



Fermented pearl millet: a product with enhanced bioactive compounds and DNA damage protection activity

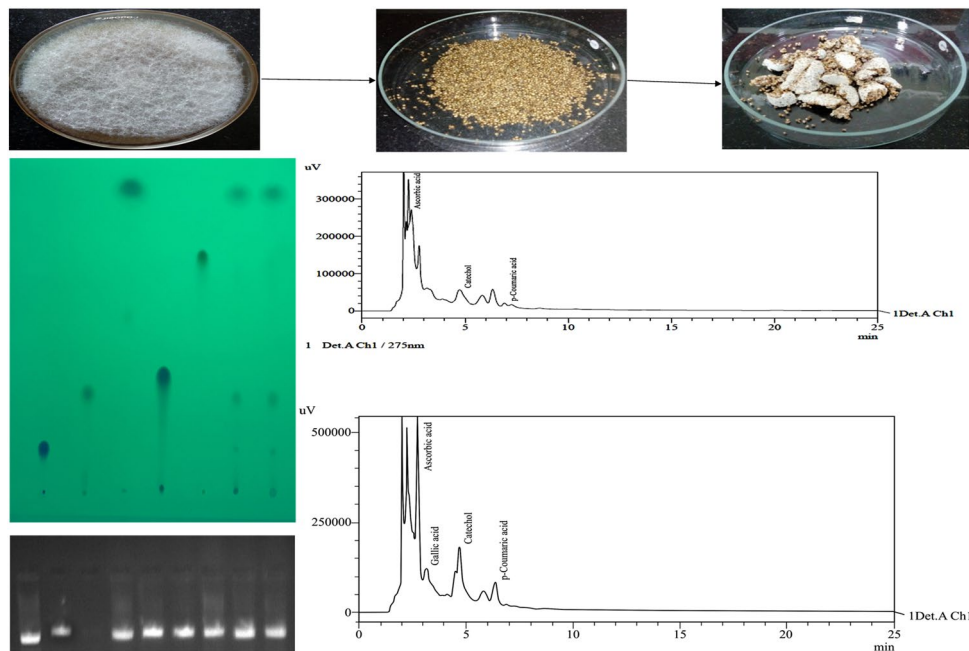
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Abstract

Pearl millet cultivar PUSA-415 was fermented for a period of 10 days using *Rhizopus azygosporus* as a starter culture. Aqueous ethanol (50%) was used as extraction phase to extract bioactive constituents from *Rhizopus azygosporus* fermented millet (RFPM) and unfermented millet (UFPM) flours at 44.5 °C for 23 min. Extracts were evaluated for the presence of bioactive compounds, antioxidant potential, enzymatic activities and DNA damage protection activity (DDPA). Solid state fermentation significantly ($p < 0.05$) enhanced the bioactive constituents with the highest values being observed on 10th day of fermentation. The ranges for different bioactive properties [Total phenolic content (TPC), Condensed tannin content (CTC)] were, TPC [6.6–21.8 mg gallic acid equivalent/g dry weight basis (mg GAE/g dwb), CTC (101.3–176.7 mg catechin equivalent/100 g dwb)] and enzymatic activities [α -amylase (61–201 IU /gds), β -Glucosidase (4–86 IU /gds) and xylanase (7–77 IU /gds)]. Maximum DDPA was observed in RFPM extract (10th day). Thin layer chromatography confirmed the presence of catechin, catechol and ascorbic acid in both UFPM and RFPM. HPLC also confirmed the presence of specific bioactive compounds *viz.* *p*-Coumaric acid, catechol, ascorbic acid and gallic acid in extracts studied.

Graphical Abstract



Keywords Solid state fermentation · Bioactive constituents · Antioxidant potential · DNA damage protection activity · Enzymes

Introduction

Functional food products are in demand due to their bioactive properties which combat oxidative stress (OS) conditions. OS occurs in response to imbalance between the production of free radicals (FR's) and antioxidants. FR's generation can result in occurrence of chronic diseases, early ageing and DNA damage in living organisms [1–3]. In response to reactive oxygen species (ROS) and OS, human body generates/requires different kind of bioactive constituents especially polyphenols and phenolic derivatives. Bioactive constituents are well known for slowing down the damaging effects resulting from OS and FR's [4]. Antioxidant rich fruits [5, 6]; processed food products [7]; fortified food products [8, 9] and fermentation based functional food products [10, 11] can be used to combat FR's formation, generation of ROS and OS related chronic problems [12–14].

Edible natural resources could be enriched with potent antioxidants and health benefiting properties through solid state fermentation (SSF) process. Generally recognized as safe (GRAS) microorganisms especially fungi are used in SSF for the improvement of nutritional profile of edible resources [15–17]. Microorganisms itself [18–20] or in response to specific substrates could results in release of bioactive constituents from their bound form to free form [21]. Fermentation of natural resources (cereal grains, fruits, agricultural waste) can be carried out in two states: (i) SSF (ii) submerged fermentation (SmF) [22]. SSF allows the growth of starter culture on sterilized soft substrates with limited amount of moisture, just enough to support the biochemical reactions. As compared to SmF, SSF has gained more interest from the researchers, as the process is efficient, cost-effective and environment friendly. However, SSF is also a complex practice as it requires the selection of appropriate substrate, starter culture, fermentation conditions and optimization of extraction phases [23–31].

Starter cultures especially fungi's induce remarkable changes in natural resources. Fungal strains could be used for the production of alcoholic beverages, antibiotics, specific enzymes (α -amylase, β -glucosidase, phytase and xylanase), antioxidant rich fermented food products and acids for industrial purposes. Fungal strains belonging to the *Rhizopus* group are widely used on a variety of natural resources. Various researchers have reported SSF using *Rhizopus* spp. on chokeberry pomace [28]; fava bean [32]; rice bran [33–35]; green coffee beans [36]; okara [37]. In our earlier published work Indian pearl millet cultivars

of commercial importance were studied for their bioactive properties. Among cultivars studied the best results in terms of bioactive properties was observed in PUSA-415. SSF using PUSA-415 as substrate and *Aspergillus oryzae* and *Aspergillus sojae* as starter cultures have already been studied by our research group. These two fungi's had different effects on substrate (PUSA-415). *Rhizopus* spp. is widely used as a starter culture for enhancement of bioactive properties of natural resources; however, its action on pearl millet has not been studied. This prompted us to study the effect of fermentation duration of *Rhizopus azygosporus* on bioactive constituents, antioxidant potential (AP) and DNA damage protection activity (DDPA) of pearl millet. Further, thin layer chromatography (TLC) and HPLC analysis were carried out to evaluate the changes in specific bioactive compounds.

Materials and methods

Substrate for SSF

Pearl millet cultivar PUSA 415 (substrate) was selected for SSF. It was procured from Indian Agriculture Research Institute (IARI) PUSA, New Delhi, India. Grains were washed, dried in an oven (NSW-143, Delhi, India) at 45 °C for 48 h.

Starter culture for SSF

Starter culture (*Rhizopus azygosporus* MTCC 10195) for SSF was purchased from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. Starter culture was maintained on potato dextrose broth (PDB) at 25 ± 2 °C. Spores suspension was prepared using sterilized distilled water. An aliquot (1 mL) containing approximately 1×10^5 spores were used for fermenting the sterilized pearl millet grains. Chemicals and solvents used during the present study were of analytical grade.

Solid state fermentation (SSF)

For SSF, dried pearl millet grains (50 g) were soaked in PDB at room temperature and left for 12 h. After soaking, excess PDB was decanted and pearl millet grains were autoclaved (121 °C for 15 min, Yorco, India) and then subsequently cooled. Spores suspension (1 mL) was sprayed on to the surface of sterilized pearl millet grains, mixed properly and incubated in Bio-Oxygen demand (BOD) incubator (NSW-152, Delhi, India) at 25 ± 2 °C for 10 days. The unfermented

pearl millet (UFPM) was prepared without the addition of spore's suspension.

Extraction of enzymes

UFPM and *Rhizopus azygosporus* fermented pearl millet (RFPM) were suspended in an adequate amount of double distilled water (1:10 w/v, Millipore) and agitated at 120 rpm (Orbitek shaker, Scigenics Biotech Private Limited, Chennai, India) for 15 min and filtered through Whatman no. 1 filter paper. The filtrate was then centrifuged at 4200 rpm for 10 min at room temperature. The clear supernatant (UFPM and RFPM) was assessed for the presence of enzymatic activities (α -amylase, β -glucosidase and xylanase). Standards used for the estimation of enzymatic activities were glucose, p-nitrophenol and xylose.

α -Amylase assay

α -amylase activity in extracts (UFPM and RFPM) were assayed by mixing 0.25 mL of appropriately diluted enzyme with 0.2 M acetate buffer (0.5 mL, pH 5.0) and soluble starch (1%, 1.25 mL). After incubation at 50 °C for 10 min, the concentration of glucose liberated from starch by action of α -amylase was determined spectrophotometrically (GENESYS™ 10 S UV-Vis, Thermo Scientific, India) at 575 nm following the method described by Miller [38]. One international unit (IU) of α -amylase activity is defined as the amount of enzyme that releases 1 μ mol of reducing sugar (glucose) per minute under assay conditions and the results were expressed as IU/g dry substrate (IU/gds).

β -Glucosidase assay

β -Glucosidase activity in extracts prepared from unfermented and fermented millet flour were determined using 1.0 mL reaction mixture containing 5 mM p-nitrophenyl- β -D glucoside (PNPG), 100 mM acetate buffer (pH 5.0), and an appropriately diluted enzyme extract [38]. The mixture was incubated at 50 °C for 30 min. The reaction was terminated by adding 1 M Na₂CO₃ (2 mL), and the p-nitrophenol release was estimated spectrophotometrically at 400 nm. One international unit (IU) of β -glucosidase activity was defined as the amount of enzyme that liberates 1 μ mol of p-nitrophenol/minute under assay conditions. The results were expressed as IU/gds.

Xylanase assay

Xylanase activity in extracts (UFPM and RFPM) was determined by mixing 0.025 mL of appropriately diluted enzyme solution with 0.475 mL of 0.2 M acetate buffer (pH 5.0) and

0.5 mL of 1.0% (w/v) xylan. Reaction mixture (RM) was incubated for 10 min at 50 °C [38]. The amount of xylose liberated from xylan by xylanase was evaluated spectrophotometrically at 575 nm. One international unit (IU) of xylanase activity was defined as the amount of enzyme that liberates 1 μ mol of xylose per min under assay conditions and the results were expressed as IU/gds.

Extraction of bioactive compounds

RFPM was taken out from Erlenmeyer flasks at every 48 h intervals and dried in an oven (NSW-143, Delhi, India) at 45 °C for 24 h. Seeds from dried RFPM and UFPM were milled by grinding in a mixer grinder (Sujata, Delhi, India). Defatting of RFPM and UFPM flours was carried out with hexane (1:5 w/v thrice, 120 rpm, 5 min) at ambient temperature on Orbitek shaker (Scigenics Biotech Private Limited, Chennai, India). Extraction of UFPM and RFPM flours were carried out with aqueous ethanol (50%) (1:20 w/v) at 44.5 °C for 23 min [24].

Experimental assays

Total phenolic content (TPC)

Extracts (UFPM and RFPM) were evaluated for the presence of TPC using Folin-Ciocalteu reagent (FCR) method as described by Yu et al. [39]. Extracts (100 μ L) were allowed to react with FCR (500 μ L) followed by vortex mixing and an addition of aqueous sodium carbonate solution (1500 μ L, 20%). RM was vortexed (Vortex mixer CM-101, Remi, Mumbai, India) and left at room temperature for 15 min. After incubation period, final volume (10 mL) was prepared using distilled water. Absorbance of dark blue colored complex was recorded at 765 nm. Gallic acid was used as standard during the assay.

2,2-Diphenyl-1'-picrylhydrazyl radical scavenging assay (DPPH)

The radical scavenging capacity (RSC) of different extracts (UFPM and RFPM) was measured by the DPPH radical scavenging method as described by Yen and Chen [40]. Briefly, 100 μ L of each extracts (UFPM and RFPM) were allowed to react with DPPH solution (3 mL) in storage vials at room temperature under dark conditions. Change in absorbance was recorded at 517 nm after 30 min and RSC was calculated.

RSC was calculated using formula:

$$\text{RSC (\%)} = (A_C - A_E / A_C) \times 100$$

where A_C and A_E are the absorbance of control and extracts, respectively.

ABTS radical cation decolorization assay (ABTS)

RSC in extracts (UFPM and RFPM) was estimated according to method described by Re et al. [41]. ABTS⁺ reagent has prepared 16 h prior to its use for the assay. The reagent was prepared by dissolving ABTS⁺ (17.2 mg) and K₂S₂O₈ (3.3 mg) in double distilled water (5 mL), left for 16 h under dark conditions. Final volume (300 mL) was prepared by dissolving incubated solution in double distilled water (1:60 v/v). Absorbance was recorded at 732 nm after an interval of 10 min against a blank.

RSC was calculated using formula:

$$\text{RSC (\%)} = (A_C - A_E / A_C) \times 100$$

where A_C and A_E are the absorbance of control and extracts, respectively.

Hydroxyl free radical scavenging activity (HFRSA)

AP of extracts (UFPM and RFPM) against hydroxyl radicals was analyzed by following the method described by Smirnoff and Cumbes [42]. UFPM and RFPM extracts (100 μ L) were allowed to react with Smirnoff reagent (3 mL) followed by incubation at 37 °C for 30 min. Extraction phase was used as a negative control to check the AP of extracts (UFPM and RFPM). Absorbance of colored complex was recorded at 562 nm.

$$\text{Scavenged OH}^- \% = [(A_C - A_E) / A_C \times 100]$$

where, A_C is absorbance of control and A_E is absorbance of extract.

Cupric reducing antioxidant capacity (CUPRAC)

CUPRAC was measured by following the method described by Apak et al. [43]. Reagent for the measurement of CUPRAC was prepared by adding ammonium acetate buffer (1 M), cupric chloride (10 mM) and neocuproine (7.5 mM) in ratio (1:1:1). UFPM and RFPM extracts (100 μ L) was added to different vials followed by addition of CUPRAC reagent (3 mL). RM was incubated at room temperature for 30 min. Ascorbic acid was used as standard and the absorbance of colored complex was recorded at 450 nm. CUPRAC value was expressed in mg ascorbic acid equivalent (AAE)/g dry weight basis (dwb).

Total antioxidant capacity (TAC)

TAC of extracts (UFPM and RFPM) was determined by following the method described by Prieto et al. [44] and absorbance was recorded at 695 nm. UFPM and RFPM extracts (100 μ L) were added in to test tubes followed by addition of TAC reagent [conc. sulphuric acid (0.6 M), ammonium molybdate (4 mM) and sodium hydrogen orthophosphate (28 mM) in ratio (1:1:1)] (3 mL) and RM was warmed at 95 °C for 90 min. Ascorbic acid (mg/mL) was used as standard and absorbance was recorded at 695 nm.

Reducing power assay (RPA)

RPA was measured using the method described by Oyaizu [45]. Extracts (100 μ L) from UFPM and RFPM were mixed separately with aqueous potassium ferricyanide solution (100 μ L, 1%) followed by incubation in water bath at 50 °C for 30 min. The resulting mixture was cooled at room temperature followed by addition of trichloroacetic acid (100 μ L, 1%) and aqueous ferric chloride solutions (100 μ L, 0.1%). RM was incubated at ambient temperature for 15 min for the development of specific dark green colored complex. The colored RM was diluted with distilled water to prepare final volume (10 mL). Absorbance of green colored complex was recorded at 700 nm. Quercetin (mg/mL) was used as standard to compare the RPA of extracts (UFPM and RFPM).

Condensed tannins content (CTC)

CTC of extracts (UFPM and RFPM) was estimated by using vanillin-HCl method as reported by Julkunen-Titto [46] with some modifications. Aliquot of extracts (100 μ L) was added separately in each test tube followed by addition of Vanillin (1.5 mL, 4% methanolic solution) and conc. HCl (750 μ L). RM was incubated at room temperature for 15 min for the purpose to develop specific color in RM. Catechin (mg/mL) was used as standard during the assay and results were expressed as mg catechin equivalent/g dry weight basis (mg CE/g dwb). The absorbance against blank was read at 500 nm.

DNA damage protection activity (DDPA)

The ability of extracts (UFPM and RFPM) to protect plasmid DNA from damaging effects of Fenton's reagent was analyzed according to method as described by Kumar et al. [47] with slight modifications [15]. Fenton's reagent for the degradation of plasmid DNA (pBR 322) was prepared using H₂O₂ (30 mM), ascorbic acid (500 μ M) and FeCl₃·6H₂O (800 μ M) in ratio (1:1:1). RM was prepared using 2.5 μ L DNA (0.25 μ g), extract (5.0 μ L), Fenton's reagent (10 μ L) followed by addition of nuclease free double distilled water

(2.5 μL) and incubated at 37 °C for a period of 45 min. After incubation period, 2.5 μL of loading buffer (0.25% bromophenol blue and glycerol 50%) was added to the reaction mixture. Electrophoresis of extracts was done using 1% (w/v) agarose gel prepared in Tris-acetate-EDTA (TAE) buffer. Stock solution of TAE buffer (50 \times) was prepared using Tris base (242 g), acetic acid glacial (57.1 mL) and EDTA (0.5 M). Working solution was prepared using 20 mL of TAE buffer (50 \times) and final volume (1 L) was prepared using double distilled nuclease free water. Aliquot of ethidium bromide (4 μL) was added to agarose gel prepared in TAE buffer to visualize plasmid DNA after electrophoresis. Quercetin (mg/mL prepared in ethanol) was used as a positive control. Specific bands were visualized under transillumination of UV light using a gel documentation system (BIO-RAD, Tokyo, Japan).

Thin layer chromatography (TLC) and HPLC analysis

UFPM and RFPM flours were extracted with ethyl acetate for 45 min in a separating funnel. The extracts were filtered through 0.45 mm Supor1-450 membrane disc filter (Pall Gelman Laboratory, USA) and thin layer chromatography was performed on silica gel plates using chloroform:aqueous ethanol (50%):formic acid (84:15:1; v/v/v) as mobile phase and visualized under shortwave (254 nm). Specific phenolic compounds in UFPM and RFPM extracts were determined (qualitatively and quantitatively) using HPLC method as described by Salar et al. [15].

Statistical analysis

The data generated from UFPM and RFPM analysis were subjected to one-way analysis of variance (ANOVA) using Minitab Statistical Software version 14 (Minitab, Inc, USA). Principle component analysis (PCA) of measured bioactive constituents, AP and enzymatic activities was also carried

out to provide a ready means of visualizing the differences and similarities among these properties.

Results and discussion

TPC and enzymatic activities

Effect of fermentation duration (days) on TPC values of extracts (UFPM and RFPM) are presented in Table 1. Significant differences ($p < 0.05$) were observed in TPC values of RFPM (21.8 mg GAE/g dwb, 10th day) as compared to UFPM (6.6 mg GAE/g dwb). However, no significant ($p < 0.05$) difference was observed among fermented samples (2nd, 4th, 6th and 8th day). Approximately 3.3-folds increase in TPC was observed on 10th day of fermentation (21.8 mg GAE/g dwb) as compared to UFPM (6.6 mg GAE/g dwb). Percent increase in TPC value of RFPM as compared to UFPM was 231%. Similar 3.06-folds (206%) increase in TPC of pearl millet fermented with *Aspergillus oryzae* has been reported in our previous study [10]. As fungal strain (starter culture) used in both the studies were different, this might be the reason for the difference in amount of TPC and enzymes produced during the fermentation process. Dulf et al. [28] reported that fermentation of chokeberry (*Aronia melanocarpa*) pomace with *Rhizopus oligosporus* resulted in enhancement of TPC value by more than 1.8-folds. During the 6th day of fermentation the TPC value in unfermented pomace increased from 9.50 mg GAE/g dw to 17.78 mg GAE/g dw. Bei et al. [31] reported 1.17-folds increases in phenolic compounds during fermentation of oats with *Monascus anka*. The difference in TPC value may be due to nature of starter culture as well as anatomy of substrate selected. Abd Razak et al. [33] reported enhancement of TPC up to eightfolds and twofolds during SSF of rice bran with starter cultures (*R. oligosporus* and *M. purpureus*, respectively).

Table 1 Effect of SSF on enzymatic activities and TPC of UFPM and RFPM

Fermentation duration (days)	TPC (mg GAE/g dwb)	α -Amylase (IU/gds)	β -Glucosidase (IU/gds)	Xylanase (IU/gds)
UFPM (control)	6.58 \pm 0.22 ^a	61 \pm 0.83 ^a	4 \pm 0.60 ^a	7 \pm 0.12 ^a
RFPM (2nd day)	14.82 \pm 0.29 ^b _{↑125}	67 \pm 0.59 ^b _{↑10}	19 \pm 0.31 ^b _{↑375}	28 \pm 0.19 ^b _{↑300}
RFPM (4th day)	15.43 \pm 0.15 ^b _{↑134}	103 \pm 1.10 ^c _{↑69}	26 \pm 0.47 ^c _{↑550}	43 \pm 0.51 ^c _{↑514}
RFPM (6th day)	15.55 \pm 0.23 ^b _{↑136}	117 \pm 0.94 ^d _{↑92}	27 \pm 0.20 ^c _{↑575}	49 \pm 0.24 ^d _{↑600}
RFPM (8th day)	15.81 \pm 0.36 ^b _{↑140}	156 \pm 0.53 ^c _{↑155}	32 \pm 0.71 ^d _{↑700}	54 \pm 0.26 ^c _{↑671}
RFPM (10th day)	21.78 \pm 0.27 ^c _{↑231}	201 \pm 0.87 ^f _{↑229}	86 \pm 0.34 ^e _{↑2050}	77 \pm 0.67 ^f _{↑1000}
Correlations (R^2) between TPC and enzyme activities	–	0.825	0.895	0.912

SSF solid state fermentation, TPC total phenolic content, UFPM unfermented pearl millet, RFPM *Rhizopus* fermented pearl millet

Mean \pm SD values followed by similar superscript in a column do not differ significantly ($p < 0.05$). Subscripts denote the percentage increase (\uparrow) from UFPM for corresponding properties

The production of various enzymes during SSF might be responsible for an increase in TPC as enzymatic action results in structural breakdown of complex bioactive constituents. Highly positive correlations were found for TPC with xylanase ($R^2 = 0.912$ $p < 0.01$), β -glucosidase ($R^2 = 0.895$ $p < 0.05$) and α -amylase ($R^2 = 0.825$ $p < 0.05$). With increase in duration of fermentation (days) the larger quantity of enzymes was produced. α -amylase in RFPM was significantly ($p < 0.05$) higher than UFPM. Both enzymes (β -glucosidase and xylanase) increased with duration of fermentation (days); with 10th day of fermentation resulting in their maximum concentrations (86 IU/gds and 77 IU/gds, respectively).

Bioactive profile of UFPM and RFPM

TLC profiles of extracts prepared from UFPM and RFPM are shown in Fig. 1. TLC analysis was carried out using five standards (ascorbic acid, catechol, catechin, gallic acid and quercetin) and all standards showed specific band lengths. UFPM and RFPM extracts showed the presence of three major compounds *viz.* ascorbic acid, catechin and catechol. Further, HPLC analysis was carried out to check the presence of specific bioactive compounds (qualitative and quantitative) in UFPM and RFPM extracts. The results of qualitative HPLC analysis are shown in Fig. 2a–c. Figure 2a represents the chromatogram of compounds present in UFPM whereas Fig. 2b shows the chromatogram of RFPM (10th day). Figure 2c represents the combined chromatograms of standards, UFPM and RFPM. In Fig. 2c, Data 1 represents standard compounds; Data 2 represents the UFPM whereas Data 3 represents the RFPM chromatogram.

Effect of SSF on antioxidant potential (AP) and CTC

Fermentation using microbial consortia is well known process to induce the biochemical changes within the steam sterilized substrates and these changes ultimately results in increment of antioxidant properties of extracts. Bioactive constituents with antioxidant properties could be measured using various antioxidant assays like DPPH, ABTS⁺, HFRSA, CUPRAC, TAC and RPA [48–50]. The activity of the extracts prepared from UFPM and RFPM was calculated and expressed in terms of % inhibition. AP of UFPM and RFPM extracts are presented in Table 2. SSF significantly ($p < 0.05$) increased the antioxidant activity of extracts against DPPH radical. RSC was found significantly ($p < 0.05$) higher for 8th and 10th day of fermentation. On 8th and 10th day of fermentation, the activities observed were 95.2% and 95.5%, respectively, as compared to UFPM (89.3%). Abd-Razak et al. [33] performed solid state fermentation of rice bran with *Rhizopus oligosporus* for 12 days. They observed the increment in DPPH activity of fermented sample by 91.75% as compared to unfermented substrate, i.e. 87.82%. ABTS⁺ RSC of extracts obtained from UFPM and RFPM is shown in Table 2. Among fermented and unfermented samples, the value of ABTS⁺ ranged from 97.8 to 99.1%, the highest and the lowest being observed for RFPM (10th day) and UFPM, respectively. The higher value of TPC in RFPM as compared to UFPM might be responsible for the AP in the extracts. Our study revealed that an increase in fermentation time up to 10th day resulted in increased AP of the extracts. Dey and Kuhad [13] evaluated the effect of fermentation on wheat and observed fivefold increases in ABTS activity as compared to unfermented wheat. Increase in DPPH activity of rice bran after solid state fermentation was observed by Abd-Razak and co-workers [51]. They

Fig. 1 TLC profile of extracts of UFPM and RFPM (10th day)

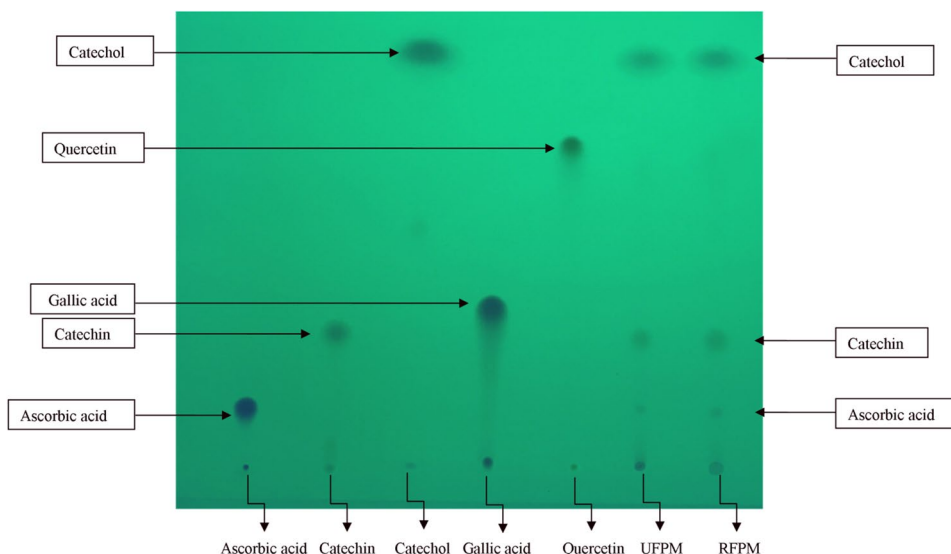
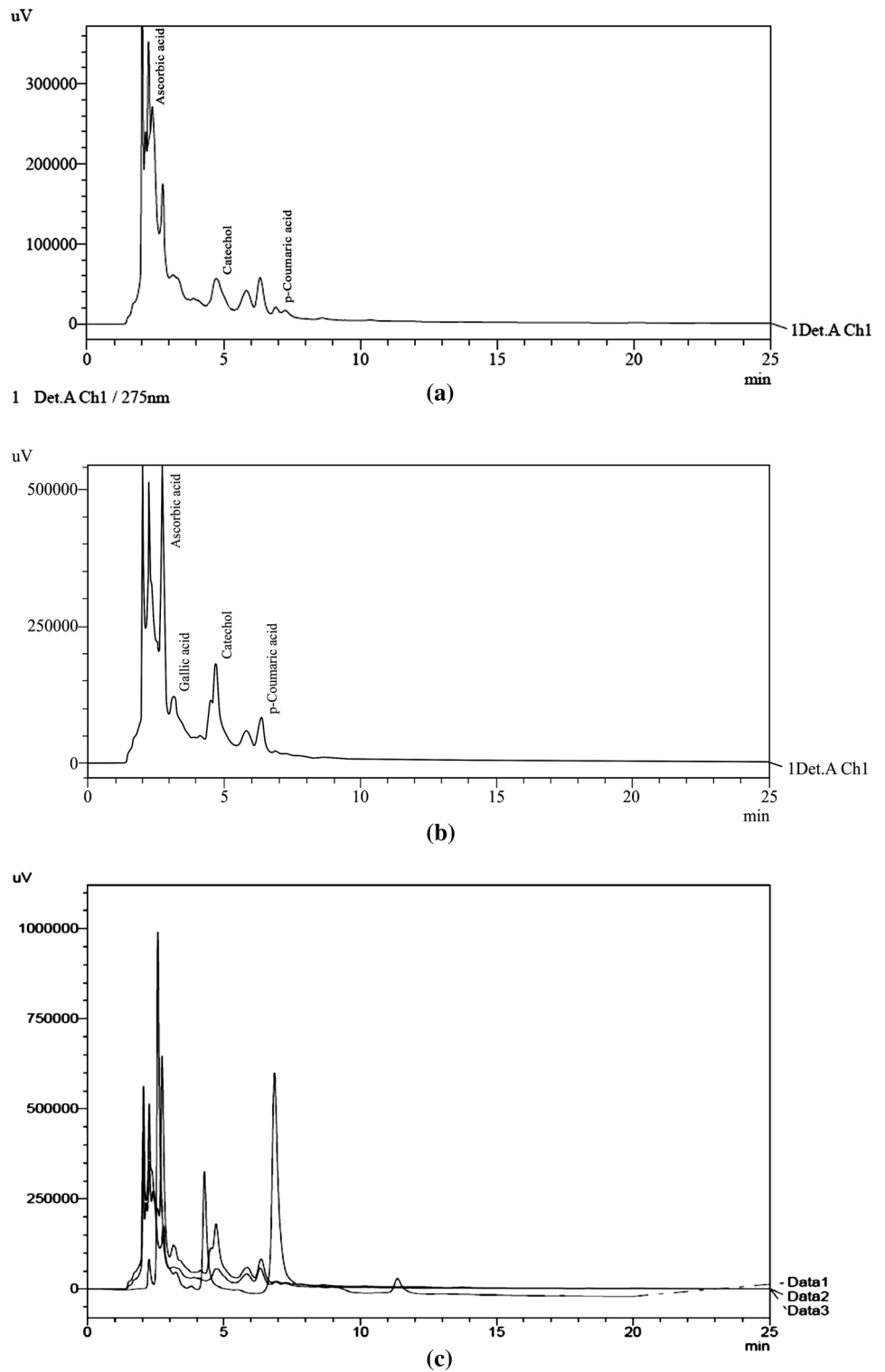


Fig. 2 HPLC chromatograms of UFPM, RFPM and superimposed chromatogram with standard compounds. **a** Chromatogram of UFPM; **b** chromatogram of RFPM and **c** combined chromatograms of UFPM, RFPM along with standards used during HPLC



observed change in DPPH activity of rice bran from 87.82 to 93.41% upon fermentation with *Rhizopus oryzae*. HFRSA observed in UFPM and RFPM ranged from 2.3 to 9.6%. Maximum HFRSA value (%) was observed on 10th day of fermentation (9.6%) whereas minimum was for UFPM

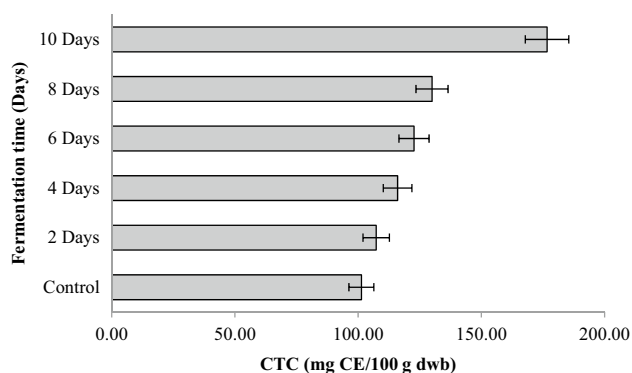
extract (2.3%). CUPRAC value of the extracts (UFPM and RFPM) is presented in Table 2 with its value ranging from 17.4 to 23.8 (mg AAE/g dwb). Salar et al. [15] reported that fermentation of pearl millet with *Aspergillus sojae* results in constant increase in HFRSA value (31.5%) till the 10th

Table 2 Effect of SSF on antioxidant potential of extracts prepared from UFPM and RFPM

Fermentation duration (days)	DPPH (%)	ABTS (%)	HFRSA (%)	CUPRAC (mg AAE/g dwb)	TAC (mg AAE/g dwb)	RPA (mg QE/g dwb)
UFPM (control)	89.3 ± 0.17 ^a	97.8 ± 0.09 ^a	2.3 ± 0.05 ^a	17.4 ± 0.07 ^a	20.1 ± 0.15 ^a	1.9 ± 0.17 ^a
RFPM (2nd day)	94.1 ± 0.26 ^b _{↑5.4}	98.1 ± 0.13 ^a _{↑0.3}	2.9 ± 0.01 ^a _{↑27.4}	17.9 ± 0.11 ^{ab} _{↑2.9}	21.6 ± 0.09 ^b _{↑7.5}	2.0 ± 0.22 ^a _{↑5.3}
RFPM (4th day)	94.5 ± 0.60 ^b _{↑5.8}	98.5 ± 0.07 ^{ab} _{↑0.7}	4.8 ± 0.10 ^b _{↑113.7}	18.2 ± 0.06 ^b _{↑4.6}	22.3 ± 0.12 ^{bc} _{↑10.9}	2.1 ± 0.09 ^a _{↑10.5}
RFPM (6th day)	94.9 ± 0.41 ^b _{↑6.3}	98.8 ± 0.11 ^{ab} _{↑1.0}	5.1 ± 0.18 ^b _{↑125.2}	18.3 ± 0.05 ^b _{↑5.2}	22.6 ± 0.08 ^c _{↑12.4}	2.6 ± 0.08 ^{ab} _{↑36.8}
RFPM (8th day)	95.2 ± 0.29 ^b _{↑6.6}	98.9 ± 0.04 ^b _{↑1.2}	5.2 ± 0.14 ^b _{↑131.9}	20.4 ± 0.14 ^c _{↑17.2}	22.7 ± 0.05 ^c _{↑12.9}	2.7 ± 0.04 ^b _{↑42.1}
RFPM (10th day)	95.5 ± 0.45 ^b _{↑6.9}	99.1 ± 0.05 ^c _{↑1.3}	9.6 ± 0.09 ^c _{↑323.5}	23.8 ± 0.05 ^d _{↑36.8}	29.2 ± 0.16 ^d _{↑45.3}	3.1 ± 0.13 ^c _{↑63.2}

DPPH 2,2-diphenyl-1-picrylhydrazyl, *ABTS* 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, *HFRSA* hydroxyl free radical scavenging activity, *CUPRAC* cupric reducing antioxidant capacity, *TAC* total antioxidant capacity, *RPA* reducing power activity

Mean ± SD values followed by similar superscript in a column do not differ significantly ($p < 0.05$). Subscripts denote the percentage increase (↑) from UFPM for corresponding properties

**Fig. 3** Effect of solid state fermentation on CTC of UFPM and RFPM

of fermentation. CUPRAC value of fermented millet was 35.0 mg AAE/g as compared to unfermented millet, i.e. 30.0 mg AAE/g. Increment in TAC value was 1.64-fold as compared to unfermented millet. TAC of UFPM and RFPM extracts was measured as equivalent to ascorbic acid/g dry weight basis. Among all extracts (fermented and unfermented) RFPM (10th day) showed higher (29.2 mg AAE/g dwb) value whereas lower was shown for UFPM (20.1 mg AAE/g dwb). The values of RPA of UFPM and RFPM extracts varied from 1.9 to 3.1 (mg QE/g dwb). Up to 4th day of fermentation, the RPA value did not differ significantly ($p < 0.05$) as compared to UFPM, with the highest being observed on 10th day. Yang et al. [52] reported that fermentation of soybean with *Acetobacter* sp., *Lactobacillus* sp., *Saccharomyces* sp. and *Streptomyces* sp. exhibited higher RPA value as compared to their non-fermented counterparts. Fermentation (2nd–10th days) showed significantly ($p < 0.05$) higher CTC values as compared to UFPM. Steep increase in CTC value was observed after 8th day of fermentation, i.e. on 10th day. The value of CTC on 10th day of fermentation was 176.6 mg CE/100 g dwb (Fig. 3). Determination of AP of natural and processed food products could be an important tool for food scientists as these are affected

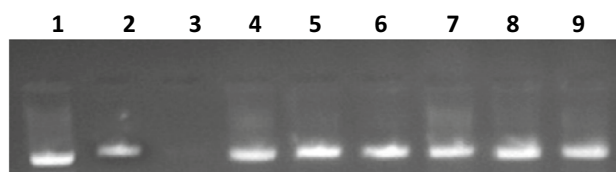


Fig. 4 DNA damage protecting activity of UFPM and RFPM extracts of PUSA-415 against hydroxyl radicals induced DNA damage of pBR322. *Lane 1*: native pBR 322 plasmid DNA; *Lane 2*: DNA + Fenton's reagent + Quercetin (mg/ml positive control); *Lane 3*: DNA + Fenton's reagent; *Lane 4*: DNA + Fenton's reagent + PUSA-415 (UFPM) extract; *Lane 5*: DNA + Fenton's reagent + PUSA-415 (RFPM 2nd day) extract; *Lane 6*: DNA + Fenton's reagent + PUSA-415 (RFPM 4th day) extract; *Lane 7*: DNA + Fenton's reagent + PUSA-415 (RFPM 6th day) extract; *Lane 8*: DNA + Fenton's reagent + PUSA-415 (RFPM 8th day) extract; *Lane 9*: DNA + Fenton's reagent + DNA + Fenton's reagent + PUSA-415 (RFPM 10th day) extract

by processing, extraction conditions and genetic modifications [53, 54]. AP of natural food and processed products are useful in planning dietary strategy for functional food formulations [55, 56]. Enhanced AP of extracts might be due to the increased amount of enzymes during SSF. Increase in enzymes activities (IU/gds) of α -amylase, β -glucosidase and xylanase were 3.29-folds (229%), 21.5-folds (2050%) and 11-folds (1000%), respectively after fermentation. Enzymes are thus responsible for microbial transformation of TPC and bioactive properties. Bhanja et al. [57] reported that hydrolytic enzymes produced by fungi during SSF, catalyze the release of aglycones from wheat koji thereby resulting in an increase in TPC as well as AP. They reported high correlation ($R^2 = 0.977$) among TPC-DPPH and TPC-ABTS ($R^2 = 0.947$) for *A. awamori* fermented wheat.

DNA damage protection activity (DDPA)

The extracts of UFPM and RFPM were evaluated for their capacity to prevent DNA damage induced by Fenton's

reagent. pBR322 was used as model DNA and the bands formed during the agarose gel electrophoresis showed DDPA. Sharpness of bands is an indicator for the presence of DDPA in extracts whereas faint bands indicate the minimal activity. DDPA of UFPM and RFPM is represented in Fig. 4. Lane 1 represents the pure plasmid DNA; Lane 2 represents the DNA damage protection activity of standard (quercetin) and Lane 3 represents the damaging effect of Fenton's reagent on DNA model. Difference observed among extracts for DDPA was non-significant. The presence of DDPA in extracts could be useful for planning diets for patients suffering from cancer, diabetes and neurodegenerative diseases. The effectiveness of the extracts to prevent the scission of DNA strands is a reflection of their positive effects against many diseases of biological systems. Xiao et al. [58] reported the DDPA in mung beans fermented with *Cordyceps militaris* SN-18. The findings of their study indicate that acetone extracts had weak DDPA as compared to water, ethanol and methanol. Further their study strongly suggests that not only the amount of TPC affects the DDPA of extracts, the type of bioactive constituents present in the extracts also have the affects.

Conclusions

The results revealed that *Rhizopus azygosporus* significantly ($p < 0.05$) increased TPC, CTC, DPPH, ABTS⁺, HFRSA, CUPRAC, TAC, RPA, DDPA and enzymatic activities (α -amylase, β -glucosidase and xylanase). Bioactive properties and enzymes studied showed the maximum increase till fermentation was carried out (10th day). SSF thus can be powerful method for modulation of bioactive compounds and enzymes. Further studies, however, are required to optimize the inoculums size, age of starter culture, extraction phase type, extraction phase concentration, extraction temperature and time.

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

References

1. M. Chandrasekara, F. Naczka, Shahidi, Food Chem. **133**, 1 (2012)
2. R.K. Salar, P. Sharma, S.S. Purewal, Tang **5**, 1 (2015)
3. P. Kaur, S.B. Dhull, K.S. Sandhu, R.K. Salar, S.S. Purewal, Food Measure. **12**, 1530 (2018)
4. B.N. Singh, B.R. Singh, R.L. Singh, D. Prakash, R. Dhakarey, G. Upadhyay, H.B. Singh, Food Chem. Toxicol. **47**, 1109 (2009)
5. G.A. Pereira, H.S. Arruda, D.R. Morais, M.N. Eberlin, G.M. Pastore, Food Res. Int. **108**, 264 (2018)
6. I.A. Neri-Numa, R.A.S. Sancho, A.P.A. Pereira, G.M. Pastore, Food Res. Int. **103**, 345 (2018)
7. K.S. Nithya, B. Ramachandramurthy, V.V. Krishnamoorthy, J. Biol. Sci. **7**, 643 (2007)
8. P. Kaur, P. Sharma, V. Kumar, A. Panghal, J. Kaur, Y. Gat, J Saudi Soc. Agric. Sci. (2017) <https://doi.org/10.1016/j.jssas.2017.12.004>
9. L. Seczyk, M. Swieca, D. Dziki, A. Anders, U. Gawlik-Dziki, Food Chem. **214**, 32 (2017)
10. R.K. Salar, S.S. Purewal, Biocatal. Agric. Biotechnol. **8**, 221 (2016)
11. A. Zabrano, O. Kotogan, T. Bencsik, C. Papp, K.C. Vagvolgyi, J. Mondal, M. Krisch, Tako, LWT Food Sci. Technol. **89**, 457 (2018)
12. H.B. Singh, B.N. Singh, S.P. Singh, C.S. Nautiyal, Bioresour. Technol. **101**, 6444 (2010)
13. T.B. Dey, R.C. Kuhad, Lett. Appl. Microbiol. **59**, 493 (2014)
14. F.V. Dulf, D.C. Vodnar, C. Socaciu, Food Chem. **209**, 27 (2016)
15. R.K. Salar, S.S. Purewal, K.S. Sandhu, Food Res. Int. **100**, 204 (2017)
16. P.D. Postemsky, M.A. Bidegain, R. Gonzalez-Matute, N.D. Figlas, M.A. Cubitto, Bioresour. Technol. **231**, 85 (2017)
17. P.D. Postemsky, N.R. Curvetto, Int. Biodeterior. Biodegrad. **100**, 52 (2015)
18. H. Smith, S. Doyle, R. Murphy, Food Chem. **185**, 389 (2015)
19. R.C. Llarena-Hernandez, E. Renouf, X. Vitrac, J.M. Merillon, J.M. Savoie, in *Fungal Metabolites*, ed. by J.M. Merillon, K. Ramawat. Reference series in phytochemistry. (Springer, Cham, 2015), https://doi.org/10.1007/978-3-319-19456-1_35-1
20. R.K. Salar, S.S. Purewal, K.S. Sandhu, 3 Biotech. **7**, 164 (2017). <https://doi.org/10.1007/s13205-017-0834-2>
21. R.K. Salar, M. Certik, V. Brezova, Biotechnol. Bioprocess Eng. **17**, 109 (2012)
22. Pandey, Biochem. Eng. J. **13**, 81 (2003)
23. F. Liyana-Pathirana, F. Shahidi, Food Chem **93**, 47 (2005)
24. R.K. Salar, S.S. Purewal, M.S. Bhatti, Resour. Eff. Technol. **2**, 148 (2016)
25. H.S. Arruda, G.A. Pereira, G.M. Pastore, Food Anal. Methods **10**, 100 (2017)
26. N. Thavamoney, L. Sivanadian, L.H. Tee, H.E. Khoo, K.N. Prasad, K.W. Kong, J Food Sci. Technol. **55**, 2523 (2018)
27. P. Arigo, P. Cesla, M.L. Silarova, L. Calabro, Ceslova, Food Chem. **245**, 829 (2018)
28. F.V. Dulf, D.C. Vodnar, E.H. Dulf, Z. Diaconeasa, C. Socaciu, LWT Food Sci. Technol. **87**, 241 (2018)
29. P. Chranioti, D. Kotzekidou, Gerasopoulos, LWT Food Sci. Technol. **89**, 403 (2018)
30. L. Wang, Y. Luo, Y. Wu, Z. Wu, J Funct. Foods **41**, 183 (2018)
31. Q. Bei, G. Chen, Y. Liu, Y. Zhang, Z. Wu, J Funct. Foods **47**, 512 (2018)
32. R. Randhir, D. Vattam, K. Shetty, Innov. Food Sci. Emerg. Technol. **5**, 235 (2004)
33. D.L. Abd Razak, N.Y. Abd Rashid, A. Jamaluddin, S.A. Shari-fudin, K. Long, Biocatal. Agric. Biotechnol. **4**, 33 (2015)
34. C.G. Schmidt, E.B. Furlong, Bioresour. Technol. **123**, 36 (2012)
35. K.C. Massarolo, T.S. Denardi, C.C. Collazzo, E.F. Badiale, L.A. Souza Soares, Food Chem. **228**, 43 (2017)
36. L.W. Lee, M.W. Cheong, P. Curran, B. Yu, S.Q. Liu, Food Chem. **211**, 925 (2016)
37. W.C. Vong, X.Y. Hua, S.Q. Liu, LWT Food Sci. Technol. **90**, 316 (2018)
38. G.L. Miller, Ann. Chem. **31**, 426 (1959)
39. L. Yu, S. Haley, J. Perret, M. Harris, Food Chem **86**, 11 (2004)
40. G.C. Yen, H.Y. Chen, Food Chem. **43**, 27 (1995)

41. R.N. Re, A. Pellegrini, A. Proteggente, M.Y. Pannala, C.R. Evans, *Free Radic. Biol. Med.* **26**, 1231 (1999)
42. N. Smirnov, Q.J. Cumbes, *Phytochem.* **28**, 1057 (1989)
43. R. Apak, K. Guclu, M. Ozyurek, S.E. Karademir, *J. Agric. Food Chem.* **52**, 7970 (2004)
44. P. Prieto, M. Pineda, M. Aguilar, *Anal. Biochem.* **269**, 337 (1999)
45. M. Oyaizu, *Jpn. J. Nutr.* **44**, 307 (1986)
46. R. Julkunen-Titto, *J. Agric. Food Chem.* **33**, 213 (1985)
47. V. Kumar, M. Lemos, M. Sharma, V. Shriram, *Free Radic. Antiox.* **3**, 55 (2013)
48. S.B. Dhull, P. Kaur, S.S. Purewal, *Resour. Eff. Technol.* **2**, 168 (2016)
49. R.K. Salar, S.S. Purewal, K.S. Sandhu, *Biocatal. Agric. Biotechnol.* **11**, 201 (2017)
50. R.K. Salar, S.S. Purewal, *Food Measure* **11**, 126 (2017)
51. D.L. Abd-Razak, N.Y. Abd-Rashid, A. Jamaluddin, S.A. Sharifudin, A. Abd-Kahar, K. Long, *J. Saudi Soc. Agric. Sci.* **16**, 127 (2017)
52. J.H. Yang, J.L. Mau, P.T. Ko, L.C. Huang, *Food Chem.* **71**, 249 (2000)
53. R. Kaur, M. Kaur, S.S. Purewal, *J. Food Process Preserv.* **42**, e13585 (2018)
54. S. Singh, M. Kaur, D.S. Sogi, S.S. Purewal, *Food Measure* (2018). <https://doi.org/10.1007/s11694-018-9950-x>
55. A.K. Siroha, K.S. Sandhu, M. Kaur, *Food Measure.* **10**, 311 (2016)
56. A.K. Siroha, K.S. Sandhu, *Food Measure* **11**, 872 (2017)
57. T. Bhanja, A. Kumari, R. Banerjee, *Bioresour. Technol.* **100**, 2861 (2009)
58. Y. Xiao, Q. Zhang, J. Miao, X. Rui, T. Li, M. Dong, *Innov. Food Sci. Emerg. Technol.* **31**, 216 (2015)

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