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RESEARCH PAPER

Assessment of Fermentation Based Enrichment of Bioactive Compounds and Antioxidant Activity of Commonly Used Cereals

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ABSTRACT

Cereals i.e. wheat, rice, oat, maize and sorghum were fermented by using GRAS fungal strain *A. awamori* to study the effect of fermentation on upgradation of phenolic level of cereals. Significant ($P < 0.05$) increase in phenolics, flavonoids, DDPH (2, 2-diphenyl-1-picrylhydrazyl) and ABTS (2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt radical scavenging potential of all the fermented cereals was observed mainly on 4th and 5th day of incubation. Increased levels of polyphenols and antioxidants after fermentation was observed maximum in *O. sativa* and *T. aestivum* followed by *S. bicolor* > *A. sativa* > *Z. mays* which was mainly due to high enzymatic activities as observed during their fermentation. A positive correlation was obtained between total phenolic and flavonoids content with antioxidant activity while linear correlation obtained between total phenolic and flavonoid contents with enzymatic activities,justified the role of α-amylase, xylanase and β-glucosidase enzymes in release of polyphenols and antioxidants during solid state fermentation (SSF).

Keywords: *A. awamori*, SSF, phenolic, flavonoids, cereals, bioactive compounds, antioxidant

The use of natural antioxidants from cereals, fruits and vegetables has gained much attention from consumers because they are considered as safer than synthetic antioxidants. Phenolic acids in cereal grains occur primarily in bound form as conjugates with sugars, fatty acids, or proteins and act as effective natural antioxidants (Wyen *et al.* 2000). These antioxidants either scavenge the free radicals or can greatly reduce the damage by neutralizing the free radicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA thus, lowering the risk of developing cardiovascular disease, certain kinds of cancer and age-related degenerative processes (Maillard *et al.* 1996; Deighton *et al.* 2000; Sadh *et al.* 2018a). Various studies justify the use of natural sources of antioxidants over synthetic drugs (Saharan *et al.* 2012; Saharan and Duhan, 2013; Duhan *et al.* 2011a; 2011b; 2013; 2015; Rana *et al.* 2014; Duhan *et al.* 2016a). Antioxidant capacity of different cereals such as wheat (Duhan *et al.* 2016b), rice (Sadh *et al.* 2017c) and ready-to-eat breakfast cereals has been reported previously (Prajapati *et al.* 2013).

Now-a-day, due to harmful effects of preservation of food by chemical, interest is increasing towards physical and biological processing like dehulling, decortication, malting, enzymatic treatment, fermentation and thermal processing of cereals to maximize the retention of level of phenolic compounds and antioxidants. Various researchers like Prajapati *et al.* (2013) (Cereals), Taylor and Duodu (2014) (sorghum and millets), Chakraborty and

Bhattacharyya (2014) (Pulses) and Saharan *et al.* (2017) (cereals) studied the effect of processing on phytochemicals and antioxidant activity of cereals and pulses. In recent years, among all the food processing options, solid state fermentation (SSF) has received more and more interest from the researchers, since several studies for colorants (Johns and Stuart, 1991), flavours (Ferron *et al.* 1996), enzymes (Pandey *et al.* 1999), bioactive compounds (Sadh *et al.* 2017d) and other substances of interest to the food industry have shown that SSF can give higher yields (Tsuchiya *et al.* 1994). It dramatically affects the quantity of phenolics present in bound form in plant materials, like Martins *et al.* (2011) reported beneficial effects of solid-state fermentation (SSF) on the production and extraction of antioxidant phenolics mainly from pulses and cereals. Liang *et al.* (2009); Katina *et al.* (2007); Huang *et al.* (2011) also used SSF for improvement of food products in order to increase their palatability, digestibility, nutritional quality, bioactivity and antioxidant activity. Similarly, Chawla *et al.* (2017) used *Vigna unguiculata* (Black-eyed Pea) as substrate to know the impact of SSF on various functional properties and mineral bioavailability with *Aspergillus oryzae*.

Recovery of phytochemicals needs the demolition of the plants cell wall and enzymatic hydrolysisis one of the most studied approaches for this purpose. Different hydrolytic enzymes produced during fermentation convert macromolecules i.e. starch, protein, phosphorus of cereals into their monomers units called glucose, free amino nitrogen, and free phosphorous, for the production of different value added products (Melikogolu, 2012). Enhancement of phenolic compound and antioxidant activity through SSF has been already reported in fava bean (Randhir *et al.* 2004), soybean (Rashad *et al.* 2011), rice bran (Oliveira *et al.* 2012), wheat (Zhang *et al.* 2012) and peanut press cake (Sadh *et al.* 2018b).

Therefore, the present study was planned to investigate the role of carbohydrate-cleaving enzymes (β-glucosidase, α-amylase, xylanase)secreted by *A. awamori* in release of phenolics during SSF of cereals i.e. wheat (*T. aestivum*), rice (*O. sativa*), oat, (*A. sativa*) maize (*Z. mays*) and sorghum (*S. bicolor*). In addition, effect of polyphenols on antioxidant properties of ethanol extracts of cereals was also evaluated.

MATERIALS AND METHODS

Cereals

Certified varieties of five cereals i.e. *T. aestivum* (PBW-550), *O. sativa* (HKR-47), *A. sativa* (HARYANA JAVI 8), *Z. mays* (HQPM-5), and *S. bicolor* (SUPER-7707) were collected from Sirsa and Hisar, Haryana (India). Cereals were washed and shade-dried before subjected to SSF as substrate.

Chemicals

2, 2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) di-ammonium salt (ABTS), p-nitrophenyl-β-D-glucoside, xylan from birchwood, gallic acid and quercetin were purchased from Sigma-Aldrich (Bangalore, India) while 2, 2-diphenyl-1-picrylhydrazyl (DPPH), p-nitrophenol, starch, L-ascorbic acid, sodium nitrate, sodium carbonate, sodium hydroxide, sodium potassium tartarate, dinitrosalicylic acid (DNSA), aluminium chloride, potassium dihydrogen phosphate, potassium chloride, magnesium sulfate (dihydrade) were purchased from Himedia (Mumbai, India). Hexane was obtained from Merck (Mumbai, India) and all other reagents used were of analytical grade.

Microorganisms used and inoculum preparation

GRAS fungal strain i.e. *Aspergillus awamori* (MTCC-548) was procured from Microbial Type Culture Collection (MTCC) Institute of Microbial Technology, Chandigarh and was used as the starter organisms for SSF. *A. awamori* was cultured and maintained on potato dextrose agar slants. Spore suspension with spore count of approximately 1×10^6 spores/ml in sterilized water was used as inoculum.

Solid state fermentation conditions

Each cereal was weighed 50 g in 500 ml Erlenmeyer flasks and 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. Substrates (cereals) in the conical flasks were steam-cooked in an autoclave at 121°C for 15 min only after removing the excess medium. On subsequent cooling, a suspension of spores [10% (w/v)] of fungal strains was sprayed separately onto the surface of the steamed substrates, mixed properly and incubated for 6 days at 30°C. The fermented mass was shaken and mixed everyday starting after 17 h of inoculation to release excess fermentation heat. The non-fermented substrates were prepared without the addition of spore suspension and were used as control.

Enzyme extraction

Mixture of enzymes from the fermented mass were extracted with water (1:10 (w/v)) after every 24 h interval. The supernatant was assayed for carbohydrate-cleaving enzymes *viz*., β-glucosidase, α-amylase and xylanase.

Extract preparation

After every 24 h interval, fermented mass was taken out of flask and dried in an oven at 60°C temperature. Both fermented and non-fermented (control) substrates were ground to obtain fine powder. Powdered substrates were defatted with hexane (1:5 w/v, 5 min, thrice) at room temperature and then, air dried for 24 h to remove hexane completely. Defatted substrate samples were stored at -20°C for further processing. The dried powder was extracted with 54% ethanol at 61°C for 64 min following optimized conditions of Liyana-Pathirana and Shahidi (2005). Extracts were filtered through Whatman No.1 filter paper and finally used for evaluation of total phenolic content and antioxidant property.

Enzyme assays

***Alpha-amylase assay*:** Alpha-amylase activity was determined by incubating reaction mixture containing 0.5 ml of appropriately diluted enzyme extract, 0.5 ml of 0.2 M acetate buffer (pH 5.0) and 1.0 ml of soluble starch (1%) at 50°C for 10 min. The quantification of the glucose released from starch by the action of α-amylase was estimated spectrophotometrically at 575 nm. One unit (U) of amylase activity was defined as the amount of enzyme that liberates one micromole of reducing sugar (glucose) per min under the assay conditions. Results were expressed as U/g dry substrate i.e. U/gds.

***Xylanase assay*:** Xylanase activity was determined by mixing 0.5 ml of appropriately diluted enzyme source with 0.5 ml of 0.2 M acetate buffer (pH 5.0)and 1.0 ml of 1.0% (w/v) xylan (from birchwood; Sigma, St. Louis,USA). The reaction was carried out at 50°C for 10 min and was stopped by adding 2.0 ml of DNS solution further heating the tube in a boiling water bath for 10 min. The amount of xylose released was measured spectrophotometrically at 575 nm. One unit of xylanase activity was defined as the amount of enzyme that liberates 1.0 μmol of xylose per min under the given assay conditions. Results were expressed as U/gds.

β-Glucosidase assay: It was measured by preparing reaction mixture of 0.25 ml enzyme extract, 0.25 ml acetate buffer (100 mM, pH 5.0) and 0.5 ml of 5.0 mM p-nitrophenyl-β-Dglucoside (pNPβG). The mixture was incubated at 50°C for 30 min and the reaction was stopped by adding 1.0 ml of 1 M sodium carbonate (Na_2CO_3) solution. The amount of released p-nitrophenol was estimated by measuring the absorbance at 400 nm using a spectrophotometer. One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1.0 μmol of p-nitrophenol per min under the assay conditions. Results were expressed as U/gds.

***Determination of total phenolic (TPC)*:** TPC was determined by following method of Singh *et al.* (2007). To 0.2 ml of ethanolic extracts of both fermented and non-fermented substrates, 1.0 ml of Folin-Ciocalteu reagent (Sigma Aldrich)and 0.8 ml of sodium carbonate Na_2CO_3 (7.5%) was added. The contents were mixed properly and allowed to stand for 30 min at room temperature. Absorbance was measured spectrophotometrically at 765 nm. Total phenolic content was calculated as gallic acid equivalent (GAE) mg/g.

Total flavonoid content (TFC)

TFC was estimated according to method given by Ordonez *et al.* (2006). An aliquot of 0.5 ml ethanolic extract was mixed with 0.5 ml of 2% $AlCl_3$ (prepared in ethanol) and after incubating for 1 h at room temperature, the absorbance was measured at 420 nm. Quercetin was used as a standard for calculation of TFC.

Antioxidant activity testing assays

DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging assay

The DPPH free radical scavenging activity was measured using the method of Brand-Williams *et al.* (1995). Two milliliter of DPPH solution with 0.1 mM concentration in methanol was added to 0.2 ml extract. After 15 min incubation in dark, the absorbance was measured at 517 nm by using spectrophotometer. The L-ascorbic acid (vitamin C) was used as the standard and DPPH radical scavenging activities of fermented and non-fermented extracts were expressed as vitamin C equivalent antioxidant capacity (VCEAC) in µmol/g cereal substrate.

ABTS (2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) assay

In ABTS assay, antioxidant activity was measured according to Re *et al.* (1999) using 7.6 mM $ABTS^+$ solution and 2.6 mM potassium persulphate solution in 5.0 ml of distilled water. The resulting solution was left to stand for 12 to 16 h in dark at room temperature. Working solution was prepared by mixing 1.0 ml of this reaction mixture with 60 ml water. Briefly, 20 µl extract were taken and mixed with 2.0 ml of ABTS solution and after 1 min of incubation at room temperature, the absorbance was measured at 734 nm by using spectrophotometer. The L-ascorbic acid was used as the positive control. The ABTS radical scavenging activity was measured by calculating the vitamin C equivalent antioxidant capacity (VCEAC) in µmol/g cereal substrate.

Statistical analysis

All the experiments were performed in triplicates, the mean values and the standard deviations were calculated from the data obtained from three separate experiments. Analysis of variance was performed by paired sample t-test using SPSS statistics viewer 16.0. Statistical differences at $P < 0.05$ were considered to be significant. Co-relation coefficient (R^2) was calculated using MS Excel in order to determine the relationship between two variables.

RESULTS AND DISCUSSION

Total phenol content (TPC)

The total phenolic content of ethanol extracts of cereals was calculated as gallic acid equivalent (GAEµM/g) from standard curve. As shown in Fig. 1, fermentation of cereals with *A. awamori* significantly ($p<0.05$) increased the TPC content of cereals. Highest TPC value was recorded in *T. aestivum* from 13.85±1.29 µM/g GAE (control) to 56.92 ± 1.67µM/g GAE (fermented) on 5th day of incubation but when compared to the control (unfermented) sample, the best effect of fermentation (more than seven times increase in TPC) was found with *O. sativa* (Fig. 1).

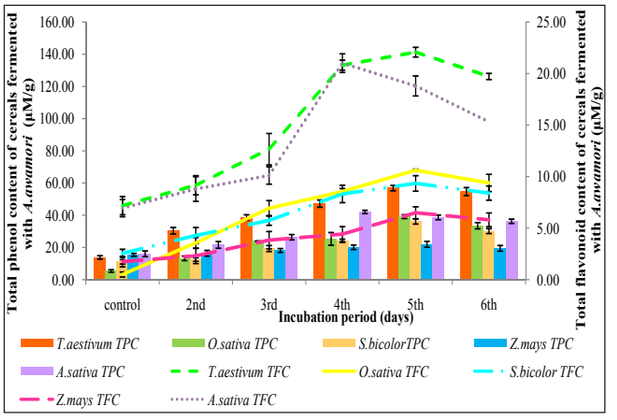

Fig. 1: Total phenol and flavonoid content of cereals fermented with *A. awamori* at different incubation days (Error bar represents SD, n=3)

On the other hand, the least enhancement was recorded in *Z. mays* i.e. 15.47 ± 0.89 µM/g GAE (control) to 22.04 ± 1.79 µM/g GAE (fermented) on 5th

day of incubation. The effect of fermentation on TPC level of cereals was observed in order *T. aestivum* > *O. sativa* > *A. sativa* > *S. bicolor* > *Z. mays*. This increase in TPC during SSF may be due to the increase in the extractability of bounded phenolics by the enzymatic degradation of cellular constituents as also observed by Oliveira *et al.* (2012) in rice bran. Similar results of fermentation on total phenolic content was also observed by Ajila *et al.* (2011) in apple pomace, Bhanja and Kuhad (2014) in wheat.

Total flavonoid content (TFC)

Quercetin was used to prepare standard calibration curve, which in turn was used for calculation of flavonoids content. The flavonoid content of fermented and unfermented cereals is presented in Fig. 2. Similar to TPC, the maximum TFC was also observed in *T. aestivum* (22.07 ± 0.47μM/g QE) followed by *O.sativa* (10.62 ± 0.24 μM/g QE). But the role of fermentation in improvement of flavonoids level was observed as *O. sativa* > *Z. mays* > *S. bicolor* > *T. aestivum* > *A. sativa*. This increase in TFC was attributed to the release of bound flavonoids after SSF. Our results are supported by outcomes of Zhang *et al.* (2012) where flavonoid contents of the *Cordyceps militaris* fermented wheat extract are significantly higher than the respective extracts of the unfermented wheat.

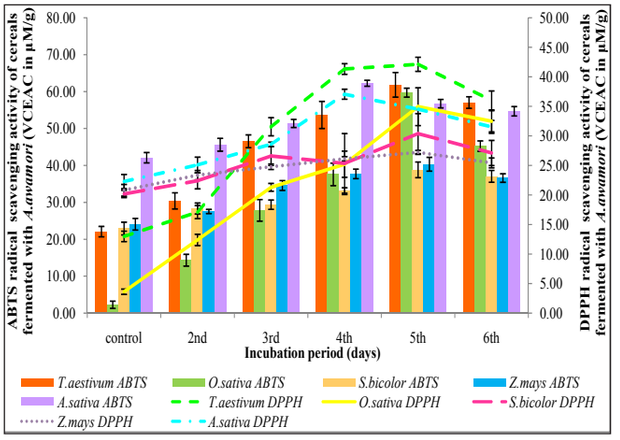

Fig. 2: ABTS and DPPH radical scavenging activity of cereals fermented with *A. awamori* at different incubation days (Error bar represents SD, n=3)

Antioxidant capacity

DPPH assay

The extraction of antioxidants from cereals can be partial, which can lead to misinterpret their actual biological availability and activity (Perez-Jimenez and Saura-Calixto, 2005). In order to maximize the extraction of antioxidant components naturally, enzymes were considered as the best suited tools and during fermentation these enzymes release bound phenolic which directly scavenge free radicals. The antioxidant activity of cereal extracts was calculated as Vitamin C equivalent antioxidant capacity (VCEAC) from L-ascorbic acid standard graph. The level of reducing capacity of DPPH ions was recorded high in fermented extracts of all cereals as compared to non-fermented extracts in following order *T. aestivum* (42.11 ± 0.90 μM VCEAC /g) > *O. sativa* (34.95 ± 3.20 μM VCEAC /g) > *A. sativa* (37.05 ± 0.06 μM VCEAC /g) > *S. bicolor* (30.38± 3.38 μM VCEAC /g) > *Z. mays* (27.22 ± 0.34 μM VCEAC /g). Although maximum VCEAC value was found in *T. aestivum* but as compared tothe control (non-fermented) peak effect of fermentation was observed in *O. sativa* (Fig. 2). The difference in effect of SSF on antioxidant potential of cereals is due to the fact that antioxidants present in cereals differ in their structure and mode of action (Duhan *et al.* 2016b; Sadh *et al.* 2017a; Saharan *et al.* 2017).

Table 1: Correlation (r^2 value) between total phenolic content, total flavonoids content and antioxidant activity of cereal extracts fermented with *A. awamori*

Cereals	TPC/ DPPH	TFC/ DPPH	TPC/ ABTS	TFC/ ABTS
T. aestivum (Wheat)	0.851b	0.925a	0.855b	0.914a
O. sativa (Rice)	0.987a	0.980a	0.981a	0.951a
S. bicolor (Sorghum)	0.870b	0.808b	0.928a	0.933a
Z. mays (Maize)	0.924a	0.717c	0.946a	0.801b
A. sativa (Oat)	0.808b	0.963a	0.807b	0.925a

[a]*(Highly significant),* [b] *(Significant),* [c] *(Non significant)*

The *in vitro* antioxidant potential of cereal is generally correlated with their polyphenol content (Gani *et al.* 2012). Table 1 represents correlation coefficient (r^2)

estimated between TPC and TFC with DPPH free radical scavenging activity of ethanol extracts from fermented cereals. All the cereal extracts, especially *O. sativa* i.e. r^2 = 0.987, *Z.mays* with r^2 = 0.924 (Table 1) establish very good correlation between TPC and DPPH scavenging ability. Similar is the case of TFC and DPPH, where maximum correlation was attained by *O. sativa* i.e. r^2 = 0.980 followed by *A. sativa* r^2 = 0.963 (Table 1). These correlation values are strong evident to prove that antioxidant activities of cereals are based on phenolic and flavonoids content of cereals. Singh *et al.* (2010) also observed that phenolic and flavonoid compounds are the reason behind stronger antioxidant activity of soybean products fermented by SSF with *T. harzianum* than unfermented products.

ABTS assay

A linear standard curve equation of L-ascorbic acid was used for VCEAC calculation. The ABTS scavenging capacity of fermented cereals was significantly increased ($p<0.05$) as compared to unfermented (control) ones.

As shown in Fig. 2, ABTS scavenging property of *O. sativa* increases maximum after fermentation on 5th day of incubation (varied within 2.28 ± 0.97 to 59.71± 1.21 μM VCEAC /g) followed by *T. aestivum* on 5th day of incubation (varied within 22.09 ± 1.41 to 61.83± 3.66 μM VCEAC /g) while the least increase was observed in *S. bicolor* on 5th day of incubation (22.91 ± 1.71 to 38.81 ± 2.12μM VCEAC /g).

When total phenol content and ABTS radical scavenging capability of cereals was correlated, highest correlation i.e. 0.981 was observed in *O.sativa* (Table 1) followed by *Z.mays* (0.946, Table 1). *O.sativa* with r^2 = 0.951 also attained good correlation between flavonoid content and ABTS activity while remaining cereals do not show as much correlation as observed in case of TPC. Along with anti-oxidant properties, flavonoids are reported to have anticancer, anti-allergic, anti-inflammatory, anticarcinogenic activity and gastroprotective ability (Yao *et al.* 2004).

Fermentation based gradual increase in antioxidant potential of cereals is much more evident than that of other physical and chemical processing parameters because during SSF, bound polyphenols are simply released *via* enzymatic hydrolysis which automatically enhances antiradical properties of cereals. All the cereals under present study possessed varying antioxidant properties and these results are supported by the fact that plant polyphenol content and antioxidant property depend on a number of factors such as variety, location and environmental conditions (Yu *et al.* 2011; Klepacka *et al.* 2011).

Evaluating the effect of enzymes on release of bound polyphenols

During fermentation many biochemical changes take place due to which there is a change in nutritive and anti-nutritive components of plants which in turn affects the properties such as bioactivity and digestibility (Katina *et al.* 2007; Sadh*et al.* 2018c). During fermentation, fungus releases various enzymes (Kumar *et al.* 2011; Kumar *et al.* 2013). Phenolic compounds, especially phenolic acids, are partly responsible for insoluble cell wall structures of cereal kernels by forming cross-links between polysaccharides and lignin. The carbohydrate cleaving enzymes are required to break such cross linkage of bound phenolics and that is why the role of α-amylase, xylanase and glucosidase enzymes in enrichment of polyphenols and antioxidants during SSF of cereals was studied. The standard graphs of glucose, p-nitrophenol and xylose were made for α amylase, β-glucosidase and xylanase enzyme assays (Data not shown). Highest αamylase (45.00 ± 0.45 U/gds), xylanase (50.60 ± 1.08 U/gds), and β-glucosidase (11.25 ± 0.21 U/gds) activities were observed in *O. sativa* on 5th day of fermentation (Fig. 3). Sadh *et al.* (2017a; 2017b) also stated the role of α-amylase in increasing phenolic content of fermented rice and *Lablab purpureus* samples than that of non-fermented samples, respectively.

While correlating enzyme activity with TPC content, maximum correlation i.e. r^2 = 0.990 with *O. sativa* and *T. aestivum* was observed with xylanase enzymes while r^2 = 0.906 and r^2 = 0.864 was recorded in *O. sativa* with β-glucosidase and amylase respectively (Table

Table 2: Correlation (r^2 value) between total phenolic content, total flavonoids content and enzyme activity of cereal extracts fermented with *A. awamori*

Cereals	TPC/ Amylase	TPC/ Xylanase	TPC/ β-Glucosidase	TFC/ Amylase	TFC/ Xylanase	TFC/ β-Glucosidase
T. aestivum	0.603c	0.989a	0.783c	0.756c	0.929a	0.540c
O. sativa	0.864b	0.990a	0.906a	0.920a	0.971a	0.942a
S. bicolor	0.852b	0.875a	0.585c	0.988a	0.896b	0.726c
Z. mays	0.803b	0.748c	0.757c	0.960a	0.847b	0.762c
A. sativa	0.752c	0.791c	0.503c	0.974a	0.795c	0.390c

[a](Highly significant), [b](Significant), [c](Non significant).

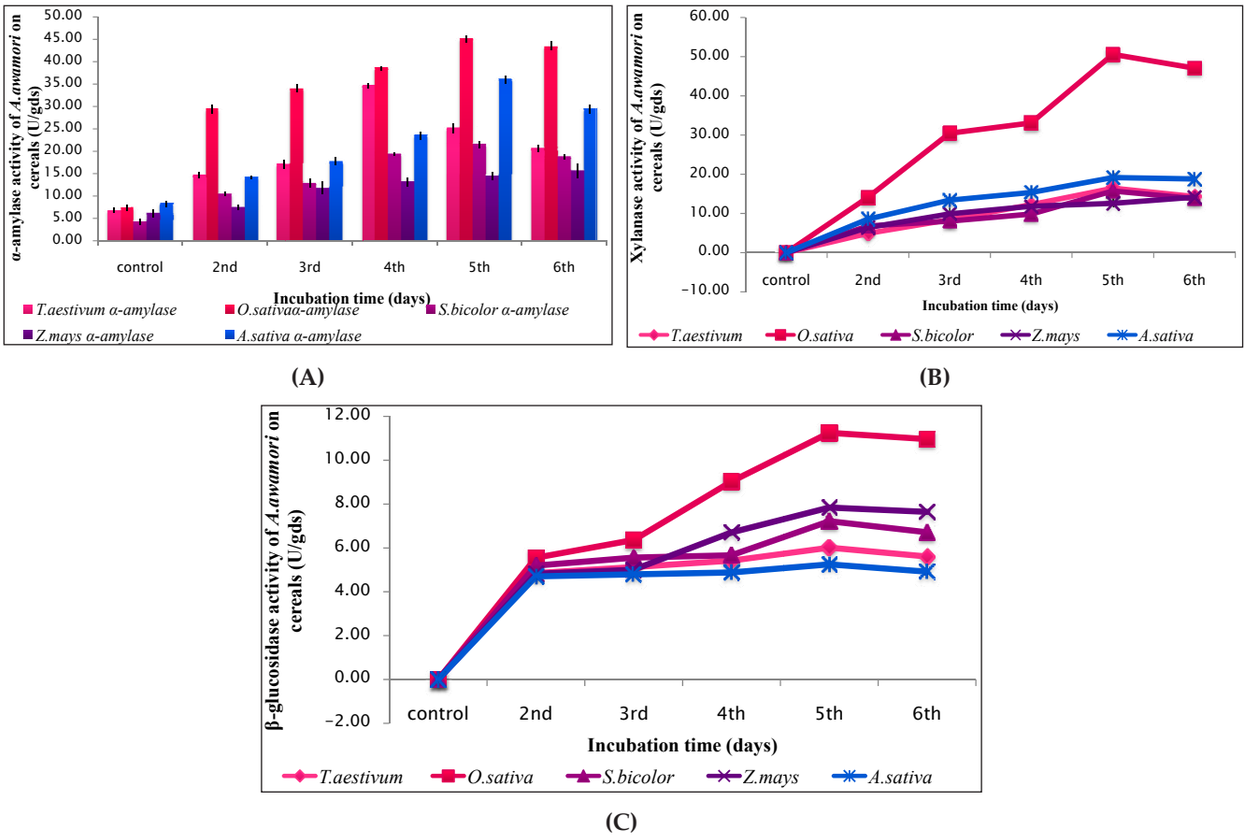

Fig. 3: (A) Effect of α-amylase **(B)** Xylanase and **(C)** β-glucosidase enzymes released by *A. awamori* on cereals during fermentation at different incubation days

2). Least correlation was found in β-glucosidase (r^2 = 0.503 in *A. sativa*) (Table 2). Similarly, results in Table 2 also showed that amylase and xylanase enzymes exhibited best correlation i.e. r^2 = 0.988 and r^2 = 0.971 with TFC in *S. bicolor* and *O. sativa* respectively. On the other hand, β-glucosidase showed good correlation with *O. sativa* only while with remaining cereals poor correlation was observed. Finally, it was concluded that amylase and xylanase are highly involved in enzyme-assisted extraction of polyphenolic compounds while β-glucosidase have least role. The effect of enzymatic hydrolysis on phenolic antioxidant extraction was also observed by Ribeiro *et al.* (2013).

CONCLUSION

Under SSF, fungi produce carbohydrates cleaving enzymes which are responsible for the degradation of polysaccharides linkages and hence, releasing the carbohydrate bound polyphenolics. Among studied cereals, maximum effect of fermentation was found in *T. aestivum* and *O. sativa* followed by > *A. sativa* > *S. bicolor* > *Z. mays* which is mainly due to high enzyme activities as observed during their fermentation. In fermented cereals, released phenolics and flavonoids contribute to their antioxidant capacity. Hence, the fermented products of these cereals can be used as a health food or ingredient with multifunctional properties as a natural antioxidant in order to avoid or minimize degenerative diseases.

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Conflict of interest: There is no conflict of interest in present investigation.

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