Journal of Cell and Molecular Research (2018) 10 (1), 32-39 DOI: 10.22067/jcmr.v10i1.73844

Research Article

Desaturase Genes Expression and Fatty Acid Composition of *Pleurotus ostreatus* in Response to Zinc and Iron

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Received 1 July 2018 Accepted 6 September 2018

Abstract

The influence of $ZnSO_4$, $FeSO_4$, ZnO, and Fe_2O_3 at 80 µM on the expression of desaturase genes and fatty acid composition of *Pleurotus ostreatus* was investigated. Fatty acid was extracted by lipid extraction and methylation procedure using acidic methanol: normal saline: hexane solution followed by gas chromatography-mass spectrometry (GC-MS). The most prominent fatty acids in *P. ostreatus* were linoleic acid (44.7%), palmitic acid (8.6%), oleic acid (8.5%), stearic acid (2.9%), pentadecanoic acid (2.6%) and heptadecanoic acid (2.3%). ZnO strongly and Fe_2O_3 slightly lead a decrease in unsaturated fatty acid (UFA), polyunsaturated fatty acid (PUFA) and omega-6 and an increase in the monounsaturated fatty acid (MUFA), omega-7 and omega-9 content. Accordingly, our results considerably confirmed the different effects of $ZnSO_4$ and ZnO on fatty acid content. These differential effects for $FeSO_4$ and Fe_2O_3 were not significant. Δ9-desaturase and Δ12-desaturase expression but not Δ15-desaturase were upregulated in the seeds cultured in media containing ZnO and Fe_2O_3.

***Keywords*: *Pleurotus ostreatus,* essential fatty acid, desaturase**

Introduction

In recent years, mushrooms have been appreciated for texture, flavor, nutritional and medical applications. They have been reported as therapeutic food in preventing diseases such as hypertension, hypercholesterolemia, atherosclerosis, diabetes mellitus and cancer. They presented potent cholesterol-lowering, glucose-lowering, antitumor, antiviral, antithrombic and immunomodulating effects (Rathore et al., 2017; Roncero-Ramos and Delgado-Andrade, 2017). Biological and functional characteristics of mushroom are mainly due to their chemical composition, since they are characterized as a useful source of carbohydrates, fibers, proteins, essential amino acids, non-essential amino acids, lipids, unsaturated fatty acids, essential fatty acids, vitamins, vitamin precursors, minerals and a large variety of secondary metabolites like organic acid, alkaloids, terpenoids, steroids and phenolic compounds (Stephan et al., 2018; Zhang et al., 2017).

Fatty acids, especially unsaturated fatty acids and essential fatty acids, are used as indicators of nutritional, medicinal and pharmacological purposes of foodstuff. Linoleic and linolenic acids are essential fatty acids for synthesis of other polyunsaturated fatty acids during biological pathways in mammals. They are building blocks of cell membranes; support the defensive barrier and also are involved in cholesterol metabolism. Moreover, it has been shown that essential fatty acids can reduce the rate of cancer colony formation, prevent breast cancer, regulate blood pressure, decrease the level of cholesterol, improve diabetes, and ameliorate the body's immunological resistance against antigens (Freitas et al., 2017; Zarate et al., 2017).

Among mushrooms, *Pleurotus ostreatus* is a culinary and medicinal mushroom with beneficial biological and medicinal properties. *P. ostreatus,* as a nutritional and therapeutic mushroom, is a valuable source of carbohydrates, vegetable proteins and amino acids, fibers, vitamins and vitamin precursors, minerals, organic acids, phenolic and flavonoid compounds and low levels of lipid and essential fatty acids with low calories (Poniedziałek et al., 2017; Gasecka, et al., 2016). Unlike plants, mushrooms do not require agricultural land, and their resulting metabolite profile could be manipulated and changed substantially by simply varying their growth conditions. In this study, we decided to evaluate the influence of $ZnSO_4$, $FeSO_4$, ZnO, and Fe_2O_3 on the expression of desaturase genes and fatty acids profiling in *P. ostreatus*.

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Materials and Methods

Materials

Glucose (CID: 79025), $ZnSO_4$ (CID: 24424), $FeSO_4$ (CID: 24393), ZnO (CID:14806), Fe_2O_3 (CID:14833), KH_2PO_4 (CID:516951), $MgSO_4$ (CID: 24083), NaCl (CID: 5234), 2,2 Dimethoxypropane (CID: 6495), Methanol CID: 887, Sulfuric acid (CID: 1118), Hexane (CID: 8900), Formic acid.

Mushroom Strain and Preparation of Seed Culture

P. ostreatus edible-medicinal mushroom was obtained through Arian's Mushrooms Research Company (Tehran, Iran). The seeds were grown in 200 mL of basal medium (glucose 50.0 g/L, yeast extract 10.0 g/L, casein hydrolysate 10.0 g/L, KH_2PO_4 1.0 g/L, distilled water 1 L, and pH 6.0) in 500 mL Erlenmeyer flask at 25°C, on a rotary shaker, at 110 rev/min for 7 days. The obtained seed culture was used for inoculation (Turlo et al., 2010).

Preparation of Basal Liquid Media Containing $ZnSO_4$, $FeSO_4$, ZnO, and Fe_2O_3

The composition of the basal liquid medium for mycelium cultivation was beet molasses 50 g/L (beet sugar factory, Isfahan, Iran), soybean flour 5 g/L (IPP company, Isfahan, Iran), KH_2PO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 1 g/L (Merck, Germany) and distilled water 1 L with initial pH 6.0. The liquid media was enriched with 0.08 mM of $ZnSO_4$, $FeSO_4$, ZnO, and Fe_2O_3. All liquid culture media were sterilized by autoclaving at 121°C for 20 min. After cooling, cultivation had been carried out in polypropylene containers containing 95 mL of liquid medium and inoculated with five mL of the seed culture (Poursaeid et al., 2015).

Biochemical Analysis

The total lipid content was quantified by sulphuric acid-phosphoric acid-vanillin reagent using sesame oil (1mg/mL in ethanol) as standard and monitoring light absorbance at 530 nm. Briefly, 100 mg of powder carefully was suspended in 500 µL of ethanol. 1 mL of concentrated sulfuric acid (98%) was added to the samples and then heated at 100°C for 10 min. After cooling in room temperature (25-27°C), 1 mL phosphoric acid-vanillin reagent (0.6 g vanillin in 100 mL deionized water and 400 mL concentrated phosphoric acid) was added and the sample incubated for 15 min at room temperature (Mishra et al., 2014).

Fatty Acids Methylester Profiling by GC-MS

Mushroom powder (200 mg) was put into extraction glass test tubes. Then 1.0 mL of acidic methanol (methanol: sulfuric acid in 85:15 v/v) was added to glass tubes. The tubes were placed in shaking water bath at 80°C for 120 min. Next, tubes were placed in the vortex until they come to room temperature. 1.0 mL of normal saline was added to tubes and vortexed for 5 min. Afterward, 1.0 mL of hexane was added to tubes, and vortexed for 5 min then centrifuged for 20 min at 3000g. The upper phase (hexane phase) was extracted and transferred to gas chromatography vials for gas chromatography - mass spectrometry (GC-MS) (Agilent 7890B GC 7955A MSD) analysis according to manual instruction (Woldegiorgis et al., 2015).

Real-Time Quantitative PCR Analysis

P. ostreatus that were grown in different liquid media were frozen in liquid nitrogen and then stored at -70°C for RNA isolation. Total RNA was extracted from 100 mg of mushroom using the RNX-Plus buffer (Cinnagen, Tehran, Iran) according to the manufacturer's instructions. Quantification of total RNA was performed with a Nano Drop ND 1000 spectrophotometer at 260 nm (Thermo Fisher Scientific, Wilmington, DE, USA). DNase treatment was carried out using a Fermentas DNase Kit (Fermentas, Hanover, MD, Germany) using the manufacturer's protocol. The integrity of RNA was checked by visual observation of 28S rRNA and 18S rRNA bands on an agarose gel electrophoresis before real-time PCR analysis (Figure S1 in the supplemental file). 5 µg of DNase-treated RNA was converted to cDNA with a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas) using the manufacturer's protocol in a 20 µL final volume.

Primers for the target (D9-desaturase, D12-desaturase, D15-desaturase) and internal control (beta-actin) genes were designed using Allele ID 7 software (Table 1). Primers for the PCR reactions were designed to have a melting temperature of about 55°C to 65°C and to give a PCR product between 100 and 200 bp. Real-time PCR was performed using a line Gene K Thermal cycler (Bioer Technology Co, Hangzhou, China). The cDNA samples were diluted 1:5 by using nuclease-free water, and 5 µL of cDNA was used for real-time PCR. The final volume for relative real-time PCR was 20 µL containing 4 pmol of each primer, 5 µL (diluted) of the first-strand cDNA and 1x SYBR Premix Ex Taq TM Π (Takara, Japan). The initial denaturing time was 5 min., followed by 40

Table 1. Primer used in this study

Genes	Primer sequence	TM	Length
Δ9 Desaturase F	TCATTGCCTTGTGTAACTTCA	62	21
Δ9 Desaturase R	TGTCTCTGCCTCCTTCTG	62	18
Δ12 Desaturase F	TGGGTCAACCAyTGGCT	57	17
Δ12 Desaturase R	TCAATACCAGAGTCGCTGG	57	19
Δ15 Desaturase F	TGGATTTTGGCyCATGAATG	57	20
Δ15 Desaturase R	ATGGCCAGTAGCTTTATGATG	57	21
18S rRNA F	CAGCGAAATGCGATAAGTAAT	55	21
18S rRNA R	CCAACAATCCAAACATCACAA	55	21

Primers for target genes and internal control gene were designed using Allele ID 7 software.

PCR cycles consisting of 94 °C for 10 s, annealing temperatures of each primer 15 s, and 72 °C for 30 s. A melting curve was recorded after the PCR cycles followed by heating from 50 to 95 °C. A Proper control reaction was carried out without the reverse transcriptase treatment. For each sample, the subsequent real-time PCR reactions were performed in twice under identical conditions.

For real-time data analysis, the relative expression of the target gene in each sample was compared with the control sample, and was determined with the delta-delta Ct method. The Ct for each sample was calculated using the Line-gene K software (fqdpcr ver. 4.2.00), where Ct refers to the threshold cycle determined for each gene in the exponential phase of PCR amplification. In this analysis method, the relative expression of the target gene in the control sample was equal to one (2^0) by definition (Livak and Schmittgen, 2001).

Results and Discussion

Fatty Acid Composition

Biochemical analysis of *P. ostreatus* cultivated in beet molasses, and soybean flour using sulfuric acid-phosphoric acid vanillin (SPV) as chromogen confirmed a total fatty acid content of 5660 mg/100 g dried weight (Table 2). Our experimental analysis clearly demonstrated that $ZnSO_4$ and ZnO or $FeSO_4$ and Fe_2O_3 had no significant effects on the total fatty acid content but GC-MS analysis revealed a significant difference between the qualities of the fatty acid composition. It has been shown that cultivation of *Mortierella ramanniana* mushroom in the presence of dextrose-yeast extract with $FeCl_2$ and $ZnSO_4$ lead to a significant increase and decrease in the biomass, respectively (Dyal et al., 2005). Tan et al. (2017) reported that metal ions such as $ZnSO_4$ and $FeCl_3$ increased biomass and lipid content in Mortierella. Sajbidor et al. (1992) examined the effects of various concentrations of calcium, magnesium, manganese and iron ions on lipid synthesize by Mortierella. Based on the results, all above-mentioned ions exception manganese inhibit lipid accumulation and arachidonic acid production. Moreover, iron had an inhibitory effect on arachidonic acid production by Mortierella. Hansson and Distalek (1988) indicated that Cu_2^+ and Zn_2^+ have positive effects on lipid accumulation and gamma-linolenic acid production by Mortierella. Accordingly, there are various reports in the literature for the stimulatory effects of metal ions on the production of biomass or lipid content in the mushroom. The difference may be related to mushroom species, basal medium composition, inoculation condition, type of metal ion salts (for example; $ZnSO_4$, ZnO, FeCl2, $FeCl_3$, $FeSO_4$, FeO, Fe_2O_3).

GC-MS analyzed of *P. ostreatus* indicated different components in the mushroom including fatty acids (73%) and other metabolites (27%) including limonene, organic acid, and phenol compounds (Table 3). $FeSO_4$ and $ZnSO_4$ had not a significant effect on total fatty acid content while ZnO and Fe_2O_3 significantly reduced fatty acid content. Fe_2O_3 significantly increased while ZnO significantly reduced secondary metabolite of *P. ostreatus* while $FeSO_4$ and $ZnSO_4$ did not display significant effect. The most prominent fatty acids in *P. ostreatus* were linoleic acid (44.7%), palmitic acid (8.6%), oleic acid (8.5%) stearic acid (2.9%), pentadecanoic acid (2.6%) and heptadecanoic acid (2.3%) (Table 3). The most prominent fatty acid in $ZnSO_4$ enriched *P. ostreatus* were linoleic acid (44.7%), palmitic acid

Table 2. Total fatty acid content of cultivated *P. ostreatus* in medium enriched by $ZnSO_4$, ZnO, $FeSO_4$ and Fe_2O_3.

Treatments	Lipid (mg/100g)
Control	5660 ± 10
$ZnSO_4$	6820 ± 12
ZnO	5240 ± 11
$FeSO_4$	5840 ± 14
Fe_2O_3	5120 ± 12

Values are means with triplicate determination. Means sharing the same superscript across column are not significantly different at P< 0.05.

(7.5%), oleic acid (7.35%), pentadecanoic acid (4.2%), heptadecanoic acid (2.8%), and stearic acid (1.8%) (Table 3). The most prominent fatty acids in *P. ostreatus* enriched with ZnO were linoleic acid (25%), oleic acid (14.45%), palmitic acid (12.4%), palmitoleic acid (3.95%), and stearic acid (2%) (Table 3). The most prominent fatty acid in *P. ostreatus* enriched with $FeSO_4$ were linoleic acid (38%), palmitic acid (9.6%), oleic acid (8.85%), pentadecanoic acid (5.4%), myristic acid (3.6), heptadecanoic acid (2.95%), and stearic acid (1.85%) (Table 3). The most prominent fatty acid in *P. ostreatus* nriched with Fe_2O_3 were linoleic acid (35.85%), palmitic acid (9.93%), oleic acid (8.85%), palmitoleic acid (3.25%) and stearic acid (2.4%) (Table 3).

Yilmaz et al. (2006) reported that the main fatty acid composition of *P. ostreatus* were linoleic acid (44%), oleic acid (20%), palmitic acid (12%), and stearic acid (5%). Woldegiorgis et al. (2015) reported that the main fatty acid composition of *P. ostreatus* were linoleic acid (1663 mg/g), oleic acid (323 mg/g), palmitic acid (310 mg/g), and stearic acid (39 mg/g). Pedneault et al. (2007) reported that the main fatty acid composition of *P. ostreatus* were linoleic acid (79%), palmitic acid (11%) and oleic acid (5.6%). Ergonul, et al. (2013) reported that the main fatty acid composition of *P. ostreatus* were linoleic acid (65.3%), palmitic acid (12.4%), oleic acid (10.4%) and stearic acid (3.7%). Based on the above-mentioned studies, the proportion of unsaturated fatty acids were higher than saturated fatty acids in *P. ostreatus* which is consistent with our data.

The results of individual fatty acids including saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), as well as omega-6 fatty acids, omega-7 fatty acids, and omega-9 fatty acids have shown in Table 4. PUFA especially omega-6 fatty acid constitute the most proportion of fatty acids of *P. ostreatus.* ZnO strongly and Fe_2O_3 slightly lead a decrease in UFA, PUFA and omega-6 and an increase in the MUFA, omega-7 and omega-9 content that were close to the values already reported by Pedneault, et al., (2008), Ogwok, et al., (2017) and Warude, et al., (2006).

Linoleic acid (C18:2n6) as an omega-6 fatty acid, is the precursor of 1-octen-3-ol, known as the alcohol of fungi. Aromatic compounds which exist in most fungi and might contribute to mushroom flavor originates from 1-octen-3-ol (Ribeiro et al., 2009). Palmitic acid (16:0) is the most common saturated fatty in human food. This saturated fatty acid is the precursor of long fatty acids like oleic acid (18:1) (Ohlsson, 2010). Oleic acid (C18:1n9) is a bioactive compound that decreases cholesterol serum level. This fatty acid is a good substrate for the liver enzyme known as Acyl CoA:cholesterol acyltransferase (ACAT), a liver enzyme. ACAT transfer a fatty acid (such as oleic acid) from coenzyme A to the hydroxyl group of cholesterol and convert it to cholesterol ester (the hydrophobic form of cholesterol) (Won et al., 2007). Stearic acid (C18:0) as compared to cholesterol-raising SFAs decreases LDL while raises LDL as compared with unsaturated fatty acids (Mensink, 2005). Pentadecanoic acid (15:0) is the member of odd chain saturated fatty acids (OCS-FA). It has been shown that consumption of OCS-FAs rich foods, such as dairy fats, could reduce the risk of developing metabolic diseases (Jenkins et al., 2015). Accordingly, the biological action of *P. ostreatus* fatty acid extract on the above-mentioned activities must be investigated.

Desaturase Gene Transcription

Fatty acid desaturases play a critical role in the biosynthesis of PUFA by catalyzing the addition of double bond in specific positions of fatty acid chain. The most predominant fatty acids analyzed

Table 3. Fatty acid composition (percent of total fatty acid) of cultivated *P. ostreatus* in medium enriched by $ZnSO_4$, ZnO, $FeSO_4$ and Fe_2O_3.

Fatty acid	Control	$ZnSO_4$	ZnO	$FeSO_4$	Fe_2O_3
Dodecanoic acid (C12:0)	1.00	1.00	1.00	1.00	1.85
Tridecanoic acid (C13:0)	0.01	0.01	0.01	0.01	0.01
Tetradecanoic acid (C14:0)	0.20	0.15	1.50	3.60	0.50
9-Tetradecenoic acid (C14:1n5)	0.02	0.02	0.02	0.02	0.02
Pentadecanoic acid (C15:0)	2.60	4.19	1.23	5.42	0.96
Hexadecanoic acid (C16:0)	8.60	7.55	12.40	9.60	9.93
9-Hexadecenoic acid (C16:1n7)	1.28	1.20	4.00	1.60	3.25
7,10-Hexadecadienoic acid (C16:2n6)	0.03	0.03	0.03	0.03	0.03
7,10,13-Hexadecatrienoic acid (C16:3n3)	0.04	0.04	0.04	0.04	0.04
Heptadecanoic acid (C17:0)	2.26	2.80	0.60	2.95	0.35
Octadecanoic acid (C18:0)	2.90	1.79	1.95	1.85	2.40
9-Octadecenoic acid (C18:1n9 Trans)	0.04	0.04	0.04	0.04	0.04
9-Octadecenoic acid (C18:1n9 Cis)	8.50	7.35	14.45	8.85	8.85
10,13-Octadecadienoic acid (C18:2n5)	0.02	0.02	0.02	0.02	0.02
9,12-Octadecadienoic acid (C18:2n6 Trans)	0.03	0.03	0.03	0.03	0.03
9,12-Octadecadienoic acid (C18:2n6 Cis)	44.70	44.65	25.00	37.90	35.85
9,12,15-Octadecatrienoic acid (C18:3n3)	0.03	0.03	0.03	0.03	0.03
6,9,12-Octadecatrienoic acid (C18:3n6)	0.20	0.45	0.37	1.20	0.35
Docosanoic acid (C22:0)	0.40	0.35	0.10	0.20	0.08
Total lipid	**72.86**	**71.70**	**62.81**	**74.39**	**64.58**

The most prominent fatty acids are linoleic acid > palmitic acid > oleic acid.

Table 4. Saturated and unsaturated fatty acid composition (percent of total fatty acid) of cultivated *P. ostreatus* in medium enriched by ZnSO4, ZnO, $FeSO_4$ and Fe_2O_3.

Fatty acid	Control	$ZnSO_4$	ZnO	$FeSO_4$	Fe_2O_3
Saturated fatty acid (SFA)	17.77	17.69	17.29	21.03	15.57
Unsaturated fatty acid (UFA)	54.89	53.86	44.03	49.76	48.51
Monounsaturated fatty acid (MUFA)	9.84	8.61	18.51	10.51	12.16
Polyunsaturated fatty acid (PUFA)	45.05	45.25	25.52	39.25	36.35
Omega-3 fatty	0.07	0.07	0.07	0.07	0.07
Omega-5 fatty	0.02	0.02	0.02	0.02	0.02
Omega-6 fatty	44.96	45.16	25.43	39.16	36.26
Omega-7 fatty	1.28	1.20	4.00	1.60	3.25
Omega-9 fatty	8.54	7.39	14.49	8.89	8.89

The most prominent UFA are PUFA especially omega-6.

in this study were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n9) and linoleic acid (18:2n6). The conversion of palmitic acid to stearic acid catalyzed by elongase. Δ9-desaturase catalyzes the conversion of stearic acid to oleic acid, an omega-9 fatty acid. Oleic acid then converted to, linoleic acid (an omega-6 fatty acid) by the action of Δ12-desaturase. Δ15-desaturase then converts linoleic acid to α-linolenic acid (18:3n3), an omega-3 fatty acid. Δ12-desaturase and Δ15-desaturase was not found in animals but present in plants, algae and fungi. Accordingly, linoleic and linolenic acid are essential fatty acids and must be obtained from diet. Our experimental results showed ZnO and Fe_2O_3 upregulated Δ9-desaturase but $FeSO_4$ and $ZnSO_4$ did not significant effects on the upregulation of these genes (Figure 1). *P. ostreatus* has the potential to be a strain for production of omega-6 and omega-3 polyunsaturated fatty acid. The biosynthesis of the omega-6 and omega-3 fatty acid is catalyzed by

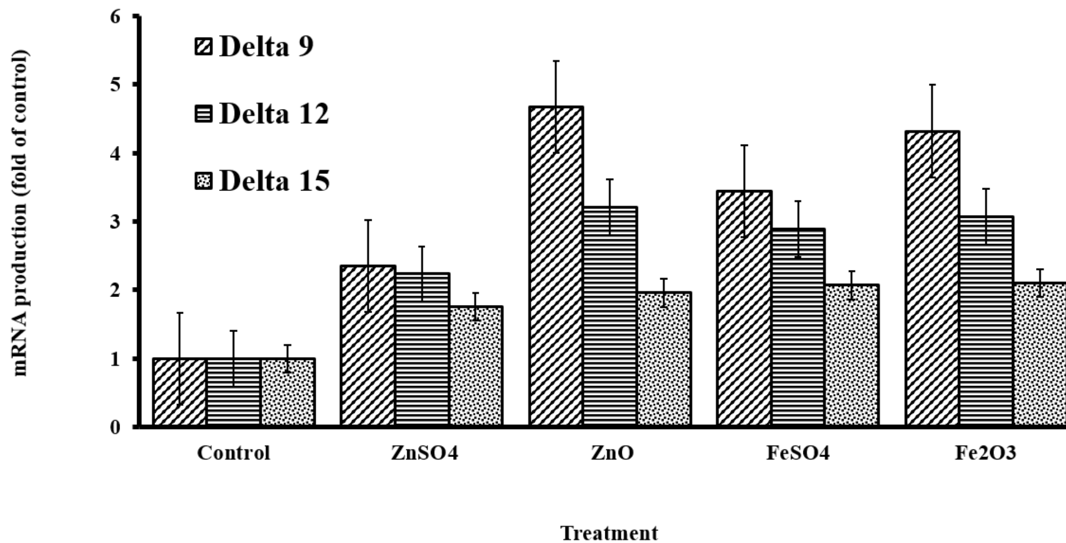

Figure 1. Stimulatory effect of $ZnSO_4$, $FeSO_4$, ZnO and Fe_2O_3 at concentration of 80 µM on the expression of delta 9 desaturase, delta 12 desaturase and delta 15 desaturase in cultivated *P. ostreatus*.

desaturase enzymes, including Δ9-desaturase, Δ12-desaturase, Δ15-desaturase, and Δ6-desaturase. The 5′ upstream flanking sequence of desaturase genes contain regulatory elements, including metal-responsive element. The presence of this special cis-acting response elements in the promoter region of desaturase genes implies that their transcription is regulated by metal ions (Lee, et al., 2016). Tan et al. (2017) reported that metal ions such as $ZnSO_4$ and $FeCl_3$ could stimulate polyunsaturated fatty acid production and upregulate Δ9-desaturase, Δ12-desaturase, Δ5-desaturase and Δ6-desaturase gene transcription in Mortierella sp.

Conclusion

Our results clearly revealed that the most predominant fatty acids in *P. ostreatus* were linoleic acid, palmitic acid, oleic acid and stearic acid. This mushroom also can be considered as an edible source of PUFA and omega-6 fatty acid content. ZnO strongly and Fe_2O_3 slightly lead to a decrease in UFA, PUFA and omega-6 and an increase in the MUFA, omega-7, and omega-9 content. Our results considerably revealed differential effects of $FeSO_4$, $ZnSO_4$, ZnO, and Fe_2O_3 on fatty acid content of the mushroom that may be attributed to differential co-factor activity of zinc and iron on the biochemical pathway for fatty acid synthesis.

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