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Resource-Efficient Technologies 2 (2016) 148–157

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Research paper

# Optimization of extraction conditions and enhancement of phenolic content and antioxidant activity of pearl millet fermented with *Aspergillus awamori* MTCC-548

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Received 29 May 2016; received in revised form 20 August 2016; accepted 20 August 2016

Available online 16 September 2016

**Abstract**

The present study envisaged two stage optimization of conditions using RSM for extraction of total phenolic compounds from pearl millet koji prepared with *Aspergillus awamori*. Antioxidant activity was determined by employing DPPH and radical cation of ABTS. In phase-1, fermentation time (5–8 days), extraction temperature (40–60 °C), extraction time (45–60 min.) and solvent (ethanol, 50%; 0.5 ml HCL + 99.5 ml methanol) were tested for maximizing extraction process. The optimum conditions of phenolic recovery were achieved at 8 days fermentation time, 40 °C extraction temperature, 45 min. extraction time with 50% ethanol as solvent, with values of 169.19 mg GAE/g for TPC, 262.7 VCEAC µmol/g for DPPH and 281.86 VCEAC µmol/g for ABTS. TPC were found to be positively correlated ($p < 0.05$) with DPPH and ABTS under these conditions. In phase-2, a central composite design was applied for design of experiments and model building using extraction time and extraction temperature as process variables for further maximizing the extraction of TPC. The optimized conditions using RSM for maximizing the extraction of total phenolic compounds were: ethanol concentration, 50%; extraction temperature, 44.5 °C and extraction time, 23.8 mins. Under these conditions, 176.82 mg GAE/g of total phenolic compounds were extracted which was very close to the predicted value of 173.2 mg GAE/g. The model was validated at these optimal points.



*Keywords:* Koji; Phenolics; Antioxidants; SSF; RSM

## 1. Introduction

There has been an increasing interest over the use of natural antioxidants primarily due to free radical scavenging ability of a variety of phytochemicals, especially phenolic compounds [1]. Grains, fruits, herbs, spices and other dietary sources containing hundreds of phenolic compounds have been demonstrated to be effective antioxidants in model systems [2–5]. These compounds have attracted the attention of food biotechnologists and medical scientists due to their strong *in vitro* and *in vivo* antioxidant activities and their ability to scavenge free radicals. The later are highly reactive and unstable molecules are produced as a result of normal metabolism in higher organisms. These are also involved in organism's vital activities including phagocytosis, regulation of cell proliferation, intracellular signaling and synthesis of biologically active compounds [6]. However, excess production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion radical, and the hydroxyl radical causes damage in cells leading to etiology of many chronic diseases including atherosclerosis, diabetes and cancer [7,8].

Pearl millet (*Pennisetum glaucum*) is the most widely grown millet crop. India is the leading producer of pearl millet which is primarily cultivated and consumed in the states of Haryana, Rajasthan, Gujarat and Madhya Pradesh. Locally known as '*bajra*' it is a principal source of energy, protein, vitamins and minerals for millions of the poorest people in the regions where it is cultivated. It is also a major food staple in semi-arid and arid lands of Africa and Asia and is well adapted to drought and sandy acid soil of low fertility. The crop ranks as worlds' fourth most tropical food cereal. The nutrient composition of pearl millet indicates that it is a good source of energy and protein

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http://dx.doi.org/10.1016/j.reffit.2016.08.002

[9]. Essential amino acid profile revealed that it is 40% richer in lysine and methionine and 30% richer in threonine compared to corn protein [10]. Nutritional studies indicated that metabolized energy of pearl millet for non-ruminant animals is approximately equal to that of maize [11]. As with other cereals, certain nutritional inhibitors, such as enzyme inhibitors, phytic acid and tannins are also associated with pearl millet. These factors affect the nutritional value of the grain by inhibiting protein and starch digestibility and mineral bioavailability.

Considering the dietary needs of humans, the nutritional improvement of grains is seen an added advantage in diminishing the damaging effects of free radicals. Most phenolic acids in cereals occur in bound form as conjugate with sugars, fatty acids or proteins [12]. In the recent past solid state fermentation of cereal grains with GRAS filamentous fungi [4,13,14] yeasts and lactic acid bacteria [15] has gained interest for bioaugmentation of phenolic contents and antioxidant activity. However, an industrial process will be economical if high extraction efficiency is attained. Traditionally, process optimization could be achieved by either empirical or statistical methods [16]. One-factor-at-a-time approach, in which one factor varies at a time and all others are kept constant, has several limitations including the inability to determine interactions between the variables, time consuming, expensive and less effective [17].

Response surface methodology (RSM), originally described by Box and Wilson [18], is a mathematical and statistical tool that allows evaluation of the influence of several input process variables and their interaction on output response [19]. RSM has been widely employed to find a combination of factor levels that produce an optimum response. Therefore, RSM has become most popular optimization technique and has been successfully used to model and optimize biochemical and biotechnological processes. It has been used for optimizing extraction of phenolic compounds from a number of dietary sources [2,16,20,21].

The objectives of the present investigation were twofold. Firstly, to enhance the phenolic content and antioxidative property of pearl millet (millet koji) prepared through solid state fermentation using *Aspergillus awamori* Nakazawa (traditionally used in the preparation of many oriental food products) as the starter organism. Secondly, to model and optimize the extraction conditions (solvent concentration, extraction time, temperature) in order to maximize the yield of total phenolic content (TPC) and antioxidant activity of pearl millet using RSM.

## 2. Materials and methods

### 2.1. Plant material

Whole grains of pearl millet (HHB-197) were obtained from the Bajra section, Chaudhary Charan Singh Haryana Agricultural University, Hisar, India. The millet grains were washed thoroughly to remove any debris or dust particles and dried in shade followed by drying at 40 °C in an oven for 24 hrs. The grains were stored in airtight containers until used for further studies.

### 2.2. Chemicals and glasswares

All the solvents used were of analytical grade and obtained from Fischer Scientific. The compounds, DPPH (2, 2-diphenyl-1-picrylhydrazyl) and 2, 2′-azino-bis [3-ethylbenzothiazoline-6-sulfonate (ABTS)] were obtained from Hi-media Pvt. Ltd., Mumbai and Sigma respectively. Glasswares used in the experimental work were of Borosilicate. Before using, glasswares were washed with labolene detergent and rinsed with distilled water and sterilized in an oven at 180 °C for three hours.

### 2.3. Microorganism and inoculum

*Aspergillus awamori* (MTCC-548) used for preparation of millet koji was obtained from Microbial Type Culture Collection, Chandigarh and maintained in the laboratory on Czapek agar slants at 4 °C. Spore suspension was prepared by washing the mycelium grown on Czapek agar for 4 days at 30 °C, with an aqueous solution of 0.1% (w/v) Tween 80. Spore suspension obtained as such containing approximately $1 \times 10^5$ spores/ml was used for inoculating the autoclaved millet grains.

### 2.4. Preparation of millet koji

Solid state fermentation, as described in our earlier study [4], was performed in 250 ml Erlenmeyer flasks to prepare millet koji. Briefly, washed and dried millet grains (50 g) were first soaked overnight at room temperature in 50 ml Czapek solution. After decanting the solution, the millet grains were steam cooked in an autoclave (121 °C, 15 min). After cooling, the steamed millet substrate was inoculated with 1 ml spore suspension of *A. awamori* under aseptic conditions. The inoculated millet substrate, after thorough mixing, was incubated at 30 °C up to 10 days. During the cultivation period, the millet grains were stirred and mixed after 24 h and 36 h of cultivation to accelerate the release of fermentation heat. The unfermented millet grains were prepared without the addition of spore suspension.

### 2.5. Preparation of millet extracts

The fermented millet grains were taken out of the Erlenmeyer flask at 2 days interval and dried in an oven at 40 °C for 24 h. The dried substrates were ground in an electric grinder to obtain a fine powder. All samples were defatted by blending the finely ground substrate with hexane (1:5 w/v, 5 min, thrice) in a waring blender at ambient temperature. Defatted millet samples were air dried for 24 h and stored at −20 °C until further analysis.

One gram of defatted substrate was extracted with 20 ml of extraction solvents [ethanol 50%, methanol 50% and HCl/methanol (1:99, 0.5:99.5 {v/v})] separately at 60 °C for 60 min in a water bath. Extracts were filtered and the supernatants were collected, pooled and dried in rotary vacuum at 40 °C [4]. Residues containing extracted phenolics/antioxidants were dissolved in 2 ml of the respective solvent for determination of TPC and antioxidant property. All extracts were stored at −20 °C in the dark until further analysis.

### 2.6. Total phenolic content (TPC) determination

Total phenolic contents of the extracts were determined using Folin–Ciocalteu (FC) reagent following Yu et al. [22] with minor modifications. Briefly, 100 µl of extracts were mixed with 0.5 ml of FC reagent in a 10 ml volumetric flask. Subsequently 1.5 ml aqueous solution (20%, w/v) of sodium carbonate anhydrous was added, vertexed and incubated for 15 min at room temperature. The flask was filled with distilled water to volume. The absorbance was read at 765 nm, subtracting the value of a control solution consisting of distilled water instead of extracts. The amount of total phenolic content was calculated as gallic acid equivalents (GAE) from the standard calibration curve of gallic acid and expressed as mg gallic acid equivalents per gram of sample.

### 2.7. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The radical scavenging capacity of different samples was measured using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) according to Yen and Chen [23] with slight modifications. The mixture was prepared by taking 200 µl of extract in a spectrophotometric cell and then 3 ml of 100 µM DPPH (4 mg DPPH in 100 ml methanol) were added. The changes in absorbance at 519 nm upon time during 10 minutes were recorded using a visible spectrophotometer. In the reference sample, 200 µl of solvent were used instead of extracts. The absorbance values in minute 10 relative to the reference sample were used for evaluation of antioxidant activity. The DPPH radical scavenging capacities were quantified using ascorbic acid as reference antioxidant and the results were expressed as vitamin-C equivalent antioxidant capacity (VCEAC) in µmol/g of sample.

### 2.8. 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfate) (ABTS) radical cation depolarization assay

Antioxidant activity was measured by using a modified method of Re et al. [24] and Arts et al. [25]. 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfate) (ABTS, Sigma) was used for production of corresponding radical cation ($ABTS^+$) by dissolving 17.2 mg ABTS and 3.3 mg $K_2S_2O_8$ in 5 ml distilled water, and the resulting solution was left to stand for 16 h in dark at room temperature. A stock solution of $ABTS^+$ was prepared by mixing 1 ml of this reaction mixture with 60 ml water. The concentration of $ABTS^+$ was determined spectrophotometrically using the characteristic value of molar absorption coefficient at 732 nm, $1.5 \times 10^4$ $mol^{-1} \cdot l \cdot cm^{-1}$. After rigorously mixing 2.5 ml $ABTS^+$ solution with 50 µl of extracts, the absorbance was taken after 10 minutes. Spectrum of initial $ABTS^+$ solution measured against distilled water was taken as a reference spectrum. The difference in the absorbance in 10th min at 732 nm relative to reference spectrum was used to calculate the antioxidant activity. The ability of samples to eliminate $ABTS^+$ is expressed as VCEAC in µmol/g of sample.

### 2.9. Experimental design for response surface methodology

In this study two step optimization of total phenolic compounds extraction from millet koji employing three independent response variables (solvent concentration, extraction temperature and extraction time) was carried out using response surface methodology (RSM). The maximization of extraction was taken as response. Half normal probability plot was used for selection of process variables in phase-1 and ANOVA model was constructed in phase-2 which best fits the response data. D-optimal design consisting of 22 experimental runs followed by center composite design (CCD) were employed. The design variables were the solvent composition (%, v/v, water/methanol, water/ethanol, HCL/methanol), extraction temperature (A, °C) and extraction time (B, min.) while response variable was total phenolic content (TPC).

## 3. Results and discussion

The use of different GRAS (generally recognized as safe) filamentous fungi during solid state fermentation is recognized to enhance the phenolic content and antioxidative properties of various food grains [4,14,26,27]. Generally, the efficiency and effectiveness of extraction of polyphenol compounds is governed by multiple variables such as temperature, time and solvent concentration and composition [2,28]. The influence of extraction variables such as extraction temperature, time and solvent concentration and composition on the recovery of phenolic compounds in millets has not been reported yet. Thus, the present study was divided into three parts. The initial study included determination of fermentation time (in days) for maximal mobilization of phenolic contents of millet koji. Second part of the study involved optimization of extraction conditions *viz*., temperature, time and solvent concentration and composition using response surface methodology. Finally, the experiments were verified based on predicted conditions and actual values of experimental data were obtained.

### 3.1. Effect of fermentation on phenolic compounds mobilization

Cereal grains possess phenolic compounds and their derivatives in bound form. Fungal fermentation may help in mobilizing these phenolic compounds from their bound form to free state by producing various enzymes during fermentation. In the present investigation, TPC in millet koji were measured in terms of GAE in mg/g dw. Accordingly, a calibration curve of gallic acid was constructed. The calibration equation for gallic acid was $y = 0.001x + 0.045$ ($R^2 = 0.998$). The results reported in this study were computed from this equation. *A. awamori* has been frequently used to ferment various food grains *viz*., wheat [14], black bean [27,29]. In the present study, *A. awamori* (MTCC 548) was used to enhance phenolic contents in millet under SSF up to 10 days. The total phenolic contents of fermented millet koji increased manifold up to 8 days of fermentation (85.12 mg GAE/g dw) as compared to unfermented millet (18.00 mg GAE/g dw) (Fig. 1). Thus 8 days was the optimum time for fermentation to release bound phenolics from millet grains and in all future experiments SSF was carried out up to 8 days.

### 3.2. Selection of solvent for extraction of TPC

The choice of extraction solvents is critical as it usually determines the type and amount of phenolic compounds being extracted. Aqueous solvents such as acetone, ethanol and

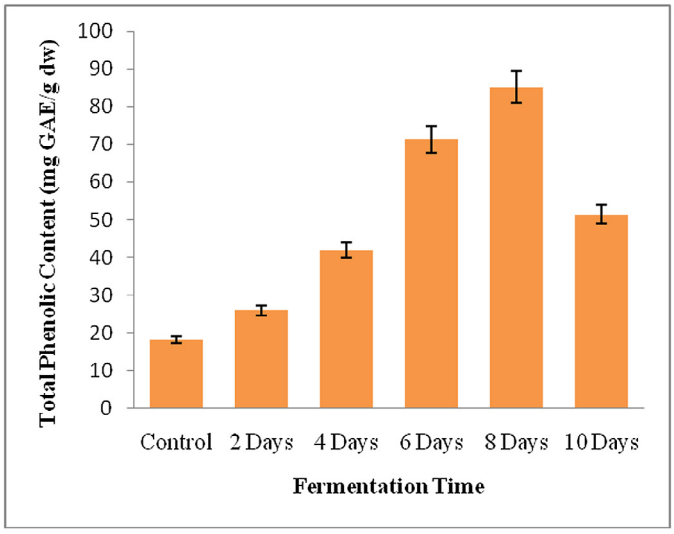


Fig. 1. Total phenolic content of pearl millet extracts prepared in ethanol (50%).

methanol are commonly employed in phenolic extraction from plant sources [30,31]. In the present investigation, the initial step of the preliminary experiment was to select a suitable extraction solvent system for millet phenolics. Three different solvent systems *viz*., ethanol (50%), methanol (50%) and HCl:methanol (in ratio of 0.5:99.5, 1:99) was used. The crude phenolic extracts were prepared by extracting the ground millet (unfermented and fermented) samples (1 g) with 20 ml of solvent at a varying temperature range of 45–75 °C for 45–90 minutes using D-optimal design to optimize the extraction of phenolic compounds. Based on the results obtained (Table 1), two best solvents and their composition were chosen.

Maximum extraction of total phenolic content i.e. 152.51 mg/g was observed at 45 °C and 45 minutes with HCl:methanol in ratio 0.5:99.5. Whereas minimum extractions of total phenolic content i.e. 43.53 mg/g was observed at 75 °C and 90 minutes with HCl:methanol in ratio 0.5:99.5 (Table 1). It is clear from the results that either ethanol 50% or HCl:methanol in ratio of 0.5:99.5 would be the best solvent for the extraction of phenolic compounds from pearl millet. Further, to compare the effect of both solvents on extraction, a series of 22 experiments were again conducted in which moderate temperatures of 40, 50 and 60 °C were chosen as lower, middle and upper levels, respectively and a time durations of 45, 52.5 and 60 minutes as lower, middle and upper levels, respectively to be applied in RSM optimization.

According to new design generated by RSM, millet grains were again fermented with *A. awamori* for a period of 5 days, 6.5 days, and 8 days. Screening was done for the detection of conditions for extraction of maximum phenolic compounds from the fermented millets. Results of this investigation are presented in Table 2 and reveal that maximum amount of phenolic content was obtained from 8 days fermented sample. Finally, 8 days fermented samples were selected to investigate the best extraction conditions. It is clear from the results that maximum extraction of TPC could be possible with 50% ethanol. Experimental observations indicate the extraction of maximum phenolic compounds (169.19 mg/g) at 40 °C for 45 minutes prepared in 50% ethanol and minimum extraction of phenolic content (94.83 mg/g) at 60 °C for 45 minutes prepared in HCl:methanol in a ratio of 0.5:99.5 (Table 1). Cacace and Mazza [32] revealed that maximum total phenolics in black currant extracts was obtained at about 60% ethanol followed by a decrease with further increase in concentration. Nepote et al. [33] also established that increasing the ethanol concentration

Table 1
Screening of solvent, temperature and time for maximizing total phenolic content (TPC) using D-optimal design for variable duration of fermentation time.

| Exp. No. | Fermentation time Days | Solvent | Extraction temp. °C | Extraction time minutes | Total phenolic content (mg GAE/g DW) |
|---|---|---|---|---|---|
| 1 | 5 | Ethanol 50% | 60 | 60 | 120.6 |
| 2 | 8 | Ethanol 50% | 40 | 45 | 169.19 |
| 3 | 8 | Ethanol 50% | 60 | 60 | 117 |
| 4 | 8 | Ethanol 50% | 40 | 60 | 148.5 |
| 5 | 5 | Ethanol 50% | 60 | 45 | 138 |
| 6 | 5 | Ethanol 50% | 40 | 60 | 130.6 |
| 7 | 5 | Ethanol 50% | 40 | 45 | 129.08 |
| 8 | 6.5 | Ethanol 50% | 50 | 52.5 | 130.1 |
| 9 | 8 | Ethanol 50% | 60 | 45 | 125.85 |
| 10 | 6.5 | Ethanol 50% | 50 | 52.5 | 133.14 |
| 11 | 6.5 | Ethanol 50% | 50 | 52.5 | 127.52 |
| 12 | 5 | 0.5 ml HCl + 99.5 ml Methanol | 40 | 60 | 131.13 |
| 13 | 5 | 0.5 ml HCl + 99.5 ml Methanol | 60 | 45 | 113.29 |
| 14 | 8 | 0.5 ml HCl + 99.5 ml Methanol | 40 | 60 | 137.83 |
| 15 | 6.5 | 0.5 ml HCl + 99.5 ml Methanol | 50 | 52.5 | 119.01 |
| 16 | 8 | 0.5 ml HCl + 99.5 ml Methanol | 60 | 45 | 94.83 |
| 17 | 5 | 0.5 ml HCl + 99.5 ml Methanol | 40 | 45 | 134.36 |
| 18 | 5 | 0.5 ml HCl + 99.5 ml Methanol | 60 | 60 | 103.68 |
| 19 | 8 | 0.5 ml HCl + 99.5 ml Methanol | 40 | 45 | 152.36 |
| 20 | 6.5 | 0.5 ml HCl + 99.5 ml Methanol | 50 | 52.5 | 133.61 |
| 21 | 6.5 | 0.5 ml HCl + 99.5 ml Methanol | 50 | 52.5 | 126.2 |
| 22 | 8 | 0.5 ml HCl + 99.5 ml Methanol | 60 | 60 | 97.64 |

Table 2
Effect of temperature and time on total phenolic content (TPC) for 8 days fermentation time with ethanol 50% as solvent.

| Sr. no. | Fermentation time | Solvent | Temp. (°C) | Time | TPC (mg/g dwb) |
|---|---|---|---|---|---|
| 1 | 8 days | Ethanol 50% | 37 | 30 | 137.5668 ± 0.40* |
| 2 | 8 days | Ethanol 50% | 37 | 30 | 137.7764 ± 0.25 |
| 3 | 8 days | Ethanol 50% | 52 | 30 | 121.3673 ± 0.38 |
| 4 | 8 days | Ethanol 50% | 52 | 30 | 121.8843 ± 0.19 |
| 5 | 8 days | Ethanol 50% | 37 | 60 | 143.5825 ± 0.39 |
| 6 | 8 days | Ethanol 50% | 37 | 60 | 143.358 ± 0.44 |
| 7 | 8 days | Ethanol 50% | 52 | 60 | 109.4938 ± 0.41 |
| 8 | 8 days | Ethanol 50% | 52 | 60 | 108.5818 ± 0.41 |
| 9 | 8 days | Ethanol 50% | 33.9 | 45 | 121.6272 ± 0.60 |
| 10 | 8 days | Ethanol 50% | 33.9 | 45 | 122.068 ± 0.83 |
| 11 | 8 days | Ethanol 50% | 55.1 | 45 | 107.0189 ± 0.61 |
| 12 | 8 days | Ethanol 50% | 55.1 | 45 | 107.9659 ± 0.45 |
| 13 | 8 days | Ethanol 50% | 44.5 | 23.8 | 176.8298 ± 0.33 |
| 14 | 8 days | Ethanol 50% | 44.5 | 23.8 | 176.2238 ± 0.23 |
| 15 | 8 days | Ethanol 50% | 44.5 | 66.2 | 173.3333 ± 0.32 |
| 16 | 8 days | Ethanol 50% | 44.5 | 66.2 | 173.1002 ± 0.17 |
| 17 | 8 days | Ethanol 50% | 44.5 | 45 | 173.7063 ± 0.36 |
| 18 | 8 days | Ethanol 50% | 44.5 | 45 | 173.4266 ± 0.44 |
| 19 | 8 days | Ethanol 50% | 44.5 | 45 | 173.8462 ± 0.11 |
| 20 | 8 days | Ethanol 50% | 44.5 | 45 | 173.7063 ± 0.54 |
| 21 | 8 days | Ethanol 50% | 44.5 | 45 | 173.7529 ± 0.55 |

* ±Standard deviation.

beyond 70% dramatically reduces the amount of phenolics extracted from peanut skins. A remarkable drop in TPC at 100% ethanol revealed that absolute solvent does not ensure a good recovery of phenolic compounds as compared to aqueous ethanol [33].

### 3.3. Effect of extraction time on extraction of TPC

The extraction time is also crucial for extraction of phenolic compounds and can be as small as few minutes or very long and extending up to 24 hours [34,35]. In our study, the range of extraction time was premeditated on practical and economical aspects. Increase in extraction time from 23.8 to 45 min led to a small rise in TPC from 158.9 to 173.713 mg GAE/g (Fig. 3b). No further significant increase in extraction of millet phenolics was observed after 45 min. This observation was supported by Fick's second law of diffusion, which states that "final equilibrium will be achieved between the solute concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent) after a certain time", hence, a longer extraction time was not useful to extract more phenolic antioxidants [36]. Furthermore, extended extraction process might lead to oxidation of phenolic compounds owing to prolonged light or oxygen exposure. Thus, taking into account all these facts, an extraction time of 23.8–66.2 min was chosen for RSM optimization.

### 3.4. Response surface methodology experiments

#### 3.4.1. Screening of process variables

In phase-1, fermentation time (5–8 days), extraction temperature (40–60 °C), extraction time (45–60 min) and solvent (ethanol 50%, 0.5 ml HCl + 99.5 ml methanol) were tested for maximizing extraction of TPC. The half probability plot predicted fermentation time, temperature and solvent as important process variables. ANOVA model was significant at 99.99% with F-value of 16.37. The predicted $R^2$ was close to adjusted $R^2$ with signal to noise ratio greater than 4 (permissible level). Also, model has the non significant lack of fit.

#### 3.4.2. Analysis of response surface plot

Fig. 2 illustrated three-dimensional response surface plots by presenting the response in function of two factors and keeping the other constant at its middle level. Each figure revealed the effects of the selected parameters on phenolic content. The predicted response surface depicts the effect of fermentation time and extraction temperature on total phenolic content at constant solvent concentration *i.e.*, 50% ethanol. Fig. 2 also depicts higher amount of phenolic content yield in 8 days fermented sample. Both fermentation time and extraction temperature showed significant quadratic effects on phenolic content (Fig. 2). Therefore, the phenolic content gradually mounted up with the increase of fermentation time up to 8 days. In general, the polarity of ethanol–water mixture would increase continuously with the addition of water to ethanol.

#### 3.4.3. Center composite design optimization

In phase-2, CCD was used for design of experiments and model building using extraction temperature and extraction time as process variables. The model was generated with few non significant model terms. Thus, model was again generated after eliminating model terms with p-value >0.05. The reduced model was again significant with F-value of 16.37 at 99.99% significance level. The lack of model was not significant at p-value of 2.86. Thus, model can be navigated in the design space. The relationship between the tested independent variables and the response was explained by applying multiple regression analysis (Equation 1):

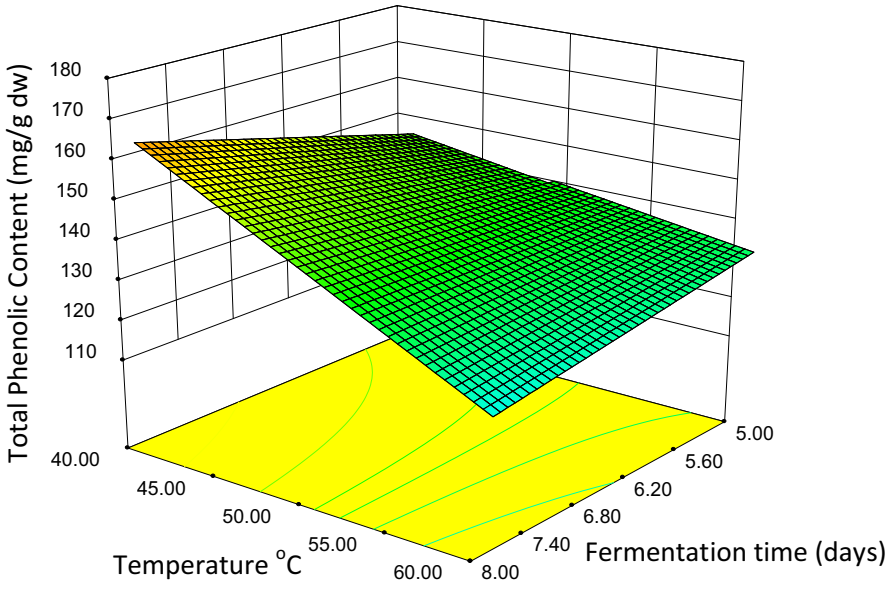


Fig. 2. Response surface plot for the effect of fermentation time and temperature at a constant solvent concentration (ethanol 50%) on total phenolic content of millet koji.

Final equation in terms of coded factors:

$$Rl = +173.23 - 10.17 \times A - 0.47 \times B - 28.83 \times A^2 - 9.26 \times B^2$$

Final equation in terms of actual factors: (1)

$$Rl = -863.26065 + 44.25937 \times A + 3.67093 \times B - 0.51253 \times A^2 - 0.041135 \times B^2$$

The suitability of the model equation for predicting the response values was verified by conducting the extractions under the recommended optimal conditions. In this experiment, an optimization method was adopted to find a point that maximizes the response. The experimental and predicted values of TPC were compared in order to determine the validity of the model. To confirm the results, runs were carried out in triplicates under the selected optimized conditions. Extraction temperature and time demonstrated quadratic effect on total phenolic content present in millet extracts prepared in ethanol 50% (Table 3).

The relationship of extraction temperature and extraction time with TPC is shown in Fig. 3a and b. Both the factors displayed significant linear and quadratic effect (at least at $p < 0.05$) on TPC (Table 4) with reference to extraction temperature, TPC of millet extracts increased readily with increasing temperature up to 44.5 °C followed by a slight decrease subsequently. This suggested that incubation up to this temperature did improve phenolics extraction, yet was gentle enough to avoid heat degradation of the target phenolic antioxidants. Mild heating might soften the plant tissue, weaken the cell wall integrity, hydrolyze the bonds of bound phenolic compounds (phenol–protein or phenol–polysaccharide) and enhance phenolics solubility, as a result more phenolics would dispense to the solvent [37–39]. At optimum extraction temperature (44.5 °C), higher amounts of phenolic contents were recovered with short extraction time. This shows that long extraction time may recompense the beneficial effects of moderate temperature by inducing oxidation or degradation of phenolic compounds, yielding low TPC [40]. By combining the results presented in Fig. 3a and b, it was concluded that extraction temperature and time had the most critical role in the extraction of phenolic compounds from millet followed by ethanol concentration. Modulation of phenolic compounds could be enhanced using an aqueous ethanol over a limited compositional range. In general, it was observed that ethanol concentration ranging from 40 to 60% had greater effectiveness in the extraction of polyphenol compounds compared to pure ethanol. This seems to be agreed with 50% ethanol reported in the present study. On the contrary, extrac-

Table 3
Two factor and five level central composite design (CCD) with experimental and predicted values under different extraction conditions.

| Runs | Solvent type and conc. | Independent variables | | Dependent variable total phenolic content (mg GAE/g DW) | |
|---|---|---|---|---|---|
| | | A, Temperature (°C) | B, Time (mins.) | Experimental | Predicted |
| 1 | Ethanol 50% | 37.0 | 30.0 | 143.1 | 145.8 |
| 2 | Ethanol 50% | 52.0 | 30.0 | 121.8 | 125.4 |
| 3 | Ethanol 50% | 37.0 | 60.0 | 140.3 | 144.8 |
| 4 | Ethanol 50% | 52.0 | 60.0 | 126.0 | 124.5 |
| 5 | Ethanol 50% | 33.9 | 45.0 | 134.1 | 130.0 |
| 6 | Ethanol 50% | 55.1 | 45.0 | 101.7 | 101.2 |
| 7 | Ethanol 50% | 44.5 | 23.8 | 158.9 | 155.4 |
| 8 | Ethanol 50% | 44.5 | 66.2 | 155.2 | 154.1 |
| 9 | Ethanol 50% | 44.5 | 45 | 176.4 | 173.2 |
| 10 | Ethanol 50% | 44.5 | 45 | 174.6 | 173.2 |
| 11 | Ethanol 50% | 44.5 | 45 | 167.6 | 173.2 |
| 12 | Ethanol 50% | 44.5 | 45 | 178.4 | 173.2 |
| 13 | Ethanol 50% | 44.5 | 45 | 169.1 | 173.2 |

Table 4
Analysis of variance (ANOVA) of the model for total phenolic contents of millet.

| | | Degree of freedom | Mean squares | F-value | p-Value |
|---|---|---|---|---|---|
| Source | | | | | Prob>F |
| Model | 6825.2 | 4 | 1706.31 | 84.72 | <0.0001* |
| A: Extraction temp. | 827.7 | 1 | 827.72 | 41.10 | 0.0002* |
| B: Extraction time | 1.8 | 1 | 1.75 | 0.09 | 0.7755# |
| $A^2$ | 5782.1 | 1 | 5782.08 | 287.10 | <0.0001* |
| $B^2$ | 595.9 | 1 | 595.90 | 29.59 | 0.0006* |
| Lack of fit | 74.0 | 4 | 18.51 | 0.85 | 0.5605# |
| **Model statistic** | | | | | |
| | Std. dev. | 4.49 | $R^2$ | 0.977 | |
| | Mean | 149.79 | Adjusted $R^2$ | 0.965 | |
| | C.V.% | 3.00 | Predicted $R^2$ | 0.933 | |
| | PRESS | 467.08 | Adequate precision | 25.9 | |

* Significant at $p < 0.001$.
\# Not-significant at $p < 0.05$.

tion temperature and time were important variable to be optimized in order to minimize the energy cost of the process. The results revealed that extraction carried out at moderate temperature (44.5 °C) for shorter time (23.8 min.) was enough to saturate the solutions with phenolic compounds (Table 3). Additionally, this condition was able to minimize the possible impact on phenolics present in millet which might be heat and light sensitive.

#### 3.4.4. Verification experiments

Using the best solvent type and the best solvent concentration selected in single factor experiments, samples were extracted at various extraction temperatures ranging from 37 to 55.1 °C and time ranging from 23.8 to 66.2 minutes. The best extraction temperature was selected according to the amount of TPC obtained. Based on the results of single factor experiment, the ranges of three factors (solvent concentration, extraction temperature and extraction time) were determined for RSM (Table 2). Verification experiments were performed with the predicted conditions derived from the response surface and contour plots of RSM. It demonstrated that experimental values were reasonably close to the predicted values confirming the validity of the performed models (Table 3).

### 3.5. Effect of extraction temperature on extraction of TPC

The final step in a sequence of single factor experiments was to select an appropriate extraction temperature. The extraction of phenolic compounds increased moderately when extraction temperature was increased from 37 to 44.5 °C (Fig. 3a) However, it is imperative to note that increasing the temperature beyond certain limit may prop up decomposition of phenolic compounds which were already mobilized at low temperature or may even lead to degradation of phenolics still left over in the extracts. Moreover, high temperature may also promote solvent loss through vaporization thereby increasing the cost of extraction. Hence, moderate extraction temperature of 30 °–55.1 °C (Fig. 3a) was selected as the lower and upper limits for optimization using RSM.

### 3.6. Verification of predictive model

CCD employed a correlation between the actual experimental values and the predicted values that are generated by the response surface methodology. Table 3 showed that the experimental results were very close to the predicted one. This implied that there was a high fit degree between the values observed in experiment and the values predicted from the regression model. Hence, the response surface modeling could be applied effectively to predict extraction of phenolic compounds from millet.

To fit the response function and experimental data, the linearity and quadratic effect of the independent variables, their interactions and regression coefficients on the response variables were evaluated by analysis of variance (ANOVA) (Table 4). The ANOVA of the regression model showed that the model was highly significant due to a very low probability value ($p < 0.0001$). The fitness and adequacy of the model were judged by the coefficient of determination ($R^2$) and the significance of lack-of-fit. $R^2$ which is defined as the ratio of the explained variation to the total variation was a measure of the degree of fit [41]. The closer the $R^2$ value to unity, the better the empirical model fits the actual data [42]. By referring to Table 4, $R^2$ value for the model of TPC was 0.977, which was close to 1. This suggested that the predicted models defined well the real behavior of the system. In addition, the value of adjusted $R^2$ (0.965) was also very high to advocate for a high significance of the model. The adjusted $R^2$ was a corrected value for $R^2$ after the elimination of the unnecessary model terms. If many non-significant terms have been included in the model, the adjusted $R^2$ would be remarkably smaller than the $R^2$ [43]. In this study, the adjusted $R^2$ was very close to the $R^2$ value. Besides, the absence of any lack of fit ($p > 0.05$) also strengthened the reliability of the model.

The P-values were used as a tool for checking the significance of each coefficient, which in turn might indicated the interaction patterns between the variables [44]. The smaller the P-value, the more significant was the corresponding coefficient. It could be observed from Table 2 that both the linear and quadratic terms of all parameters (ethanol concentration, extraction temperature, and extraction time) had significant (at least at $p < 0.05$) effect on TPC. In addition, TPC was also significantly influenced by the interactions between extraction temperature and extraction time (Fig. 3). Among all the three extraction parameters studied, temperature had the most critical role in the extraction of phenolic compounds from millet followed by extraction time and ethanol concentration.

### 3.7. Antioxidant activity

Obtaining the enhanced concentration of TPC in fermented millet as compared to control, antioxidant activities of both the extracts were also determined using DPPH and ABTS anti-free radical assays.

### 3.8. Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical

Hydrogen/electron transfer is considered as an important mechanism of antioxidant action, consequently DPPH was used

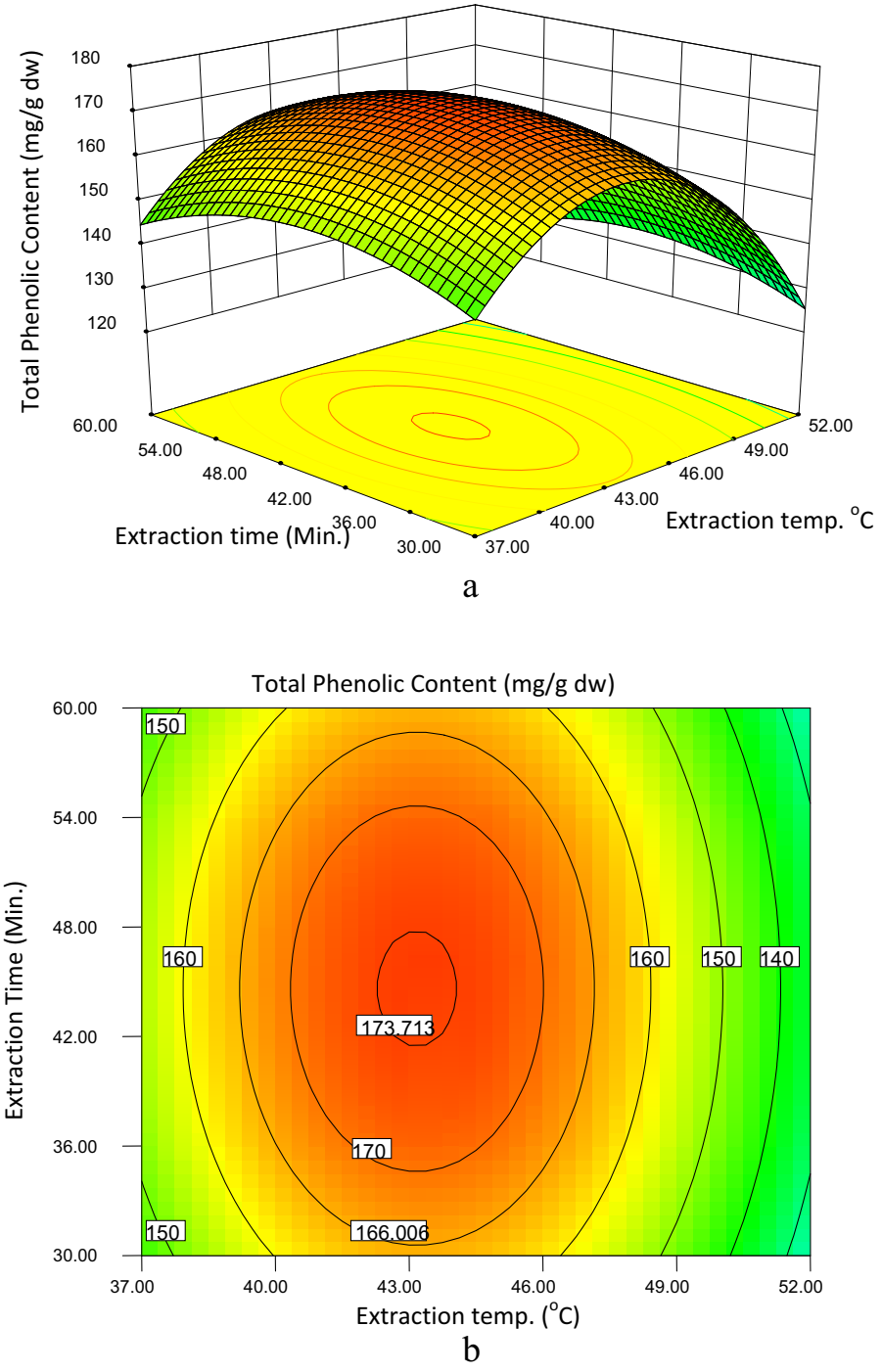


Fig. 3. Response surface plot (a) and contour plots (b) for the effect of extraction time and extraction temperature at a constant solvent concentration (ethanol 50%) on total phenolic content of millet koji.

to determine the scavenging action of fermented and unfermented millets. The DPPH radical has been widely used to test the free radical scavenging activity of various natural products [45] and has been accepted as a model compound for free radicals originating in lipids [46,47]. The purple color of DPPH solution disappears in the presence of antioxidants. The extent of discoloration indicates the scavenging potential of the extracts. In the present investigation, extracts prepared from fermented millet showed increased scavenging of the DPPH radicals. The DPPH scavenging activity of fermented millet extracts prepared in 50% ethanol was highest (262.7 µmol VCEAC/g dw) as compared to unfermented millet (233.36 µmol VCEAC/g dw) on 8th day of fermentation (Table 5). Free and bound phenolic compounds are present in grains with the levels of the later being highest in corn (85%) > oats and wheat (75%) > rice (62%) [12]. The contribution of bound phytochemicals to free radical antioxidant activity was 90% in wheat, 87% in corn, 71% in rice and 58%

Table 5
Antioxidant activity of fermented and unfermented millet extracts (VCEAC µmol/gm) prepared in 50% ethanol.

| Fermentation time | (DPPH assay) | (ABTS assay) |
|---|---|---|
| Control | 233.36 ± 0.00 | 244.28 ± 0.12* |
| 2 days | 241.7 ± 0.01 | 251.95 ± 0.85 |
| 4 days | 248.53 ± 0.01 | 255.36 ± 0.53 |
| 5 days | 254.28 ± 0.19 | 254.86 ± 0.40 |
| 6 days | 252.53 ± 0.02 | 264.11 ± 0.10 |
| 8 days | 262.7 ± 0.23 | 281.86 ± 0.31 |
| 10 days | 242.11 ± 0.02 | 249.45 ± 0.50 |

* ±Standard deviation.

in oats [12]. As investigated, SSF process offers great advantage over chemical and enzymatic processes to release the bound form of phytochemicals present in millet and thus increasing the product's antioxidant ability.

### 3.9. Scavenging of ABTS radical

The ABTS radical solution possesses a distinctly blue green color which disappears quickly in the presence of antioxidants. In the present study, ABTS radical decolorization assay also showed similar results as reported for the DPPH assay. As revealed in Table 5, the highest ABTS radical scavenging activity (281.86 µmol VCEAC/g) was observed on 8th day of fermentation of millet extracts as compared to control (244.28 µmol VCEAC/g). The obtained results of vitamin C equivalent antioxidant capacity per gram of millet were slightly higher as compared to DPPH assay. This may be explained by the fact that both lipophilic and hydrophilic antioxidants are involved in ABTS radical assay. The results were found to be significant at $P < 0.05$ level. Thus, higher antioxidant activities of fermented millet in the present study could be positively related to higher total phenolic contents which were modulated during SSF by *A. awamori*.

## 4. Conclusions

This investigation corroborates the benefit of RSM over traditional methods for optimizing the extraction conditions for phenolic compounds from fermented millet grains. RSM results depicted that the extraction of phenolic compounds from millet was mostly influenced by ethanol concentration, followed by extraction time and extraction temperature. The optimized conditions using RSM for the extraction of maximum phenolic compounds were: ethanol concentration, 50%; extraction temperature, 44.5 °C and extraction time, 23.8 mins. Under these preconditions of optimization, 176.82 mg GAE/g of phenolic compounds was extracted which was very close to the predicted value of 173.2 mg GAE/g. Thus, solid state fermentation could be successfully used to improve the nutraceutical properties of millet. The results suggested that fermented millet (millet koji) may be a healthy food supplement rich in antioxidants compared to unfermented millet. Further work is in progress to elucidate the solo or blended flour of fermented millet for preparation of various millet based food products rich in antioxidants. However, careful examination of the other nutritional parameters is also warranted.

## Acknowledgements

The work was supported by grant F. No. 41-541/2012 (SR) received from University Grants Commission (UGC), New Delhi, India for a major research project awarded to Dr. R.K. Salar.

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