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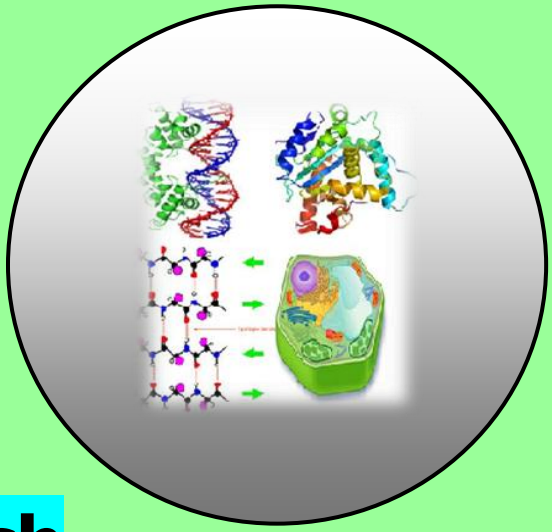

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RESEARCH PAPER

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Pleurotus ostreatus as a Biodegradator for Organophosphorus Insecticide Malathion

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ABSTRACT

Excessive and continuous use of organophosphorus insecticides as well as malathion has led to the contamination of ecosystems in several rejoins of the world. For its degradation in the ecosystems usage of the microbes has been proved to be the effective method for controlling ecosystems pollution. Biodegradation of pesticides by using microbes have more advantages over the conventional methods. In the present investigation therefore attempts have been made to make use of white rot fungus Pleurotus ostreatus for malathion degradation. Malathion rest quantity at 5, 15 and 25 days was 40.5, 22.6 and 11.8(µg/100mL) with degradation % 19.0, 54.8 and 76.4 respectively at initial concentration(50µg/100mL). Lignin peroxidase, manganese peroxidase, and laccase production was stimulated with the presence of malathion. At 25 µg/100mL malathion, productivity of lignin peroxidase, manganese peroxidase, and laccase was 0.51, 0.57 and 4.30 U/ml respectively compared with the control (growth medium without malathion). At low concentrations of malathion (25 and 50 µg) P. ostreatus productivity of these enzymes was more, but at high concentration of malathion the productivity was less than control. Addition of lignin compound induced the productivity of ligninolytic enzymes and therefore the biodegradation of malathin was increased .GC/MS analysis revealed the presence of many products as a result of malathion biodegradation including diethyl mercaptosuccinate, methy l, 2-(Dimethoxyphosphoryl)-2-(1,2,3-thiadiazol-4-yl) acetate, 3,4-dihydrothienyl (3,4,b)-5-carboxythiophene, Butanedioic acid, 2,2'-Thiodisuccinic acid; 1,1-Dimethyltetradecyl hydrosulfide and Disulfide, di-tert-dodecyl.

Key words: Whit rot-fungus, Pleurotus ostreatus, Biodegradator and Malathion.

INTRODUCTION

Organophosphorus compound poisoning is a worldwide health problem with around three million poisonings and 200000 deaths annually (Karalliedde&Senanayake, 1999; Sogorb et al., 2004). Continuous and excessive use of organophosphorus compounds has led to the contamination of several ecosystems in different parts of the world (McConnell et al., 1999; Cisar& Snyder, 2000; Tse et al., 2004).Malathion[S-(1,2-dicarbethoxyethyl)-O,O-dimethyldi-thiophosphate], also known as maldison, carbophosand mercaptothion is a nonsystemic, wide-spectrum organophosphorus insecticide used to control the household and agricultural pests. According to Singh et al. (2012)malathion was recognized as the first organophosphorous insecticide with highly selective toxicity. Malathion is an organophosphate insecticide and acaricide that has been suited for the control of suckingand chewing insects on field crops, fruits, vegetable, livestock,and also extensively used to prevent mosquitoes, flies, household insect, animal parasites, and head bodylice as substitute for DTT compound (Chambers,1992; Barlas, 1996). Malathion in soil undergo a variety of transformations that provide a complex pattern of metabolites and its fate in soil is controlled by chemical, biological and physical dynamics of this matrix. Products resulted from malathion degradation include dimethyl phosphate, dimethyl dithiophosphate, dimethylthiophosphate, isomalathion, malaoxon and malathion mono and dicarboxylic acid due to enzymatic activity of cutinase, carboxylesterase, phosphatase(Singh and Walker, 2006). Malathion itself is of low toxicity, it mainly concentrates in peel and may not readily removed by washing in water alone (Nath and Srivastava 1999) but easily enter the body through absorption through the skin,ingestion and inhalation results in its metabolism to malaoxon which is substantially more toxic (Edwards 2006).Long-term exposure to oral ingestion of malaoxon in rats, showed 61 times more toxic than malathion.

Today, microorganisms are used in bioremediation of environmental pollutants. Using microorganisms is natural process, very effective when compared to the other methods and applicable for *in situ* bioremediation.Biodegradation of organophosphate compounds releases phosphate as one of the end product (Pradnya et al., 2004). Fungi (Chalamala et al., 2012) are capable of degrading malathion. Ahmed et al. (2007) found that the degradation rate of malathion by bacterial was slower than the fungal isolates .White rot fungi as a group include *Phanerochaete chrysosporium, Trametes versicolor, Pleurotus ostreatus, Bjerkandera adusta,* and *Pleurotus tuberregium.* On the basis of their ability to secrete extracellular lignin-degrading enzymes, these fungi have been applied in bioremediation studies (Arora et al. 2002). White-rot fungi can degrade a wide spectrum of recalcitrant organopollutants, often carcinogenic, mutagenic or toxic, that arise from industrial operations, petroleum released into environment and plant protection (Jadwiga and Agnieszka 2013). *Pleurotus ostreatus* is a commercially important edible mushroom (Sanchez 2010).Most of the white-rot fungi secretes various extra cellular enzymes that degrade a large range of natural and anthropogenic compounds that have structural and chemical similarities to the lignin substructure. Among them, peroxidase or ligninolytic enzymes, predominantly lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Presence of nonspecific enzyme system, enable fungi to degrade natural complex aromatic polymers of lignin as well as complex aromatic polymers that share structure with lignin, such as pesticides, PAHs, PCBs and dyes (Cameron et al., 2000).

Several studies stated that potential applications of white-rot fungi and their enzymes are gaining increasing importance in the detoxification of industrial waste waters, biodegradation, biopulping, degradation and detoxification of recalcitrant substances (Esposito et al., 1991; Reddy 1995; D'Annibale et al., 1998; Tortella et al., 2008; Zhang et al., 2013).The present work was aimed at studying the ability of a white-rot fungus *Pleurotus ostreatus*, as a biodegratator of insecticide malathion. Optimal conditions for the biodegradation were also explored.

MATERIALS AND METHODS

Biodegratator used

Pleurotusostreatus (obtained from agricultural ministry, Egypt) was cultivated on Malt agar plates for 10 days at 30°C. Mycelium agar plugs 9 mm in diameter (cut along the edge of an actively growing colony) were used as inocula.

Fungal degradation of organophosphrus insecticides at different incubation period

Mineral salt liquid medium supplemented with 50 µg/100mL concentration of analytical grade standard of Malathion (Fig.1) was used for biodegradation test at 5,15 and 25 days.

Effect of different concentrations of malathion on fungal growth

Different concentrations of malathion 25, 50 and 100µg/100mL were added to growth medium. Fungal mycelia (disc 0.5 cm) were transferred to growth medium and incubated at 28±2°C for 10 days.

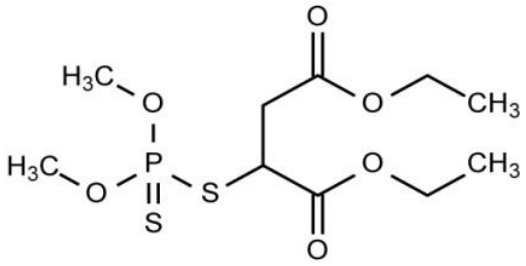

Figure 1. Malathion chemical structure.

Lignin as inducer to malathion degradation at different concentrations and ligninolytic enzymes detection

Mineral salt liquid medium supplemented with various concentrations of malathion (25, 50 and 100 µg/100mL) was autoclaved. Then, 0.2 g % of lignin was added to growth medium, in order to test the ability of the fungus to produce ligninolytic enzymes which enhance the biodegradation of malathion. Flasks containing medium were inoculated with fungus and then incubated at 30°C for 15 days. After incubation period the malathion rest quantity was extracted for quantitative analysis and the metabolized medium was filtrate through filter paper, and used for assay of three enzymes lignin peroxidase (LIP) , laccase (phenol oxidase), manganase peroxidase (MnPase). LIP activity was assayed using veratryl alcohol as a substrate. LIP catalyzes the oxidation of veratryl alcohol by H_2O_2 to veratryl aldehyde. The aldehyde was absorbed strongly at 310 nm after incubation for 1 h at 37 °C.

The reaction medium contained: 1 mL of the enzyme solution (culture filtrate), 0.2 mL of 2 mmol/L veratryl alcohol, 0.2 mL of 0.4 mmol/L H_2O_2, 0.2 mL of 0.25 mmol/L tartaric acid.Laccase activity is determined by the oxidation of 2, 2¢-azino-bis (3-ethylthiazoline-6-sulfonate), i.e., ABTS at 37°C at A420 (Buswell and Odier 1987). One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 mMol of ABTS per minute.Reaction mixture of 1ml contained 2 mM of ABTS in a Mcllvaine buffer (pH 5.0). To the assay mixture,100 µl of centrifuged extracellular supernatants were added. The enzymatic activity was estimated in IU by monitoring the adsorbance change at 420 nm (ABTS), Є=36 $Mm^{-1}cm^{-1}$ by spectrophotometer (UVikon XS/60/99-90289; Volt 110/240v)at 30°C. MnPase activity was carried out in 1 cm quartz cuvette. The reaction mixture of 1 ml contained 2 mM of ABTS and 1mM Mn^{2+} in a Mcllvaine buffer (pH 5.0). To the assay mixture, 100 µl of centrifuged extra cellular fluids (supernatants) were added .The peroxidase activity was then initiated by the addition of 0.4 mM H_2O_2 (Field *et al*.,1996 and Garzillo*et al*., 2001). The enzymatic activity was estimated in IU by monitoring the absorbance change at 420 nm (ABTS), Є=36 $mM^{-1}cm^{-1}$ by spectrophotometer at 30 °C.

Gas Chromatography/mass Spectrometry (GC/MS) Analysis of malathion degradation

The concentrated extract of metabolized medium was extracted with using methanol, then filtrated and concentrated into 1 ml and was placed in GC auto-sampler vials until they were analyzed. A Varian Star 3400 Cx Ion Trap GC/MSShimadzu GCMS-QP 5050 A. software class 5000. Searched library: Wiley 229 LIB. Column: DBI, 30m,053 mm ID; 1.5 um film. Carrier gas: Helium (flow rate 1 ml/min.).Ionization mode: El (70 ev).Temperature program: 70 (static for 2 min) then gradually increasing (at a rate of 2 /min) up to 220 (staticfor 5min).Detector temperature 250 injector temperature 250 The chromatographs were compared andindividual peaks were identified by comparing mass spectra to the library references. At the Regional Centerfor Mycology and Biotechnology AL-Azhar University).

RESULT AND DISCUSSION

Investigation of microbial degradation is useful for developing insecticide degradation strategies using microorganisms. In the current study, *Pleurotus ostreatus* growth decreased with increasing malathion concentration (Table 1&Fig.2). At 25, 50 and 100 µg/100mL malathion,the inhibition % was 4.62,35.48 and 64.62% respectively. Recently, Abbas and Yadegar (2015) found that the efficacy of the pesticides on the inhibition of the fungal growth and spore germination showed that they were both reduced with the increasing concentration of the insecticide.

Table 1. *P. ostreatus* growth and degradation % at different concentration of Malathion.

Malathion Concentration	Fungus growth (cm)	Inhibition %
0	6.5	0.00
25	6.2	4.62
50	4.2	35.48
100	2.3	64.62

Figure 2. Fungal growth from right to left at control, 25, 50 and 100 µg/100mL.

GC/MS analysis of malathion degradation (Fig.3) with using *P. ostreatus* at different incubation periods up to 25 days indicated that biodegradation increased with increasing incubation period. Malathion rest quantity at 5, 15 and 25 days was 40.5, 22.6 and 11.8(µg/100mL) with degradation % 19.0, 54.8 and 76.4 respectivelyat initial concentration(50µg/100mL)(Fig 4). Chalamala et al. (2012) worked on mycodegradation of Malathion by using *A. niger* and got 86.72 % of degradation. Recently, research activities in this area have shown that a diverse range of microorganisms are capable of degrading malathion (Baljinder et al., 2014). White-rot fungi tolerate considerable amounts of toxic pollutants, have high surface growth rates (Sang et al. 2002) and produce extracellular enzymes and other mediators that can reach contaminants that are poorly bioavailable (Bumpus et al., 1985).

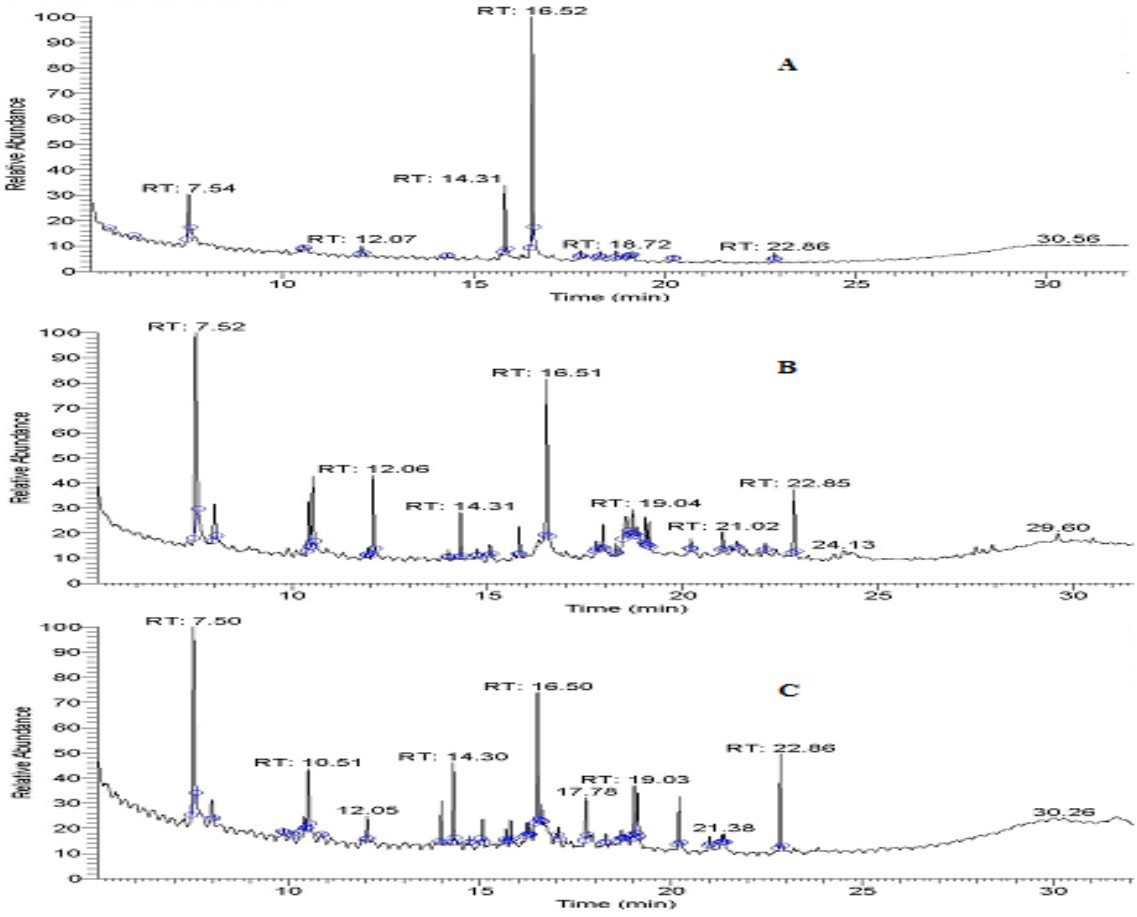

Figure. 3 GC/MS of Malathion degradation after different incubation Periods (A,5days;B,15 days; C,25 days).

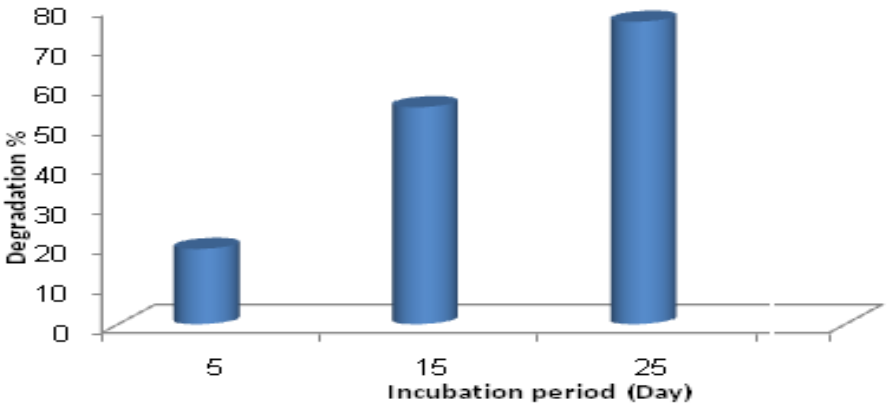

Figure 4. Malathion degradation at different incubation periods at initial concentration (50µg/100mL).

P. ostreatus secretes enzymes such as lignin peroxidase, manganese peroxidase, and laccase. These ligninolytic enzymes have been reported to be versatile and nonspecific in their degradation characteristics and can be used in degrading a broad range of aromatic contaminants (Rodriguez et al., 2004). In the current study, lignin peroxidase, manganese peroxidase, and laccase production was stimulated with the presence of malathion. At 25 µg/100mL malathion, productivity of lignin peroxidase, manganese peroxidase, and laccase was 0.51,0.57 and 4.30 U/ml respectively compared with the control (growth medium without malathion) (Table 2). In previous studies (Mougin et al., 2002; Kollmann et al., 2003), production of lignolytic enzymes is indeed known to be stimulated by toxic phenols released during lignin degradation or by toxins released by other organisms and by the presence of aromatic xenobiotic compounds. At low concentrations of malathion (25 and 50 µg) *P. ostreatus* productivity of these enzymes was more, but at high concentration of malathion the productivity was less than control. Addition of lignin compound induced the productivity of ligninolytic enzymes and therefore the biodegradation of malathion was increased (Table 2).

Table 2. Degradation of malathion at different concentrations in growth medium amended with lignin.

Malathion Concentration (µg/100mL)	Lignin	Malathion Rest quantity	Malathion Degradation %	Lip (U / ml)	Laccase (U/ml)	MnPase (U/ml)
Control	-	0.00	0.00	0.39±0.02	3.78±0.3	0.56±0.12
	+	0.00	0.00	0.76±0.01	6.95±0.03	0.88±0.10
25	-	5.56	77.76	0.51±0.03	4.30±0.03	0.57±0.09
	+	4.05	83.80	0.79±0.05	8.32±0.05	0.89±0.07
50	-	14.60	70.80	0.57±0.01	3.22±0.06	0.59±0.12
	+	12.89	74.22	0.99±0.02	8.21±0.03	0.87±0.10
100	-	41.89	58.11	0.36±0.04	3.21±0.04	0.47±0.08
	+	35.67	64.33	0.93±0.04	5.90±0.03	0.62±0.06

Control, growth medium without malathion; (+) growth medium with Lignin;(-), growth medium without Lignin; peroxidase (Lip), manganese peroxidase (MnPase).

According to many authors (Cerniglia 1992; Lang et al., 1996; Rodriguez et al., 1999; Pointing 2001) biodegradation and bioremediation of a large array of organic pollutants, including polycyclic aromatic hydrocarbons, insecticides, polychlorinated aromatic compounds, and synthetic dyes with using white-rot fungi have been successfully. Enzymatic systems of white-rot fungi are capable of oxidative depolymerization and subsequent mineralization of lignin-related compounds (Nerud et al., 1991). Fungi and bacteria produce intracellular or extracellular enzymes including hydrolytic enzymes, peroxidases, oxygenases (Van Eerd et al., 2003, Ortiz-Hernández et al., 2011). According to Xie et al. (2009), malathion was completely decomposed within 29 h at the concentration of 12.860 g/L. and decreased from 100 to 0 mg/L in 84 h with using *Acinetobacter johnsonii*.

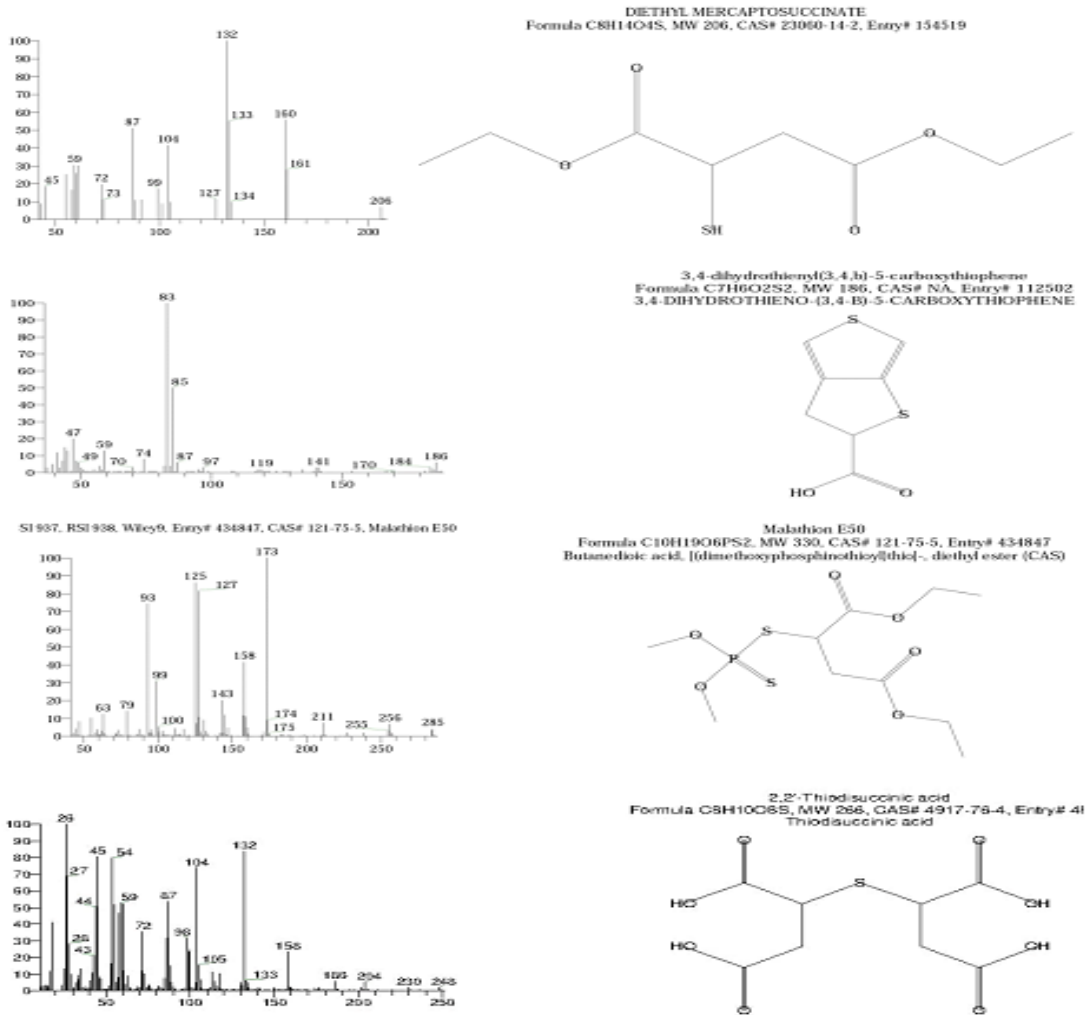

Figure. 5 Mass spectrum of molecules as a result of malathion degradation.

In the present study, GC/MS analysis (Fig. 5) revealed that many products as a result of malathion biodegradation including diethyl mercaptosuccinate, methy l, 2-(Dimethoxyphosphoryl)-2-(1,2,3-thiadiazol-4-yl) acetate, 3,4-dihydrothienyl (3,4,b)-5-carboxythiophene, Butanedioic acid, 2,2'-Thiodisuccinic acid; 1,1-Dimethyltetradecyl hydrosulfide and Disulfide, di-tert-dodecyl.

Kanade et al. (2012) reported that six products were obtained with varied functional groups as phosphorothioic acid, o,o,strimethyl ester, phosphorodithioic acid, o,o,strimethyl ester, Butanedioic acid, mercaptoderivative, Succinic acid mercapto diethyl ester, sester with o-s-dimethyl phosphorodithioate, Butanedioic acid [(dimethoxyphosphinothioyl) thio]- , diethyl ester and Succinic acid mercapto diethyl ester, s-ester with o,s-dimethyl phosphorodithioate as a result of microbial degradation of malathion. Our results agreement with Abo-Amer (2007), who observed the formation of diethylsuccinate and succinate metabolites when the malathion used as a sole carbon source for *Pseudomonas aeruginosa* growth. Recently studies (Andleebet al., 2013; Andleeb et al., 2014) reported that organophosphate pesticides are degraded by a number of microorganisms through hydrolysis and/or microbial cleavage utilizing phosphatase, oxidoreductases, phosphatases, esterase, hydrolase, and oxygenase into a variety of metabolites like malaoxon, diethylphosphorothioate with subsequent conversion into salt of succinic acid.

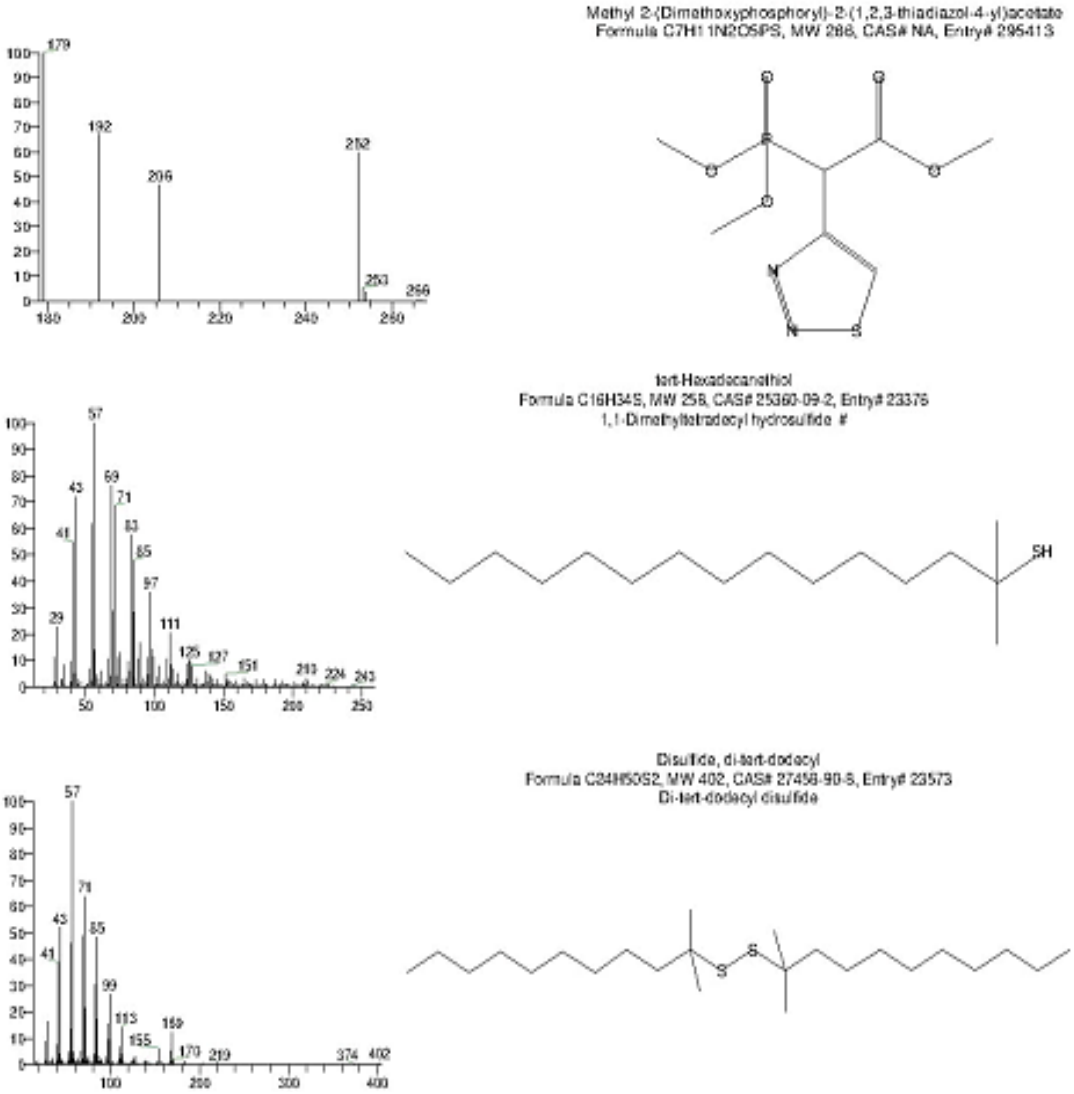

Figure. 5 (Contd.) Mass spectrum ofmolecules asa result of malathion degradation.

CONCLUSIONS

White-rot fungus possesses complex and efficientlignolytic enzyme system. They have beensuccessfully applied in treatment and decompositionof insecticide malathion on the laboratory level.

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Regional Center for Mycology and Biotechnology ALAzhar University, Cairo, Egypt.

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