [32]. ABTS solution was freshly prepared by dissolving 2,20-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid in water to make 7 mM concentration stock solution. ABTS radical cations ($ABTS^{+}$) were produced by mixing 2.45 mM potassium persulfate with ABTS stock solution in 1:1 ratio (v/v) and left in the dark at room temperature for 12–16 h before use. $ABTS^{+}$ solution was diluted in 95% ethanol to obtain absorbance between 0.7 ± 0.02 at 734 nm. The spectrophotometric assay was conducted by mixing 190 μl of the ABTS reagent with individual 10 μl of the raw and fermented meal extracts and incubated for 15 min at room temperature in the dark. In reaction with antioxidants, the $ABTS^{+}$ reduced its color and absorbance of the solution. The lower the absorbance, the stronger the free radical scavenging ability of the sample [33].

The radical scavenging activity of the extracts was measured by comparing with the scavenging activity of α-tocopherol and calculated using the following Eq. 3.

$$\text{ABTS} = 1 - \frac{(A_c - A_s)}{A_c} \times 100 \tag{3}$$

where A_c represents the absorbance of the control and A_s represents the absorbance of extracts.

Ferric Reducing Antioxidant Power Assay of Raw and Fermented Meal Extracts FRAP assay of all the raw and fermented seed meal extracts was carried out separately according to Othman et al. [34], by monitoring the reduction of $Fe3^{+}$ of tripyridyltriazine (TPTZ) to blue colored $Fe2^{+}$ of TPTZ. The FRAP reagent was prepared by mixing three solutions: 300 mM acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM $FeCl_3$, $6H_2O$ in a ratio of 10:1:1. The reaction mixtures contained 1 ml each of raw meal extracts diluted with hexane and 3 ml of FRAP reagent. FRAP values of each extract were measured separately at 0 min and 4 min of interval and the color reduction was read at 593 nm. FRAP value of the extracts of the fermented meals was measured in the similar way described above. Antioxidant potential of the sample was measured by comparing with the activity of ascorbic acid (AA) standard. Ferric reducing power was evaluated as µM of Fe (II) released per gram of dry weight. Results were expressed as µmol AAE/ml.

FRAP value was calculated by using the following Eq. 4.

$$\text{FRAP} = \frac{\text{Sample } (t_0 - t_4)}{\text{Standard } (t_0 - t_4)} \times \text{FRAP value } \textit{of} \text{ ascorbic acid (1000 uM)} \tag{4}$$

where t_0 is the absorbance at 0 min and t_4 is the absorbance at 4 min of interval.

Statistical Analysis

All experiments were conducted in triplicates. Results were reported as their mean values ± standard deviation. Determination of the significance was carried out by analyzing one-way analysis of variance (ANOVA) of the data using Origin 2018 software. Tukey's honest significant difference (HSD) test was done to determine the *p* value. $p \leq 0.05$ was considered statistically significant.

Results and Discussion

Screening of Lipid Containing Fungi

Twelve fungal strains were isolated from the soil collected from rice bran oil industry, from which three fungal strains were screened as oleaginous by Sudan black B staining technique. Though this staining technique could not give precise insight into cellular lipid content, as it stained only the lipidic matter surrounding the cytoplasmic membrane, but the presence of blue black colored fat globules within the cell of three fungi denoted that the isolated fungal strains were oleaginous in nature [8]. These three strains were designated as fungus 1, 2, and 3.

Isolation and Identification of the Fungal Strains

For the identification of three oleaginous fungal strains, 18S rDNA sequence plays a vital role to determine the phylogenies of fungal species. The comparison of the 18S rDNA gene sequences allows differentiation between organisms at the genus level. The gel picture of the amplicons of the identified fungal species is depicted in Fig. 1.

The assembled DNA sequence of fungus 1 was as follows:

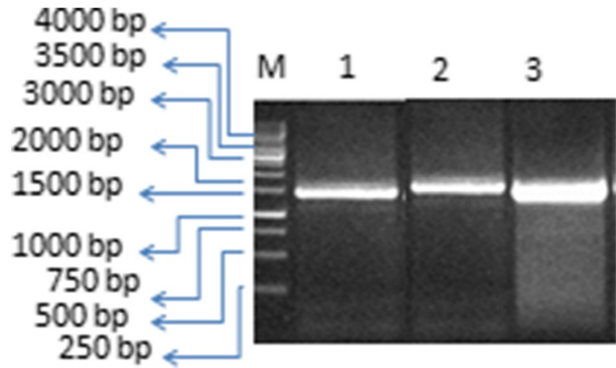

Fig. 1 The gel picture of the amplicons of the isolated fungal cultures, where M, 1 kb ladder of the marker; lane 1, PCR product of fungus 1; lane 2, PCR product of fungus 2; and lane 3, PCR product of fungus 3

CAGATAGATGAAAAGTATCGCATATTCAAGAGCGGGCAAACTGTTGTTGAC CTTGTATGACAGCCGCAACCTTTGATATCCCATAGCCATATTTGTTAACAGTAC AATCACACCTTATAGGATATGCCCCTGGATCCTGGTCTCAAGTACGTCAAGTA TTAAGTAAATATTATATCCCAACTGACTCTATGCTACTAGGTAGCAGTAGCTCG AACGCAACCCCATGGCCGGGTTTTGGGCGTCGACATCATCCCAGCGCAGCCTCC CAAAGGGGTATCACAATTCAAGGGAATTTTCTGGACCCTCATGTCCAGTCTTAT GTGCGTGAATTTGTGCGTGACAGCAATAGAGGCCGGCCGCGTCCACCAGAA TCAACGTCTAATATCGAACACAATTCGAGGATGCTTGAGTCTGTGAACGCCAGC TCAGAAAATGCAATAGTAGATGTGGTTCTAAGTGATATGTCTGCTCCCTGGGAT CAAACCACTGGATTTGGGAAGAGAAGTCTAAGTGATCCCTATAACAGGATGGTG AACACCAGCGGCATTAGTTTCAGAGATCATGCAGGTAGTATGGTATGTCAACCA GATCAGCCGGGAAACTATTGCGCTGAACCATATTACCAGGATCTTTGCCGCGCA GCATTGAGCTTCAGTCAAGAAGTTCTCCGGACCGGTGGCCATTTCGTCTGCAAA TTCTA.

The assembled DNA sequence of fungus 2 was as follows:

GGGCTGCGGAAGGATCATTACCGAGTGCGGGTCCTTTGGGCCCAACCTCCC ATCCGTGTCTATTATACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCC GGGGGGGCGCCTTTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACG AACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACT TTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGA TAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATT GCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCA AGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCC GAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTC ACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTTTTTCC AGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCATAA.

The assembled DNA sequence of fungus 3 was as follows:

AGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTCT ATCGTACCTTGTTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAG GCCTTGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGT TCTGAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCAACAA CGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT GTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCC TGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGC

TTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCA
GCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGT
AGGCCCGGCCGGCGCCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCG
GATCAGGTAGGGATACCCGC.

The molecular analysis of fungus 1 shows 100% identity to *Aspergillus heteromorphus CBS 117.55*, fungus 2 shows 100% identity to *Aspergillus niger st YLAC-69*, and fungus 3 shows 100% identity to *Aspergillus fumigatus st SZJO1* (Table 1).

Proximate Composition of Defatted Oil Seed Meals

Total carbohydrate, crude protein, moisture content, and ash and fiber content were measured after total extraction of oil from flaxseed, black mustard seed, and rice bran. Percentages of these compositions are shown in Table 2.

From the proximate analysis experiment, it was observed that defatted flaxseed meal contained 29.78% carbohydrate and 28.27% protein along with 6.4% fiber and 3.2% ash content, whereas another study reported higher amount of ash (5.12%) and carbohydrate (40.4%), when they examined the proximate composition of cold-pressed flaxseed cake used as a co-substrate of fermentation for nutritional enhancement of tempeh [35].

It was observed that de-oiled mustard meal contained high protein, carbohydrate, and fiber, but much higher protein (41.03%), carbohydrate (32.73%), and ash (10.23%) contents with similar moisture (8.07%) and fiber (10.63%) contents were found in a study [36].

The nutrient content of de-oiled rice bran meal composed of very high amount of carbohydrate (47.1%) and fiber (13.2%). Higher fiber content (34.9%) along with lower protein (15.6%) and ash (9.86%) content was observed in a study, when they used rice bran for the formulation of cookies. Recently, reported fiber and ash contents of defatted rice bran coincide with our data, which were 13.1% and 11.6% respectively along with higher carbohydrate (61.46%) and lower protein content (13.8%) [37].

Biomasses and Lipid Production by the Fungi Using Defatted Seed Meals

Biomasses and lipid contents of the individual fungal isolates were measured from the fungal mycelium grown individually on control media and fermentation media containing 1% each of FSM, MM, and RBM. The results are shown in Table 3.

From Table 3, we observed that all the fungal cultures were able to produce high amount of intracellular lipid. *A. niger* produced highest amount of biomass 7.98 g/l and 23.29% lipid, followed by *A. fumigatus* and *A. heteromorphus*. As all the isolated strains produced more than 20% lipid in their biomasses, they all designated oleaginous. In a similar study, 50 fungal isolates were screened from soil and only 30 oleaginous fungi were able to produce 20–44% lipid by converting glucose [38].

All the oleaginous fungal strains utilize meals and oil production was increased while using these oil seed meals as supplements. Fermentation media containing FSM helped the fungal strains to produce highest amount of oil. Maximum 26.07% increase in oil production using FSM was observed by *A. niger*. Biomass production was also increased from 7.98 (in the control media) to 11.80 g/l by using FSM.

A. fumigatus produced highest amount (31.30%) of lipid in the MM containing fermentation media. Biomass production was increased from 7.30 to 8.95 g/l in the MM containing media.

Table 1 BLAST reports of the fungi

Description	Maximum score	Total score	Percent query coverage	E value	Percent identity
Aspergillus heteromorphus CBS 117.55 23S ribosomal RNA methyl transferase (BO70DRAFT 346,317), partial mRNA	701	994	76%	0.0	100%
Aspergillus niger st YLAC-69 small subunit ribosomal RNA gene, partial sequence, internal transcribed spacer 1	1072	1072	100%	0.0	100%
Aspergillus fumigatus st SZJO1 small subunit ribosomal RNA gene, partial sequence, internal transcribed spacer 1	1013	1013	100%	0.0	100%

Table 2 Proximate composition of defatted meals

Seed meal	Carbohydrate (%)	Protein (%)	Moisture (%)	Ash (%)	Fiber (%)
FSM	29.78 ± 0.3	28.27 ± 0.7	12.70 ± 0.6	3.2 ± 0.1	6.4 ± 0.2
MM	18.39 ± 0.6[a]	31.46 ± 0.9[a]	7.2 ± 0.1[a]	8.6 ± 0.4[a]	9.8 ± 0.3[a]
RBM	47.1 ± 1.1[ab]	17.8 ± 0.2[ab]	8.3 ± 0.1[ab]	11.7 ± 0.6[ab]	13.2 ± 0.7[ab]

Data expressed as mean±SD (n=3). For comparison between samples, one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was performed. $p<0.05$ was considered significant. [a]$p<0.05$ compared to FSM..[b]$p<0.05$ compared to MM

Table 3 Biomasses and lipid production by the fungi using meals

Seed meals	Fungus	Dry wt. of the biomass (g/l)	Oil yield (g/l)	Lipid content (%)
Control	*A. niger*	7.98 ± 0.3	1.91 ± 0.1	23.93 ± 0.5
	A. heteromorphus	6.49 ± 0.2	1.36 ± 0.1	20.98 ± 0.2
	A. fumigatus	7.30 ± 0.2	1.67 ± 0.2	23.01 ± 0.7
FSM	*A. niger*	11.80 ± 0.9 [a]	5.90 ± 0.3[a]	50.0 ± 0.6[a]
	A. heteromorphus	8.40 ± 0.5 [a]	2.10 ± 0.1[a]	25.0 ± 0.1[a]
	A. fumigatus	9.27 ± 0.8 [a]	3.05 ± 0.1[a]	32.9 ± 0.3[a]
MM	*A. niger*	5.23 ± 0.4 [ab]	1.56 ± 0.1 [b]	29.8 ± 0.1[ab]
	A. heteromorphus	8.21 ± 0.4 [a]	2.47 ± 0.1[ab]	30.1 ± 0.2[ab]
	A. fumigatus	8.95 ± 0.1 [ab]	2.80 ± 0.1[ab]	31.3 ± 0.4[ab]
RBM	*A. niger*	11.90 ± 0.8[abc]	4.99 ± 0.3[abc]	41.9 ± 0.2[abc]
	A. heteromorphus	11.21 ± 0.4[abc]	3.87 ± 0.1[abc]	34.6 ± 0.5[abc]
	A. fumigatus	10.41 ± 0.9[abc]	4.56 ± 0.2[abc]	43.9 ± 0.9[abc]

Data expressed as mean±SD (n=3). For comparison between samples, one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was performed. $p<0.05$ was considered significant. [a]$p<0.05$ compared to control. [b]$p<0.05$ compared to FSM..[c]$p<0.05$ compared to MM

Highest biomass production was found in the RBM containing media. *A. niger* was observed to produce maximum 11.90 g/l biomass with 4.99 g/l oil yield, in this media, whereas highest 43.9% lipid content was observed in *A. fumigatus* in the medium containing RBM.

Overall, 25–50% SCO productivity was found among all kinds of meals. The oil industry byproducts were proved to be suitable media for single cell oil production by the fungi. In another study, Khot et al. evaluated the microbial conversion of coconut oil waste into SCO, where copra cake was used as a low-cost renewable substrate for the growth of an oleaginous fungus, *Aspergillus terreus IBB M1*, resulted in maximum biomass production of 8.2 g/l with 0.25 g/l oil production [9].

Effect of Fungal Fermentation on the Nutrient Composition of the Meals

Defatted oil seed meals used in the fermentation media for production of SCO showed significant changes in their nutrient compositions itself, after the completion of fermentation process. The results of nutritive values such as carbohydrate, protein, and ash contents

Table 4 Effect of fermentation on nutrient composition of meals

Seed meal	Fungus	Soluble carbohydrate (%)	Total protein (%)	Ash (%)
FFSM	*A. niger*	21.28 ± 0.3	37.11 ± 0.5	6.1 ± 0.9
	A. heteromorphus	24.73 ± 0.9	34.27 ± 0.1	5.8 ± 0.1
	A. fumigatus	24.11 ± 0.4	32.65 ± 0.3	4.23 ± 0.3
FMM	*A. niger*	12.29 ± 0.1 [a]	37.40 ± 0.2	12.10 ± 0.1 [a]
	A. heteromorphus	14.62 ± 0.7 [a]	35.20 ± 0.6	10.3 ± 0.4[a]
	A. fumigatus	11.10 ± 0.9 [a]	34.74 ± 0.2[a]	11.32 ± 0.3 [a]
FRBM	*A. niger*	41.02 ± 0.3 [ab]	26.58 ± 0.1[ab]	15.04 ± 0.1[ab]
	A. heteromorphus	42.9 ± 0.7 [ab]	22.71 ± 0.7[ab]	14.02 ± 0.8[ab]
	A. fumigatus	41.8 ± 0.5 [ab]	25.6 ± 0.7[ab]	14.68 ± 0.4[ab]

Data expressed as mean±SD (n=3). For comparison between samples, one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was performed. p<0.05 was considered significant. [a]p<0.05 compared to FFSM..[b]p<0.05 compared to FMM

of the meals, after fermentation by various strains of *Aspergillus* sp., have been shown in Table 4.

Effect of Fermentation on the Protein Content of the Meals

Protein is the most important nutrient present in the meals. From Table 4, it was observed that fermentation increased total protein content of all the seed meals studied. Highest improvement in the total protein content was observed from 28.27 (in FSM) to 37.11% (in FFSM), fermented with *A. niger*, followed by *A. fumigatus* and *A. heteromorphus*. Similar result was observed in flaxseed cake fermented with *A. niger* and *Candida utilis* for duckling feed. The initial protein content was 37.08% which increased up to 42.94% after fermentation [14].

The protein content of MM after fermentation was ranged from 34.74 to 37.40%, while only 31.46% protein was observed in MM before fermentation. *A. niger* showed highest result followed by *A. heteromorphus* and *A. fumigatus*.

A. niger also increased the amount of protein content in the RBM, from 17.80 present in the meal before fermentation to 26.58% after fermentation. *A. fumigatus* and *A. heteromorphus* also increased the protein content in FRBM.

In another study, a 3-day solid state fermentation using *A. niger* increased crude protein content of guar meal from 39.63 to 41.13% along with very slight increase in total ash content [39]. Similar result was found in another study of solid state fermentation of rapeseed cake with *A. niger*, resulted with increased crude protein content from 31.84 to 39.17% [19]. Another study with flaxseed cake revealed that when the seed meal was fermented with the strains of *Rhizopus oligosporus*, ATCC 64,063, and *Rhizopus oligosporus*, DSM 1964, the protein content increased up to 10% by both strains in the first 4 days of fermentation [2].

Effect of Fermentation on Ash Content of the Meals

From Table 4, we observed that ash content of the meals also showed an ascending trend. FFSM contained 6.10% ash, when fermented with *A. niger*, while only 3.20% ash

content was found in FSM. The increase in ash content of FFSM was also achieved by *A. heteromorphus* and *A. fumigatus*. A study with flaxseed cake provided the same trend of increase in ash content from 6.26 to 7.62%, when fermented with *Rhizopus oligosporus* [2].

Enhancement of ash content was observed highest in the mustard meal. From 8.60% ash content before fermentation to 12.10% after fermentation was reported in the mustard meal, by *A. niger*. The improvement was followed by *A. fumigatus* and *A. heteromorphus*.

A. niger also increased the ash content of RBM, from 11.70 to 15.04%. This effect was also achieved when fermentation was done using *A. fumigatus* and *A. heteromorphus*. Other studied meals also showed improvement in their ash contents by fungal fermentation. The ash content of flaxseed cake was also increased from 6.26 to 7.03% and 7.62% respectively on 6th day of fermentation, when incubated with *Rhizopus oligosporus*, ATCC 64,063, and *Rhizopus oligosporus*, DSM 1964 [2].

Effect of Fermentation on Carbohydrate Content of Meals

It was observed from Table 4 that the carbohydrate content of these fermented seed meals showed an inverse result. Soluble carbohydrate decreased proportionately with fermentation. In the case of FFSM, lowest carbohydrate content (21.28%) was observed when fermented with *A. niger*, followed by *A. heteromorphus* and *A. fumigatus*. Another study of flaxseed oil cake fermented with *Rhizopus oligosporus* DSM 1964 and ATCC 64,063 showed slight increase in the carbohydrate content in different incubation periods [2].

MM was best utilized by *A. fumigatus*. Hence, maximum reduction of carbohydrate from 18.39 to 11.10% was observed in FMM, fermented with this fungal strain. Reduction in carbohydrate content of FMM was also achieved by *A. niger* and *A. heteromorphus*.

Carbohydrate content of RBM was reduced the most by *A. niger*. Maximum 6.08% reduction was observed in FRBM by *A. niger*, followed by *A. fumigatus* and *A. heteromorphus*.

Another study showed similar effect of natural fermentation on carbohydrate content, when a mixture of cereals and legumes (sorghum, amaranth, millet, and pumpkin, in various ratios) is fermented for improvement of nutritional quality. They showed a significant decrease in carbohydrate content in the mixtures after fermentation than the raw mixtures [40].

Each seed meal contains different amounts of nutrients. Microorganisms require sugar for their growth and metabolism which caused a decrease in carbohydrate content. Increase in protein content may be due to conversion of these carbohydrates into smaller sized peptides via fermentation. Enzymes like α-amylase and cellulase which are produced by the fungi helped to utilize the carbohydrate present in the medium and increase the protein content of the seed meals [41]. Fungal fermentation helps to release some complex mineral-degrading enzymes which liberate minerals and might help to increase the ash content [42]. Different amounts of carbohydrate affect the growth cycle of microorganisms differently; hence, the protein content differs for various substrates [40]. *A. niger* showed highest enhancing effect on the nutrient content among the seed meals studied. This data along with many others was found witnessing the enhancing effect of fermentation by *A. niger*, on the nutritional quality of various oil seed meals. But, to the best to our knowledge, there are no previous studies found showing the effect of fermentation by *A. heteromorphus* and *A. fumigatus* on the mustard and rice bran meals.

Enzyme Activity of the Fungal Isolates

Enzymes are needed to hydrolyze the indigestible carbohydrates like starch and cellulose present in the oil seed meals which were used as substrates in the fermentation media. Enzyme activity of each fungal strain is shown in Table 5.

Amylase Activity of the Fungal Strains

Amylase is the one primary enzyme responsible for the hydrolysis of starch present in the medium. It produced the necessary sugar source needed for the growth and metabolism of the microbes. From Table 5, it was observed that all three *Aspergillus* strains produced amylase. In the control media, the highest amylase producing strain was found to be *A. niger* (5.89 IU/ml), followed by *A. heteromorphus* and *A. fumigatus*. These values increased when the fermentation media contained various seed meals as substrate.

In the media containing flaxseed meal, the highest amylase activity was observed by *A. niger*, which showed 9.29 IU/ml amylase activity, followed by *A. fumigatus* and *A. heteromorphus*.

An increase in amylase activity of *A. niger* from 3.31 to 6.23 IU/ml was observed in the media using mustard meal as substrate. Improvement in the enzyme activity was also achieved by *A. heteromorphus* and *A. fumigatus*.

Amylase activity lower than the control media was observed in the media containing RBM. Only 0.98 to 1.29 IU/ml enzyme activity was observed, in which *A. niger* showed highest activity, followed by *A. heteromorphus* and *A. fumigatus*.

In the media containing FSM, *A. niger* showed highest extracellular amylase activity. *A. niger* RN produced 8.90 U/ml in Czapek-Dox broth after 7 days of incubation [43]. In another study, *A. niger* showed highest 0.08 IU/ml, when wheat bran was used as a substrate [44].

Table 5 Enzyme activity of the fungal isolates

Fungal culture	Defatted seed meals	Enzyme activity	
		Amylase (IU/ml)	Cellulase (IU/ml)
A. niger	Control	5.89 ± 0.3	0.29 ± 0
	FSM	9.29 ± 0.4^{a}	0.81 ± 0.03^{a}
	MM	6.23 ± 0.4^{b}	0.33 ± 0^{b}
	RBM	6.29 ± 0.1^{b}	0.37 ± 0.01^{b}
A. heteromorphus	Control	2.96 ± 0.1	0.98 ± 0.02
	FSM	6.34 ± 0.2^{a}	1.41 ± 0.1^{a}
	MM	3.59 ± 0.1^{ab}	1.68 ± 0.1^{a}
	RBM	3.13 ± 0.06^{ab}	2.52 ± 0.2^{bc}
A. fumigatus	Control	3.04 ± 0.1	0.41 ± 0.01
	FSM	7.87 ± 0.6^{a}	0.50 ± 0.01
	MM	3.31 ± 0.1^{b}	0.48 ± 0
	RBM	3.98 ± 0.02^{ab}	0.47 ± 0.01

Data expressed as mean±SD (n=3). For comparison between samples, one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was performed. p<0.05 was considered significant. [a]p<0.05 compared to control. [b]p<0.05 compared to FSM..[c]p<0.05 compared to MM

Cellulase Enzyme Activity of the Fungal Strains

Cellulase is an enzyme, primarily produced by fungi to act upon cellulase containing substrates and polysaccharides. In the control media, *A. heteromorphus* showed highest activity of cellulase (0.98 IU/ml) followed by *A. fumigatus* and *A. niger*. From Table 5, we observed that the enzyme activity increased with the addition of seed meals in the fermentation media.

FSM containing media showed highest 1.41 IU/ml cellulase activity by *A. heteromorphus*, followed by *A. niger* and *A. fumigatus*.

In the fermentation media containing mustard meal, *A. heteromorphus* showed highest 1.68 IU/ml cellulase activity followed by *A. niger* and *A. fumigatus*.

RBM containing media was the highest cellulose containing media, in which *A. heteromorphus* showed highest cellulase activity (2.52 IU/ml), followed by *A. fumigatus* and *A. niger.*

Among all the strains, *A. heteromorphus* showed highest cellulase activity in the media containing RBM. Another study observed that *A. niger* showed cellulase activity of 0.51 IU/ml in submerged fermentation using rice bran but the enzyme activity increased to 3.46 IU/ml on 4th day of fermentation [21]. In another study, *A. heteromorphus* showed very high cellulase activity (83.00 IU/ml) in the media containing wheat straw [45].

To the best of our knowledge, there are no previous studies that reported the analysis of extracellular enzyme activities of these *Aspergillus* strains using flaxseed, mustard, and rice bran meals.

Effect of Fermentation on Antioxidant Content of the Extracts of Meals

Total Phenolic Content (TPC) of the Phytochemical Extract of Raw and Fermented Meals

TPC value of the seed meal extracts, before and after fungal fermentation, was shown in Table 6.

It was observed from Table 6 that the TPC values of all the seed meal extracts increased after fermentation. *A. niger* showed highest change in FFSM extract. It was increased from 21.26 of FSM to 29.34 mg GAE/g in FFSM, followed by *A. fumigatus* and *A. heteromorphus*.

In FMM extract, highest increase in TPC value was achieved by *A. fumigatus*. The content became 27.37 mg GAE/g after fermentation with the fungus, from 21.72 mg GAE/g of the extract of MM, before fermentation. This value was followed by *A. niger* and *A. heteromorphus*.

TPC value of the extract of RBM was increased mostly when fermented with *A. heteromorphus*, which was observed to be 28.36 mg GAE/g. The increase in TPC value of the extract of FRBM was followed by *A. fumigatus* and *A. niger*.

Highest TPC value was observed in *A. niger*-fermented flaxseed meal. Another study conducted with rice bran fermented with *A. awamori* and *A. oryzae* showed that TPC value was increased by fermentation to 19.77 mg GAE/g and 28.21 mg GAE/g respectively [19]. This study reported first time the TPC value of the extract of flaxseed, mustard, and rice bran meal, fermented with these *Aspergillus* strains. TPC value of the seed extracts was correlated with their antioxidant potential and increases their reducing properties and

Table 6 TPC and TFC values of raw and fermented seed meals

Seed meals	Organism	TPC content of 100 µg/ml seed meal extract (mg GAE/g)	TFC content of 100 µg/ml seed meal extract (mg CE/g)
FSM		21.26 ± 0.8	242.93 ± 0.5
FFSM	*A. niger*	29.43 ± 0.1[abc]	452.35 ± 0.7[abc]
	A. heteromorphus	24.17 ± 0.3[abc]	489.61 ± 0.7[abc]
	A. fumigatus	25.29 ± 0.2 [ab]	461.27 ± 0.8[abc]
MM		21.72 ± 0.9	118.0 ± 0.2
FMM	*A. niger*	26.20 ± 0.7[abc]	515.41 ± 0.9[abc]
	A. heteromorphus	25.94 ± 0.9 [ab]	503.6 ± 0.7[abc]
	A. fumigatus	27.37 ± 0.1[abc]	486.39 ± 0.8[abc]
RBM		25.54 ± 0.3	130.11 ± 0.6
FRBM	*A. niger*	26.41 ± 0.6 [ab]	318.24 ± 0.9[abc]
	A. heteromorphus	28.36 ± 0.6[abc]	344.16 ± 0.3[abc]
	A. fumigatus	28.07 ± 0.2[abc]	351.37 ± 0.2[abc]

Data expressed as mean±SD (n=3). For comparison between samples, one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was performed. $p<0.05$ was considered significant. [a]$p<0.05$ compared to FSM. [b]$p<0.05$ compared to MM..[c]$p<0.05$ compared to RBM

singlet oxygen quenching activity, allowing them to be active as hydrogen donor [46]. The changes that occur in the TPC values may be due to the metabolic process of the fungi during fermentation. Microorganisms which release saccharolytic enzymes like α-amylase and cellulase during fermentation catalyze the substrates and produced phenol which increases the phenolic content [5]. In our study, the strains of *Aspergillus* produced various amounts of amylase and cellulase enzymes in the fermentation media, which might be the reason for increase of this much amount of phenolic content in the seed meals.

Total Flavonoid Content (TFC) of the Phytochemical Extracts of Raw and Fermented Meals

Flavonoid is a group of phytochemical which increases the bioactivity and functional properties of the meals. The effect of fermentation on the TFC values of the meal extracts is shown in Table 6.

Increase in the flavonoid contents of the meal extracts was observed after fermentation. In the extract of FFSM, highest increase in the TFC value from 242.93 to 489.61 mg CE/g was observed when fermented with *A. heteromorphus*, followed by *A. fumigatus* and *A. niger*.

Best improvement in the flavonoid content was observed in the extract of FMM. Fermentation by *A. niger* increased the TFC value from 118.0 to 515.41 mg CE/g. Fermentation by *A. heteromorphus* and *A. fumigatus* also showed improvement in the TFC value.

A. fumigatus showed highest increase in the TFC value of the extract of FRBM from 130.11 to 351.37 mg CE/g, followed by *A. heteromorphus* and *A. niger*.

Maximum increase in the TFC value was observed in FMM, fermented with *A. niger*. This increase in flavonoid contents may also contribute to the enhancement of antioxidant activity of the seed meals after fermentation. Another study with rice bran fermented with *Monascus pilosus* KCCM 60,084 found similar result, where TFC value was increased from 123, found in the raw seed meal, to 518 µg QE/g [46]. This study reported first time

the effect of *Aspergillus* strains on the TFC values of the extract of FFSM, FMM, and FRBM.

Effect of Fermentation on Antioxidant Activity of the Meal Extracts

Effect of fermentation on antioxidant activity of the phytochemical extracts of the meals, before and after fermentation, was measured in terms of IC_{50} values. DPPH, ABTS, and FRAP activities of *Aspergillus*-fermented meal extracts along with the antioxidant activity of the raw meal extracts are shown in Table 7.

DPPH Radical Scavenging Activity of Raw and Fermented Meal Extracts

DPPH activity is used to determine the free radical scavenging activity of a product. Free radicals are harmful substances that are produced during metabolic process of our body, and they produced various degenerative diseases in the body. Substrate with substantial DPPH activity is beneficial for health, as they can prevent chronic diseases that occur due to oxidative stress, such as diabetes, cancer, and cardiovascular diseases. The method involves reduction of methanolic DPPH solution and the scavenging activity is believed to increase with increased concentration. From Table 7, it was observed that *Aspergillus*-fermented seed meal extracts showed better DPPH activity.

FSM extract showed 50% scavenging activity in 243.78 µg/ml concentration, which increased after fungal fermentation for 7 days. Extract of FFSM fermented with *A. heteromorphus* showed 50% scavenging activity at 193.21 µg/ml. Lower IC_{50} value was also observed in the FFSM extract fermented with *A. fumigatus* and *A. heteromorphus.*

Table 7 Effect of fermentation on DPPH, ABTS, and FRAP activity of the meal extracts

Seed meals	Organism	DPPH (IC_{50}) (µg/ml)	ABTS (IC_{50}) (µg/ml)	FRAP (IC_{50}) (µg/ml)
FSM		243.78 ± 0.8	95.65 ± 0.3	159.45 ± 0.9
FFSM	*A. niger*	212.39 ± 0.5	90.12 ± 0.6^{abc}	90.89 ± 0.3^{ac}
	A. heteromorphus	193.21 ± 0.5^{abc}	86.79 ± 0.6^{abc}	84.91 ± 1.2^{abc}
	A. fumigatus	201.20 ± 0.4 ^{ab}	82.31 ± 0.9^{abc}	87.27 ± 0.8^{abc}
MM		57.47 ± 0.7	102.28 ± 0.7	92.30 ± 0.3
FMM	*A. niger*	50.78 ± 0.1^{abc}	97.30 ± 0.3^{abc}	51.47 ± 0.7^{abc}
	A. heteromorphus	47.62 ± 0.1^{abc}	92.19 ± 0.3^{abc}	50.29 ± 0.2^{abc}
	A. fumigatus	48.30 ± 0.3^{abc}	93.47 ± 0.1^{abc}	53.36 ± 0.9ab^{c}
RBM		208.37 ± 0.2	114.28 ± 0.1	145.45 ± 0.4
FRBM	*A. niger*	192.46 ± 0.8^{abc}	98.63 ± 0.8^{abc}	121.79 ± 0.4^{abc}
	A. heteromorphus	180.30 ± 1.1^{abc}	87.21 ± 0.7^{abc}	109.50 ± 0.8^{abc}
	A. fumigatus	187.72 ± 0.9^{abc}	89.16 ± 0.7^{abc}	111.29 ± 0.1^{abc}
Ascorbic acid		20.59 ± 0.03	-	-
α-Tocopherol		-	12.12 ± 0.02	

Data expressed as mean±SD (n=3). For comparison between samples, one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was performed. p<0.05 was considered significant. ^{a}p<0.05 compared to control. ^{b}p<0.05 compared to FSM. ^{c}p<0.05 compared to MM

MM extract showed highest scavenging activity (IC_{50} value, 57.47 μg/ml) of DPPH. This activity was increased (IC_{50} value, 47.62 μg/ml) in the FMM extract, fermented with *A. heteromorphus*, followed by *A. fumigatus* and *A. niger*.

RBM extract showed IC_{50} value of 208.3 μg/ml. Increase in scavenging activity was observed in FRBM extract fermented with *A. heteromorphus* (IC_{50} value, 180.30 μg/ml), followed by *A. fumigatus* and *A. niger*.

Highest DPPH scavenging activity was found in mustard meal extract, after fermentation with *A. heteromorphus*. In a study conducted by Monica et al., it was reported that fermented flax exhibited more free radical scavenging activity of 65.25%, than raw flaxseed, which showed only 51.35% reduction [16]. The effective concentration value of DPPH activity of methanolic extract of naturally fermented kimchi was ranged from 9.9 to 10.6 mg/ml before fermentation, which became 25.5 mg/ml after 2 months of fermentation [47]. Another study conducted with rice and seim flour, fermented with *A. awamori* and *A. oryzae*, showed an increase in inhibitory percentage of 80.66% and 80.61% respectively from 66.97% found in the unfermented flours [48]. To the best to our knowledge, there was no study reported for the DPPH activity of the studied meals after fermentation with the isolated fungal cultures used in this study.

ABTS Activity of the Raw and Fermented Meal Extracts

$ABTS^{+}$ scavenging activity is also a color reduction assay like DPPH, which reduces the free radicals and reactive oxygen species produced by oxidative stress. Fermentation increased ABTS activity significantly. From Table 7, we observed that FSM extract showed high ABTS activity (IC_{50} value, 95.65 μg/ml), which increased to IC_{50} value of 82.31 μg/ml in FFSM extract fermented with *A. fumigatus*, followed by *A. heteromorphus* and *A. niger*.

MM extract showed 50% scavenging activity at 100.28 μg/ml concentration. Lower IC_{50} value was observed in FMM extract, fermented with *A. heteromorphus* (IC_{50} value, 92.19 μg/ml), followed by *A. fumigatus* and *A. niger*.

RBM extract contained IC_{50} value of ABTS activity, 114.28 μg/ml. This value was lowered as ABTS activity increased in ERBM extract, fermented with *A. heteromorphus* containing IC_{50} value, 87.21 μg/ml. This value was followed by *A. fumigatus* and *A. niger*.

Highest ABTS activity was observed in flaxseed meal fermented with *A. fumigatus*. Cheng et al. showed 20% increase in ABTS radical scavenging activity in rice bran fermented with *Monascus pilosus* KCCM 60,084 than unfermented rice bran [46]. Fermentation of rice and seim flour by *A. awamori* and *A. oryzae* showed the highest 84.01% and 73.43% scavenging activity of $ABTS^{+}$ ions respectively [48]. To the best of our knowledge, this study reported first time the ABTS activity of FFSM, FMM, and FRBM, fermented with the *Aspergillus* strains isolated in this study.

FRAP Activity of Raw and Fermented Meal Extracts

From Table 7, we observed that FRAP values were also increased in the fermented meal extracts, as a positive effect of fermentation, also, denotes their ability to convert ferric ($Fe3^{+}$) ions into ferrous ($Fe2^{+}$) ions. This electrons donating reaction terminate the free radical chain reactions. FRAP value of FSM was found to be IC_{50} value, 159.45 μg/ml. *A. heteromorphus*-fermented FSM extract showed highest FRAP activity (IC_{50} value, 84.91 μg/ml), followed by the FFSM extract fermented with *A. fumigatus* and *A. niger*.

MM extract showed 50% reduction value of FRAP activity at 92.30 µg/ml concentration. This value was increased to IC_{50} value of 50.29 µg/ml in the FMM extract fermented by *A. heteromorphus*, followed by the extracts of FMM, fermented by *A. niger* and *A. fumigatus*.

RBM extract contained 50% reduction ability of FRAP at 145.45 µg/ml concentration. Higher reduction capacity was observed in the extract of FRBM fermented with *A. heteromorphus*, which showed 50% reduction at 109.50 µg/ml concentration. *A. fumigatus* and *A. niger* also showed high ferric reduction potential.

Highest FRAP value was observed in *A. heteromorphus*-fermented mustard meal extract. Fermentation of flaxseed showed ferric reducing activity increased to 80.1% compared to 66.22% of raw flaxseed [16]. There was no previous literature found reporting the FRAP activity of FSM, MM, and RBM, fermented with the oleaginous *Aspergillus* strains isolated in this study.

Conclusion

From the above study, we can conclude that the isolated fungal strains were oleaginous in nature and they have the ability to produce high amount of oil using oil industry residues as substrate. Effect of fermentation on nutritional value of various agro-industrial waste products like defatted flaxseed, mustard, and rice bran meals, by the oleaginous strains of *Aspergillus* sp., was done for the first time and it showed significant improvement in the nutritional quality of the meals as well as their antioxidant potential. The enzymes produced by the fungal species could be the reason for these effects, as they helped the fungi to utilize the nutrients present in the seed meals and increased their nutritive value. These meals can also be used as poultry feed, as well as low-cost substrate for nutrient dense food production The improved antioxidant content can help to prevent against the dreadful effects of reactive oxygen species and other degenerative diseases like diabetes, cardiovascular disease, aging, and various carcinogenic effects. In the future, after analyzing the amino acid and other properties, protein isolates can be produced and utilized in food fortification.

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Author Contribution Ruma Dutta: investigation, formal analysis, validation, writing original draft.
Saheli Ghosal: formal analysis, validation.
Dipak K. Bhattacharyya: conceptualization, methodology, supervision, writing—review and editing.
Jayati Bhowal: conceptualization, supervision, writing—review and editing.

Data Availability The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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