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Bioaugmentation of phenolics and antioxidant activity of *Oryza sativa* by solid state fermentation using *Aspergillus* spp.

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Abstract

In the present study, solid state fermentation was carried out by using GRAS filamentous fungi i.e. *Aspergillus awamori* MTCC 548 and *Aspergillus oryzae* MTCC 3107. It was found that fermented seed and flour of rice extracted with ethanol showed highest total phenolic content on 4th of fermentation with *A. awamori* i.e. 281.67±1.63 µM GAE/100g, 264.95±1.57 µM GAE/100g) and on 5thday with *A. oryzae* i.e. 212.57±1.77, 213.88±2.16 while antioxidant activity was maximum on 4thday of incubation with *A. oryzae* i.e. 1120.17± 5.79 µM VCEAC /100g and on 3rdday with *A. awamori* i.e.1025.89±15.50 µM VCEAC/100g, respectively. Higher antioxidant activity in seed may be due to the presence of higher total phenolic content (TPC) in seed as compared to flour because phenolics are responsible for antioxidant activity of plants. Amylase activity in fermented samples was also found higher than that of non-fermented samples which indicates that increase in phenolic content of fermented rice samples was attributed to the enzymatic action of amylase. This study demonstrated that fermented seed and flour of rice may be a good source of natural antioxidants as compared to non-fermented rice.

Introduction

Antioxidants play a major role in the prevention and treatment of a variety of diseases. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. The free radicals produced by oxidation reactions start chain reactions that damage cells. Activated oxygen in free radicals can cause oxidative injury to living organisms and thus play an important role in many lifestyle-related diseases such as arthritis, atherosclerosis, emphysema and cancer (Kehrer, 1993; Jacobs *et al*., 1999). Antioxidant terminates these chain reactions by removing free radical intermediates and inhibits other oxidation reactions by being oxidized themselves (Sies, 1997). The generated reactive oxygen species (ROS) are detoxified by the antioxidants present in the body. Antioxidants function in the body as free-radical scavengers, complexes of pro-oxidant metals, reducing agents and quenchers of singlet-oxygen formation (Andlauer and Furst, 1998). Lin and Yen (1999) suggested that the intake of food-derived antioxidants may reduce oxidative damage and have a corresponding beneficial effect on human health. Cereal-based food contains antioxidants that may contribute to the health benefits by reducing the incidence of aging-related chronic diseases, heart diseases, cancer and diabetes (Miller *et al*., 2000). In recent years, several undesirable disorders have developed due to the side-effects of the use of synthetic antioxidants commonly applied in the food and flavoring industries.

Cereals and legumes i.e. barley, corn, nuts, oats, rice, sorghum, wheat, beans, pulses etc. are the main sources of dietary polyphenols (Scalbert *et al.*, 2000; Escarpa and Gonzalez, 2001; Prakash *et al.*, 2012). Phytochemicals present in cereals are responsible for their antioxidant activity (Awika and Rooney, 2004; Belobrajdic and Bird, 2013). In plant foods phytochemicals believed to exert health beneficial effects, as they combat oxidative stress in the body by maintaining a balance between oxidants and antioxidants, therefore, food industry is concentrating on plant phenolics, as they retard oxidative degradation of biomolecules like lipids, DNA and proteins (Jacobs *et al*., 2001). Much attention has been paid on the use of antioxidants, especially natural antioxidants to inhibit lipid peroxidation or to protect the human body from the oxidative damage by free radicals (Yang *et al*., 2000; Duhan *et al*., 2011a; Duhan *et al*., 2011b; Saharan *et al*., 2012; Saharan and Duhan, 2013; Rana *et al.*, 2014; Duhan *et al.*, 2015). Phenolic compounds are plant-derived antioxidants that possess metal-chelating capabilities

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and radical-scavenging properties (Bors and Saran, 1987; Lopes *et al*., 1999). Phenolic acids are the major phenyl propanoid components in cereals and different levels of these phenolics are found in different fractions of cereals. Phenolic acids have been reported to selectively block the biosynthesis of leukotrienes, components involved in immune regulation diseases, asthma and allergic reactions (Koshihara *et al.,* 1984).

Therefore, the search for new products with antioxidative properties is very active domain of research. As cereals are main dietary component, so it is necessary to explore cereals and pseusdocereals for their level of phenolic content.Fermentation is used to create foods with nutritional value far superior to that of the things most modern peoples eat, and to preserve these foods without freezing or canning. Solid-state fermentation (SSF) is shown to be particularly suitable for the production of enzymes by filamentous fungi because they provide the conditions under which the fungi grow naturally (Pandey *et al*., 1999, Duhan *et al*., 2016). Among cereals, rice is most important crop as it is used as a staple food for more than three billion people in the world. The ayurvedic treatise records show the existence of several medicinal rice varieties in India. The vitamin and essential amino acid content of rice products significantly increases during fermentation and remains at a superior level to the one existing in rice, even if fermented rice is used as raw material for producing rice crackers, chips, snacks (Tongnual and Fields, 2006) or ready-to-eat breakfast cereals.

In recent years the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Akpan *et al*., 1999; Abu *et al.*, 2005). Regarding importance of rice as routine diet part, main objective of this study was to enhance the phenolics compound and antioxidant activity of *Oryza sativa* through solid state fermentation by using two GRAS fungi i.e. *Aspergillus oryzae* (MTCC 3107) and *Aspergillus awamori* (MTCC 548).In present investigation, the phenolic as well as antioxidant potential of ethanolic extracts of fermented and non-fermentedOryza sativawere compared. Antioxidant potential was assayed by DPPH (1, 1-Diphenyl-2-picrylhydrazyl) and ABTS (2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay.

Materials and Methods

Microorganisms

The microorganisms used for fermentation were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh. The generally recognized as safe (GRAS) fungal strains i.e. *Aspergillus oryzae* MTCC 3107 and *Aspergillus awamori* MTCC 548 were used for solid state fermentation in present study. The fungal strains were cultivated and maintained on potato dextrose agar (PDA) plates.Spore suspension was prepared having a spore count of 1×10^6 spores/ml.

Medium and chemicals

Oryza sativa (Mini mogra basmati rice) was used as substrate for SSF. The organic solvents (ethanol, methnol, hexane) used in the present study was from Qualigens. All other chemicals like DPPH (1,1-Diphenyl-2-picrylhydrazyl), ABTS(2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid), gallic acid, Folin reagent, L-ascorbic acid, sucrose, sodium carbonate etc. used in this study were of Hi Media.

Solid state fermentation

Substrate was first washed and dried before use. Fifty grams of substrate (rice) 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 overnight. After decanting the excess media, the substrate was autoclaved (121^0C, 15 minutes) and subsequently cooled before inoculation. The autoclaved substrate was inoculated with 5.0 ml spore suspension (1×10^6 spores/ml) of selected fungal strains, mixed properly and incubated for 6 days at 30^0C. The non-fermented rice was prepared without the addition of spore suspension or taken as control.

Extraction of enzymes

Fermented samples were taken at an interval of 24 h.The enzymes were extracted from fermented rice with distilled water (1:10 w/v). Extracted samples were filtered through Whatman filter paper No.1.The supernatant was assayed for α-amylase activity.

Extraction of phenolic compounds

The fermented rice samples were taken out from the Erlenmeyer flask at an interval of 24 h and dried in oven at 60^0C 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 minutes, thrice) at room temperature. Defatted samples were air dried for 24 h and stored at -20^0C for further analysis. Defatted samples were extracted with 54% ethanol at 61^0C for 64 minutes (Liyana and

Shahidi, 2005). The extracted samples were filtered through Whatman filter paper No.1. The filtrate was used for determination of total phenolic content and antioxidant properties.

Determination of total phenolics

Total phenolic content was determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). 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 minutes at room temp. The absorbance was measured at 765 nm (Systronic 2202 UV–VIS spectrophotometer) (Singh *et al*., 2007).Total phenol value was obtained from the regression equation and expressed as µM/g gallic acid equivalent using the formula (Akinmoladun *et al*., 2007).

$C = c.V / M$

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 substrate i.e. ethanolic extract (g).

Alpha amylase assay

Alpha-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 minutes of incubation at 50^0C, the concentration of glucose liberated from starch by the action of α-amylase was estimated spectrophotometrically at 575 nm (Miller, 1959). 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.

DPPH radical-scavenging effect

The free radical scavenging activity was measured by DPPH assay, following Brand-Williams *et al.* (1995) 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 (200 µl) of ethanolic extract was mixed with 2.0 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. 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 minutes was measured and plotted.The antioxidant activity of ethanolic extract was evaluated by calculating the % inhibition by using the formula:

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

A= absorbance of the blank

A_1= absorbance of the extract

The DPPH radical scavenging activities of rice extracts were expressed as µM/g VCEAC (Kim and Lee, 2004).Vitamin C equivalent antioxidant capacity (VCEAC) was calculated byusing this formula:-

$VCEAC = \Delta\ Abs – a / b$

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 chemicals tested after 30 minutes at 734 nm.

ABTS radical cation depolarization assay

In ABTS assay, antioxidant activity was measured using 7.6 mM (19.0 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 (Re *et al*., 1999; Arnao *et al*., 2001). Ethanolic extract (30 µl) was mixed with 3.0 ml of ABTS solution and optical density was measured at 734 nm after 1 minutes of incubation at room temperature using spectrophotometer. The reduction of ABTS was measured by evaluating the % inhibition and expressed as µM/g VCEAC as described in DPPH scavenging assay.

Statistical analysis

The mean value and standard deviation was calculated from the data obtained from the three separate experiments. Analysis of data was performed 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 value.

Results and Discussion

DPPH assay

The evaluation of total antioxidant activity using different model assay systems has become increasingly imperative. The purpose of DPPH assay was to assess the potential antioxidant activity in

rice. Due to high sensitivity and operational ease 1, 1-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay effectively evaluated the free radical-scavenging capacity of tested samples. Antioxidant compounds are responsible for scavenging of DPPH radicals and this scavenging is directly proportional to the concentration of antioxidant (Magalhaes *et al*., 2006).The DPPH radical-scavenging activity wasrepresented as vitamin C equivalent antioxidant capacity (VCEAC) and calculated by the value obtained from the standard graph. The VCEAC of ethanolic extract of rice flour was 852.04±3.69 µM/100g on 3rd day of incubation with *A. oryzae* and 850.99±6.42 µM/100g on 4th day of fermentation with *A. awamori* which was lower than that of seed i.e. 1120.17±5.79 µM/100g VCEAC on 4th day with *A. oryzae* and 1025.89±15.50 µM/100g VCEAC on 3rd day of incubation with A. awamori as shown in Figure 1 and 2.This may be due to presence of high amount of total phenolic contentin seed as compared to flour because phenolics are responsible for antioxidant activity of substrates. Similar results were obtained in mung beans where SSF significantly increased the phenolic content, thus enhances the antioxidant activity of the beans (Randhir and Shetty, 2007). Antioxidant activity of fermented samples has been reported to be higher than that of non-fermented ones in black bean (Lee *et al.*, 2008), Wheat (Duhan *et al*., 2016) which suggests that bioactive compounds produced by microorganisms in SSF are responsible for release of conjugated phenolic compounds.

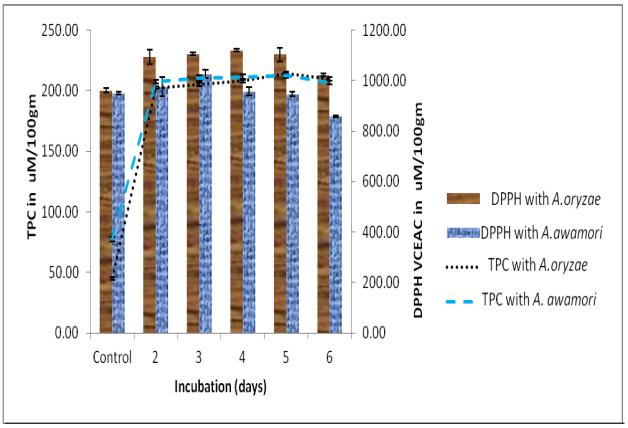

Figure1. TPC and DPPH radical scavenging property of rice seed extracts (54% ethanol) fermented with *A. oryzae* and *A. awamoriof* (Error bar represents SD, n=3)

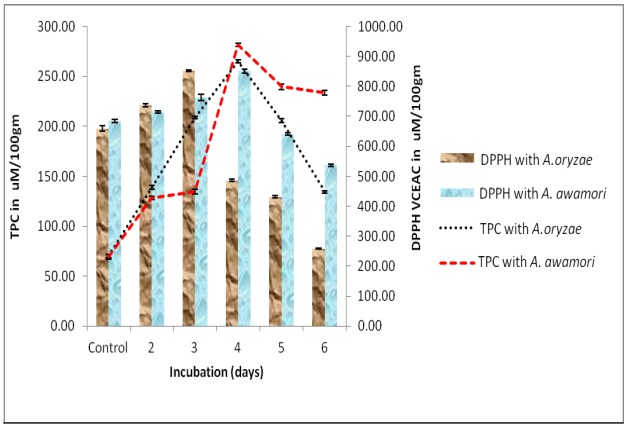

Figure 2. TPC and DPPH radical scavenging property of rice flour extracts (54% ethanol) fermented with *A. oryzae* and *A. awamori* (Error bar represents SD, n=3)

ABTS assay

Another free radical scavenging assay used was ABTS radical cation decolourisation assay, where maximum VCEAC i.e. 705.96±1.89 and 702.49±4.27 µM/100g was observed in fermented samples of rice seed on 4th day of fermentation with *A. oryzae* and *A. awamori*, while it was found to be 124.28±3.94 and 227.12±4.92 µM/100g, respectively in case of non-fermented samples (Fig.3). In fermented rice flour, ABTS assay showed maximum inhibition (464.33±3.41 and 533.41±4.86 µM/100g VCEAC) on 5th day of incubation with both the Aspergillus spp. which was quite higher than non-fermented ones (Fig.4). These results once again suggest that solid state fermentation process offers great advantage over the chemical and enzymatic processes to release the bound form of phytochemical present in rice, thus increases the product antioxidant ability. Antioxidant activity of fermented samples is (approx. times) higher than that of non-fermented ones (Lee *et al*., 2008), which suggests that bioactive compounds produced by microorganisms in SSF are responsible for release of conjugated phenolic compounds.These results were supported by thefindings of many others (Duenas *et al*., 2005; Lee *et al*., 2008; Jonnalagadda *et al*., 2011; Duhan *et al*., 2012). In black beans and mung bean similar results were obtained, where SSF significantly increased the phenolic content thus enhances the antioxidant activity of the beans (Lin *et al*., 2006; Randhir and Shetty, 2007).

Total phenol content (TPC)

Many reports are available regarding enhancement of phenolic content of cereals and pulses by SSF using GRAS fungal and bacterial strains (Randhir and Shetty, 2007; Zhang *et al.*, 2008; Lee *et al*., 2008; Starzynska-Janiszewska *et al*., 2008; Bhanja *et al*., 2009; Juan and Chou, 2010; Singh *et al*., 2010). In present study, phenolic content of seed and flour of rice fermented with *A. awamori* and *A. oryzae* was compared with non-fermented samples. Total phenolic contents (TPC) was represented as gallic acid equivalent (GAE) and was calculated by y = 0.025 x -0.063 from the standard curves of

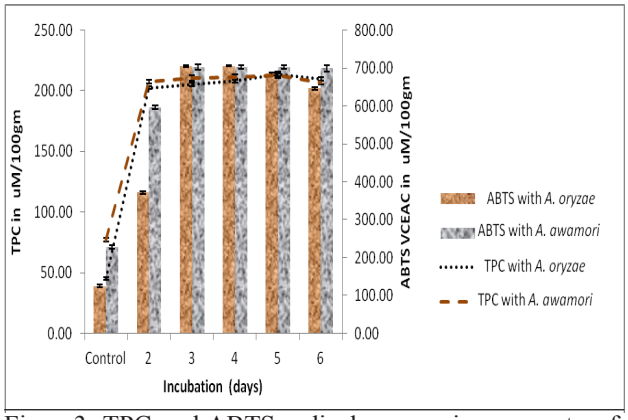

Figure3. TPC and ABTS radical scavenging property of rice seed extracts (54% ethanol) fermented with *A. oryzae* and *A. awamori* (Error bar represents SD, n=3)

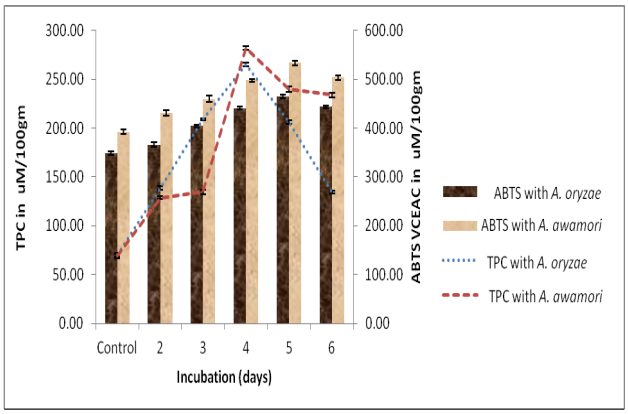

Figure 4. TPC and ABTS radical scavenging property of rice flour extracts (54% ethanol) fermented with *A. oryzae* and *A. awamori* (Error bar represents SD, n=3)

L-ascorbic acid. Maximum TPC i.e. 281.67±1.63 and 264.95±1.57 µM GAE/100g was observed on 4th day of fermentation in seed while it was 212.57±1.77 and 213.88±2.16 µM GAE/100g on 5th day of fermentation with *A. awamori* and *A. oryzae* respectively in flour. These values of fermented samples were higher than the non-fermented ones i.e. 45.11±1.18 and 77.37±1.63µM GAE/100g of fresh weight. Similarly, Lee and co-workers reported that total phenol content and antioxidant activity of black bean fermented with *A. awamori* was higher than that of non-fermented black bean.

The non-significant correlation was found between TPC and DPPH of rice flour fermented with A.oryzae and TPC and DPPH of rice seed fermented with A. awamori, whereas all other combinations of TPC and DPPH as well as $ABTS^{+}$ radical scavenging activity of rice seed and flour were found significantly ($P<0.01$) correlated. Which provide the strong evidence that the antioxidant activity in rice fractions is derived from phenolic compounds The fermented rice seed and flour have more antioxidant components than the non-fermented ones which could either react

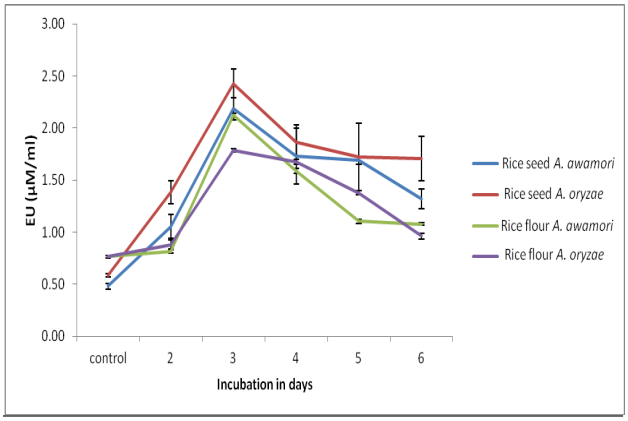

Figure 5. Alpha-amylase activity of fermented and non-fermented seed and flour of rice at different incubation periods (Error bar represents, SD, n=3).

with the free radicals and reduced almost all the free radicals to their corresponding hydroxyl groups or scavenge ROS at the stage of initiation of chain reaction and thereby terminating the auto-oxidation.

Alpha amylase assay

Numerous bioactive compounds are produced by plants in bound form but these bound phenolic compounds can be easily recovered by the action of enzymes produced by microorganisms used for SSF (Sarikaya *et al.*, 2002). Amylase activity was calculated from the value obtained from the standard i.e. y=0.008 x -0.047.There was a difference in α-amylase activity at different incubation period in solid-state fermentation as shown in figure 5. Highest amylase activity (i.e. 2.18±0.11 and 2.43±0.21 µM/ml in seed and 2.12±0.02 and 1.78±0.02 in flour) was observed on 3rd day of incubation with both strains of *Aspergillus* spp.On further incubation, the enzyme activity gradually decreased. This may be due to the depletion of essential nutrients required for the growth and enzyme production. Production of glucoamylase by *A. awamori* in solid-state fermentation was also reported by Ellaiah *et al.*, 2002; Negi and Benerjee, 2009. Likewise production of α-amylaseby Aspergillus niger MTCC-104 employing solid state fermentation has been reported by Kumar and Duhan, 2011; Kumar *et al.*, 2013.In present study, amylase activity in fermented samples was found higher than that of non-fermented samples so, the increase in phenolic content of fermented rice samples was attributed to the enzymatic action of amylase.

Conclusions

Results obtained from the present investigation showed that solid state fermentation was effective for augmentation of phenolic content, DPPH radical

and ABTS scavenging activities of seed and flour of *Oryza sativa* (rice) by using GRAS filamentous fungi i.e. *Aspergillus awamori* MTCC 548 and *Aspergillus oryzae* MTCC 3107. Findings of the study showed that the fermented ethanolic extract of seed and flour of rice will be an antioxidant rich and healthy food supplement compared to non-fermented seed and flour of rice.

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