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Research paper

Bio-enrichment of phenolics and antioxidant activity of combination of *Oryza sativa* and *Lablab purpureus* fermented with GRAS filamentous fungi ☆

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ABSTRACT

Cereal and legumes meet a considerable requirement of protein and carbohydrate of the local population. Most of the foods are cereal based but some cereal/legume or legume based foods are also common in many countries of Asia and Africa. In present study, the effect of fermentation on total phenolics, antioxidant activity and α-amylase enzyme activity of ethanolic extracts of each of seeds and flours combination (1:1) of *Oryza sativa* (rice) and *Lablab purpureus* (seim) was determined. The percentage inhibition of free radicals formation by DPPH and ABTS assays was found maximum i.e. 80.66 ± 0.21, 97.67 ± 0.35 on 4th day of incubation of combined sample of rice and seim seeds fermented with *Aspergillus oryzae* and *Aspergillus awamori,* respectively. The increased percentage inhibition of free radical formation of fermented samples was found greater than the non-fermented samples (65.88 ± 0.15, 42.00 ± 0.63). The TPC of substrate i.e. rice:seim seeds (1:1) was also found maximum i.e. 47.53 ± 0.20 on 5th day of fermentation with *A. awamori*. α-amylase activity of fermented samples was also found higher than that of non fermented samples. Almost similar results were obtained in combined flour extract of both the substrates. Increase in level of α-amylase enzyme during SSF indicates that enzymes produced by microorganisms were responsible for release of bound phenolics which may be responsible for increase in antoxidant activity of extracts of fermented seeds and flour combination a cereal and a legume.

1. Introduction

Cereals and pseudo-cereals are important sources of macronutrients. Cereal grains provide significant quantities of energy, protein and selected minerals in the animal and human diet. Chemical composition and bioavailability of nutrients vary between species and varieties of grains and may be affected by the forms of processing as feed and food [1]. Cereals also contain a wide range of chemical classes with antioxidant activity [2]. Cereal grains are rich in phenolic acids and saponins, while phytoestrogens and flavonoids are present in small quantities [1]. Legumes (poor man's meat) play an important role in human nutrition since they are rich sources of protein, calories, minerals and vitamins and therefore can be good supplements [3]. Legumes belong to the family leguminosae, which is probably the second most important source of food and fodder. The family leguminosae is however, a very important crop in terms of production system since grains and fodder can be obtained with minimal investment in terms of nitrogenous fertilizers. They are rich in lysine and tryptophan [4] but low in the sulfur-containing amino acids, methionine and cysteine [5]. It is often, therefore, emphasized that legume seed grain proteins are the natural supplement to cereal grain protein in producing and overall essential amino acid balance.

Cereal and legumes meet a considerable requirement of protein and carbohydrate of the local population. Wheat, barley, maize, rice and millet are the major cereals that are cultivated in many countries in Asia and Africa. Traditional foods prepared from major cereals are common in almost all of the Asian and African countries. Most of these traditional foods are based on rice, wheat and barley and some are also prepared from other grains. These products being highly nutritious, easily prepared and conveniently pre-

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served are very popular among the populations. Most of the foods are cereal based but some cereal/legume or legume based foods are also common in these countries [6].

It is well accepted that plants are the richest source of antioxidants. Among plants, cereals and legumes are prominent because they contain a wide array of phenolics [7]. Plant foods such as grains of cereals, vegetables and fruits contain a wide variety of biologically active phytochemicals [8]. Phenolic acids occurring in the grain of cereals, primarily, in bound form as conjugates with sugars, fatty acids, or proteins act as effective natural antioxidants [9]. The antioxidant properties of phenolic compounds in grains have been associated with the health benefits attributed to these crops and the value-added products derived from them. Antioxidants may play an important role in the chronic disease prevention by arresting oxidative damage caused by reactive oxygen species (ROS) to vital biomolecules such as DNA, lipids and proteins [10]. Cancer and cardiovascular diseases are ranked as the first two leading causes of death in many developed countries [11,12]. Unhealthy dietary habits, living habits and exposure to dangerous chemicals in the environment could lead to the production of more free radicals. Oxidative damage caused by free radicals may be related to cancer, atherosclerosis, diabetes, arthritis and other aging diseases [13]. The intake of antioxidant-containing food has been associated with a reduction in cardiovascular and other health risks [14–16].

Throughout history, fermentation has been used to improve product properties. Fermentation is an ancient method of food processing aimed at prolonging shelf-life and improving palatability. It may also improve digestibility and nutritional value of food and feed. Previous studies have shown that microorganisms start to modify plant constituents during fermentation [17]. Many biochemical changes occur during fermentation, leading to altered ratio of nutritive and antinutritive components of plants, which affect product properties such as bioactivity and digestibility [18]. To observe the changes in phenolics and antioxidant activities, a study on solid state fermentation of rice and seim individually with GRAS filamentous fungi was carried out in our laboratory [19,20] and found that the activities of fermented substrates were higher than the non-fermented ones. The present study was carried out to investigate the effect of fermentation on total phenolic and antioxidant activities in combination (1:1) of two substrates and their milling fraction.

2. Material and methods

2.1. Microorganisms and substrate

The fungal strains i.e. *Aspergillus oryzae* (MTCC 3107) and *Aspergillus awamori* (MTCC 548) used for solid state fermentation in present study were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh. These fungal strains are generally recognized as safe (GRAS), which are cultivated and maintained on potato dextrose agar (PDA) plates. Spore suspension was prepared having a spore count of 1×10^6 spores/mL. *Oryza sativa* (Mini mogra basmati rice) and *Lablab purpureus* (seim) were used as substrate for solid state fermentation and were procured from local market of Sirsa.

2.2. Chemicals and glassware's

The organic solvents (ethanol, hexane, methanol etc.) used in current study were from Qualigens (Mumbai, India). All other chemicals 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), gallic acid, Folin–Ciocalteu reagent, L-ascorbic acid, sucrose, sodium carbonate etc. used were of analytical grade and purchased from Hi-Media (St. Louis, USA). Glassware's used in the study were of borosil.

2.3. Substrate and inoculum preparation

Substrates were first washed and dried overnight in a hot air oven (Narang Scientific Instruments, NSW 143, Ambala, India). After dried it was used for fermentation. The fungal culture of *A. oryzae* and *A. awamori* were maintained on slants of potato dextrose agar were transferred to fresh PDA plates before starting the experiment. The inoculated plates were incubated at 25 °C for 120 h. Spore suspension of required inoculum was prepared in sterilized cellular grade water having a spore count of approximately 1×10^6 spores/mL by using haemocytometer (Bright-Line Z359629, Sigma).

2.4. Fermentation conditions

Rice and seim seeds as well as flour in 1:1 ratio (25 g : 25 g) each were taken in 500 mL Erlenmeyer flasks and then soaked in 50 mL Czapek-dox medium [$NaNO_3$ (2.5 g/L), KH_2PO_4 (1.0 g/L), KCl (0.5 g/L) and $MgSO_4$. $2H_2O$ (0.5 g/L)] at room temperature overnight. After decanting the excess media, the substrates were autoclaved at 121 °C and 15 lb^2 inch pressure for 15 min and then subsequently cooled before inoculation. The autoclaved substrates were inoculated with 5.0 mL spore suspension of selected fungal strains, mixed properly and incubated for 6 days at 30 °C. The non-fermented substrate (prepared without the addition of spore suspension) was taken as control.

2.5. Extraction of enzymes

Fermented samples were taken at every 24 h of interval, the enzymes were extracted from fermented substrate with distilled water (1:10 (w/v)) by rotation method. Extracted samples were filtered through Whatman filter paper No. 1. The supernatants were assayed for α-amylase activity.

2.6. Extraction of phenolic compounds

The fermented and non-fermented substrates were taken out from the Erlenmeyer flask at every 24 h of interval and dried in oven at 60 °C for 24 h. The dried substrates (fermented and non-fermented) were ground in an electric grinder. All samples were defatted by blending the ground material with hexane (1:5 w/v, 5 min, thrice) at room temperature. Defatted samples were air dried for 24 h and stored at −20 °C for further analysis. Defatted samples were extracted with 54% ethanol at 61 °C for 64 min [21]. The extracted samples were filtered through Whatman filter paper No. 1. The filtrate was used for determination of total phenolic content and antioxidant properties [22].

2.7. Determination of total phenolics

Total phenolic content was determined using Folin–Ciocalteu reagent [23]. The ethanolic extract 200 µL was mixed with 1.0 mL of Folin–Ciocalteu reagent and 0.8 mL of sodium carbonate Na_2CO_3 (7.5%). The contents were allowed to stand for 30 min at room temp. The absorbance was measured at 765 nm (Systronic 2202 UV–VIS spectrophotometer). Total phenol content was obtained from the regression equation and expressed as µM/g gallic acid equivalent using the following formula [24].

$$C = c.V/M$$

where C = total content of phenolic compounds in mg/g gallic acid equivalent

c = the concentration of gallic acid (mg/mL) established from the calibration curve
V = volume of extract
M = the weight of pure ethanol extract (g)

2.8. α-Amylase assay

α-Amylase activity was determined by mixing 0.25 mL of appropriately diluted enzyme (1:5 v/v) with 0.5 mL of 0.2 M acetate buffer (pH 5.0) and 1.25 mL of soluble starch (1%). After 10 min of incubation at 50 °C, the concentration of glucose liberated from starch by the action of α-amylase was estimated spectrophotometrically at 575 nm [25]. One unit (U) of amylase activity is defined as the amount of enzyme that liberates one micromole of reducing sugar (glucose) per min under the assay conditions. Results were expressed as EU (μM/mL).

2.9. DPPH radical-scavenging effect

The free radical scavenging activity was measured by DPPH assay, following Brand-Williams et al [26], method with some modification. Four mg of DPPH (0.1 mM DPPH) was dissolved in 100 mL of methanol to obtain working solution. An aliquot of ethanolic extract (200 μL) was mixed with 2.0 mL of 0.1 mM DPPH and incubated for 30 min in dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm. Color of DPPH was reduced from purple to yellow. The antioxidant activity of ethanolic extract was evaluated by calculating the % inhibition using the formula:

$$\%\text{inhibition} = [(A - A_1)/A] \times 100$$

A = absorbance of the blank
A_1 = absorbance of the extract

2.10. ABTS radical cation depolarization assay

In ABTS assay, antioxidant activity was measured using 7.6 mM (19 mg/5.0 mL) $ABTS^+$ solution and 2.6 mM potassium persulphate (3.5 mg/5.0 mL $K_2S_2O_8$) solution in 5.0 mL of distilled water. The resulting solution was left to stand for 16 h in dark at room temperature. Working solution was prepared by mixing 1.0 mL of this reaction mixture with 60 mL water [27,28]. Ethanolic extract (30 μL) was mixed with 3.0 mL of ABTS solution and optical density was measured at 734 nm after 1.0 min of incubation at room temperature using spectrophotometer. The reduction of ABTS was measured in the % inhibition as described in DPPH assay.

2.11. Statistical analysis

The mean value and standard deviation was calculated from the data obtained from the three replicates. Analyses of data were carried out by paired sample T test and by paired sample correlation using PASW statistics viewer 18. Statistical differences at $P < 0.05$ were considered as significant values (Table 3).

3. Results

3.1. Total phenolic content (TPC)

In the present investigation, total phenolic content was calculated with regression equation i.e. y = 0.0017x-0.0191 from standard curve of L-ascorbic acid (Fig. 1A) and expressed as gallic acid equivalent (GAE). Maximum TPC was observed on 5th day of fermentation (i.e. 47.53 ± 0.20 and 46.66 ± 0.21 GAE μM/mL) of whole seed but in fermented flour TPC was found maximum (i.e. 46.83 ± 0.26 and 39.55 ± 0.16 GAE μM/mL) on 4th day of incubation with *A. awamori* and *A. oryzae*, respectively. These values of fermented seed as well as flour were found higher than the non-fermented one (29.48 ± 0.20 and 16.16 ± 0.26) as shown in Fig. 1B.

Table 1
Effect of fermentation time on percent inhibition of free radical formation by *Lablab purpureus* and *Oryza sativa* seed and flour extracts (54% ethanol) using DPPH assay (mean ± SD).

Fermentation time (in days)	% inhibition of DPPH Seed (rice:seim)		% inhibition of DPPH Flour (rice:seim)	
	A. oryzae	*A. awamori*	*A. oryzae*	*A. awamori*
Control	65.88 ± 0.15	66.97 ± 0.35	61.37 ± 0.21	60.07 ± 0.20
2	71.20 ± 0.42	72.97 ± 0.30	64.22 ± 0.30	64.10 ± 0.28
3	72.39 ± 0.13	72.25 ± 1.29	67.39 ± 0.28	67.29 ± 0.20
4	80.66 ± 0.21	80.61 ± 0.18	74.15 ± 0.30	65.81 ± 0.18
5	63.38 ± 0.35	71.48 ± 0.26	73.28 ± 0.25	61.57 ± 0.31
6	60.29 ± 0.23	70.88 ± 0.13	63.20 ± 0.13	60.08 ± 0.27

SD, standard deviation.

Table 2
Effect of fermentation time on percent inhibition of free radical formation by *Lablab purpureus*and *Oryza sativa* seed and flour extracts (54% ethanol) using ABTS assay (mean ± SD).

Fermentation time (in days)	% inhibition of DPPH Seed (rice:seim)		% inhibition of DPPH Flour (rice:seim)	
	A. oryzae	*A. awamori*	*A. oryzae*	*A. awamori*
Control	45.22 ± 0.43	42.00 ± 0.63	46.57 ± 0.64	43.87 ± 1.22
2	63.78 ± 0.56	55.71 ± 0.45	64.29 ± 0.57	56.04 ± 0.49
3	73.89 ± 0.35	69.51 ± 0.42	73.94 ± 0.63	60.09 ± 0.57
4	95.66 ± 0.56	97.67 ± 0.64	84.01 ± 0.43	73.43 ± 0.64
5	82.98 ± 0.70	96.22 ± 0.35	71.66 ± 0.85	71.89 ± 1.14
6	95.29 ± 0.43	86.25 ± 0.77	61.17 ± 0.77	68.95 ± 0.50

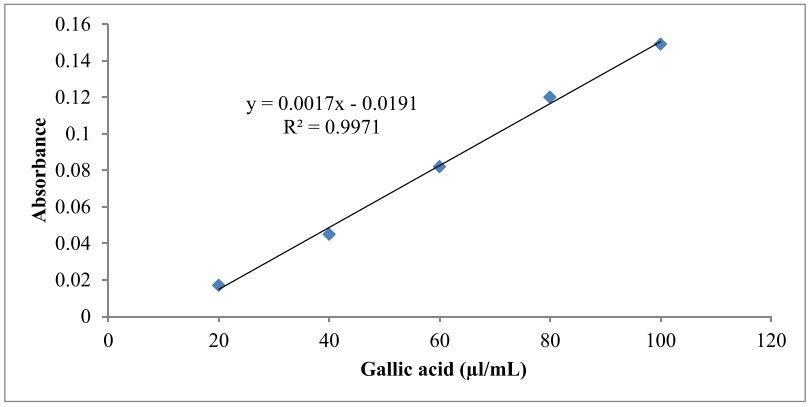
SD, standard deviation.

3.2. DPPH radical-scavenging assay

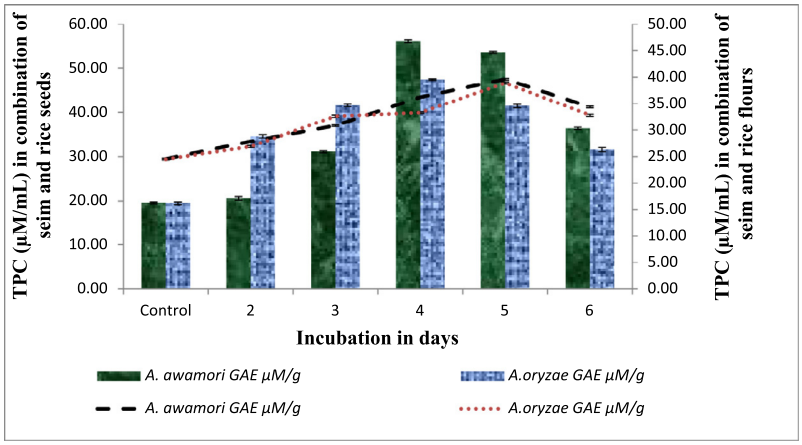
The results showed that maximum percentage inhibition (80.66 ± 0.21 and 80.61 ± 0.18) of free radicals formation was found on 4th day of incubation in combination (1:1) of seeds of rice and seim when fermented with both the fungi i.e. *A. oryzae* and *A. awamori,* respectively. The percentage inhibition of free radicals formation was more in fermented substrate than the non-fermented one. However, the inhibition of free radical formation of fermented flours combination was observed maximum on 4th and 3rd day of incubation (74.15 ± 0.30 and 67.29 ± 0.20) with *A. oryzae* and *A. awamori*, respectively, but it was less than the fermented seeds (Table 1). Both the fermented seeds as well as flours combinations of seim and rice showed the higher percentage inhibition than the non fermented ones (60.07 ± 0.20 and 66.97 ± 0.35).

3.3. ABTS radical scavenging assay

According to the results shown in Table 2 the fermented seed extract of rice and seim (1:1) showed highest (95.66 ± 0.56 and 97.67 ± 0.64) scavenging of ABTS radicals on 4th day of incubation with *A. oryzae* and *A. awamori*, respectively. Similarly in case of fermented flour combination of rice and seim the maximum inhibition i.e. 84.01 ± 0.43 and 73.43 ± 0.64 was observed on 4th day after fermentation with both the fungi. These results showed similarity with DPPH assay as the value of fermented combination of rice and seim seed as well as flour were higher than the non-fermented samples (42.00 ± 0.63 and 46.57 ± 0.64). The Table 3 showed that the total phenolic content of both seed and flour of rice and seim was significantly correlated ($P < 0.05$) with the ABTS values.



(A)



(B)

Fig. 1. **(A)** Standard graph of TPC with R^2 value **(B)** Total phenolic content (µM/mL) in combination of seim and rice seeds and flours extracts (54% ethanol) fermented with *Aspergillus oryzae* and *Aspergillus awamori* (Error bar represents SD, n = 3).

Table 3
Paired sample correlation by using PASW statistics viewer 18. Statistical differences at $P < 0.05$ were considered as significant value.

Substrate	TPC DPPH	TPC DPPH	TPC ABTS	TPC ABTS
	A. oryzae	*A. awamori*	*A. oryzae*	*A. awamori*
Combination of seeds (seim:rice)	0.089†	0.499†	0.912*	0.975*
Combination of flours (seim:rice)	0.876*	0.169†	0.992*	0.899*

* $P < 0.05$ (significant).
† $P > 0.05$ (non significant).

3.4. α-Amylase assay

The present investigation was also focused on the α-amylase activity under the solid substrate fermentation for the enhanced release of polyphenol from rice and seim seed as well as flour. Highest α-amylase activities (1.36 ± 0.02, 0.92 ± 0.01 and 1.29 ± 0.14, 0.96 ± 0.01 EU (µM/mL) of each of seed and flour of the substrates were observed on 3rd day of fermentation with *A. awamori* and *A. oryzae*, respectively. The fermented samples showed higher enzyme activity than the non-fermented ones i.e. 0.52 ± 0.02, 0.79 ± 0.01 and 0.65 ± 0.01, 0.64 ± 0.04 EU (µM/mL) in both the substrate as shown in Fig. 2.

4. Discussion

Phenolic compounds, present in all plants, are of great importance for food and beverages derived from plants, since these compounds are responsible for their organoleptic properties. As a consequence, they are closely related to the quality of such products, and thus their analysis is of considerable interest [29]. Legumes and cereals are rich sources of polyphenolic compounds. Here, in the current research the phenolic content of substrate augment with solid state fermentation and phenolic content increases with increase in incubation time (in days). Maximum phenolics was found on the 5th day of incubation (Fig. 1B) of seed combination and on the 4th day of flour combination of the substrate (rice:seim) with both the fungi i.e. *A. awamori* and *A. oryzae*.

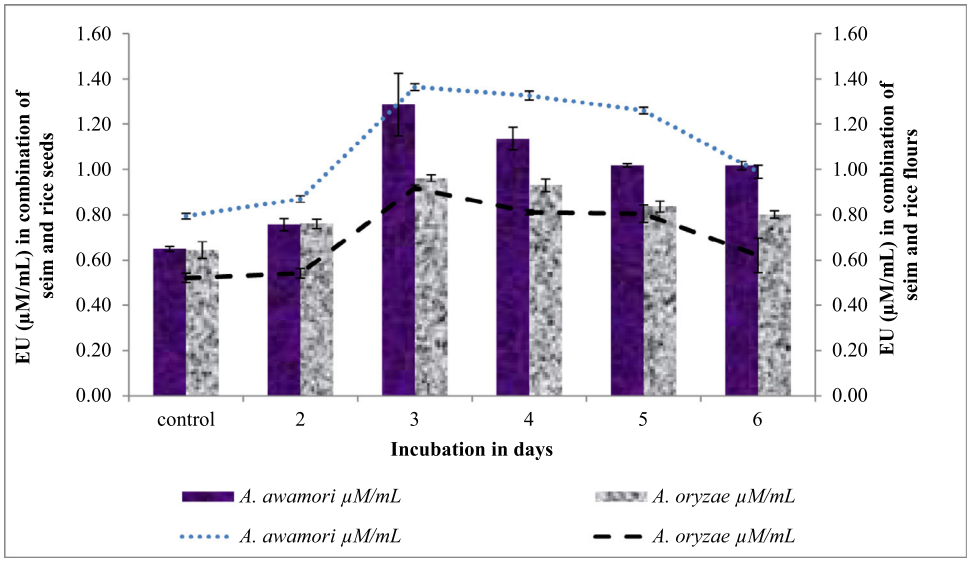


Fig. 2. α-Amylase activity of fermented and non-fermented seed and flour in combination of seim and rice at different incubation periods (Error bar represents, SD, n = 3).

After attaining the maximum value, phenolics content in the sample decreases gradually. Previous studies suggested that phenolic content of substrate is correlated with their antioxidant activities [30]. Increased phenolic content in fermented samples as compared to non-fermented samples was also found in earlier investigation with different substrates [20,31].

Cereal and legumes provide significant quantities of energy, protein and selected micronutrients in the animal and human diet. Significant levels of antioxidants have been detected in cereals, cereal-based products and legumes [32,33]. Antioxidants are present naturally in the plants and their related foods. The naturally present antioxidants increase with fermentation techniques [34–36]. In this study DPPH and ABTS radical scavenging assays were performed for measurement of free radical scavenging activities of rice and seim. A freshly prepared DPPH solution exhibited a deep purple color with a maximum absorption at 517 nm. This purple color disappears when an antioxidant is present in the medium. Thus, antioxidants molecules can quench DPPH free radicals and convert them to a colorless product, resulting in a decrease in absorbance at 517 nm [37]. The ABTS assay is also based on the ability of the antioxidants present in the sample to scavenge the cation radical of $ABTS^{+}$. This scavenging results produced a decrease in the absorbance at 734 nm [38,39]. The percentage inhibitions of free radicals formation of both the assays were found maximum at 4th day of incubation of seed and flour combination (rice:seim). The fermented seed combination showed higher inhibition then fermented flour combination when incubated with *A. awamori* and *A. oryzae*. α-Amylase is one of the most important enzymes which can be used in a number of industrial processes. Studies on fungal amylase especially in the developing countries have been concentrated on *Aspergillus* species, probably because of ubiquitous nature and non-fastidious nutritional requirements [40]. During fermentation, fungus releases various enzymes [41,42] and the same has been observed in this study where maximum amylase activity was observed on 3rd day after incubation with *A. oryzae* and *A. awamori*. The main objective i.e. to increase the phenolics, antioxidants and amylase activity through fermentation was found in this study because the resulted value of fermented samples was higher than the non-fermented samples. Earlier studies also showed that the value of fermented substrates were greater than the non-fermented samples [43,44].

5. Conclusions

Cereal and legume based foods are most popular followed by fruit, vegetable and milk based products. Results obtained from the present work showed that through SSF, the level of phenolics can be increased. Enzymatic release of bound phenolics increases the TPC values as well as the antioxidant property during fermentation. So, fermented substrate possesses more phenolics and antioxidant activities as compared to the non-fermented substrate. In this study increase in level of amylase enzyme is directly correlated with increase in TPC. So, it could be concluded from the results of present study that the fermented ethanolic extract of rice and seim seed combination as compared to flour will be an antioxidant rich and healthy food supplement compared to non-fermented seed and flour combination of rice and seim.

Conflict of interest

There is no conflict of interest in present investigation.

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