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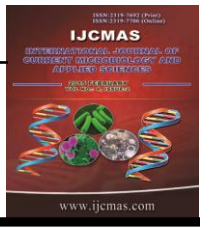

Original Research Article

Production of antioxidant by fungi using soybean milk residue (okara)

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Keywords

Soya milk waste, Okara, Fermentation, Toxicity, Aspergillus oryzae, Antimicrobial activity, Phenolic compound, Antioxidant activity, Solvent, DPPH assay, Reducing power

A B S T R A C T

Okara is the residue obtained from ground soybean after removing the water- extractable fraction used to produce tofu or soymilk. The aim of this study was to evaluate the potential use of okara and to improve the health beneficial properties of soybean waste manufacture product (Okara). In this study, Three fungal isolates were isolated from okara to proved their virulence of pathogenicity, only one fungal isolate was found to be non-toxic and safe to use, this fungal isolate was identified as *Trichoderma harzianum* using traditional mycological method of identification and nuclotide sequence analysis of the inter transcribed spacer region. The screening for the production of antioxidant was carried out on *Trichoderma harzianum* in addition to the previously provided isolate of *Aspergillus oryzea.* The antioxidants were produced by solid-state fermentation using different solvents (water, ethanol, methanol, acetoneand di-ethyl ether) for extraction of antioxidant in a period of 10 days. In comparison with non- fermented okara, the protein and ash contents increased in all fermented okara samples than non-fermented one; the level of protein increased from 6.2 to 11.1% and from 6.0 to 10.1% using *Aspergillus oryzea* and *Trichoderma harzianum*, respectively. Also, the ash contents increased from 1.03 to 2.6% and from 0.72 to 1.6% using the previously identified *Aspergillus oryzea* and *Trichoderma harzianum*. While, the fat content reduced from 3.0 to1.4% and from 3.1 to 1.3 %, using *Aspergillus oryzea* and *Trichoderma harzianum*, crude fiber content reduced from 3.4to 1.5% and from 3.3 to 1.43 % using *Aspergillus oryzea* and *Trichoderma harzianum.* The water extraction of the antioxidant revealed asignificant increase in the total phenolic compounds ranged from 9.2 to 43.8 mg gallic/gm of okara after 5 days of fermentation using *Aspergillus oryzea*, while the total phenolic compounds increased from 6.5 to 27.2 mg gallic/gm of okara using *Trichoderma harzianum.* The extraction with diethyl ether revealed the lowest content of total phenolic compound which ranged from 1.5 to 8.7 mg gallic/gm of okara using *Aspergillus oryzea* after five days of fermentation. By using DPPH assay and their reducing power the antioxidant activity revealed an increase in all fermented okara samples in comparison with non- fermented one. The HPLC analysis of the water and ethanol extracts of the fermented okara using *Aspergillus oryzea* revealed a higher content of phenolic compounds in water extract; the cholorogenic (60117.6 mg/100g), caffeine (67876.86 mg/100g) and coumarin (45940.7 mg/100g).Also, the fermented okara with *Aspergillus oryzea* had a higher content of isoflavons in water extract; forment in (863.9 mg/100g), then genistein (434.7 mg/100g) and diadzein (476.1 mg/100g), but in ethanol extract the biochanin was the highest (831 mg/100g), followed by genistein (537.9 mg/100g) and diadzein (517.7 mg/100g). The antimicrobial activity of the fermented okara extracts using *Aspergillus oryzea* and *Trichoderma harzianum*, revealed a remarkable inhibition against all the tested and pathogenic bacterial and fungal species, using disc diffusion method

Introduction

Okara is a by-product from the soymilk industry. Raw okara, also called soy pulp, is a white or yellowish insoluble material from soybean seeds, which remains in the filter sack when pure soybeans are filtered for the production of soymilk. It's a by-product of tofu, soymilk or soy protein manufacturing and is treated as industrial waste with little market value because of its short shelf life (van der Riet et al., 1989; O'Toole, 1999; Prestamo et al., 2007; Redondo-Cuenca et al., 2008).

The amount of okara reaches about7.0 x 105 tons per year in Japan (O'Tool, 1999) and is mostly burnt or dumped into landfills, establishing a utilization process is necessary to prevent possible global warming and environmental pollution. Extraction of its components is one of the utilization schemes for recycling purposes because of the large volume of residue produced, about 2 to 3 tons for each ton of soybean processed were used according to (Grizotto et al. 2006).

In fact, okara contains protein up to 25.4-28.4% (dry basis) with high nutritive quality and superior protein efficiency ratio, suggesting that it is a potential source of low cost vegetable protein for human consumption (O'Toole, 1999; Kasai et al., 2004). Several studies for improving the functional properties of okara protein is by acid deamination (Chan and Ma, 1999), and by using hydrolyzing enzymes (Kasai et al., 2004). Using of okara as nitrogen or carbon sources for the solid-state fermentation (SSF) of a microorganism was reported by (Hsieh and Yang, 2004) and `(Rashad et al., 2010).

Several health effects of okara have been assessed. In vitro experiments have indicated that okara is a potential source of antioxidants components (Amin & Mukhriza, 2006).

The nutritional quality and antioxidant of soybean meal was improved by solid state fermentation using *Aspergillus oryzae* and Bacillus subtilis (DaTeng et al., 2012).

Utilization of soybean residue by microbial fermentation has been shown to produce beneficial products such as riboflavin, lipase (Kittikun and Tani, 1986), fructofuranosidase (Hayashi et al., 1992) and single cell protein (Khare et al., 1984).

In a related study (Zhu et al. 2008) reported that the okara with a low protein- content byproduct of tofu, soymilk or soy protein was used as a substrate to obtain a functional food by fermentation with B. subtilis. Rashad et al. (2011) showed that the nutritional quality and antioxidant activities of the okara were enhanced by solid yeast treatment fermentation.

Soy bean residue was fermented by *Aspergillus oryzae*, Koji was formed and its extract contains more antioxidants than soybean residue (Wu Shi bin et al., 2010).

The aim of this study was to evaluate the potential use of okara and to improve the health beneficial properties of soybean waste manufacture product (Okara) which, produced by soild state fermentation. This process was carried out using Trichoderma harzianum isolated from okara and a previously study *Aspergillus oryzae* (Abu – Zaid, 2005).

Material and Methods

Okara was obtained from Food Technology Research Institute, soybean processing center, Agriculture Research Center, Giza, Egypt. It was freshly collected and it was freezed till used.

Isolation of fungal isolates

One gram of okara sample was suspended in 10 ml of physiological saline solution. Potato dextrose agar (PDA) medium was inoculated with 100 ml of okara suspension and incubated at 25°C. The developed colonies were then picked up and isolated as pure fungal colonies. These fungal cultures were maintained on PDA slants at 25°C. Also, *Aspergillus oryzae* was used in study, this isolate was obtained from the laboratory of Food Science and Technology, korea and was previously used in the production of soy sauce (Abu – Zaid, 2005).

Identification of fungal isolate

The macroscopic morphology of the fungal isolate was studied on Sabouraud dextrose agar medium and potato dextrose agar.

The microscopic morphology of each fungus culture was also studied by preparing slide culture technique on potato dextrose agar medium (Riddle, 1950). Identification was determined on basis of growth and microscopic morphology of each culture using the following universal manuals (De Hoog and Guarro, 1995; Kendrick, 2000).

Molecular characterization

Identification was further confirmed using nucleotide sequence analysis of specific amplification of inter transcribed spacer region (Tamura et al., 2001) by using sodium dodecyl sulphate (SDS) method.

The two oligonucleotide sequences used as primers in this study were sense and antisense characterized as described by (Weisburg et al., 1991). These primers are capable of amplifying nearly full-length 18S ribosomal DNA.

The fungus cultures were centrifuged at 20,000 rpm for 10 min and cell pellets were suspended in 100 ml phosphate – buffered saline. Cell suspension was again centrifuged at 20,000 rpm for 10 min and the pellets were used for DNA isolation.

Pellets were re-suspended in 200 µl enzyme solution contining 20 mg/ml lysozyme, 20 Mm Tris – HCl (PH 8), 2m MEDTA, and 1.2% Trion and incubated for 1 hr at 37°C. Then 25 µl of proteinase K and 200 µl of buffer AL (Qiagen) were added and the lysates were incubated at 56°C for 39 min as well as at 96 °C for 5 min. DNA was eluted with elution buffer 200µl of and stored at – 20 °C.

Display of the tree using iTOL

The tree generated from PhyML program was displayed using iTOL/ interactive tree of life online display tool.

Assessment of virulence of pathogenicity of fungal isolates

To sterile Blood Agar base, 5% (vol/vol) of sterile defibrinated blood has been added and inoculated with the test organism.

The results were recorded as beta or alpha hemolysis (Gerhardt and Philipp et al., 1994).

b- Assessment of aflatoxin and Ochratoxin

This method was carried out using High performance liquid chromatography (HPLC) (Regional Center of Food and Feed). Ochratoxin and alfatoxin of the fungal isolates were determined according to the method of (Moss, 1998), and (Elizalde-Gonzalez et al., 1998).

Fermentation of okara

Fresh okara was obtained after the removal of the liquid layer containing the water-

soluble components of soybean. The fresh okara was transferred into glass jars and was cooked at 121°C for 20 min in an autoclave. After cooling, the okara was inoculated with fungal isolates (Trichoderma harzinum and *Aspergillus oryzae*). These fungal isolates were activated by two successive transfers into potato dextrose agar (PDA, Difco, Detroit, Michigan, USA) slants and incubated at 30 °C for 3 days.

The fungal spores were harvested by flooding the surface of the agar with sterile distilled water containing 0.1% tween 80. The spore suspension was adjusted with sterile distilled water to a concentration of 106/ml which served as inoculum for the fermentation of okara. After mixing, aliquots (200 g) of the inoculated okara were placed in bamboo baskets (11 cm in diameter, 4 cm in depth), covered with wet cheesecloth and incubated at 30 °C for 10 days in an incubator. Sample was removed every day, dried (in anoven at 60 o C) and ground for analysis (Lin et al., 2006).

Assessment of chemical composition of okara

The crude protein content was determined using micro – kjeldahl method. Also, Ash, fat, crud fiber content, moisture were determined as described in A.O.A.C (2007).

Extraction of antioxidant

Water extraction of antioxidants

One gram of each sample of fermented okara was mixed with 20 ml of distilled water and homogenized for 2 min in homogenizer. The homogenized sample was boiled for 15 min to stop the enzyme activity and then kept at 60°C for 1 hr in the shaking water bath (150 rpm). After centrifugation at 3000 rpm, for15 min, the supernatant was filtered and the filtrate was used for further analysis (Wu Shi bin et al., 2010).

Solvent Extraction of antioxidants

After drying at 60°C for 24 hr, the prepared okara samples were extracted with methanol (1:5, w/v) by refluxing at 55°C for 3 hr in shaking water bath at 100 rpm. After filtering through Whatman No. 1 filter paper, the extract was vacuum concentrated and dried. The same method was used with different solvents (ethanol, acetone, diethyl ether) to carry out the extraction (Lateefet al., 2008).

a- Quantitative analysis of antioxidant by measuring of adiphenylpicrylhydrazyl (DPPH) free

The scavenging activity of DPPH free radicals was measured according to (Zhao et al., 2006). An aliquot of 1 ml of DPPH (0.1 mM) solution in ethanol and 0.5 ml of antioxidant extract (water or solvent extracts) were mixed. The mixture was shacked vigorously and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring the decrease in absorbance at 517 nm, using spectrophotometer model (T 60) and the DPPH radical scavenging was calculated according to the following equation:

% scavenging rate = [1-(A1 -A2)] x 100

Where A1 represents the absorbance of extract, A2 represents the absorbance of control.

b- Determination of antioxidant activity by reducing power measurement

The reducing power of the antioxidant (in water or solvent extracts) was determined according to Chang et al. (2002). An aliquot

of 0.5 ml antioxidant extract was added to 0.1 ml of 1% potassium ferricyanide. After incubating the mixture at 50°C for 30 min, during which ferricyanide was reduced to ferrocyanide, it was supplemented with 0.1 ml of 1% trichloroacetic acid and 0.1% FeCl3, and left for 20 min.

Absorbance was read at 700 nm to determine the amount of formed ferric ferrocyanide (Prussian blue). Higher absorbance of the reaction mixture indicated higher reducing power of the sample.

C - Determination of total phenolic content (TPC)

The total polyphenolic content was determined colorimetrically using the Folin-Ciocalteau (FC) method according to (Singleton et al., 1999) with some modifications. Test op'[sample (0.5 ml) of antioxidant extract was mixed with 0.2 ml of FC reagent and allowed to stand for 10 min then 0.6 ml of 20% sodium carbonate was added and mixed completely. The reaction mixture was incubated at 40°C for 30 min. Absorbance of the reaction mixture was measured at 765 nm. Gallic acid was used as standard.

Phytochemical analysis of antioxidant

The identification of the chemical constituents, polyphenolic compounds and phenolic acids of the antioxidants using HPLC was carried out in water and ethanol extracts of the two fermented okara using *Trichoderma harzianum* and *Aspergillus oryzae* in addition to non-fermented okara (control) (Panyaphu et al., 2012). Also, isoflavone and flavonoid contents of the fermented and non- fermented okara weredetermined by HPLC (Hewlett packard 1050, USA) according to the method of Lori et al. (1993).

Assessment of antimicrobial activity of water and ethanol extracts of antioxidant

antimicrobial activity of water and ethanol extracts of antioxidants samples was carried out using four pathogenic bacterial strains, and three pathogenic fungal strains. This method was carried out using disc diffusion method (Kotzekidou et al., 2008).

The bacterial strains used in this method (Gram positive and Gram negative) were kindly supplied by the Department of Microbiology, Faculty of Agriculture, Cairo University. These strains were Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922, Bacillus cereus ATCC 33018, Salmonella typhimurium ATCC 20231.

The bacterial cultures were maintained on nutrient agar slants at 4 o C and sub cultured on nutrient broth at 37oC for 24 hours prior to incubation. Food- borne filamentous fungus Aspergillus niger Van Tieghem was isolated from different spoilage sources (vegetables, fruits, grains) (Rizk et al., 2009).

Candida albicans CAIM-22 was obtained from MIRCEN (Microbiological Center CAIM) Ain – Shams.

Statistical analysis

The obtained data were statistically analyzed according to Snedecor and Cochran (1994) using LSD to compare the significance of the results.

Results and Discussion

Isolation of fungal isolates and assessment of virulence of pathogenicity & toxicity

Three fungal isolate from okara, in addition to the previously identified fungal isolate

Aspergillus oryzae. All fungal isolates were subjected to the assessment of virulence of pathogenicity. This experiment revealed the presence of green discoloration with two fungal isolates as shown in fig (1). Blood hemolysis occurred with two fungal isolates except for one unknown fungal isolate and *Aspergillus oryzae*. The results of HPLC analysis of alfatoxin and ochratoxin to confirm the toxicity of the unknown fungal isolate and *Aspergillus oryzae*, revealed the absence of either toxin.

Identification of the unknown fungal isolate

The surface colony color is white and scattered greenish patches become visible as the conidia are formed. The microscopic examination revealed the presence of repeatedly branched conidiophores, irregularly verticillate, bearing clusters of divergent, often irregularly bent, flask - shaped phialides. On the basis of the macroscopic and microscopic morphology, the unknown fungal isolate was identified as *Trichoderma harzianum*. The genetic identification of this strain was also performed.

In the present work, ITS1-5.8S-ITS2 rRNA region sequence of Trichoderma sp YMHS has been deposited in DDBJ (DNA Data Bank of Japan) under accession number AB610508 and aligned with related fungi. Trichoderma sp YMHS, showed 99% similarity to the species of *Trichoderma harzianum*.

Polymerase chain reaction (PCR) amplification

From the agarose gel in Figure 2, it can be seen that positive PCR amplification products were visualized for sample.

The following Automated sequencing results shown in Figure 3 were provided by VACSERA Biotechnical Services.

As shown in the results of table 1, the constituents of the sterilized okara (control) at moisture 76.60%, were protein (5.7%), fat (3.1%), fiber (2.8%) and ash (0.9%). These results are nearly resembled to the contents of fresh okara obtained by Rashad et al. (2010). The relative content of crude protein in all fermented okara samples increased than non- fermented one which improved the nutritional qualities or quantities of the okara, The protein contents of the fermented okara were increased in the range from 6.2 to 11.1% and the ash increased from 1.03 to 2.6% using Aspergillus oryzae, but with the fungal isolate *Trichoderma harzianum* the protein increased from 6.0 to 10.1% and the ash increased from 0.72 to 1.6%. The fat content reduced from 3.0% to 1.4% and crude fiber reduced from 3.4 to 1.5% using *Aspergillus oryzae* but the fungal isolate *Trichoderma harzianum* the fat content reduced from 3.1 to 1.3 % and crude fiber from 3.3 to 1.43 % as shown in Table 1. In this work, solid-state fungal fermentation significantly increased protein contents of okara which may refer to the rapid increase of fungal growth of fungi using okara as a substrate as previously mentioned on the fermented wheat bran by yeasts (Moore et al., 2007).

Matsuo (1997) observed an increase in protein content of fermented okara ranged from 22 to 27% using Neurospora inter media. Also Iluyemi et al. (2006) reported real increase in the protein content of the fermented palm kernel cake in the range from 29.4 to 54.50% using different fungal isolates. Zhu et al. (2008) reported that the okara with a low protein- content byproduct of tofu, soymilk or soy protein was used as substrate to obtain a functional food by

fermentation with B. subtilis. Fermentation of the okara led to the reduction in the crude fat contents. In a similar study, the fat content of fermented okara by N. intermedia was reduced from 15 to 9% (Matsuo, 1997). Previous studies have shown a reduction in the lipid content of different substrates fermented with different microorganisms (Das and Weeks, 1979; Iluyemi et al., 2006; Lateef et al., 2008). They attributed these results to the accumulation of lipids by the fungal strains and during fungal processing, some lipolytic strains assimilate lipids from substrates for biomass production leading to a general reduction of the overall lipid content of the substrate.

The reduction in the crude fiber contents of the fermented substrates is an indication of secretion of cellulose/hemicellulose-degrading enzymes by the yeasts during fermentation (Lateef et al., 2008), and suggested that individual yeasts may differ in their ability to modify soluble and insoluble fibers in the tested solid state reaction systems. Moore et al. (2007) stated that, this reduction may be explained by the fact that individual yeast preparations may have different enzyme activities and interact differently with soluble and insoluble fiber components. Several organisms including A. oryzae, *R. oligosporus* and baker's yeast have been reported to degrade cellulose/hemicellulose in a similar manner (Matsuo, 1989a,b; Moore et al., 2007). The ash contents of all the fermented okara substrates were improved (Table, 1). This indicated that all the fungal strains were behaved similarly in increasing ash contents. Since the determination of ash content is a measure of mineral levels in the substrates, it can be inferred that fermentation contributed to the higher levels of the obtained minerals. Similar improved levels of ash content, following fermentation have been reported by O'Toole (1999) using okara as a substrate.

From the above results, it is clear that the value of okara could be improved through fermentation process. In this study the solid-state fermentation may have potential application in improving the bioavailable nutritional properties of okara.

cavenging activity of DPPH free radicals of various solvent extracts of antioxidants during ten days of fermentation of okara was determined. The extracts at a dosage of 2 mg/ml showed various degrees of scavenging effect of DPPH radicals depending on the starter organism and the solvent used. In comparison, the control sample (non -fermented sterilized okara extract) showed a scavenging effect of 42.7 % for DPPH-free radical. The results in table (2) showed the degree of DPPH with different solvents; the water extracts showed a highest value of DPPH 92.8%, while diethyl ether showed the lowest values in both fungal isolates 69.2 %. The maximum values were obtained after 5 days of incubation, after that these values were decreased but still in a higher degree more than zero time, generally, the *Aspergillus oryzae* recorded the highest value of DPPH in all solvents than *Trichoderma harzinum*.

There were more antioxidant components present in fermented okara than in nonfermented one, which could react rapidly with DPPH radicals, and reduce almost all DPPH radical molecules corresponding to available hydroxyl groups (Brand- Williams et al., 1995). These results are similar with that of soybean koji using various organisms (Lin et al., 2006) and fermented okara by *Bacillus subtilis*, which had high antioxidant in water extract after 12h of fermentation (Zhu et al., 2008). Also, *Aspergillus oryzae* in koji had the highest antioxidant activity in cold water extract more than ethanol (Toshiyuki Kawasumi et al., 1999)

In the present study, assay of reducing activity was based on the reduction of Fe3+/ferricyanide complex to the ferrous form in the presence of reluctant (antioxidants) in the tested samples. The Fe2+ was then monitored by measuring the formation of Perl's Prussian blue at 700 nm (Oyaizu, 1986). The results in table 3 showed the values of the reducing power of the various fermented okara extracts and non-fermented one (control). All the fermented okara extracts showed a higher absorbance than did the non-fermented okara extract (0.110) at the same dosage level (2 mg/ml). after five days of incubation, the water extract of fermented okara using *Aspergillus oryzae* had a relatively higher reducing power (2.38). While the water extracts of *Trichoderma harzianum* had a reducing power ranged from (0.52 – 2.2).

Evidently, only fermented okara exhibited excellent reducing power. Fermented okara might produce certain metabolites with superior reducing power during fermentation, creating a major discrepancy between fermented okara and non-fermented okara. The same results of the enhanced reducing power of fermented bean and by products have been previously reported by Berghofer et al. (1998); Yang et al. (2000); Chung et al. (2002); Wang et al. (2004); Zhu et al. (2008). The observed increased reducing power may be due to the formation of reluctant that could react with free radicals to stabilize and terminate radical chain reactions during fermentation Yang et al. (2000). In addition, the intracellular anti-oxidants, peptides of the starter organism and their hydrogen-donating ability may also contribute to this increased reducing ability Yang et al. (2000).

Phenolics are secondary metabolites, and in part, are produced as a result of the plant's interaction with the environment (Snyder and Nicholson, 1990). They have biological properties such as antioxidant, anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, antiatherosclerosis, cardiovascular protection, improvement of the endothelial function, as well as inhibition of angiogenesis and cell proliferation activity. Most of these biological actions have been attributed to their intrinsic reducing capabilities (Harborne et al., 2007).

In the table 4, the total phenolic content of the water extract of fermented okara has a higher value than other solvent extracts. The fermented okara with Aspergillus oryzae had a higher phenolic content ranged from (9.2 to 43.8) mg gallic acid equivalents/g dry okara in all extracts after 5 days of fermentation. All samples of fermented okara had a higher phenolic content than that of the control samples (non -fermented).

Lin et al. (2006) reported that in comparison to the fermented soybean using A. awamori and A. oryzae, the total phenolic content and antioxidant activities were increased more than non-fermented soybean. Bhanja et al. (2009) demonstrated that fermented wheat grain is a better source of phenolics incomparison to non-fermented wheat. The total phenolic content of the non-fermented okara is 0.82 mg gallic acid equivalents/g dry okara

The water extract and ethanol extract of fermented okara using Trichoderma harzinum and *Aspergillus oryzae* were subjected to HPLC analysis of phenolic compounds, flavnoid and isoflavones. as shown in the results of table (5) the fermented okara with *Aspergillus oryzae* had a higher content of phenol in water extract more than *Trichoderma harzianum*, the chlorogenic acid, caffeine and coumarin were found in a fermented okara in a

remarkable higher amount more than other standards, the cholorogenic 60117.6 mg/100g, caffeine 67876.86 mg/100g and coumarin 45940.7 mg/100g Malberg and Theander (1985) found that the by HPLC analysis the chlorogenic acid gave a highest values. Fridman (1997) reported that chlorogenic acid was underwent time and light dependent change in methanolic and ethanol extract of potato.

Some authors have been suggested that during fermentation at least a partial cleavage or change in the phenolic glycosides takes place in association with the enzymatic breakdown of plant cell wall by fungi and, as a consequence, the aglycones are released. Those phenolic aglycones have exhibited a stronger antiradical capacity than corresponding glycosides (Vattem and Shetty, 2003; Starzynska-Janiszewska et al., 2008).

As previously reported by Lafka et al. (2007), the majority phenolic compound present in wine-making wast is gallic acid, along with catechin and epicatechin. The gallic acid content increased immediately after 6 h of culture. The highest amounts of gallic acid, 9±0.61 and 6.7±0.23 mg/g were recorded at 12 h for *A. niger* (GH1) and (PSH) respectively. In comparison, for *A. niger* (Aa-20) and (ESH) gallic acid content was 5.9±0.51 and 6.3±0.42 mg g at 9 h and 15 h, respectively.

The increased amount of total isoflavones in soybeanproducts after fermentation is consistent with findings reported by other investigators (Chen et al., 2000 and Shen et al., 2007).
However, the amount of isoflavones depends on the substrate, fungal inoculum and fermentation period (Chaiyavat Chaiyasut et al., 2010).

Soybean products fermented by SSF with *Trichoderma harzianum* showed stronger antioxidant activity than unfermented products, which was probably related to the markedly higher contents of phenolic acids, flavonoids and aglycone isoflavone with more free hydroxyl groups achieved during SSF (Singh et al., 2010).

Two different filamentous fungi (*Aspergillus oryzae* and Aspergillus awamori) used in SSF were very effective for the improvement of phenolic content and antioxidant properties of wheat grains.

In this study, fermented wheat grains were considered to be antioxidant richer and healthier food supplement compared to non-fermented wheat grains (Bhanja et al., 2009).

Assessment of antimicrobial activity

As showen in table 7 the ethanolic extract of antioxidant had a higher antimicrobial effect than water extract because the last one easy desirable and contaminated faster, also the phenolic compound more stable in solvent extract than water extract. The results agree with (Rauha et al., 2000; Estevinho et al., 2008), Rodriguez et al. (2010) who reported that the phenolic compound affect on microbial growth according to their constitution and concentration (Almedia et al., 2006) reported that chlorogenic acid and caffeic acid exhibited an inhibition on *Staphylococcus aureus, Bacillus cereus* and other food borne pathogens.

Table.1 Chemical analysis of various components of fermented okara using *Trichoderma harzianum* and *Aspergillus oryzae* in wet weight

Incubation time (days)	Protein			Fat			Crude fiber			Ash		
	control	*Trichoderma harzianum*	*Aspergillus oryzae*	control	*Trichoderma harzianum*	*Aspergillus oryzae*	control	*Trichoderma harzianum*	*Aspergillus oryzae*	control	*Trichoderma harzianum*	*Aspergillus oryzae*
Zero time	5.74	6.0	6.2	2.9	3.1	3.0	3.2	3.4	3.4	0.66	0.68	1.05
1 day	5.74	6.2	6.7	2.9	2.9	2.9	3.1	3.3	3.3	0.66	0.71	1.28
2 days	5.73	6.5	7.1	2.7	2.8	2.8	3.1	3.1	3.1	0.65	0.76	1.4
3 days	5.74	7.2	8.3	2.6	2.6	2.7	3.1	2.8	3.0	0.65	0.88	1.6
4 days	5.71	7.8	8.7	2.7	2.5	2.5	3.1	2.7	2.8	0.65	0.97	1.7
5 days	**5.70**	**8.1**	**9.0**	**2.5**	**2.1**	**2.4**	**3.1**	**2.5**	**2.6**	**0.66**	**1.1**	**1.8**
6 days	5.71	9.2	9.3	2.7	2.0	2.1	3.06	2.3	2.4	0.64	1.3	1.8
7 days	5.68	9.3	9.5	2.7	1.9	1.9	3.05	2.1	2.1	0.64	1.4	2.0
8 days	5.66	9.7	9.6	2.66	1.77	1.6	3.00	1.9	2.0	0.64	1.52	2.2
9 days	5.63	9.9	9.7	2.7	1.63	1.5	3.00	1.8	1.7	0.67	1.64	2.4
10 days	5.64	10.2	10.0	2.7	1.50	1.4	3.00	1.6	1.5	0.7	1.7	2.6

Table.2 Assessment of scavenging activity of DPPH free radicals of various solvent extracts of antioxidants of fermented okara using *Trichoderma harzinum and Aspergillus oryzea*

tnevloS **Incubation time (days)**	**Non fermented okara**	**Water**		**Ethanol**		**Methanol**		**Acetone**		**Diethyl ether**		**L.S.D**
		Trichoderma harzianum	*Aspergillus oryzae*	*Trichoderma harzianum*	*Aspergillus oryzae*	*Trichoderma harzianum*	*Aspergillus oryzae*	*Trichoderma harzianum*	*Aspergillus oryzae*	*Trichoderma harzianum*	*Aspergillus oryzae*	
Zero time	42.7	47.1	49.7	45.1	45.1	43.0	43.5	42.1	42.8	41.3	41.6	
1 day	42.7	50.1	58.3	48.8	52.3	46.4	49.8	44.7	48.4	44.1	45.3	
2 days	42.5	61.9	71.6	55.4	61.8	51.7	57.3	51.4	60.5	49.1	50.5	
3 days	42.7	69.5	77.4	62.6	68.9	59.8	65.2	58.5	71.3	53.2	54.8	
4 days	42.6	78.5	89.9	71.5	79.8	70.1	74.5	67.8	79.8	62.3	68.3	
5 days	**42.6±0.2^{i}**	**85.80±0.1^{c}**	**92.8±0.16^{a}**	**81.7±0.2^{e}**	**88.4±0.2^{b}**	**81.2±0.3^{e}**	**84.2±0.3^{d}**	**79.1±0.1^{f}**	**81.4±0.1^{e}**	**69.2±0.33^{h}**	**75.7±0.3^{g}**	**0.3462**
6 days	42.6	85.2	92.4	81.5	88.2	80.9	83.8	78.7	81.1	68.7	75.3	
7 days	42.5	85.1	92.2	81.0	88.0	80.2	83.1	78.0	80.6	68.3	75.0	
8 days	42.6	84.6	92.1	80.3	87.7	79.6	82.8	77.4	80,3	67.3	74.8	
9 days	42.5	84.1	92.0	78.5	87.5	77.7	82.5	75.1	80.0	66.1	74.2	
10 days	42.6	83.7	91.8	78.2	87.1	75.5	82.2	74.7	79.6	65.7	73.2	

Significant difference at $p < 0.05$, ± standard deviation (SD) are mean of triplicat

Table.3 Assessment of reducing power of various solvent extracts of antioxidants in water and solvent extracts using *Trichoderma harzianum* and *Aspergillus oryzae*

tnevloS Incubation time (days)	Non fermented okara	Water		Ethanol		Methanol		Acetone		Diethyl ether		L.S.D
		Trichoderma harzanum	*Aspergillus oryzae*	*Trichoderma harzianum*	*Aspergillus oryzae*	*Trichoderma harzianum*	*Aspergillus oryzae*	*Trichoderm harzianum*	*Aspergillus oryzae*	*Trichoderma harzianum*	*Aspergillus oryzae*	
Zero time	0.15	0.32	0.45	0.20	0.36	0.18	0.30	0.18	0.26	0.11	0.18	
1 day	0.15	0.43	0.61	0.26	0.43	1.9	0,38	0.19	0.31	0.15	0.21	
2 days	0.15	0.81	1.1	0.43	0.81	0.28	0,60	0.28	0.51	0.22	0.34	
3 days	0.13	1.02	1.6	0.74	1.01	0.48	0,91	0.48	0.78	0.39	0.51	
4 days	0.15	1.51	1.9	1.15	1.42	0.71	1.16	0.71	0.93	0.57	0.72	
5 days	0.15±0.01[j]	**1.93±0.21[b]**	**2.38±0.03[a]**	**1.52±0.16[d]**	**1.8±0.32[c]**	**0.92±0.35[g]**	**1.52±0.04[e]**	**0.92±0.27[g]**	**1.47±0.1[f]**	**0.73±0.01[i]**	**0.88±0.20[h]**	**0.3143**
6 days	0.14	1.87	2.98	1.43	2.38	0.87	1.98	0.87	1.42	0.68	1.10	
7 days	0.14	1.80	2.8	1.39	2.31	0.82	1.93	0.82	1.40	0.66	1.00	
8 days	0.13	1.72	2.79	1.31	2.30	0.79	1.90	0.79	1.38	0.61	0.98	
9 days	0.12	1.70	2.73	1.28	2.27	0.72	1.88	0.72	1.32	0.54	0.93	
10 days	0.10	1.69	2.7	1.2	2.22	0.64	1.80	0.60	1.29	0.49	0.88	

Table.4 Assessment of total phenolic contents (Mg Gallic acid/gm okara) in water and various solvent extracts using *Trichoderma harzianum* and *Aspergillus oryzae*

tnevloS Incubation time (days)	Non fermented okara	Water		Ethanol		Methanol		Acetone		Diethyl ether		L.S.D
		Trichodermaharzianum	*Aspergillusoryzae*	*Trichoderma harzianum*	*Aspergillusoryzae*	*Trichodermaharzianum*	*Aspergillusoryzae*	*Trichodermaharzinum*	*Aspergillusoryzae*	*Trichodermaharzianum*	*Aspergillus oryzae*	
Zero time	0.82	7.1	9.2	3.3	5.4	2.4	4.8	1.7	3.7	1.1	1.5	
1 day	0.82	11.3	13.6	7.4	8.2	3.5	6.9	2.6	5.4	1.5	2.1	
2 days	0.84	15.9	24.1	10.5	13.1	6.4	8.5	4.6	10.3	2.2	4.8	
3 days	0.83	20.3	29.7	14.4	18.4	10.8	13.7	7.2	12.7	3.9	5.9	
4 days	0.82	22.6	38.3	17.4	20.6	14.4	17.8	8.3	15.3	5.7	8.8	
5 days	**0.82±0.1[k]**	**26.5±0.32[c]**	**43.8±0.07[a]**	**21.3±0.1[d]**	**30.7±0.04[b]**	**16.2±0.1[g]**	**20.3±0.41[e]**	**14.9±0.01[h]**	**18.9±0.21[f]**	**7.3±0.56[j]**	**9.7±0.23[i]**	**0.3051**
6 days	0.83	26.3	43.7	20.4	30.5	15.5	19.9	14.1	18.6	6.8	9.5	
7 days	0.81	25.9	43.1	20.1	30.1	15.1	19.7	13.6	18.3	6.6	9.3	
8 days	0.81	25.1	42.8	19.7	29.8	14.9	19.6	13.1	18.2	6.1	9.2	
9 days	0.82	24.9	42.5	19.3	29.2	14.3	19.4	12.8	17.8	5.4	8.8	
10 days	0.82	24.2	42.2	18.9	28.8	13.7	19.3	12.2	17.3	4.9	8.7	

Significant difference at $p < 0.05$, ± standard deviation (SD) are mean of triplicate.

Table.5 Detection the types of phenolic compounds in fermented and non fermented okara (mg/100g)

standards	Non fermented okara	Water extract of *Trichoderma harzianum*	Water extract of *Aspergillus oryzae*	Ethanol extract of *Aspergillus oryzae*	Ethanol extract of *Trichoderma harzianum*
cigallE	6.5	10.9	44.87	22.6	2.1
cillaG	10.1	15.6	30.3	18.9	9.9
Pyrogallol	122.5	212.7	471.9	347.4	124.8
Protocatchin	23.3	47.8	172.4	65.1	23.6
Catchein	55.4	155.2	277.6	289.7	182.3
Cinnamic	2.8	6.1	7.7	4.5	2.3
Catechol	10.8	16.2	29.5	21.9	8.6
Cholorogenic	14623.7	23656.7	60117.6	64599.4	24459.9
Vanilic	34.9	66.7	287.7	183.1	33.8
Caffeic	1.9	6.5	8.4	8.9	6.9
Ferulic	88.5	148.2	115.8	69.8	130.8
Salycleic	19.6	39.6	147.	215.3	69.2
Caffein	20043.7	25538.6	66754.7	67876.8	19515.9
Coumarin	29976.1	43038.3	45940.7	21765.8	19515.9
Benzoic	9.6	20.7	31.1	28.7	19.5

Table.6 Isoflavons and flavnoid contents of (water and various solvents) extracts of non fermented and fermented okara (mg/100g)

Standards	Non – fermented okara	Water extract of *Aspergillus oryzae*	Ethanol extract of *Aspergillus oryzae*	Water extract of *Trichoderma harzianum*	ethanol extract of *Trichoderma harzianum*
Isorohmntin	13.2	82.9	60.5	52.2	27.4
Biochanin	88.3	501.7	831.3	197.7	99.7
Genistein	122.8	434.7	537.9	213.6	191.7
Formentin	178.7	863.9	303.5	552.4	247.4
Diadzien	139.4	476.1	517.7	288.3	290.1
Flavnoid					
Apignen	13.9	16.11	16.09	14.4	15.07
7-hydroxy flavone	6.9	14.8	16.9	14.01	14.4
Hispertin	ND	28.4	14.6	21.5	7.8
Hisperidin	156.9	446.9	234.8	239.8	187.9
Rosmarinic acid	12.02	23.9	27.8	17.5	13.3
Rutin	10.03	10.7	12.5	11.8	11.9
Quercetin	0.06	1.78	0.459	0.353	0.211
Quercitrin	0.04	0.296	0.167	0.09	0.05
Kampfrol	0.07	2.25	0.91	1.01	0.504
Luteolin	7.19	57.9	258.9	11.39	8.49

Table.7 Assessment of antimicrobial effect of water and various solvent extracts of fermented okara using *Aspergillus oryzae* and *Trichoderma harzianum* against some pathogenic strains, inhibition zone diameter (mm)

Pathogens **Phenolic extracts**	***Salmonella typhimurium***	***Staphylooccus aureus***	***Bacillus cereus***	***Eschrichia coli***	***Candida albicans***	***Aspergillus niger***
	Inhibation zone (mm)					
Water extract of fermented okarausing*Aspergillus oryzae*	15	13	12	14	12	13
Water extract of fermented okarausing*Trichoderma harzianum*	12	12	12	12	10	11
Ethanol extract of fermented okarausing*Aspergillus oryzae*	22	19	18	18	15	16
Ethanol extract of fermented okarausing*Trichodermaharzinum*	20	16	17	15	13	14

A *B* *C* *D*

Fig.1 Blood hemolysis of fungal isolates ; A,B represent the alpha, beta blood haemolysis caused by unknown fungal isolates, C,D represent the non- hemolysis of the blood cultures inoculated with C (unknown fungal isolate), and D; *Aspergillus oryzae***.**

Fig.2 A Photograph of an agarose gel loaded showing PCR amplification products for (lane 1) 1kbp marker, S1 isolate (lane 2)

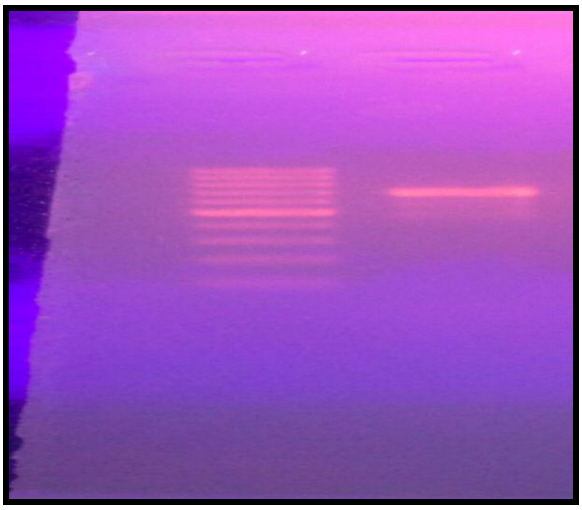


Fig.3 BLAST alignment of the nucleic acid sequence derived from unknown 18S rRNA gene sequences found on the NCBI database. Identities = (100%)

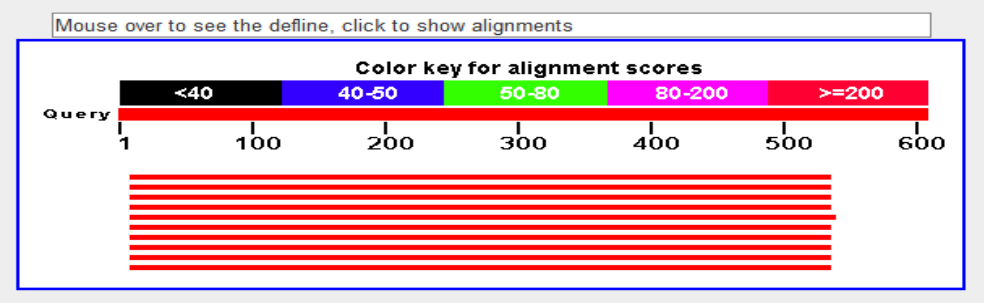


Fig.4 Phylogenetic dendrograms for fungal isolate reconstructed according to the Bootstrap Neighbour Joining (NJ) analysis function of CLUSTAL X. Bootstraps were done using 1000

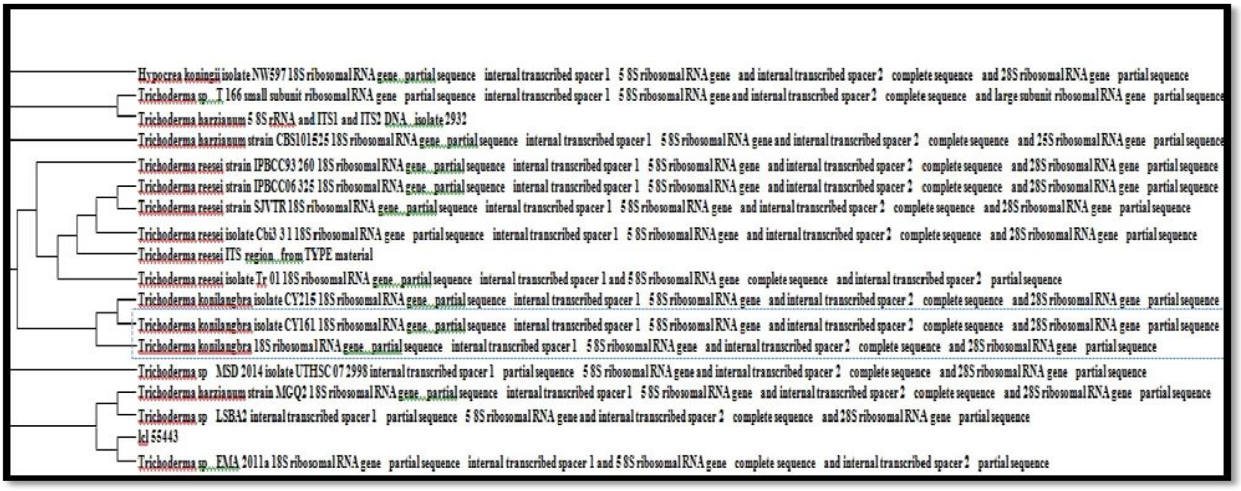
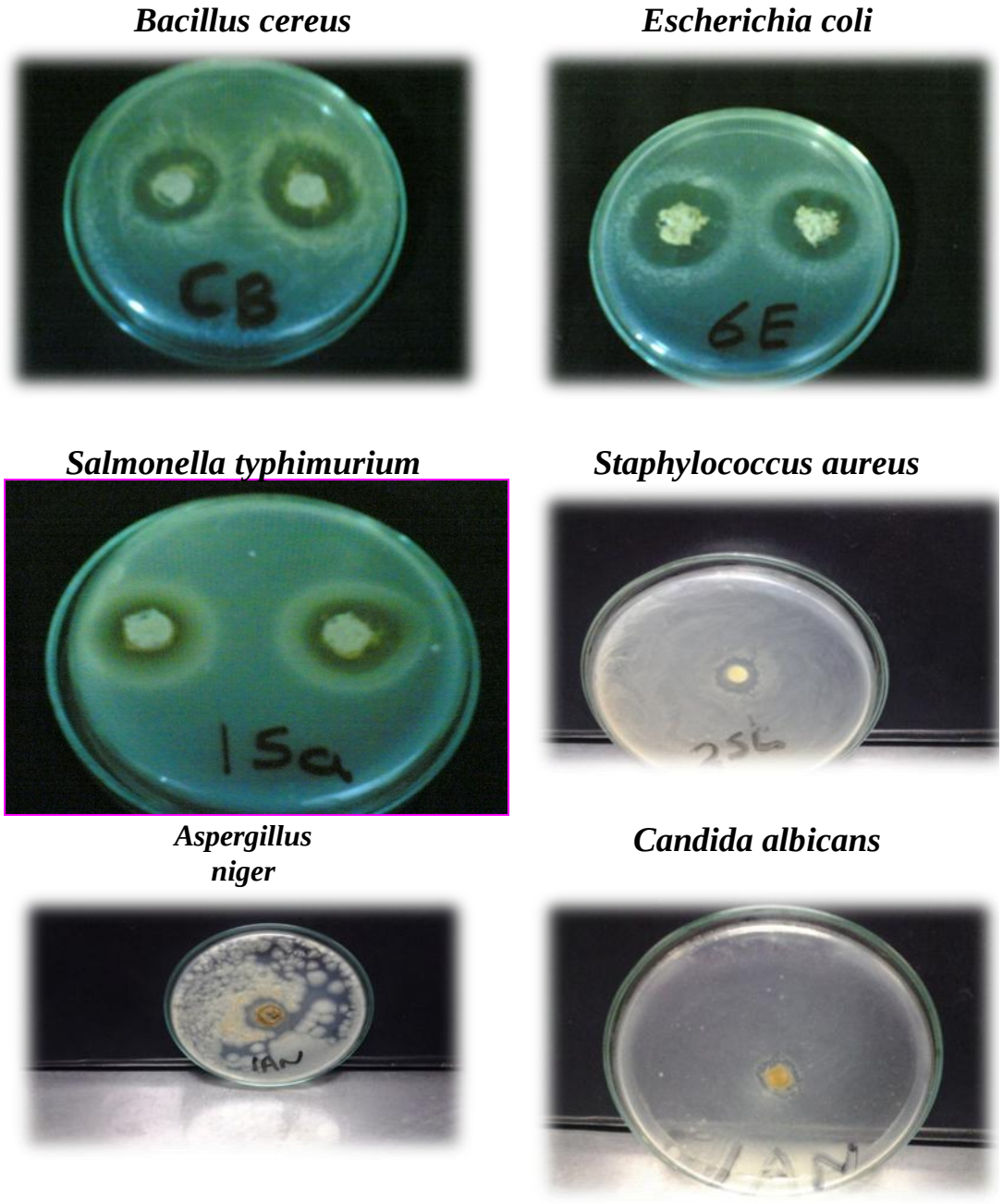


Fig.5 Assessment of antimicrobial effect of water and various solvent extracts of fermented okara using *Aspergillus oryzae* and *Trichoderma harzianum* against some pathogenic strains, (inhibition zone diameter mm)



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