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Biotechnological Production of Volatile and Non-Volatile Antioxidant Compounds From Fermented Soy Bean Meal with *Trichoderma sp*.

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ABSTRACT

Three strains of *Trichoderma sp.* were screened for *β*-glucosidase activity and their potential for the breakdown of isoflavone glucosides to the biologically active aglycones in defatted soy bean meal. The main flavonoid products of fermentation process were evaluated by qualitative and quantitative HPLC analysis. Also, the production of volatile compounds in both the crude soy meal (CSM) and defatted soy meal (DSM) cultured by *Trichoderma harzianam F.555* were determined by GC-MS. Isoflavones in defatted soy meal fermented by *Trichoderma viride,* F-516 or *T. harzianam* F-555 or *T. reesei* F-417 at 37 °C for 48 h were determined. Daidzein and genistein aglycones concentrations were significantly increased after fermentation to 162% and 362.4 % for *T. reesei*; 151.3% and 944 % for *T. harzianam* and up to 80.6% and 711 % for *T. viride*, respectively. Results showed that fermented defatted soy meal by *T. harzianam* F-555 *T. viride* F-516 and *T. reesei* F-417 increased levels of bioactive genistein (10.44, 8.11 and 4.62 folds, respectively). Moreover, defatted soy meals were fermented separately by *T. reesew, T. harzianam* F-555 and *T. viride* F-516 showed highly significant increase of daidzein (2.64, 2.51 and 1.82 folds, respectively) in comparison to unfermented defatted soy meal (DSM). The levels of aglycones increased, while the corresponding levels of glucosides decreased. Furthermore, in fermented defatted soy meal (FDSM) with *T. harzianam*, compounds such as Butylated Hydroxytoluene (38.19 %), 1,4-Di-anhydro-mannitol (14.25 %) and 2-Dimethyl (isopropyl) silyloxy methyl tetra –hydrofurane (9.62 %) were present at high concentrations as dominant volatiles. While, the major volatile compounds in fermented crude soy meal (FCSM) were 9,12-Octadecadienoicacid (Z,Z), methyl ester (28.08 %), 2,2-Bis[4-(4,6-dichloro-1,3,5-triazin-2-yl)oxy]phenyl]1,1,1,3,3,3hexafluoropropane (10.61 %), Oleic Acid (10.61 %), and 9-Octadecenoic acid-3-[(1-oxohexadecyl)oxy]-2-[(1-oxooctadecyl)-oxy] propyl ester (9.08 %). Total phenolic content and *in vitro* antioxidant activity of ethanolic extract of (FDSM) with *T. harzianam* were higher than that in (CSM), (FCSM) and (DSM). Moreover, the major volatile antioxidant-active compound was butylated hydroxytoluene. The current findings indicate that fermentation process carried out in (DSM) inoculated with different microorganisms produced significant changes in flavonoids and volatile contents. Therefore, both volatile and non volatile products of (FDSM) are considered as prospective bioactive and functional by-products of soybean oil production. These data suggest that microbial fermentation of (DSM) might be valuable sources of antioxidant that can be applied in food and pharmaceutical industry.

Key words: ***Isoflavones, HPLC, Volatile compounds, GC/MS, Soy bean, Trichoderma sp.***

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INTRODUCTION

Soybeans and the foods made from them are known to have good nutritional and functional qualities, not only for their high protein and oil content, but also because they are a source of phytochemicals. A group of phytochemicals that can be found in soybeans is the isoflavones. These phytochemicals can potentially prevent chronic diseases, such as cancer, osteoporosis and some heart problems and are recommended for alleviation of post-menopause syndrome [1-3]. The content of isoflavones in soybeans is affected by the preparation and processing of the beans. Isoflavones, which occur mainly in soybeans, are the most studied class of phytoestrogens. Daidzein, glycitein and genistein are the three basic aglycone isoflavones without sugar moiety. Daidzin, glycitin and genistin are *β*-glucoside isoflavones, which are derivative forms from their corresponding aglycones. The *β*-glucoside isoflavones are the major form of isoflavone found in unprocessed soy flour [4]. They could be decomposed by losing the *β*-glucoside group to form aglycone isoflavones during heating and fermentation [4]. Aglycone soy isoflalvones, the biologically active estrogen-like isoflavones[5], are absorbed faster and in higher amounts than their glucosides by human [6]. So far, the health function of soy isoflavones in preventing development of heart disease and cancers have been demonstrated [7-9] and received much attention from the health authorities and consumers.

Generally processing of soybeans increases the concentration of aglycones through the hydrolysis of glucosides. The aglycone form is more readily absorbed and bioavailable than the glucoside form [6]. This knowledge has led to the development of aglycone enriched products by fermentation using *bifidobacteria* [10]. Isoflvones are generally found in soy as glycosides [11] and less as aglycone unless they have been fermented [12]. The awareness that conjugated forms have less biological effect than aglycones has motivated the development of biotechnological processes to obtain soybean products rich in isoflavone aglycones by different treatments such as addition of enzymes or by the action of different microorganisms [13-15]. Fermentation is one of the major processes used in the production of foods from soybeans. Fermentation is defined as bioprocessing using microorganisms and their enzymes to achieve desirable quality characteristics of food products. This fermentation changes the physico-chemical and organoleptic properties of soy products such as color, flavor and active components. However, there is increasing interest for fermented soybean products that possess significant health effects as well as full, complex aroma characteristics. Randhir *et al.,* and Dueñas *et al*., [16, 17] reported that fermentation processes significantly increased the free radical scavenging capacity of legumes, which could be explaned by changes in the associated phenolic composition.

Several microorganisms have been used to carry out the soybeans fermentation, mainly filamentosus fungi, Bacillus sp. and lactic acid bacteria. It was demonstrated that soybeans fermented with filamentous fungi enhanced the phenolic content and radical scavenging activity [18]. In addition, fermentation of soybeans with *Aspergillus oryzae* increased antioxidant activity in fermented material [19]. Some Lactobacilli and Bifidobacteria are known to hydrolyze beta glucosides and enhance the bioavailability of isoflavones by fermentation [20]. Soybeans not only contain isoflavones but also other phenolic compounds, such as phenolic acids, flavonols, flavanones, etc. Therefore, biofermentation could modify these components and as a result the antioxidant activity of fermented soybean [18, 21, 22]. Defatted soybean meal (DSM) is a by-product of soybeans crushed for oil. The world production of soybean meal is continuously increasing. DSM is widely available with an annual production of about 150 million tons worldwide [23]. Recently many researchers are interested in finding safe and powerful natural antioxidants, which can be substituted for current and commercial synthetic antioxidants. (DSM) has become a good candidate for sources of natural antioxidants because of its high content of isoflavones and phenolic compounds. Thus, the objective of this study was to investigate the antioxidant properties, total phenolic content, active compound composition (isoflavones glucosides and aglycones and/or volatile compounds) of (FDSM) using microbial fermentation with three bacteria specieses, *Trichoderma viride, F-516*, *T. harzianam,* F-555, and *T. reesei,* F-417. Then, the most efficient specie to enrich (DSM) in isoflavones aglycones content was used to study the resulting volatile components as a result of biofermentation of (CSM) and (DSM). This study will lead to the selection of an adequate microorganism that could be used as a starter to obtain fermented product with high increased levels of bioactive phenolic and volatile compounds in order to develop functional materials, potential medicinal agents and natural antioxidants for food and pharmaceutical industry.

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MATERIALS AND METHODS

Chemicals and reagents

Daidzin, genistin, indicators, 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) and Folin-Ciocalteu reagent, gallic acid and *β*-carotene were purchased from Sigma Chemical (St. Louis, MO). Trifluoroacetic acid and liquid chromatography grade acetonitrile, methanol, and n-hexane were obtained from Merck (Darmstadt, Germany).

Standard isoflavones solution preparation

The standard isoflavone (1.0 mg) was dispersed in 10 mL methanol and stirred for 10 min. The mixed solution was transferred to a 500-mL volumetric flask after it was passed through a filter paper (0.45 µm x diameter 15 mm, Whatman, Maidstone, UK). This solution was stored at -20 °C until use.

Microorganisms and culture conditions

Trichoderma viride F-516 *Trichoderma harezinum* F-555 and *Trichoderma ressei* F-417 were obtained from Microbial Chemistry Lab. National Research center, Dokki, Cairo.Egypt, and maintained on potato dextrose agar slants at 30^{o}Cfor 72 hrs. The spore suspensions were prepared by adding 10 ml of sterilized water to slant cultures and the surface was gently rubbed with a sterilized wire loop. The fermentation was carried out in 250 ml Erlenmeyer flasks containing 5g of defatted soybean flour moistened to50 % (v/w) with distilled water. One milliliter of spore suspension (10^{6} spores) was used as inoculum. The cultures were incubated at 30^{o}C for 3 days by solid state fermentation.

Soybean extraction

Volatiles and non volatiles (phenolic) compounds were extracted from crud and defatted soy meal samples with a modification, as reported [24, 25]. Each soybean sample [crude soy meal (CSM), fermented crude soy meal (FCSM), defatted soy meal (DSM), and fermented defatted soy meal (FDSM)] was ground individually into powder using a coffee bean blender. The ground soy part (5.00 g) was weighed into a 25mL test tube and extracted using 15 mL ethanol. the solvent layer was separated from the solid residue by centrifuging at 2000 ×g for 10 minutes. The clear supernatant was transferred to a clean test tube. Then the solid residue was extracted with another 15 mL of ethanol. The separated ethanol layers were combined and dried using a vacuum evaporator at less than 50°C. The dried soybean extract was weighed and stored at -20°C until samples were subjected to:

- Identification of volatiles compounds by GC/ MS
- Determination of Total Phenolic Content
- Determination of Antioxidant Activity (Free Radical Scavenging Capability) Using the DPPH Method and *β*-Carotene-Linoleate Scavenging Assay.

Gas chromatographic-mass spectrometric analysis (GC/ MS)

The analysis was carried out by using a coupled gas chromatography Hewlett-Packard model (5890) / mass spectrometery Hewlett-Packard-MS (5970). The ionization voltage was 70 eV, mass range m/z 39-400 a.m.u. The oven temperature was maintained initially at 50°C for 5 min., and then programmed from 50 to 250°C at a rate of 4°C/min. Helium was used as the carrier gas, at flow rate of 1.1 ml/min. The injector and detector temperatures were 220 and 250°C, respectively. The isolated peaks were identified by matching with data from the library of mass spectra (National Institute of Standard and Technology) and compared with those of authentic compounds and published data. The quantitative determination was carried out based on peak area integration. Interpretation on mass spectrum GC-MS was conducted using the database of (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The Name, Molecular weight and structure of the components of the test materials were ascertained.

Determination of total phenolic content (TPC)

The level of total phenols in the extracts was determined by using Folin–Ciocalteu reagent and external calibration with gallic acid [26]**.** The absorbance was measured at 760 nm using a spectrophotometer (Shimadzu, Japan). The concentration of the total phenolics was calculated as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The determination of total phenolic compounds in the extracts were carried out in triplicate and the results were averaged.

HPLC analysis of isoflavone

Isoflavones were extracted from defatted soy meal (DSM) and fermented defatted soy meal (FDSM) samples as reported [27]. Samples were extracted with 20 ml of 80 % methanol and stirred at 60 °C for 1 h. The insoluble residue was separated by centrifugation (5,000 ⍰⍰g for 10 min), and the insoluble pellets were dried under vacuum. The dried pellet was dissolved in 5 ml of 50 % methanol and then extracted 4 times with 20 ml of n-hexane to remove residual fat. After the n-hexane was removed with a pipette, the pellet was again dried. The insoluble residue was re- dissolved in 10 ml of 80 % (v/v) methanol. The samples were filtered through a 0.45 ⍰m millipore PVDF filter membrane (Schleicher and Schuell, GmbH, Dassel, Germany) prior to HPLC analysis. HPLC analysis was performed according to Wang and Murphy (1994) with modifications, using a Hitachi Model L-6200 equipped with an ODS-AM-303 column (250 ⍰⍰4.6 mm i.d., 5 ⍰m) and an ultraviolet spectrophotometer L-2000 (Hitachi Ltd.). The mobile phase consisted of 0.1 % (v/v) glacial acetic acid in water (A) and 0.1 % (v/ v) glacial acetic acid in acetonitrile (B). The gradient was as follows: 85 % A was at 20 min, followed by a decrease to 76 % A over 10 min, maintained for 10 min, followed by a decrease to 65 % A over 4 min, steady for 8 min, and then increased to 85 % A over 5 min prior to the next injection. The flow rate was 1.0 ml/min. The isoflavone components were detected at 254 nm. Quantitative data for each isoflavones was obtained by comparison to known standards.

Determination of antioxidant activity

β-Carotene-Linoleate Scavenging Assay

The antioxidant activity of the extracts were evaluated using *β*-carotene-linoleate model system. 0.1 mg of β-carotene in 0.2 mL of chloroform, 10 mg of linoleic acid and 100 mg of Tween-20 were mixed. The solvent was removed at 40 ^{0}C under vacuum and the resulting mixture was diluted with 10 mL of water and was mixed well. To this mixture, 20 mL of oxygenated water was added. Four milliliter aliquots mixtures were pipetted into different test tubes containing 200 µL of each extract (50, 100, 200 and 400 µg/ml) and TBHQ (50, 100, 200and 400 µg/ml) in ethanol. TBHQ was used for comparative purposes. A control containing 200 µL of ethanol and 4 mL of the above emulsion was prepared. The tubes were placed at 50 ^{0}C in a water bath and the absorbance at 470 nm was taken at zero time (t= 0). The absorbance was continued to be measured until the colour of *β*-carotene disappeared in the control tubes (t =60 min) at an interval of 15 min[28]. A mixture prepared as mentioned above without *β*-carotene served as blank. All determinations were carried out in triplicate. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the *β*-carotene using the following formula,

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

Where: A_B: absorption of blank sample (t=0 min).

A_A**:** absorption of sample solution (t=60 min).

Radical scavenging activity using DPPH assay

Antioxidant activity was also determined by DPPH assay using spectrophotometer at 517 nm.[29]. Each extract of different concentrations (50, 100, 200 and 400 µg/ml, respectively) and TBHQ (50, 100, 200and 400 µg/ml) were taken in different test tubes. Four milliliter of 0.1 mM methanol solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at room temperature for 30 min. The control was prepared as the same without any extract and MeOH. The changes in the absorbance of the prepared samples were measured at 517 nm. Radical scavenging activity was estimated as the inhibition percentage and was calculated using the following formula,

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% Inhibition = $[(A_B-A_A)/A_B]$ X100 Where: A_B: absorption of blank sample (t=0 min),

A_A: absorption of sample solution (t=30 min).

Statistical analysis

The results are reported as Mean ± Standard deviation (S.D.) for at least three times experiments. Statistical differences were analyzed by one way ANOVA test.

RESULTS AND DISCUSSION

Isoflavone concentration in DSM fermented with *T. reesei*, *T. viride* and *T. harzianam* are shown in Table 1. Isoflavone aglycones concentrations (Daidzein and Genistein) had a significant increase in defatted soy bean meal after being fermented to 162 and 362.4 % for *T. reesei*; 151.3 and 944 % for *T. harzianam* and up to 80.6 and 711 % for *T. viride*, respectively. Similar results were reported by Tsangalis *et al.* [30] that *Bifidobacterium animals* Bb- 12 was grown in soymilk increasing hydrolysis of isoflavone glucosides to aglycones from 8 % to 50 % of total isoflavones. *Bifidobacterium breve*, *Bifidobacterium bifidum* and *L. casei* subsp. rhamnosus were also been shown to produce high content of isoflavone aglycone in fermented soybean milk[31]. Isoflavone glucosides in soybeans are hydrolyzed by - glucosidase produced by microorganisms, thereby increasing isoflavone aglycones during fermentation. *Lactobacillus* and *Bifidobacteria* in the intestinal microflora can produce active-glucosidase for enzyme induced biotransformation of isoflavone glucosides to aglycones [32-34]. Soymilk fermented with *L. paraplantarum* KM was 6 and 7- folds of daidzein and genistein, respectively after 6 h of fermentation [35]**.** Our results showed that *T. harzianam* increased level of bioactive genistein (10.44- folds) compared to *T.reesei* and *T.viride* (4.62 and 8.11 – folds, respectively). On the other hand, *T. harzianam* and *T. reesew* showed significant increase of mean values of daidzein (2.51 and 2.64 – folds, respectively) higher than *T. viride* (1.86- folds). Comparing genistein with daidzein, they both have 4'-OH and a 7-glucoside group. Genistein has an extra –OH at 5 site of a ring. In turn, genistein showed a slightly higher degradation rate than daidzein (Figure 1). The reason might due to their different molecular structure. Such these differences in isoflavones glucosides hydrolysis capabilities according to microbial strains were in agreement with results reported by Pham and Shah [36] which showed that *B. animalis* B hydrolyzed higher level of isoflavone glycosides to aglycones than *B. animalis* A in skim milk powder. These results may be useful for selecting the suitable microbial strains to produce the isoflavones aglycones from soybean meal for future applications in food industry and pharmaceutical products.

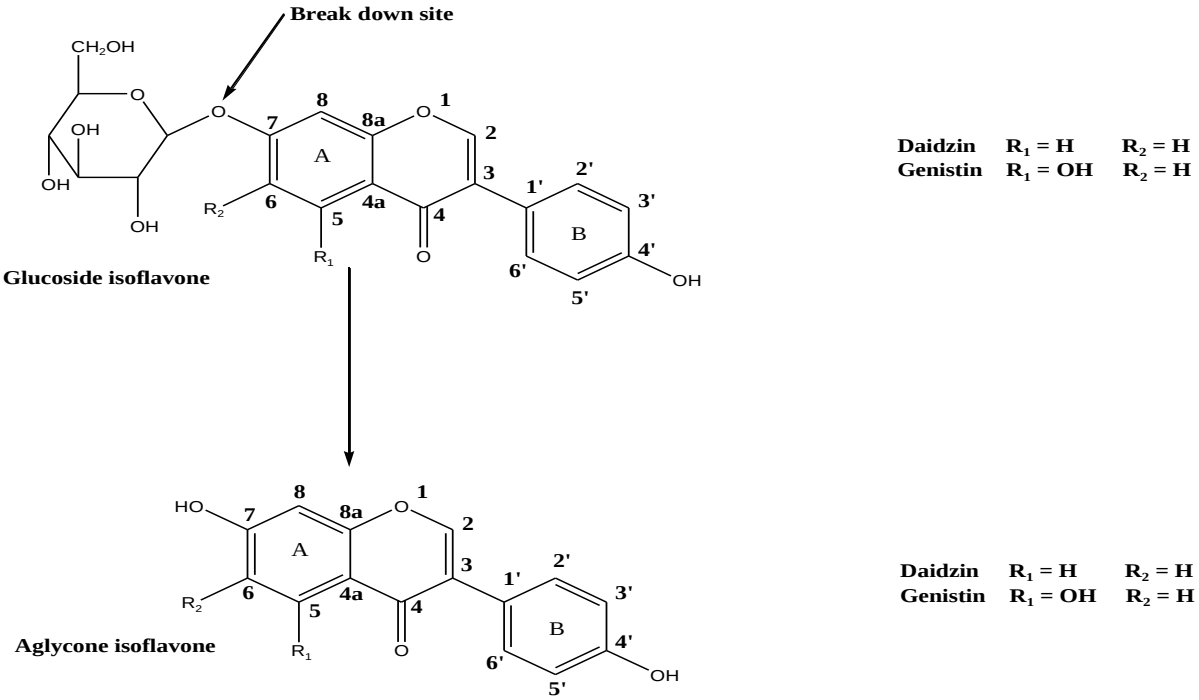

Figure 1: Chemical structure of glucoside and aglycone isoflavones

On the other hand, the volatile components in (CSM) and (DSM) as well as the produced volatile products of the biofermentation of (FDSM) and (FCSM) by *Trichoderma harzianam F.555*, were investigated. This specie was used in the fermentation process because it was the most efficient one to enrich defatted soybean meal in isoflavones aglycones. Greater qualitative and quantitative differences in the identified volatile compounds were observed between the analyzed samples. Most of the compounds were formed as a consequence of the technological process of fermentation. Tables 2 and 3 list the identified volatile components in (DSM) and (FDSM) samples by their chemical classes, peak areas and retention time, respectively. The results of GC-MS of (FDSM) showed that most of the volatile compounds in the culture extract were different than the (DSM) at R_t values and mass data. A total of 11 volatile components were characterized in the (DSM). Metabolites were identified as 1 acid, 3 alkanes, 3 ketones, and 4 benzene derivatives by R_t values and mass data (molecular weight and molecular formula) Table 2. While, the volatiles in (FDSM) were 20 compounds, including 2 acids, 3 acid salts, 3 alcohols, 2 alkanes, 4 aromatic heterocyclic, 1 ketone, 3 nitrogenous and 1 phenolic compounds. The most abundant compound in (FDSM) was Butylated Hydroxytoluene, which accounted for approximately 38.19 % of the total volatiles on average. Additionally, 1,4-Di-anhydro-mannitol and 2-Dimethyl(isopropyl)-silyloxy-methyl-tetra-hydrofurane were major volatiles, comprising 14.25% and 9.62 % of the total quantified volatiles, respectively.

Butylated hydroxytoluene (BHT) is one of the synthetic antioxidant agents commonly used for food additives to prevent lipid oxidation [37]. Butylated hydroxytoluene is primarily used as a food additive that exploits its antioxidant properties. It is approved for use in European Union under E321 and in the U.S. by the Food and Drug Administration via regulation. BHT is also documented as an antioxidant additive in such diverse products as cosmetics, pharmaceuticals, rubber, electrical transformer oil (at 0.35%),[38] **a**nd embalming fluid. In addition, ingestion of this synthetic antioxidant may decrease the risk of developing the chronic diseases[39,40]. BHT is marketed as a health food supplement in capsule form. It has been reported to have anti-viral effects, particularly in use against herpes family viruses and in combination with L-lysine and vitamin C. [41,42]. Closely related phenol antioxidants exhibit low toxicity, For example, the LD_{50} of 2,6-di-tert-butylphenol is greater than 9 g/kg. [43]. Therefore, the search for natural antioxidants as a substitute for synthetic ones has become an important goal for the food technology industry in recent years. Study has found that *T. harzianam* F. 555 is capable of producing this compound from defatted soy bean. Confirmation was made via gas chromatography–mass spectrometry analysis.

Among the identified volatile chemicals, 1,4-Di-anhydro-mannitol has antimicrobial activity[24]. In addition, closely structured related compound 2,5-anhydro-mannitol (100 to 200 mg/kg) decreased blood glucose by 17% to 58% in fasting rats, streptozotocin-diabetic mice, and genetically diabetic mice [44], so, it is recommended to test this compound as antidiabetic agent. 2-Dimethyl- (isopropyl)-silyloxy-methyl-tetra-hydrofurane is closely related in structure to 2-methyl-tetra -hydrofurane which is usable as a biofuel [45]. However, with the exception of these three mentioned components, there were no quantitatively dominant volatile compounds. The comparison of volatile compounds in (FDSM) and (DSM) revealed that no one of detected volatiles was found in both samples. So, it could be indicated that the volatile constituents in (FDSM) extract were products of the biotransformation process.

A total of 8 volatile compounds were characterized in the crude sample without fermentation (CSM). Chromatographic peaks were identified according to retention times, and mass spectra compared with data base as 1 acid, 1 alkenes, 4 aromatic heterocycle, and 2 benzene derivatives (Table 4). The volatiles in fermented crude soybean meal (FCSM) were extracted using ethanol and then analyzed by GC–MS, Table 5 lists the identified volatile components in the FCSM sample by their chemical classes, relative concentrations and retention time. These compounds were grouped into 1 acid, 3 aromatic heterocycle, 4 esters, 1 ketone, and 1 benzene derivatives according to their chemical structures. 9,12-Octadecadienoicacid (Z,Z), methyl ester (28.08 %), Hexadecanoic acid, ethyl ester (13.17 %), 2,2-Bis-[4-[(4,6-dichloro-1,3,5-triazin-2-yl)oxy] phenyl]-1,1,1,3,3,3-hexafluoropropane (10.61 %), Oleic Acid (10.21 %) and 9-Octadecenoic acid -3-[(1-oxohexadecyl)oxy]2-[(1-oxooctadecyl) oxy] propyl ester (9.08 %) were present in higher proportions in the fermented crude soya. 9,12-Octadecadienoic acid (Z,Z), methyl ester has many pharmacological effects, hepato-protective, anti-histaminic, hypo-cholesterolemic, and antieczemic. Hexadecanoic acid-ethyl ester has the property of antioxidant, Hypo-cholesterolemic, Nematicide, Pesticide, Lubricant, Antiandrogenic, Flavor, Hemolytic and 5-Alpha reductase inhibitor[46]. Oleic acid has antiinflammatory, antidiabetics [47].

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Table 1: Isoflavonoid content (mg/100g) of (DSM) and (FDSM).

	Daidzein	Genistein	% Change of aglycones after fermentation	
			Daidzein	Genistein
(DSM)	12.18±0.91	1.81±0.01		
(FDSM) by:				
T. reesei	32.18±0.93	8.37±0.21	162	362.43
T. harzianam	30.61±2.12	18.91±1.01	151.31	944.03
T. viride	22.22±1.02	14.68±0.90	80.62	711.05

Table 2: GC-MS analysis of volatile chemical compounds of (DSM)

Type of compounds	Identified compounds	Area %	Rt
Acids	Fumaric acid	7.21	37.43
Alkanes	2-Pentadecyl-1,3-dioxolane	6.41	44.33
	5-Methoxy-1-(3-methylbuta-1,3-dienyl)-7 oxabicyclo heptane	6.01	29.58
	Nonacosane	7.28	23.85
Ketones	3-Bromophenyl-7-chloro-10-hydroxy1-9-acridinedione	5.90	26.97
	7,9-Ditertbutyl-1-oxaspiro-4,5-deca-6,9-diene-2,8-dione	19.16	33.18
	3,9-Methano-azonine-10,11-dione	5.39	33.31
Benzene drivatives	11,12-Dimethoxy1-methyl-3,4-dihydronaphtho-isoquinoline	5.40	6.82
	5-methylstannyl-3-methylphenylpyridine	6.17	10.92
	10-Hydroxy-1-methoxymethylbenzo-phenanthroline	5.38	14.64
	2,3-dicyano-7,7-dimethyl-5,6-benzodiene	23.80	24.32
Total (%)		98.20%	

Table 3: GC-MS analysis of volatile chemical compounds of (FDSM) by *T. harzianam* F.555

Type of compounds	Identified compounds	Area %	Rt
Acids	Pterin 6-carboxylic acid	0.66	39.71
	Hydroxy-dodecanoic acid	0.65	45.00
Acid salts	D-Mannopyranoside,methyl, cyclic 2,3:4,6-bis (butylboronate)	5.51	25.07
	Ethyl isoallocholate	1.31	51.44
	Trans-2-phenyl-1,3-Dioxolane-4-methyl octadec-9,12,15- trienoate	0.97	48.54
Alcohols	1,4-Di-anhydro-mannitol	14.25	18.36
	2,5-Methylene,l-rhamnitol	2.50	23.34
	24,25-Dihydroxychole calciferol	0.97	51.58
Aldehyde	9,10- Dideutero-octadecanal	0.93	51.65
Alkanes	2,6,10-Trimethyl-tetradecane	3.37	13.80
	Trifluoroacetoxydodecane	1.86	15.17
Aromatic heterocycle	3,7-Diacetamido-7-H-s-triazolo[5,1-c-]s-triazole	3.25	5.25
	2-amino-5-[(2-carboxy) vinyl] Imidazole	0.68	12.84
	2-Dimethyl(isopropyl) silyloxy methyl tetra –hydrofurane	9.62	19.32
	1,4-Diacetyl-3-acetoxymethyl-2,5-methylene-l-rhamnitol	2.25	20.91
Ketones	3-[2-Diethylaminoethyl]-2,4-pentanedione	3.43	18.89
Nitrogenous compounds	Tetra-acetyl-d-xylonic-nitrile	0.65	41.49
	Oxanamide	1.11	27.22
	2,4-Dihydroxy-6-methyl-amino-S-riazine	5.78	17.17
Phenolic	Butylated Hydroxytoluene	38.19	26.38
Total (%)		97.93%	

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Table 4: GC-MS analysis of volatile chemical compounds of (CSM)

Type of compounds	Identified compounds	Area %	Rt
Acids	Propanoic acid	14.79	54.58
Alkenes	1,1-Diethoxy-2-butene	9.58	57.48
Aromatic heterocycle	Dodeca-chloro-3,4-benzo phenanthrene	8.41	50.53
	3,4,5,6-Tetrakis(p-chlorophenoxy)1,2-dicyanobenzene	27.74	50.58
	2,5-Bis[6-(4-bromophenyl)1,2,4-triazin-3-yl 4-oxide]pyridine	7.47	52.27
	Tri-methyl-silylmethyl-2-(2,4,5-trichlorophenoxy)acetate	10.55	65.76
Benzene drivatives	4-Bromophenyl-bis-2,4-dibromophenyl-amine	10.69	26.46
	1,2-Dichloro-4,9,10-triphenylanthracene	7.31	63.08
Total (%)		96.54%	

Table 5: GC-MS analysis of volatile chemical compounds of (FCSM) by *T. harzianam* F.555

Type of compounds	Identified compounds	Area %	Rt
Acids	Oleic Acid	10.21	45.82
Aromatic heterocycle	Isobutyl phthalate	4.48	41.36
	Hexa-methylated anthraquinono-porphyrin	5.87	16.79
	5,5"-Dibromo-3,3'',4,4''-tetrabutyl-2,2':5',2"-terthiophene	2.67	40.44
Benzene drivatives	2,2Bis[4[(4,6-dichloro-1,3,5-triazin-2-yl)oxy] phenyl] 1,1,1,3,3,3-hexafluoropropane	10.61	16.72
Ester	9-Octadecenoic acid -3-[(1-oxohexadecyl)oxy] 2-[(1-oxooctadecyl) oxy] propyl ester	9.08	16.54
	Octanoic acid, ethyl ester	7.0	41.95
	9,12-Octadecadienoic acid (Z,Z), methyl ester	28.08	45.70
	Hexadecanoic acid, ethyl ester	13.17	16.88
Ketone	3,3'-dihydroxy-á,áCarotene-4,4'-dione	6.85	14.62
Total (%)		98.01%	

Total phenolic content (TPC)

Total phenolic content was detrmined in ethanol extracts because it is the best solvent for phenolic compounds extraction due to their polarity [48]**.** As shown in **figure 2**.

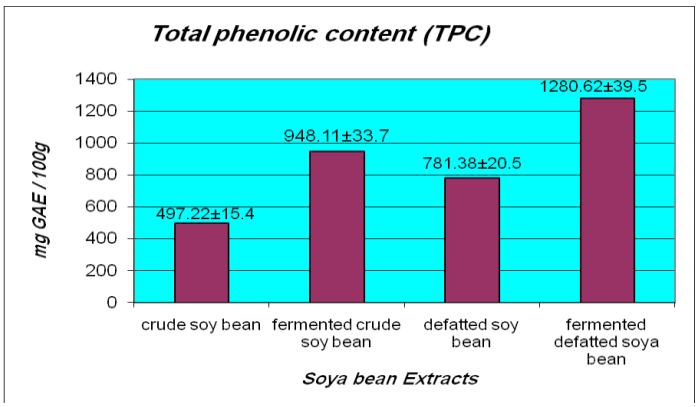

Figure 2: Total phenolic content of different ethanol extracts of soy bean

TPC values were 497.22±15.4, 781.38±20.5, 948.11±33.7 and 1280.62±39.57 mg GAE/ 100g in (CSM), (DSM), (FCSM) and (FDSM) ethanol extracts respectively . The TPC content increased in the fermented samples. However, the (FDSM) presented the highest total content in these compounds (1280.62±39.57 mg/g) with an increase of 163.89%, the (FCSM) showed the highest quantitative changes with an increase of 190.68 % in the total phenolic content (Fig. 1). Moreover, the isoflavones, other phenolic compounds, such as chlorogenic,

ferulic, caffeic, and p-coumaric acid, were found in soybean [49]. They all could significantly contribute the total phenolic content in soy bean. It is very important to point out that; there is a positive relationship between antioxidant activity and amount of phenolic compounds of the extracts[50]. The TPC could be used as an important indicator of the antioxidant capacity due to a high redox potential of the phenolic compounds allowing them to act as radical scavengers or hydrogen donors[51].

Antioxidant activities

β-Carotene-Linoleate Scavenging Assay

Table 6. shows the scavenging activities of (CSM), (FCSM), (DSM) and (FDSM) extracts in term of total antioxidant activity measured by auto-oxidation of β-carotene and linoleic acid coupled reaction. Total antioxidant effect were 10.11, 18.26, 26.15and 32.15 in (CSM); 16.66, 30.11, 44.32 and 58.95 in (FCSM); 13.51, 21.28, 28.75 and 44.85 in (DSM), while were 18.96, 40.12, 61.32 and 79.55 % in (FDSM) at 50, 100, 200 and 400 ug/ml of sample extract, respectively. We clearly observed a noticeable increase in the radical *β*-Carotene-Linoleate scavenging activity after the fermentation. Total antioxidant effect of fermented materials extracts displayed significantly high inhibition in comparison to the non fermented samples. Interestingly, the 400 ug/ml of (FDSM) sample exhibited the highest antioxidant potential in comparison to standard, with the highest antioxidant effects in all examined concentrations while, the lowest antioxidant potential was identified in the extracts of (CSM) samples **figur 3.**

Table 6: Antioxidant activity of soy bean extracts by *β*- carotene assay

Concentrations of soy bean extracts	% Inhibition				
	Crude soy meal (CSM)	Fermented crude soy meal (FCSM)	Unfermented Defatted soy meal (DSM)	Fermented defatted soy meal (FDSM)	Standard synthetic antioxidant (TBHQ)*
50 µg /ml	10.11 ± 0.9	16.66 ± 0.8	13.51 ± 0.4	18.96 ± 0.7	75.2 ± 3.1
100 µg /ml	18.26 ± 1.4	30.11 ± 1.9	21.28 ± 1.4	40.12 ± 1.1	85.0 ± 2.5
200 µg /ml	26.15 ± 1.3	44.32 ± 1.9	28.75 ± 2.0	61.32 ± 1.2	94.0 ± 2.7
400 µg /ml	32.15 ± 1.7	58.95 ± 2.1	44.85 ± 2.7	79.55 ±1.6	99.5 ± 2.7

- *TBHQ: Tert-butyl hydroquinone, standard synthetic antioxidant.
- Each value represents the mean ± S.D (Standard deviation) and mean of three replicates.

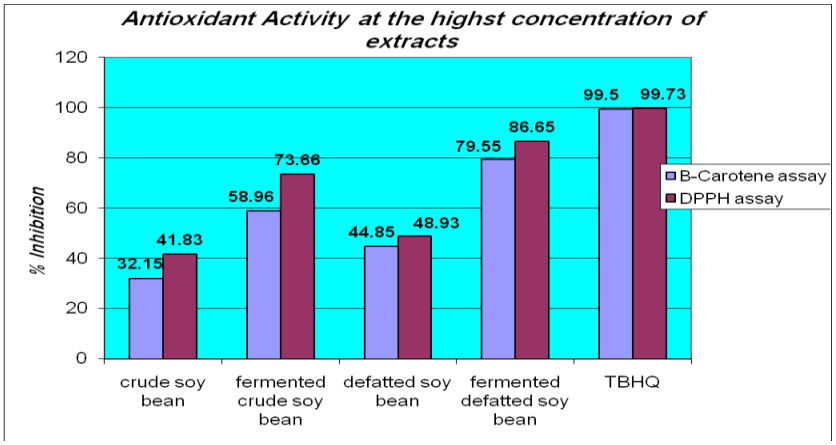

Figure 3: Antioxidant Activity of different soy bean extracts at the highest concentration (400 µg /ml)

Radical scavenging activity using DPPH assay

The free radical-scavenging activity was determined by the DPPH test. This test aims to measure the capacity of the extracts to scavenge the stable radical 2, 2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) formed in solution by donation of hydrogen atom or an electron [52]. If the extracts have the capacity to scavenge the DPPH free radical, the initial purple solution will change to a yellow colour due to the formation of

diphenylpicrylhydrazine. This reaction is used as a measure of the ability of the extracts, or any other antioxidant, such as TBHQ, to scavenge any free radical. The results from the radical scavenger assays for ethanol extracts are presented in Table 7, Fig 3. Fermented samples extracts (FCSM) and (FDSM) revealed antioxidant activity higher than non fermented sample extracts (CSM) and (UNFDSM). (FDSM) showed excellent radical scavenging activity, with 86.65% ±2.0 at 400 ug/ml. The free radical scavenging activity of ethanolic extract of (FDSM) was superior that of other samples. Furthermore, comparison of (FDSM) with the synthetic antioxidant TBHQ, showed that it is almost as effective as the known standard, in scavenging free radicals.

Table 7: Antioxidant activity of soya bean extracts by DPPH free radical scavenging method

Concentrations of soy bean extracts	% Inhibition				
	Crude soy meal (CSM)	Fermented crude soy meal (FCSM)	Unfermented Defatted soy meal (DSM)	Fermented defatted soy meal (FDSM)	Standard synthetic antioxidant (TBHQ)*
50 µg /ml	15.13 ± 1.1	21.31 ± 1.5	20.33 ± 1.1	24.61 ± 1.3	76.53 ± 2.3
100 µg /ml	24.21 ± 1.5	32.12 ± 1.2	28.51 ± 1.8	39.35 ± 1.9	83.75 ± 2.5
200 µg /ml	30.91 ± 1.7	58.33 ± 1.9	40.11 ± 2.1	66.14 ± 1.9	95.36 ± 2.6
400 µg /ml	41.83 ± 2.1	73.66 ± 2.0	48.93 ± 2.3	86.65 ± 2.0	99.73 ± 2.6

- *TBHQ: Tert-butyl hydroquinone, standard synthetic antioxidant.
- Each value represents the mean ± S.D (Standard deviation) and mean of three replicates.

CONCLUSION (PRACTICAL APPLICATION)

This study presented the significant change of fermentation process by *Trichoderma sp*. on the defatted soybean meal bioactive volatile and non volatile components. These alterations in chemical composition and as a result the biological effects (antioxidant activity) could be due to enzymes' production and activation by the Bacteria used in order to carry out the biofermentation, associated with complecated biochemical metabolism of soybean meal during this process.The current finding would be useful in preparing soy products with higher level of aglycone isoflavones and biologically active volatiles content. It could also be used in developing soy aglycone isoflavone concentrate and volatile antioxidant product for food antioxidants, additives in new food products or nutritional supplements and pharamaceutical products. This value-added post oil extraction application will increase the economic value and potential utilization of defatted soybean meal.

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