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Research paper

Bio-augmentation of antioxidants and phenolic content of *Lablab purpureus* by solid state fermentation with GRAS filamentous fungi ☆

Pardeep Kumar Sadh, Pooja Saharan, Joginder Singh Duhan*

Department of Biotechnology, Chaudhary Devi Lal University, Sirsa 125055, Haryana, India

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ABSTRACT

The present study was conducted to find out the effect of solid state fermentation on release of phenolics and subsequently on improvement of antioxidant activity of fermented seed and flour of *Lablab purpureus* (seim), using GRAS filamentous fungi i.e. *Aspergillus awamori* and *Aspergillus oryzae*. Significant increase in TPC level was observed on 5th day of fermentation of seed and flour with *A. awamori* and *A. oryzae* as compared to non-fermented ones. In DPPH and ABTS antioxidant assay, maximum activity was noticed in fermented ethanolic extract of seim seed with *A. awamori* and *A. oryzae* on 3rd and 4th day of incubation, respectively. The findings showed higher antioxidant activity formation in fermented seim seed than flour. Significant increase in enzyme activity of α-amylase was also contributed by SSF. This study demonstrated that fermented seed and flour of seim are better source of phytochemicals compared to the non-fermented ones.

1. Introduction

In developing countries, the major source of protein is legumes and the food from legumes includes pulses and oil seeds. The groups of pulses have dry seeds of cultivated legumes which are traditional food. The oil seeds group used primarily for their oil content consisting of soybean and peanut etc. The traditional processed food products from legumes can be classified into two categories: (i) non-fermented and (ii) fermented. The fermented food includes koji, milk, tofu, curd, tempeh, yuba etc. Legumes are rich source of polyphenolic complexes. Nowadays, there is considerable interest in the antioxidant activity of these complexes and in their potential health benefits, especially in the prevention of cancer and cardiovascular disease [1]. Dark colored legumes like red kidney beans, black beans, black gram and soybean have higher amount of these polyphenolic compounds [2].

Researcher performed various studies to find out the effect of solid state fermentation on different legumes with various GRAS microorganisms, and they were found that fermentation caused a marked increase in the content of bioactive compounds compared with the unfermented legumes [3]. Furthermore, the fungi-fermented legumes were noted to show an increase in the content of total extractable phenolics and anthocyanins, as well as increased antioxidative activity. The consumption of antioxidants and phytonutrient-containing foods may reduce this degenerative process [4]. Consequently, the intake of food derived antioxidants in our daily diet is widely recommended as a strategy for reducing the oxidative damage caused by free radicals, thus yielding a beneficial effect on human health [5].

Oxidative stress occurs when the generation of reactive molecules called free radicals is beyond the protective capability of the antioxidant defenses [6]. Free radicals are chemically active atoms or molecular fragments that have a charge due to an excess or deficient number of electrons. Free radicals containing oxygen known as reactive oxygen species (ROS) are most biologically significant free radicals. The oxidative damage induced by free radicals has been implicated in different diseases. Plants exhibit strong antioxidant activities [7] and have the ability to scavenge the free

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* Corresponding author. Department of Biotechnology, Chaudhary Devi Lal University, Sirsa 125055, Haryana, India. Tel.: +91 1666243147; fax: +91 1666248123.
E-mail address: duhanjs68@gmail.com (J.S. Duhan).

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radicals [8]. Antioxidants may be defined as radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ischemia, anemia, asthma, arthritis, inflammation, neurodegeneration, Parkinson's diseases, Mongolism, ageing process and perhaps dementias. Flavonoids and flavones are widely distributed secondary metabolites with antioxidant and antiradical properties [9]. The use of natural antioxidants is limited by a lack of knowledge about their molecular composition, amount of active ingredients in the source material and the availability of relevant toxicity data. Natural antioxidants tend to be safer and they also possess antiviral, anti-inflammatory, anti-cancer, antitumor and hepato-protective properties [10]. Natural antioxidants also protect the human body from the oxidative damage caused by free radicals [11–13].

In the present investigation, total phenolic contents and antioxidant potential of ethanolic extracts of fermented and non-fermented *Lablab purpureus* was evaluated using DPPH (1, 1-diphenyl-2-picrylhydrazyl) and ABTS (2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay.

2. Materials and methods

2.1. Substrate collection

Lablab purpureus (seim) was obtained from Sirsa, Haryana (India). The microorganisms used for fermentation i.e. *Aspergillus oryzae* (MTCC 3107) and *Aspergillus awamori* (MTCC 548) were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology Chandigarh, India. Potato dextrose agar and Czapek-dox medium were procured from Sigma Aldrich Co. (St. Louis, USA). The organic solvents (ethanol, methanol, and hexane) used for the present study were procured from Thermo Fisher Scientific India Pvt. Ltd., (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 in this study were of Hi Media. All chemicals used were of AR grade. Triple distilled water and acid washed glassware were used throughout the experiments.

2.2. Preparation of substrate

Substrate i.e. seim (*Lablab purpureus*) was first washed and dried overnight in a hot air oven (Narang Scientific Instruments, NSW 143, Ambala, India) at 30°C before use, then it was used directly as seed but in case of flour, it was grinded in a grinder (Sujata 2632, India) to make fine powder for fermentation, and stored in air tight container at 4–7°C temperature for further studies.

2.3. Preparation of inoculum

The fungal cultures of *Aspergillus oryzae* and *Aspergillus awamori* were maintained on slants of potato dextrose agar and were transferred to fresh PDA plates before starting of each experiment. The inoculated plates were incubated at 25°C for 120 h. Spore suspension was prepared in sterilized cellular grade water having a spore count of approximately 1×10^6 spores/ml by using hemocytometer (Bright-Line Z359629).

2.4. Fermentation conditions

Fifty grams of seed and flour was 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 (30°C) overnight. After decanting the excess media, the substrates were autoclaved (Vertical autoclave, Calton, NSW-227, India) and then subsequently cooled at room temperature before inoculation. The autoclaved substrate was inoculated with 5.0 ml spore suspension (1×10^6 spores/ml) of fungal strain, mixed properly and incubated in BOD incubator (Calton, NSW-152, India) for 0, 48, 72, 96, 120 and 144 h, respectively at 30°C. The non-fermented substrate as raw material was prepared without the addition of spore suspension.

2.5. Extraction of enzymes

Fermented samples were taken after 0, 48, 72, 96, 120 and 144 h of interval during fermentation respectively, and the enzymes were extracted from fermented substrate with triple distilled water (1:10 w/v). Extracted enzyme samples were filtered through Whatman filter paper No.1. The supernatant was used for enzyme assay.

2.6. α-amylase assay

α-amylase activity of unfermented and solid stated fermented samples 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 (1.0%). After 10 min of incubation at 50 °C, the concentration of glucose liberated from starch by the action of α-amylase was estimated using UV/VIS spectrophotometer (Systronic 106 UV–VIS spectrophotometer) at 575 nm [14]. One unit of amylase activity was calculated 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).

2.7. Extraction of phenolic compounds

The fermented samples was 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 grinded in an electric grinder (Sujata-2632, India). All the samples were defatted by blending the ground material with hexane thrice (1:5 w/v, 5 min) 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 [15]. The extracted samples were filtered through Whatman filter paper No. 1. The filtrate from extracted samples was used for determination of total phenolic content and antioxidant properties.

2.8. Determination of total phenolics

Total phenolic content was determined using Folin–Ciocalteu reagent [16]. The ethanolic extract (200 µl) was mixed with 1 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) [17]. Total phenol value was obtained from the regression equation and expressed as µM/g gallic acid equivalent using the formula [18]:

$$C = c.V/M$$

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 plant ethanolic extract (g).

2.9. DPPH radical-scavenging effect

The free radical scavenging activity was measured by DPPH assay following Brand-Williams et al. [19] 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 ml of 0.1 mM DPPH and incubated for 30 minutes in dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm (Systronic 2202 UV–VIS spectrophotometer). Color of DPPH was reduced from purple to yellow. A standard curve was prepared by using different concentrations of vitamin C. The reduction in the absorbance of DPPH solution at different concentrations of vitamin C over a period of 30 min was measured and was plotted. The DPPH radical scavenging activities of seim extracts were expressed as in µM/g VCEAC [20]. Vitamin C equivalent antioxidant capacity (VCEAC) was calculated by using this formula:

$$VCEAC = \Delta \text{ Abs} - a/b$$

Where, a: y-intercept of vitamin C standard curve

b: slope of vitamin C standard curve
Δ Abs: the initial absorbance of blank minus the resulting absorbance of samples.

2.10. ABTS radical cation depolarization assay

In ABTS assay, antioxidant activity was measured using 7.6 mM (19 mg/5 ml) $ABTS^+$ solution and 2.6 mM potassium persulphate (3.5 mg/5 ml $K_2S_2O_8$) solution in 5 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 [21,22]. Ethanolic extract (30 µl) was mixed with 3.0 ml of ABTS solution and optical density was measured at 734 nm after 1 min of incubation at room temperature using spectrophotometer (Systronic 2202 UV–VIS). The reduction of ABTS was expressed in µM/g VCEAC as described in DPPH scavenging assay.

2.11. Statistical analysis

The means and standard deviation was calculated using Microsoft Excel, 2010 (Microsoft Corp., Redmond, WA, USA) from the data obtained with three determination. Analysis of data was performed by paired sample T test by using PASW statistics viewer 18. Statistical differences at $P < 0.05$ were considered as significant value.

3. Results and discussion

3.1. Total phenol content (TPC)

Plant foods have phenolic compounds, which affect their appearance, taste, odor and oxidative stability. In cereal grains, these compounds are located mainly in the pericarp [23]. The major phenolic acids in cereals are ferulic and p-coumaric acids [24–26]. The popular usage of legumes is due to their nutritional profile. Legumes contain protein as well as polyphenolic compounds. It also contains anti-nutritional factors such as phytic acid, oligosaccharides, trypsin inhibitor etc. So, these anti-nutritional factors get minimized by fermentation with GRAS (generally recognized as safe) fungi. The nutritional value of fermented legumes and their products with additional nutritional factors is then largely enhanced [1].

In the present study, TPC of fermented and non-fermented substrates was determined by regression value i.e. $y = 0.025x - 0.063$ (Fig. 1A) and expressed as gallic acid equivalents (GAE). The maximum total phenolic content i.e. 37.03 ± 0.30, 34.61 ± 0.24 and 44.96 ± 0.47, 46.78 ± 0.17 µM/g GAE was found in 54% ethanolic extracts of *Lablab purpureus* seed and flour fermented with *A. awamori* and *A. oryzae*, respectively. The values of fermented samples were higher than that of the non-fermented seim extracts i.e. 21.90 ± 0.27, 27.63 ± 0.27 µM/g GAE. Bhanja et al. [27] reported that as compared to unfermented wheat, the total phenol content and antioxidant activity of fermented wheat was found maximum. Vattem and Shetty [28] also noted a significant increase (Pb 0.05) in total phenolic content in the kojis prepared with *A. awamori*, *R. azygosporus* and *Rhizopus* sp. No. 2 compared with the unfermented control black bean.

3.2. Antioxidant assays

3.2.1. DPPH radical-scavenging assay

The DPPH radical assay measures the capacity of antioxidants to directly react with DPPH radicals. DPPH, a protonated radical, has characteristic absorbance at 517 nm, which decreases with the scavenging of the proton radical that used to estimate the free radical scavenging result of natural antioxidants [29]. In this study, the antioxidant activity of fermented and non-fermented substrates was determined by regression value i.e. $y = 0.010x - 0.062$ (Fig. 1B) and expressed as vitamin C equivalent antioxidant capacity (VCEAC). VCEAC of fermented ethanolic extracts of seim seed and flour was observed maximum (i.e. 1130.33 ± 5.85, 1113.86 ± 4.25 and 895.15 ± 4.74, 896.91 ± 4.58) on 5th day of incubation with *A. awamori* and *A. oryzae*, respectively which was higher than the non-fermented ethanolic extracts (i.e. 901.46 ± 6.64, 965.95 ± 7.10 and 607.75 ± 4.21, 548.87 ± 10.14). Figs 2 and 3 showed the TPC and DPPH value of fermented and non-fermented ethanolic extracts of seim seed and flour respectively. From the results, it is depicted that as the value of TPC increased considerably, it also influence the antioxidant activity positively as well, in the same manner both in fermented seim seed and flour, and it was found to be significantly ($P < 0.05$) correlated ($r^2 = 0.961$, 0.845 and 0.802) (Table 1) which provides the indication that the major source of antioxidant activity is derived from phenolic compounds in seim. Different studies observed the en-

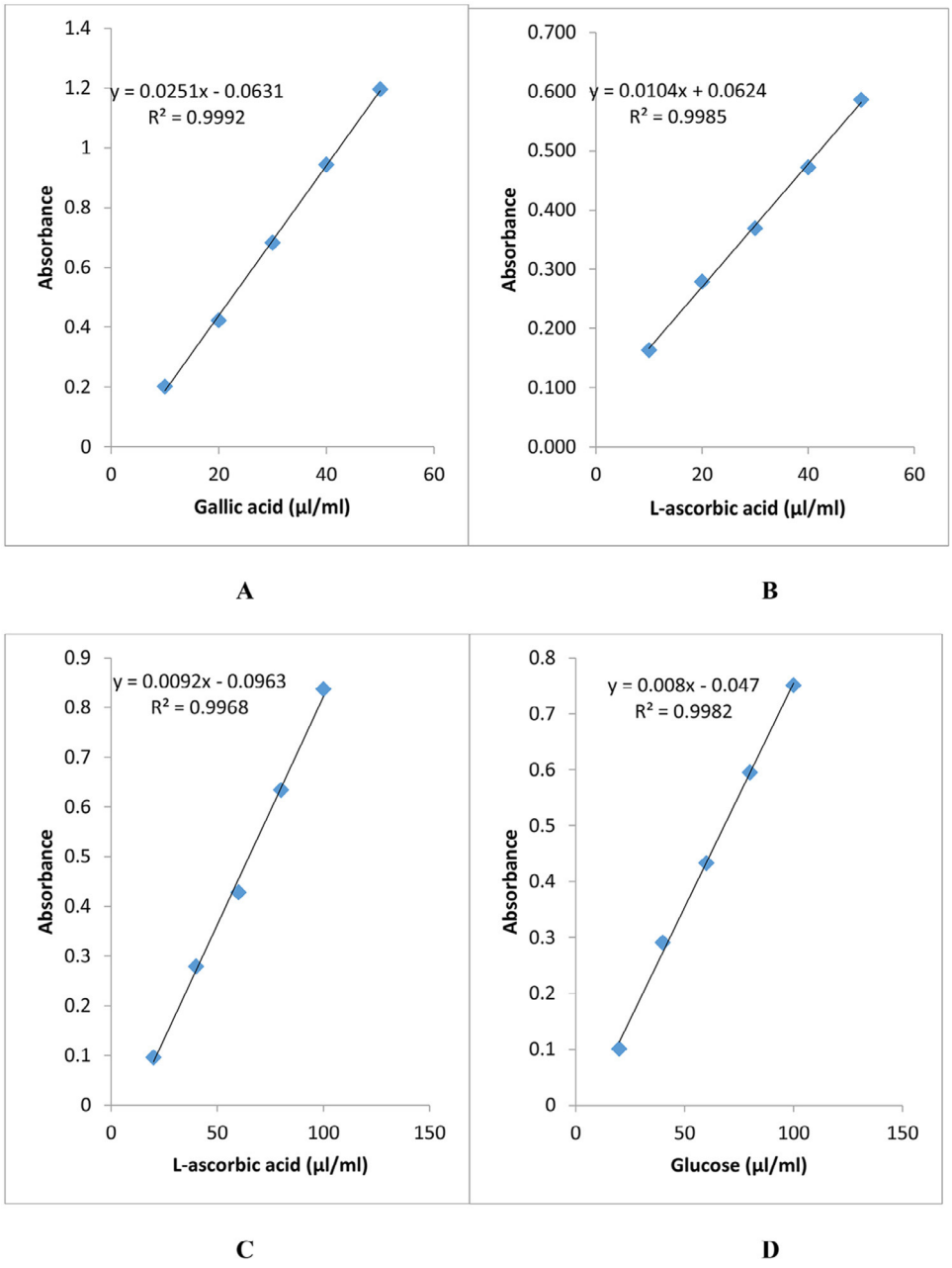

Fig. 1. Standard graphs with their R^2 value. (A) TPC (B) ABTS (C) DPPH (D) α amylase.

hanced DPPH value in form of percentage inhibition of fermented samples than the non-fermented ones [30–32].

3.2.2. ABTS radical cation assay

ABTS radical cation assay, applicable to both lipophilic and hydrophilic antioxidant also showed quite similar results compared to those obtained in DPPH assay. In the present study, $ABTS^{++}$ radical scavenging activity of fermented and non-fermented substrate were determined by regression value i.e. $y = 0.009x - 0.096$ (Fig. 1C) and expressed as vitamin C equivalent antioxidant capacity (VCEAC). The highest $ABTS^{++}$ antioxidant capacity i.e. 705.33 ± 2.89 and 702.80 ± 3.32 was observed in seim seed and 559.59 ± 3.04 and 573.16 ± 3.96 in flour on 4th day of incubation with *A. oryzae* and *A. awamori*, respectively. After that subsequently decreased in antioxidant value was recorded in both the fungi. The VCEAC values of fermented samples of seim seed and flour were higher than the non-fermented one (335.63 ± 4.76, 442.88 ± 1.89 and 322.07 ± 4.27, 294.31 ± 5.27) as shown in Figs 4 and 5. Bhanja et al. [27] also observed the enhanced VCEAC value of fermented samples. Total phenolic content and ABTS value were found significant ($P < 0.05$) and highly correlated ($r^2 = 0.940$, 0.813, 0.903 and 0.931) (Table 1), which again demonstrated that phenolics are responsible for antioxidant activity in seim seed and flour. Several researchers have reported positive correlation between antioxidant assays and total phenolic content [33–35].

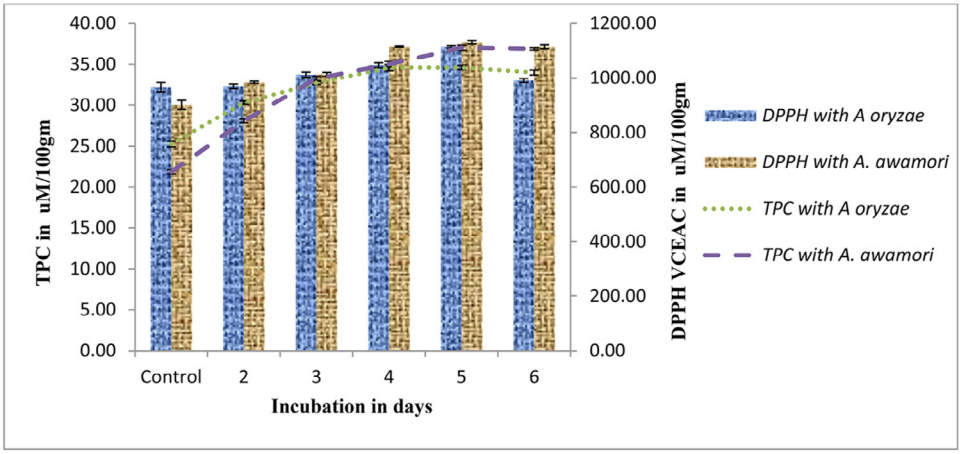

Fig. 2. TPC and DPPH values with *A. oryzae* and *A. awamori* of seim seed extracts (54% ethanol) (error bar represents SD, n = 3).

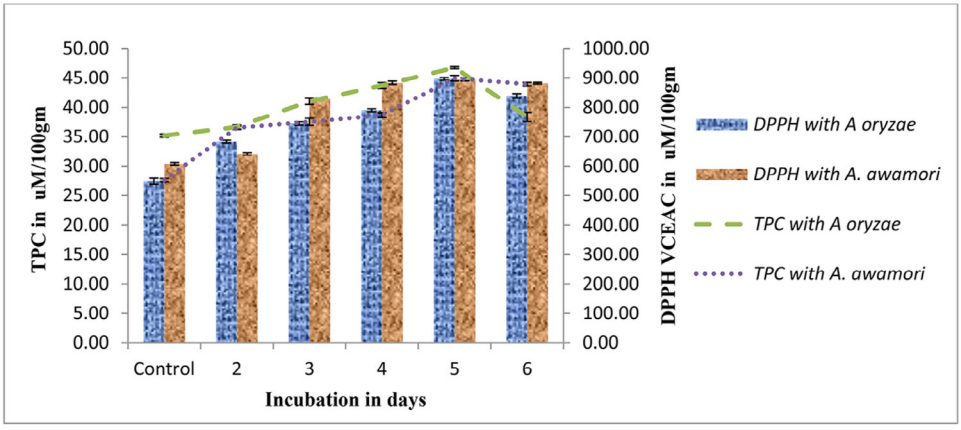

Fig. 3. TPC and DPPH values with *A. oryzae* and *A. awamori* of seim flour extracts (54% ethanol) (Error bar represents SD, n = 3).

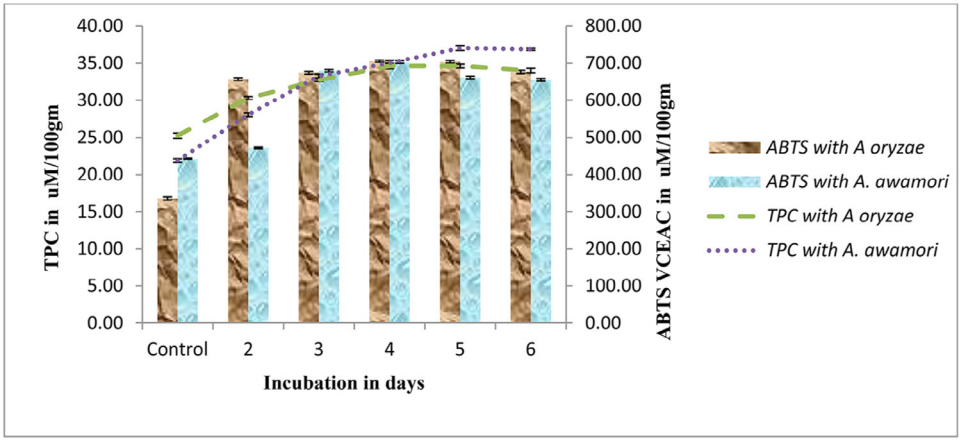

Fig. 4. TPC and ABTS values with *A. oryzae* and *A. awamori* of seim seed extracts (54% ethanol) (error bar represents SD, n = 3).

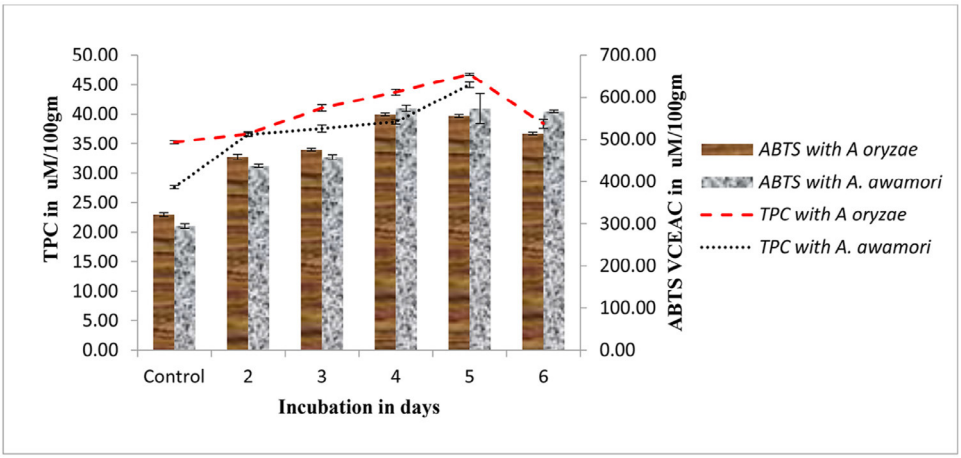

Fig. 5. TPC and ABTS values with *A. oryzae* and *A. awamori* of seim flour extracts (54% ethanol) (error bar represents SD, n = 3).

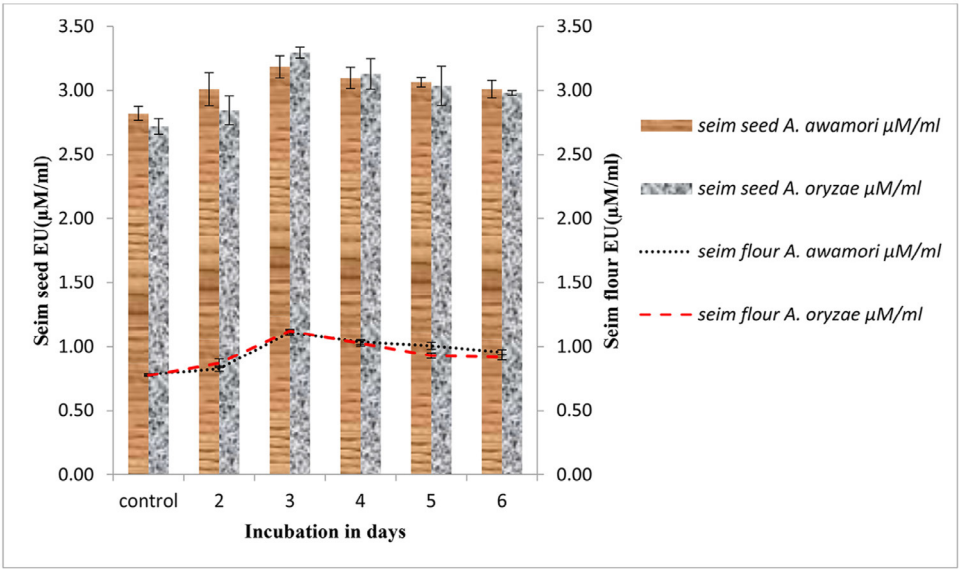

Fig. 6. α-Amylase activity of fermented and non-fermented seed and flour of seim at different incubation periods (error bar represents, SD, n = 3).

Table 1
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*
Seim seed	0.679**	0.961*	0.940*	0.909*
Seim flour	0.802*	0.845*	0.813*	0.931*

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

3.3. α amylase assay

In plants, phenolics are usually found in conjugated forms through hydroxyl groups with sugar as glycosides [36]. This condition lowers the antioxidant activity because availability of free hydroxyl group on the phenolic is used for the conversion of free radicals. In this study, phenolic content increased after fermentation, which may be due to the fact that hydrolytic enzymes produces by the fungi, catalyze the release of aglycone from the substrate and hence increase the phenolic content as well as antioxidant activity. These phenolics can be released either enzymatically or by hydrolyzing the seim koji under alkaline or acidic condition.

During solid state fermentation, fungus releases enzymes [37,38], which may be responsible for enzymatic hydrolysis of the

substrate and hence releases the phenolics from it. Keeping this in mind, amylase assay was conducted so that role of enzyme in release of phenolics can be studied. In the present study, α-amylase activity of fermented and non-fermented substrate were determined by regression value i.e. $y = 0.008x - 0.047$ (Fig. 1D) and expressed as enzyme unit (EU). Highest amylase activities of seim seeds were observed on 3rd day of incubation (3.19 ± 0.09 and 3.30 ± 0.04 µM/ml) with *A. awamori* and *A. oryzae* respectively, whereas in seim flour it was 2.12 ± 0.02 µM/ml with fungus *A. awamori* and 1.78 ± 0.02 µM/ml with *A. oryzae*. Fig. 6 showed that the fermented samples noticed higher enzyme activity as compared to the non-fermented one.

4. Conclusion

The current research work was based on fact that from SSF the level of phenolic content can increase and the result of the present study indicates that the fermented seed and flour of the seim possess more phenolics as compared to unfermented substrate which in turn responsible for its higher antioxidant activity. This study further reveals that during SSF, fungus releases several enzymes which may contribute in the enhancement of total phenolic content. Increase in level of amylase enzyme is directly correlated with increase in TPC thereby results in significant ($P < 0.05$) increase in their antioxidant activity which proves that enzymes play a key role in release of bound phenolics during solid state fermentation. The popular usage of legumes is due to their nutritional profile. The nutritional value of fermented legumes and their products with additional nutritional factors is then largely enhanced.

Conflict of interest

There is no conflict of interest in the present investigation.

Acknowledgments

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