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Effect of magnetic and electromagnetic field on spore germination and ligninolytic activities of *Ganoderma applanatum* using solid state fermentation

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Abstract

There are many attempts to increase the germination rate of basidiospores using temperature, light or chemicals. The current study has been designed to use the magnetic and electromagnetic fields to stimulate the germination of basidiospores from *Ganoderma applanatum*. Mature, healthy and fresh basidiocarpe of *Ganoderma applanatum* (Aphyllophorales: hymenochaetaceae), causes white rot of hard wood was isolated from Alsantah city (Delta Nile region of Egypt) in autumn 2018 from Casuarina tree. Basidiospores were collected from the fruiting body and cultivated on different microbial growing media. Basidiospores were obtained and exposed to magnetic field (MF) and electromagnetic field (EMF) for different times of exposure extended for one hour. Basidiospores germination was stimulated when exposed to both fields. The electromagnetic field was more stimulating than the magnetic field in basidiospores germination where the spore's germination rate reached 18.7% after exposure to the electromagnetic field for 40 min while the germination rate was 14.6% after exposure to magnetic field for 50 min. The fruiting body of the fungus was cultured to obtain fungal mycelium, and the resulting mycelium was exposed to the magnetic and electromagnetic field for different exposure times. The resulting mycelia was used to inoculate five different lignocellulosic substrate (Rice straw, Wheat straw, Wheat bran, Rice bran and Sawdust) using solid state fermentation in order to test its efficiency in these wastes degradation. Furthermore, Lignin degrading enzymes including (Laccase, Manganese-dependent peroxidases and Lignin peroxidase) were measured. The three tested enzymes showed a marked increase in their activity after exposure to the magnetic field for 20 minutes (laccase 17.8-unit, lignin peroxidase 11.1-unit and Mn dependent peroxidase 13.5- unit). Concerning the effect of electromagnetic field the activity of lignin degrading enzymes were enhanced after exposure to the electromagnetic field for 20 minutes (laccase 19.5- unit, lignin peroxidase 15.1-unit and Mn dependent peroxidase 27.4-unit). Therefore, the obtained data suggest that the using of both magnetic and electromagnetic fields could be used to break down the dormancy state of the basidiospores in order to increase the rate of spores germination and at the same time increase the activity of lignin degrading enzymes.

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Introduction

One of the real patterns in the cutting-edge mycology and biotechnology is generation of different macromycetes in the culture, preservation of them in collections and selection of highly productive strains with desired characteristics for further valuable application. This is the reason numerous researchers focus on getting pure cultures from basidiospores (Fries 1987; D'Enfert 1997; Bulesco *et al.* 2005; Kalmis and Kalyoncu, 2006; Dulay *et al.*2012) and have difficulties in their laboratory germination (Feofilova *et al.* 2004). Furthermore, in recent years, spores of macromycetes have pulled in extensive consideration regarding their utilization for medicines with valuable and regularly with interesting properties. The bioactivity of spores might be significantly higher than that of fruiting product body **(**Min *et al.* 1998; Zhu *et al.* 2000; Xin *et al.* 2002; Pei-Yu *et al.* 2012).

In the higher Basidiomycetes, *i.e.,* the Hymenomycetes and the Gasteromycetes, dispersal is based mainly on the sexually produced basidiospores. Basidiocarp formation and basidiospore germination are considering a very two critical events in the complete life cycle of these fungi. Both stages are accomplished by a sometimes very delicate interplay between internal and external factors, which is often difficult to analyse and understand (Fries 1984).

Past investigations have appeared there is a difficulty in the germination of basidiospores in view of its entrance into the dormancy stage and there have been numerous examinations on expanding the germination rate of these spores utilizing various variables. In basidiomycetous fungi, the feasibility of basidiospores is a significant part of sexual wellness. In any case, generally little is thought about the genetic and ecological components affecting basidiospore germination (Forsythe *et al.* 2016). For example, Poyedinok *et al.* (2015) studied the effects of light wavelengths and coherence on basidiospore germination of different basidiomycetous fungi. Several attempts have been made to germinate the basidiospores of higher fungi. Pandey *et al.* (2016) were able to germinate the basidiospores by using a simple technique for isolation of single basidiospores from *Pleurotus flabellatus*. Page *et al.* (2017) studied the effect of temperature and nutrient on spore germination of three species of Ganoderma. For *Ganoderma* spp. in general, it is difficult to obtain the mature basidiospores from the separated sporocarps but can be collected in the place during the flowering season (Hilton, 1961; Kadowaki *et al.,* 2010) Since the last century there have been studies on the germination of basidiospores, Mills and Eilers (1973) studied dormancy for basidiospores of *Coprinus radiates* and found that Heat and chemicals stimulate growth of basidiospores with percentage (23 % and 3 % respectively). Ganoderma spp. often causes white rot disease to some trees such as Acacia and Eucalyptus Casuarina. *Ganoderma applanatum* (Pers.ex Wallr) pat., which belongs to Polyporaceae of basidiomycetes, grows spontaneously on the branches of the broad leaf tree. G. applanatum forms semicircular carpophores on the branch in parallel. *G. applanatum* is found worldwide, including in Egypt. Where it has long been used as a medicine for the treatment of various human tumorigenic diseases (Kim *et al.*, 1980). For Ganoderma spp. in general, mature basidiospores are not easy to obtain from detached polypore sporocarps but can be collected in situ during the fruiting season (Hilton, 1961; Kadowaki *et al.,* 2010). Lim (1977) attempted germination of *G. philippii* basidiospores. There have been many studies on the effect of magnetic field on the growth of fungi, Nagy (2002) and Owen (1998) reported that enzymatic activities and growth of fungi affected by pulsed and static magnetic field, the activated and inhibitory effect of the magnetic field depends on the force of the field (Ruzic *et al.,* 1997). It was found that spore density, temperature and nutrient medium affect germination of Ganoderma spores (Page *et al.,* 2017).

Ligninolytic enzymes of the basidiomycetes assume a urgent job in the worldwide carbon cycle. The demand for application of ligninolytic enzymes complexes of white-rot fungi in industry and biotechnology is ever increasing due to their use in a

variety of processes. Ligninolytic enzymes have potential applications in a large number of fields, including agricultural, the chemical, fuel, food, paper, textile, cosmetic industrial sectors and more. This ligninolytic system of white-rot fungi is also playing avital role in the degradation of various xenobiotic compounds and dyes. Their capacities to remove xenobiotic substances and produce polymeric products make them a useful tool for bioremediation purposes (Jaqueline 2010).

Lignin degradation is caused by certain fungi as well as several bacterial species, Fungi are more efficient in the breakdown of lignin than bacteria, in which delignification is slower and more limited (Sigoillot *et al*., 2012).

Lignocellulose are regarded as the most abundant renewable organic matter on earth (Giovannozzi-Sermanni *et al.,* 2001) and are potentially a great feedstock reserve for the production of biofuels and chemicals. However, the presence of lignin as its primary constituents makes it under-utilized at present and in most cases commercially non-viable; this is because of the presence of covalent lignin-carbohydrate linkages connecting sugar hydroxyl of hemicellulose and phenylpropane subunits in lignin making the sugars less biologically available.

The primary role of lignocellulose is that it protects the plant against degradation that could happen due to the presence of autochthonous enzymes and microorganisms. The ability to break down the lignin would effectively make the carbohydrate more accessible for efficient bioconversion.

The co-cultivation of lignocellulose degrading fungi with the associated high activity of lignin modifying enzymes (Laccase (Lac), Manganese-dependent peroxidases (MnP), Lignin peroxidase (LiP) and versatile peroxidase (VP)) (Solarska, 2009, Mahajan, 2011) due to their synergistic and antagonistic actions is necessary to increase performance, efficiency and end product yield (Mata *et al*., 2005, Flores *et al.,* 2009, Bader *et al.,* 2010, Dwivedi *et al.,* 2011).

Successful lignin decomposition can only be achieved by the application of multiple enzymes due to the complexity and heterogeneity of lignin and the diverse chemical linkages it contains (Bugg and Rahmanpour, 2015). Therefore, the biodegradation of wood constituents (ligninolysis) is widely understood as a multi-enzymatic process that produces many intermediates (Leonowicz *et al.,* 1999). However, fungal ligninolysis does not involve one specific set of dedicated enzymes (Butler Day, 1989). Instead, the composition of the multi-enzymatic mixture depends on the type of fungus (Gonzalo *et al*., 2016).

Enzymes responsible for lignin degradation are known as ligninases (Butler and Day, 1998). The most common fungal ligninases are copper-containing laccases and heme peroxidases, the latter defined further as lignin peroxidases (LiP), manganese peroxidases (MnP), versatile peroxidases (VP) and dye-decolorizing (DyP-type) peroxidases (Sugano, 2009; Abdel-Hamid, 2013).

The objective of this study was to increase the germination rate of basidiospores of *Ganoderma applanatum* by using the magnetic and electromagnetic fields, at the same time increasing the production of enzymes for the fetus, which is produced by the fungus after field's exposure to different periods.

Materials and methods

The fruiting body of the fungus was collected from Casuarina tree, which was found, on the bank of stream near Alsantah city (Gharbiya Governorate). Basidiospores were collected from the fruiting body of Ganoderma applanatum (Fig. 1).

Identification: Basidiocarps of Ganoderma applanatum (Fig.1) were tentatively identified according to morphological characters as previously described by Breitenbach and Kranzlin (1986), Jah (1990) and Glen *et al*. 2009; and from mycelia as recommended by Nobeles (1965) and Stalpers (1978). Some tests of enzymes were performed on the mycelia of the pure culture. Laccase and tyrosinase were

tested according to Kaarik (1965) and peroxidase as described by Taylor (1974). All tests were performed on actively growing marginal hyphae and they were measured after 3, 24 and 72 hours, Cultivation was carried out from tissue of the fruitbodies on 2 % malt extract agar medium, to which 3 ppm benomyl and 10 ppm aureomycin were added to prevent the development of fungal and bacterial contaminations respectively.

Collection of spores

Spores were collected as method of Carmel *et al.* (2002) and Ho and Nawawi (1985). Spores were collected by exposing sterile Petri dishes under sporulating sporophores suspending a plastic plate covered with clean white butter paper 5–10 mm below the surface the pores of each basidome /fruit body. The entire fruit body and suspended plate were then enclosed in a large sheet of clean paper to minimize air-borne contamination at night. After 24 hours spores prints were collected and the fruit body was separated carefully from the tree and was carried in a clean plastic bag to the Lab. It was cleaned from dust and then prepared for cultivation and identification.

Spore germination

Five types of media were used Malt Extract Agar, Dextrose Agar, Czapeks Agar, Wood- meal agar and Water agar. Each medium was inoculated with one ml of spore suspension of Ganoderma applanatum basidiospores. The germinated spores were counted and calculated as percentage after 1, 2, 3, and four days.

Effect of magnetic and electromagnetic field on the germination of Ganoderma applanatum basidiospores

Since the Wood- meal agar medium gave the highest percent of germination so it was selected to study the effect of magnetic and electromagnetic field on the germination of Ganoderma applanatum basidiospores as follow: spore suspension was prepared from the collected basidiospores of Ganoderma applanatum and placed in tubes which were divided into three groups, one group was exposed to non-uniform magnetic field (97 gauss) Fig (2a), the second was exposed to uniform electromagnetic field (133 gauss) Fig (2b) and the third was left as control. One ml from each tube was removed at zero time, 5 ,10, 20, 30, 40, 50 and 60 minutes and placed on the surface of Petri dishes containing Wood- meal agar medium (3 replica). The Petri dishes were incubated for four days at 28 ± 1°C. The germinated spores were counted and calculated as percentage.

Cultivation of Ganoderma applanatum from sporocarp tissue

Cultivation was carried out from tissue of the fruitbodies on 2 % malt extract agar medium, to which 3 ppm benomyl and 10 ppm aureomycin were added to prevent the development of fungal and bacterial contaminations respectively.

Screening of lignin degrading enzymes from Ganoderma applanatum on different substrate using solid state fermentation

Substrate for solid state fermentation: Five different agricultural wastes (Rice straw, Wheat straw, Wheat bran, Rice bran and Sawdust), were collected from local market in Egypt, air dried then chipped, grinded and sieved to size (0.5 - 1 cm) separately materials were then stored in plastic bags at room temperature for the further experimental studies.

Solid state fermentation: SSF was performed in 500 mL Erlenmeyer flasks with 25 g of each substrate moistened with 75 ml Salt solution containing (per liter): 2 g KH_2PO_4, 0.3 g $MgSO_4$. $7H_2O$, 0.4 g $CaCl_2$. $2H_2O$ and 0.1 g yeast extract and sterilized at 121 °C for 30 min. The flasks were aseptically inoculated with 5 mycelial plugs (7 mm) of 7-day-old culture and incubated at 30°C for a period of 15 days, then dried at 105 °C, homogenized, and aliquots taken for analysis Ligninolytic activity of Ganoderma applanatum.

Effect of magnetic and electromagnetic field on ligninolytic activity of Ganoderma applanatum

Many of Ganoderma applanatum cubes (7 mm) were exposed to both the magnetic and electromagnetic fields under sterilized condition as previously described and used as a inoculum. Since the rice straw was the most suitable substrate for production of lignin degrading enzymes, it was selected to study the effect of effect of magnetic and electromagnetic field on ligninolytic activity of Ganoderma applanatum.

SSF was performed as described previously except the inoculation was done by 5 plugs (7 mm) previously exposed to the magnetic field and electromagnetic.

Enzyme assays

The fermented sawdust after 15 days of fermentation was soaked overnight in 0.05 M citrate buffer, pH 4.8 at 4 0C (10 g fermented sawdust /50 ml buffer) and then blended for 30 sec in a warring blender and centrifuged. The activities were measured in the clear supernatant.

Laccase activity was determined by method of Haars and Hutterman (1983) by measuring the change in A468 with 2,6 dimethoxyphenol as substrate as) in 0.05 M citric acid phosphate buffer (PH 4.5).

MnP activity was estimated by the methods of Kofujita *et al.* (1991) using a reaction mixture containing 0.4 mM guaiacol, 50mM Na-lactate buffer (pH 4.5), 0.2 mM $MnSO_4$ and 0.1 mM H_2O_2 in a total volume of 1 ml. The reaction was measured by monitoring the increase in absorbance of the reaction at 465 nm. MnP activity was calculated by subtracting the activity determined in the absence of Mn (II) from that in the presence of Mn (II).

LiP activity was measured spectrophotometrically as described by Tien and Kirk (1984) using a reaction mixture containing 0.8 mM veratryl alcohol, 0.1 m Na-tartarate buffer (pH 3.0), 0.25 mM H_2O_2 and enzyme solution in a final volume of 1.0 ml. The reaction was measured at a wavelength of 310 nm. One unit of MnP and LiP was defined as the amount of enzyme that increase the absorbance at 465 and 310 nm respectively by 0.1 per minute. The plates were incubated at 27 ± 1°C.

Results

The results in Table 1 indicate that spores germination was zero in all tested media after one day of incubation while the highest percentage of spore's germination was observed when using a Wood- meal water agar (7.9 %) after 4 days of incubation followed by Potato Dextrose Agar medium (4.3 %). The lowest percentage of germination was recorded by Malt Extract Agar medium (3.3 %).

Table 1. Effect of different media on germination of *Ganoderma applanatum* basidiospores as percentage after at 28 ± 1°C at different substrate.

Incubation period	1 day	2 day	3 day	4 day
Malt Extract Agar	0.0	0.0	2.1	3.3
Potato Dextrose Agar	0.0	1.0	2.8	4.3
Czapek's agar	0.0	0.0	0.0	2.9
Wood- meal agar	0.0	2.3	3.6	7.9
Water agar	0.0	0.0	1.8	3.9

From the data on Table (1) it is obvious that the highest percentage of spore germination was observed on wood- meal agar medium after 4 days (7.9 %), there no spore germination was observed after 1 day for all used medium. Concerning Czapek's agar medium, it showed the lowest percentage of germination after 4 days (2.9 %), in the same time, spores germination was not detectable during the first three days of incubation. Regarding to Malt Extract Agar and water agar medium, spores began to germinate on the third day with low percentage (2.1% and 1.8% respectively).

Table 2. Effect of magnetic (MF) and electromagnetic (EMF) on germination of *Ganoderma applanatum* basidiospores on Wood- meal water agar after 4 days of incubation at 27 ± 1°C at different Exposure time.

	Spore germination (%)						
Exposure time (minutes)	*zero*	10	20	30	40	50	60
Magnetic field	7.9	8.7	9.1	10.3	11.9	14.6	8.5
Electromagnetic field	7.9	8.9	9.8	12.3	18.7	13.6	10.2

Table 3. Effect of different lignocellulosic wastes on production of ligninolytic activities during solid state fermentation (Unit/g fermented straw)

Types of Substrate	Laccase	Lignin peroxidase	Mn dependent peroxidase
Rice straw	13.5	4.3	8.9
Wheat straw	9.6	5.3	7.5
Wheat bran	11.7	3.9	4.9
Rice bran	8.5	4.7	5.3
Wood- meal agar	10.5	5.1	6.5

Wood- meal agar was selected to study the effect of magnetic and electromagnetic field on spore's germination. The results obtained from the magnetic (MF) and electromagnetic (EMF) field effect on spores germination of Ganoderma applanatum are scheduled in Table (2). The highest increase of spore germination was observed after exposure of the spore suspension to magnetic field for 50 minutes (14.6 %), while the highest percentage after exposure to electromagnetic field was (18.7%) after 40 minutes. These percentages were more than the maximum germination of control (7.9%).

Generally the high exposure time lead to decrease in germination percentage but still more than the unexposed spores. Electromagnetic field stimulate the germination of spores more than the Magnetic field.

Table 4. Effect of magnetic (MF) on ligninolytic activities of *Ganoderma applanatum* using rice straw SSF expressed as (Unit/g fermented straw) for each assayed enzyme.

Exposure time (minutes)	laccase	lignin peroxidase	Mn dependent peroxidase
0	13.5	4.3	8.9
5	15.6	5.3	9.5
10	18.7	6.9	10.9
15	19.5	8.7	11.3
20	23.5	11.1	13.5
25	17.8	7.9	6.9
30	12.4	4.0	5.3

The results in Table 3 showed the potential ligninolytic enzymes secreted by the tested organism was different according to the utilized substrate. The activity of laccase enzyme was the highest (13.5 unit) when the fungus was cultivated on rice straw as substrate followed by Wheat bran and Wood- meal agar (11.7 and 10.5 unit respectively). Among the lignin degrading enzymes, lignin peroxidase enzyme showed the lowest activity on all used substrate and there are no big differences between the activities on all utilized substrate. Concerning Mn dependent peroxidase enzyme, the highest activity was observed on rice straw followed by wheat straw (8.9 and 7.5 unit respectively).

Table 5. Effect of electromagnetic filed (EMF) on ligninolytic activities of *Ganoderma applanatum* using rice straw SSF expressed as (Unit/g fermented straw) for each assayed enzyme.

Exposure time (minutes)	Laccase	Lignin peroxidase	Mn dependent peroxidase
0	13.5	4.3	8.9
5	15.7	6.7	11.5
10	19.8	8.5	13.9
15	22.3	11.2	15.3
20	27.4	15.1	19.5
25	18.6	7.9	14.3
30	15.4	4.9	10.5

Rice straw was selected to study the effect of magnetic and electromagnetic field on lignin degrading enzymes. Table (4) illustrates the production of lignin degrading enzymes of Ganoderma applanatum when mycelium exposed to magnetic field (MF).

Fig. 1. Fruiting body of *Ganoderma applanatum* (Pers. ex Wallr) Pat.

The data revealed that exposure of mycelium to MF accelerate the production of laccase, lignin peroxidase and Mn dependent peroxidase enzymes; the maximum production of all enzymes was obtained when the time of exposure was 20 minutes (23, 11.1 and 13.5 for laccase, lignin peroxidase and Mn dependent peroxidase respectively. It should be noted that the production of enzymes were mostly decreased after the exposure to 25 and 30 minutes of magnetic field. At zero-time laccase enzymes was the highest one (13.5 unit) followed by Mn dependent peroxidase (8.9 unit) and lignin peroxidase (4.3 unit). Although lignin peroxidase was the lowest, but it was the most affected by the magnetic field, it increased from (4.3 unit) to (11.1 unit). Lignin degrading enzymes were highly stimulated to EMF, the production of enzymes increased at 5, 10, 15- and 20-minutes exposure times, while the maximum acceleration was detected when the exposure time was 20 minutes for all enzymes (Table 5). The production of enzymes decreased when the mycelium was exposed for 25 minutes to the EMF and this decrease continued when the mycelium was exposed for 30 minutes but still higher than the control (zero time).

Discussion

This study showed that the rate of basidiospores germination of *Ganoderma applanatum* was zero after one day and this result is consistent with the study carried out by Page (2017) on three genera of Ganoderma (*Ganoderma austral*, *Ganoderma mastoporum*, and *Ganoderma philippii*). The low germination rate of non-exposed basidiospores is due to dormant phase, during which metabolism is reduced by about 50%. Several types of dormancy exist in fungi; that might be either exogenous or endogenous (Feofilova *et al.*, 2012).

Concerning magnetic field treatment, germination rate increased after 4 days from 7.9 % to 14.6 % after 50 minutes of exposure, this stimulation of the magnetic field was recorded by Jamil (2012) during his study on growth and yield of mushroom using magnetic field treatment. Vashisth and Nagarajan (2010) reported positive results in the growth of maize, chickpea and sunflower seeds exposed to static magnetic field.

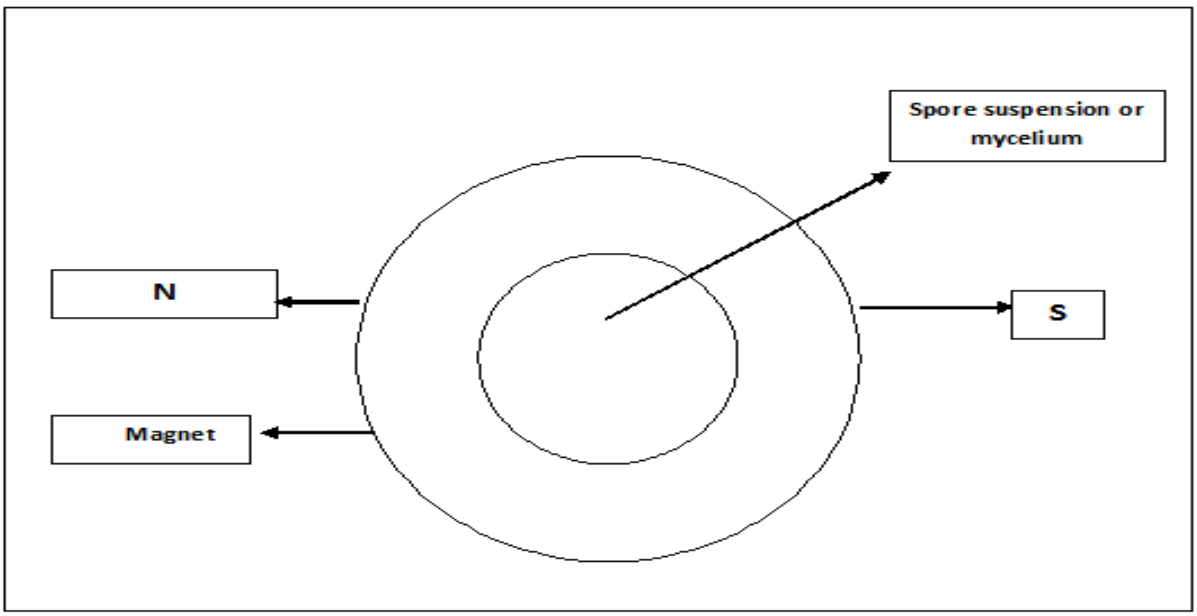

Fig. 2a. The magnet employed to study the effect of magnetic field.

The effect of magnetic field (MF) stimulation in this study is confirmed by the study of Florez *et al.* (2007) and Marks and Szecówka (2010). They reported that magnetic field treatment on biological systems stimulate germination as well as growth in later stages of development. The MF mechanisms of action on growth is not well known yet, however several theories have been proposed, including biochemical changes or altered enzyme activities (Jamil 2012).

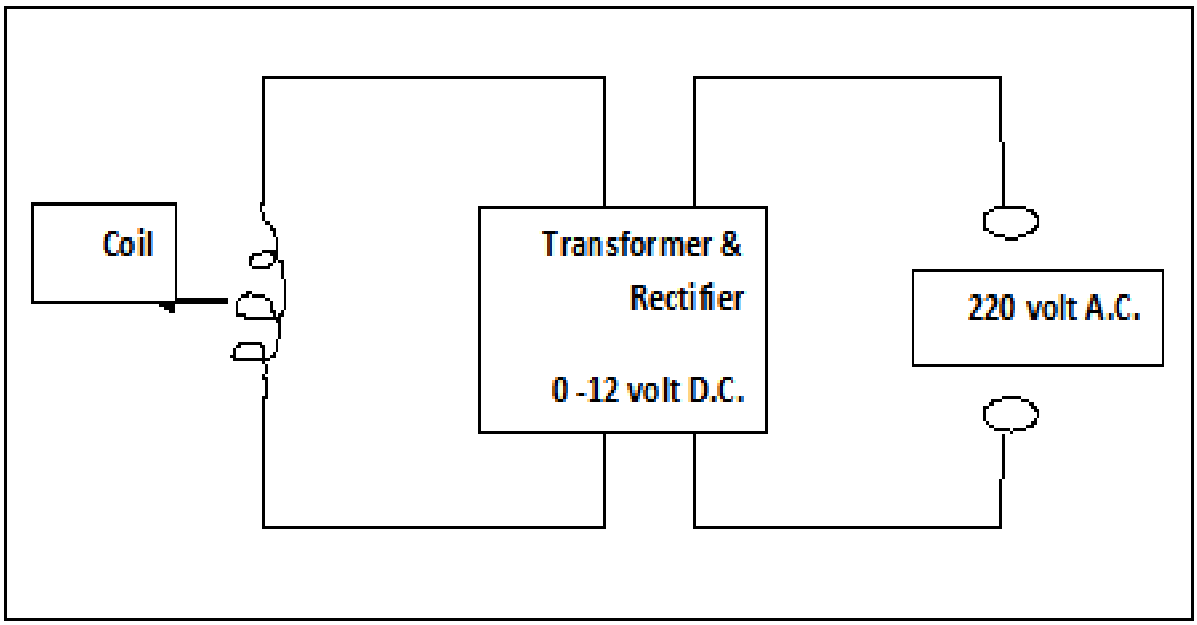

Fig. 2b. The apparatus designed to produce uniform electromagnetic field.

Compared with the control (Non-exposed spores) electromagnetic field stimulated spore germination from 7.9 % to 18.7 % after 40 minutes of exposure and this could be attributed to magnetic field treatment that might change the free radicals, concentration of ions and electrical charges without any degradation/alteration in the chemical profile of seed and makes the membranes more permeable (Iqbal *et al.* 2012). Also, the broken of spores dormancy permitting nutrients to begin to enter, or the

endogenous inhibitors to leach out of the spore (Moore *et al.*2011).

In our study, *Ganoderma applanatum* showed an increased activity of three important enzymes as response to stress with magnetic and electromagnetic field (laccase, lignin peroxidase and Mn dependent peroxidase) that might perform a good impact in agriculture ligninolysis and degradation process. Also, fungal peroxidase was recorded in biological ligninolysis. As recorded by Hammel and Cullen (2008).

In this work both magnetic and electromagnetic field enhanced enzymes production especially laccase, where a varied effect of different time exposure on the laccase activity was observed. The ability of the Ganoderma spp. to secrete the laccase enzyme in this study was acceptable for the study of Kuhar (2014) who study laccase production by two strains of *Ganoderma lucidum*. In another study carried out by Anggoro (1999) confirmed that magnetic energy can stimulate the growth of mycelium of mushroom. In this study declared that the effect of magnetic field and electromagnetic field depends on the degree of exposure of germs or mycelium to each, similar results were obtained by Adey (1992) who was found the effect of weak electric and magnetic fields could be revealed only at suitable value of magnetic field.

Conclusion

Ganoderma applanatum basidiospore's germination was stimulated when exposed to both magnetic and electromagnetic fields with highest spore germination percentage after 50 mins in case of magnetic field and 40 mins for electromagnetic filed. Also, lignin degrading key enzymes including laccase, lignin peroxidase, and Mn- dependent peroxidase activity was increased when compared at zero exposure time for both magnetic and electromagnetic fields. This might be important when dealing with agriculture waste management.

We hope that the results obtained from this work will help to improve spores germination rate and break the dormancy of the fungal spores by exposing them to the magnetic and electromagnetic field, in addition to improving the production of enzymes for lignin degrading enzymes.

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