

Original Article

## Effect of extremely low frequency magnetic field in growth, CMC<sub>ase</sub>, electric conductivity and DNA of *Aspergillus niger*

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### Abstract

In the past years the extremely low frequency magnetic field (ELF-MF) of the millimeter range has been actively used in biology, medicine, biotechnology, in this study the impact of electromagnetic radiations in growth, carboxymethylcellulase activity (CMC<sub>ase</sub>), cell conductivity and RAPD DNA pattern of *A. niger* were investigated. Experimentally, when the fungus treated with magnetic field at constant intensity 50Hz, 10 mT at different exposure times for five days (2 h/day) the total biomass dry weight inhibited by 72.2% after the first 2 h. ELF-MF showed also inhibition effect of in CMC<sub>ase</sub> activity of *A. niger*. The enzyme activity was decreased from 30 IU/ml to 22.5 IU/ml after only 2h of exposure and the treated fungus lost about 50% of its CMC<sub>ase</sub> activity after 10 h. The treated fungus showed also decreased in conductivity of fungal cells from  $1.78 \times 10^{-1}$  to  $0.51 \times 10^{-5}$  Siemens/meter. The study extended to detect the changes in RAPD patterns of *A. niger* DNA before and after exposure to the 50 Hz frequency. In conclusion, ELF-MF can be used as a safe method in control the activity of highly resistant fungi as *A. niger*.

**Keywords:** Electromagnetic radiation, CMC<sub>ase</sub>, electric conductivity, *A. niger*

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## Introduction

For many years, scientists believed that extremely low frequency magnetic field (ELF-MF) did not cause any significant biological effects. In recent decades, many scientific studies have verified that electric and/or magnetic fields of extremely low frequency (<300 Hz) can influence the biological systems. But the recent studies in the past few decades declare that non-ionising, non-thermal ELF-EMF cause a number of different biological effects (Volpe 2003, Simko 2004). These fields are emitted from electric devices, communication systems, electronic appliances, and electric transmission lines, and affect all living forms in all occupational and residential environments.

In the past years ELF-EMF has been actively used in biology, medicine, biotechnology, etc. The actual mechanism by how this type of radiation affects biological systems is under intense study, But some explanations based on its effect in cell membranes as the electromagnetic forces at the surface of a membrane could modify ligand-receptor interactions and, as result, would affect the state of the membrane molecules that control the cell transportation besides the conversion of the electromagnetic energy into acoustic-electric waves that affects the metabolic processes inside the cell (Dobson *et al.*, 2000, Pirogova *et al.*, 2009).

*A.niger* a fungus so widely distributed and ubiquitous in nature. This fungus can spread easily and colonize a wide range of substrates, causing different degree of deterioration and spoilage. Moreover, few studies have focused on the effect of magnetic field on growth and metabolisms of fungi have been published (Aiman *et al.*, 2013). So the objective of this research is explore the knowledge of ELF-MF biological effects on *A. niger*.

## MATERIALS AND METHODS

### Fungal isolate

The *A. niger* isolate used in this study was kindly provided by Assoc. Prof. Mohammad M. El-Metwally in Mycology Lab., Department of Botany and Microbiology, Faculty of Science, Damanhur University, Damanhur, Egypt.

The *A. niger* was cultivated in modified Czapek-Dox broth medium. The culture medium was prepared by dissolving the following components into one liter of distilled water: di-potassium hydrogen phosphate  $\text{KH}_2\text{PO}_4$  15g,  $(\text{NH}_4)_2\text{SO}_4$  (5g), Calcium chloride 0.6 g, magnesium sulfate heptahydrate 0.6 g, iron (II) sulfate heptahydrate 0.005g, and 10 g of carboxy methyl cellulose (CMC). In 100 ml conical flasks 20 ml of the medium sterilized by autoclavation and inoculated by 1 ml of  $10^6$  spore suspension.

### Exposure facility system

The liquid media were exposed to 50Hertz,10mili Tesla (2hrs/day, for five days) homogenous electromagnetic field generated by four solenoids of 1500 turns each of

electrically insulated 2.2 mm copper wire, wound around a copper cylindrical chamber of 17cm external diameter as shown in Figure (1) Water was pumped in a copper jacket separating the wire winding and the chamber in order to keep the temperature of the chamber constant during the exposure period. The temperature of the flowing cooling water at the outlet of the jacket and the temperature inside the irradiation chamber were periodically measured through the use of thermocouple thermometer, which can give readings for the temperature variations within  $\pm 0.1^{\circ}\text{C}$ . There was no measurable difference in temperature between the room and the chamber. The actual current passing in the solenoids was about 1Amper. The coils were connected to a variac fed from the mains (220 V and 50Hz). The magnetic field exposure system was locally manufactured. The temperature during exposure was maintained by a thermostat in the range of  $30^{\circ}\text{C}$ . The temperature ( $30 \pm 1^{\circ}\text{C}$ ) in the bottles was measured using a thin Type-K thermocouple thermometer having an accuracy of  $\pm 0.2^{\circ}\text{C}$  and 0.8 sec response time. Liquid cultures with *A. niger* was exposed in glass bottles (diameter of 60 mm with heights of 80 mm). Each glass bottle with samples was placed on a nonconductive stand with the height at the middle of the each coil. The coils were fastened to the rocking bed using bench holdfasts and an electric motor rotated the coils at 200 rpm.

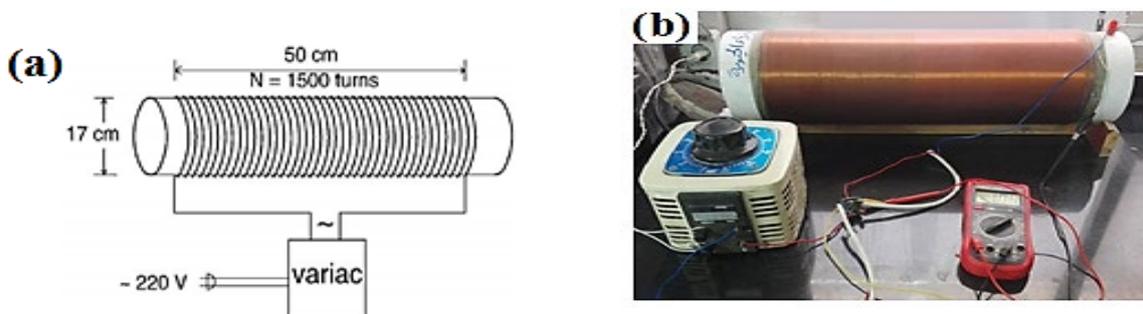


Figure (1) Schematic diagram (a) and photo (b) for exposure facility system.

### Percentage of inhibition

$[\text{Average of control} - \text{average of treated growth} / \text{average of control growth}] \times 100$

### Effect of magnetic radiation on *A.niger*

#### Effect on growth

The fungal mycelium was harvested after every 24 hours of growth, separated from the culture liquid by filtration through a Whatman No. 1 filter paper. The mycelial pellet was repeatedly washed with distilled water and dried at  $80^{\circ}\text{C}$  overnight. The dry weight of the fungus was calculated by using the following formula:

**Dry weight = (weight of filter paper + mycelium) - (weight of filter paper)**

### Assay of carboxymethylcellulase (CMCase) activity

CMCase activity was determined using the method recommended by Acharya *et al.* (2008). The reaction mixture contained 0.5 ml of 0.5% of CMC substrate prepared in 0.5 M sodium acetate buffer pH 5.5 and 0.5 ml of enzyme extract. The control sample contained the same amount of substrate and 0.5 ml of the enzyme solution heated at 100°C for 15 min. Both the experimental and control samples were incubated at 50°C for 30 min. At the end of the incubation period, tubes were removed from the water bath, and the reaction was terminated by addition of 3 ml of 3, 5-dinitrosalicylic acid (DNSA) reagent per tube (Shazia *et al.*, 2010). The tubes were incubated for 5 min in a boiling water bath for color development and then were cooled rapidly. The activity of reaction mixture was measured against a blank sample at wavelength of 540 nm. The concentration of glucose released by enzymes was determined by comparing against a standard curve constructed similarly with known concentration of glucose. Unit enzyme activity was defined as the amount of enzyme required for liberating 1 $\mu$ M of glucose per milliliter per minute.

### Conductivity measurements of *A. niger* cells

For conductivity measurement, a fungal sample (1ml) was placed into sterile micro centrifuge tube and centrifuged at 14,000 rpm at 4°C for 15 min. The pellet was then harvested and resuspended in a 1 ml volume of sterile deionized water. The tube was then centrifuged and the pellet was washed with deionized water twice more, before finally being resuspended in sterile deionized water. The dielectric measurements were carried out for the samples at frequency range 50Hz using a loss Factor Meter type HIOKI 3532 LCR Hi TESTER; version 1.02, Japan, and cell types (PW950/60) manufactured by Philips Figure (2). The cell has two parallel square platinum black electrodes of 0.8 cm side each, and area 0.64cm<sup>2</sup>, with an inter-electrode distance of 1 cm. During the measurements both the cell and the sample were kept at 25°C in an incubator (Kottermann type 2771, Germany). Each run was repeated three times.



Figure (2): Conductivity measurements (Hioki, 3532-50 LCR Hitester).

## DNA Analysis

### Isolation of DNA

DNA was extracted from 50 mg of fresh cultures of fungi (either control or exposed cultures) according to this method which is an improved method of the standard phenol/chloroform method (Neumann *et al.*, 1992). To extract the DNA from fungi, 1 ml cell suspension was centrifuged at 8000 rpm for 2 min, to pellet cells. After removing the supernatant, the cells were washed with 400  $\mu$ l STE Buffer (100 mM NaCl, 10 mM Tris/ HCl, 1 mM EDTA, pH 8.0) twice. Then the cells were centrifuged at 8000 rpm for 2 min. The pellets were resuspended in 200  $\mu$ l TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Then 100  $\mu$ l Tris-saturated phenols (pH 8.0) were added to these tubes, followed by a vortex-mixing step of 60 second to lyse cells. The samples were subsequently centrifuged at 13000 rpm for 5 min at 4°C to separate the aqueous phase from the organic phase. 160  $\mu$ l upper aqueous phases were transferred to a clean 1.5 ml tube. 40  $\mu$ l TE buffer were added to make 200  $\mu$ l and mixed with 100  $\mu$ l chloroform and centrifuged for 5 min at 13000 rpm at 4°C. Lysate was purified by chloroform extraction until a white interface was no longer present; this procedure might have to be repeated three times. 160  $\mu$ l upper aqueous phases were transferred to a clean 1.5 ml tube. 40  $\mu$ l TE and 25  $\mu$ l Proteinase K (modified instead of 5  $\mu$ l RNAs) were added and incubated at 37°C for 10 min to digest RNA. Then 100  $\mu$ l Chloroform were added to the tube, mixed well and centrifuged for 5 min at 13000 rpm at 4°C. 150  $\mu$ l upper aqueous phase were transferred to a clean 1.5 ml tube. The aqueous phase contained purified DNA and was directly used for the subsequent experiments or stored at 20°C. The purity and yield of the DNA were assessed spectrophotometrically by calculating the  $A_{260}/A_{280}$  ratios and the  $A_{260}/A_{280}$  values to determine protein impurities and DNA concentrations (HaiRong & Ning Jiang., 2006).

### Quantization of DNA sample

Two types of methods were used to measure the amount of nucleic acid in a preparation:

#### I) Spectrophotometer quantization of the extracted DNA

The ratio between the reading of optical density for the DNA samples at 260 nm and 280 nm ( $OD_{260}/OD_{280}$ ) provides an estimate of the purity of the nucleic acid was done as follow (Kaushlesh *et al.*, 2012). 1ml TE (Tris-EDTA) buffer was taken in a cuvette and calibrate the spectrophotometer at 260nm as well as 280nm. Ten  $\mu$ l of each DNA sample was added to 900  $\mu$ l TE buffer and mixed well. TE buffer was used as a blank in the other cuvette of the spectrophotometer. The  $OD_{260}$  and  $OD_{280}$  ratio was calculated. The following comments were considered that the ratio between "1.8-2.0" denotes that the absorption in the UV range is due to nucleic acids and a ratio lower than 1.8 indicates the presence of proteins and/or other UV absorbers. Also a ratio

higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol. In either case (<1.8 or > 2.0) it is advisable to reprecipitate the DNA.

The amount of DNA was quantified using the formula:

DNA concentration ( $\mu\text{g/ml}$ ) =  $[\text{OD}_{260} \times (\text{dilution factor}) \times 50\mu\text{g/ml}] / 1000$   
(Kaushlesh *et al.*, 2012)

## II) Quantization of the DNA quality by means of agarose gel

This method is based on comparing the DNA extracted sample with DNA of known concentrations and was done as follows: Prepare a 0.8% agarose gel. Add 1  $\square$  of 6X gel loading dye to 2-3  $\square$  of each DNA sample before loading the wells of the gel. Load at least 2 wells with uncut, good quality DNA or any previously quantified DNA samples (50ng and 100ng) as molecular weight standards. Run the submarine electrophoretic gel at 70V till the dye has migrated one-third of the distance in the gel. DNA can be visualized using a UV trans illuminator and quantified in comparison with the fluorescent yield of the standards.

## Random Amplified Polymorphic DNA Technique (RAPD-PCR)

Three primers were tested Codes, nucleotide sequences and G+C percentages of tested primers used in the RAPD reaction are shown in Table (1). The reaction was carried out in a DNA Thermocycler (MJ Research Inc. USA). Reactions without DNA were used as negative controls. A stock buffered solution containing 250  $\mu\text{l}$  of 10 $\times$ PCR buffer, 12.5  $\mu\text{l}$  at a concentration of 125 millimole (mM) for each dATP, dTTP, dGTP, Dctp and 100  $\mu\text{l}$  of 25 Mm  $\text{MgCl}_2$  was prepared in 1.5 ml eppendorf tube. The primers were used at a concentration of 20 pico, and double distilled water was added to bring the volume of the stock buffer solution to 1.5 ml. A volume of 5  $\mu\text{l}$  of the target DNA was added to 44  $\mu\text{l}$  of the stock solution in PCR tubes and mixed by vortexing. A volume of 1  $\mu\text{l}$  of Taq DNA polymerase (Perkin Elmer, Amersham, USA) was used at a concentration of 5.0 units. To avoid evaporation of the PCR mixture during the high temperature of the thermal cycling profiles. A drop of mineral oil was added to cover the reaction mixture (Fevzi et al, 2001).

**Table (1) Code, nucleotide sequence and G+C (%) of arbitrary primers used in the random amplified polymorphic DNA (RAPD) reaction**

primer	Sequence 5'-'3'	(%) G+C	Annealing ( $^{\circ}\text{C}$ )
P1	ATGCCC CTG T	60	32
P2	GAG CCA GTG TCT GCT TTG	56	30
P3	CGC TGT CGC C	80	28
P4	GAG CCA GTG TCT GCT TTG	56	30
P5	TGAAGGGGGGAACCCCTGTG	65	62

All PCR amplification reactions were carried out in a final volume of 50 $\mu$ l. The thermal cycling profiles were as follow a 2-min incubation at 95oC, followed by 40 cycles of 94oC for 1min, 55oC for 30 sec and 72oC for 45 sec, and a final incubation at 72oC for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler(Techne, Princeton,N.J.U.S.A) following amplifications, 20  $\mu$ L from each PCR reaction containing amplified product were loaded onto gels of 1.5% Seakemagarose (FMC Bio product, Rockland ME.,U.S.A) and electrophoresed. The gels were stained with ethidium bromide and the PCR products were identified following visualization under UV light (Kaushlesh *et al.*, 2012).

### DNA Electrophoresis

For all samples, the amplified DNA (15 $\mu$ l) was electrophoresed using electrophoresis unit (wide mini-sub-cell GT Bio-Rad) on 2% agarose containing ethidium bromide (0.5 $\mu$ l/ml), at a constant 75 volt and 60mA, and visualized with UV trans-illuminator.

### Gel Analysis

DNA gel was scanned for band, using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton, CA 92631). The different molecular weights of bands were determined against as 100,200,300,400,500,600,700,800,900,and 1000bp DNA standard (100bp DNA Ladder, Stragene, and Canda) with molecular weights..The similarity level was determined by un-weighted pair group method based on arithmetic mean (UPGMA) (Eberswalde., 2009).

## Results

### Effect of magnetic radiation in growth of *A. niger*

When *A. niger* treated with ELF-MF at constant intensity 50 Hz, 10mT at different exposure times for five days (2 h/day) the data Figure (3) revealed that as exposure time increased the total biomass dry weight decreased. After only 2 h of exposure the fungal growth inhibited by 72.2% and the inhibition degree increased with exposure time to complete inhibition after 10 h of exposure.

### Effect of magnetic field in CMCase activity of *A. niger*

The data in (Fig.4) showed the inhibition effect of magnetic radiation in CMCase activity of *A. niger*. The enzyme activity was decreased from 30 U/ml to 22.5 U/ml after only 2h of exposure and the treated fungus lost about 50% of its CMCase activity after 10 h.

### Effect of magnetic radiation in cell conductivity of *A. niger*

The results in Figure (5) show decreased in conductivity of fungal cells as the exposure time decreased from  $1.78 \times 10^{-1}$  S/m for untreated group to  $0.98 \times 10^{-3}$  S/m

after 2 h of exposure and became the value of conductivity  $0.52 \times 10^{-5}$  after 8 h hours of exposure in the 4<sup>th</sup> day.

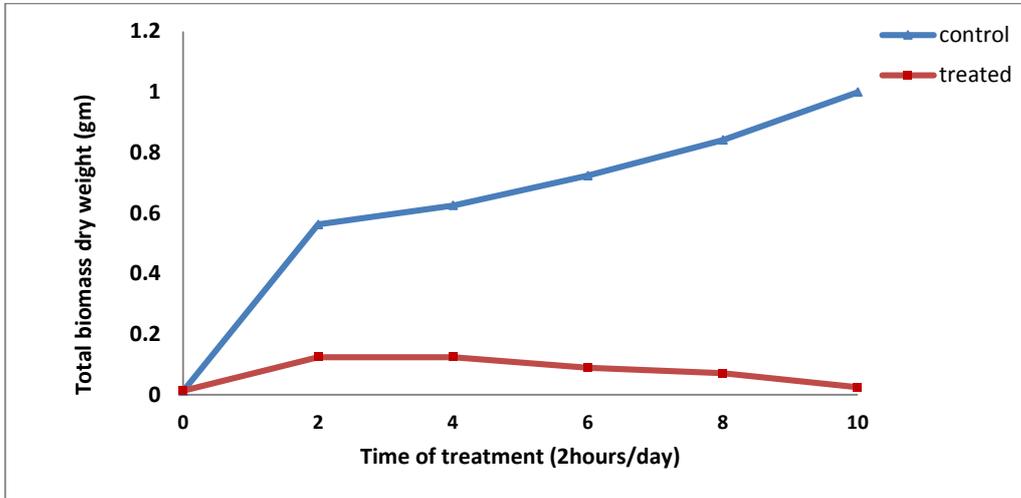


Figure (3) Total biomass dry weight of fungus *A. niger* in treated fungi as compared to negative control.

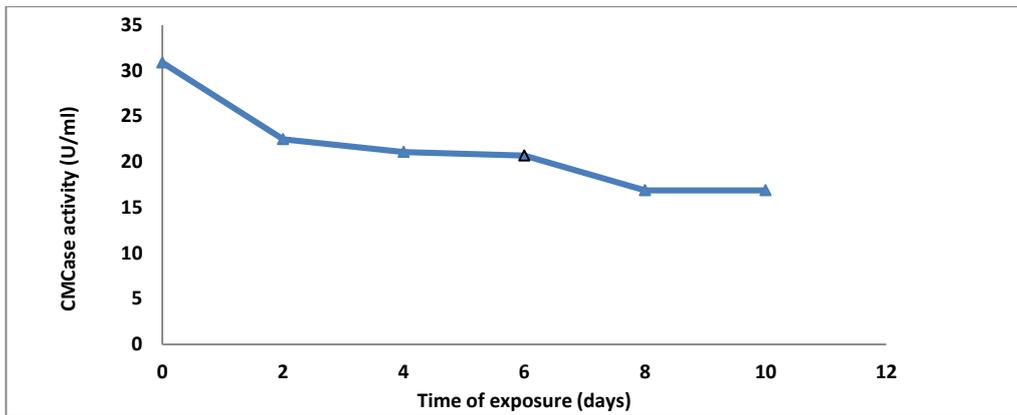


Figure (4) Effect of magnetic radiation in CMCCase activity of *A. niger* ( $B = 10$  mT).

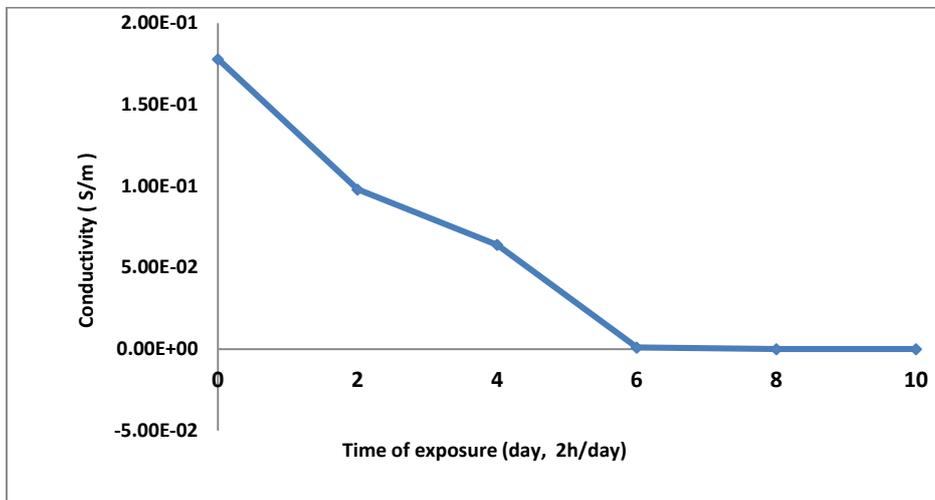
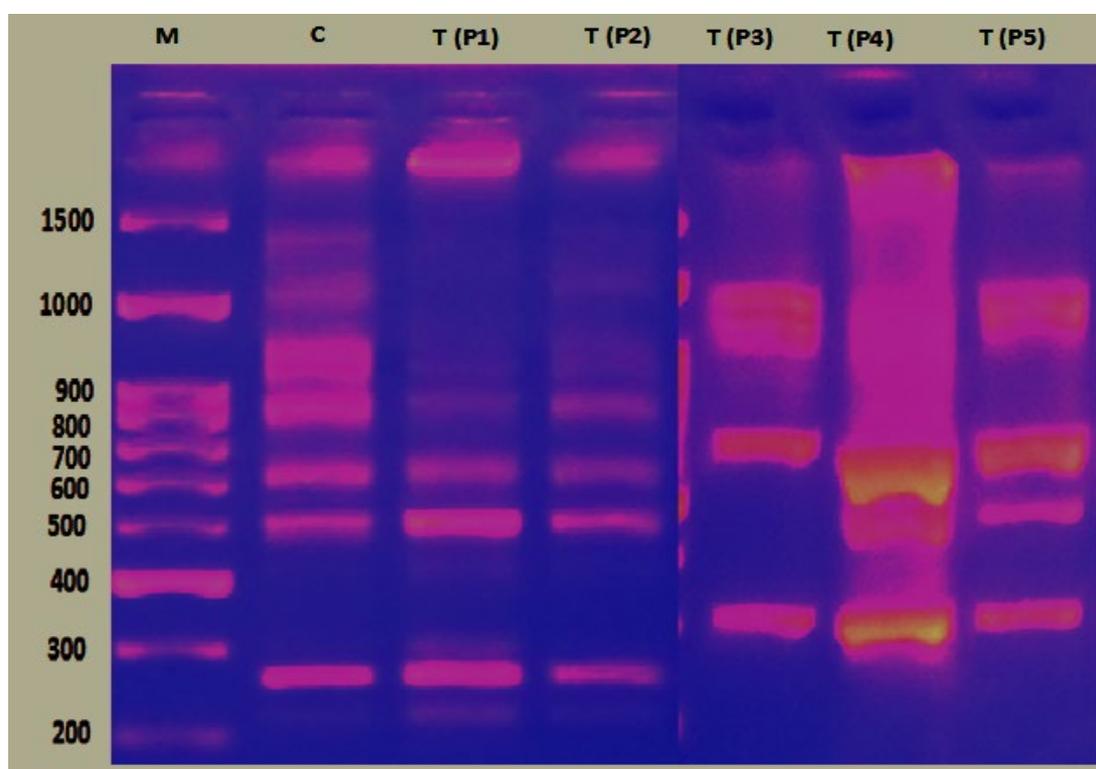


Figure (5) conductivity of *A. niger* fungi exposed to magnetic fields.

### Effect of magnetic radiation on DNA of *A. niger*

Figure (6) show electrophoresis RAPD patterns for the nuclear DNA extracted from *A.niger* before and after exposure to the frequency 50Hz after 6 days (2hours/day) of exposure. These patterns were detected using different random primers, **P1)** 5'-ATGCCC CTGT-3'/**P2)** 5'- CGC TGT CGC C -3'/**P3)** 5'- GAG CCA GTG TCT GCT TTG -3' /**P4)** 5'- GAG CCA GTG TCT GCT TTG-3' **P5)** 5'-TGAAGGGGGGAACCCCTGTG-3' respectively. The RAPD pattern for exposed and unexposed *A. niger* in Fig (6) reveal the appearance of new bands in the amplified DNA for the five primers the results shows for control *A. niger* five bands, as a result of exposure to magnetic field for 120 min these numbers of bands have been decreased to three bands. For P1 the absence of two bands between 900pb and 1000pb and for P2 the presence of one band between 800pb and 900pb respectively, for P3 the existence of three bands between 300pb and 400pb and the other at 700pb and 800pb and one band at 1000pb.Regarding P4, the presence of two bands is one at 300 pb and the other about 500 pb and 600 pb. For P5, the presence of two dense fragments trapped between 700 pb&800 pb and 900 pb&1000 pb is shown. These five primers show the presence of new bands as a result of exposure, which may be called an indicator for genetic alterations in the DNA of the exposed microorganism.



**Figure (6)** Electrophoresis RAPD Profiles of *non-irradiated* and *irradiated A. niger* with magnetic field M=DNA ladder (DNA marker), C=DNA of control sample, T= treated sample. (p1: primer 1, p2: primer 2, p3: primer 3, p4: primer 4 and p5: primer 5)

## Discussion

The number of studies of the effect of electromagnetic waves on microorganisms has increased significantly in the last decades. But little studies have been done on fungi (Nagy, 2005, Potenzo *et al.*, 2012). In the present study, the data confirm that ELF-MF have the potential to reduce the growth of *A. niger* when exposed to magnetic field at 50 Hz, 10mT. The results indicate a sharp decrease in the total biomass dry weight with variation of exposure time as compared with control culture which can be explained by changes in metabolic activity or electrostatic properties of the cell surface or both appears in the decreased in fungal cell conductivity. Knowledge about possible effect low frequency or static magnetic fields on fungi is very confusing, Nagy and Fischl (2004) for example, it has been reported that magnetic fields produce morphological changes on the conidia of *Aspergillus puniceus* and *Alternaria alternata*, as well as changes in colony pigmentation of *Aspergillus niger* (Sadauskas *et al.* 1987). In contrast, Ruiz-Gómez *et al.* (2004) demonstrated that magnetic fields have no effect on fungal growth, and others found that magnetic fields depressed it (Novak *et al.* 2007). In the same connection, Growth and sporulation of phytopathogen microscopic fungi were studied under a static magnetic field. The applied flux densities were 0,1, 0,5 and 1 mT. The magnetic field decreased the growth of colonies by 10 % using this flux density region. At the same time, the number of the developed conidia of *Alternaria alternata* and *Curvularia inaequalis* increased by 68-133 percent, but the number of *Fusarium oxysporum* conidia decreased by 79-83 percent (Nagy 2005). Al-Mayah and Ali concluded that, when magnetic field are applied at constant frequency, with high energy and for a sufficiently long period of time, their thermal effect is most likely dominant and kills fungal cells (Al-Mayah and Ali, 2010). Generally, The negative or positive effect of ELF-EMW on microorganisms depends on the strength and frequency of the electromagnetic field applied, and microbial strain used (Fojt, *et al.*, 2004, Justo *et al.*, 2006).

Fungal cells contain charged ions, polarized molecules and electric fields which obey the laws of electromagnetism and thermodynamics. For example, every cell possesses a resting transmembrane potential, and the absence of such a potential is clear evidence of a non-functional (dead) cell. The impact of the magnetic field energy lies in the stimulus to the events of significant changes in the characteristics of metabolic organisms; these are changes in the exchange of ions through the cell membrane (Li *et al.*, 2004). The main theories based on the possible effects on the permeability of the ionic channels in the membrane; this can affect ion transport into the cells and result in biological changes (Matthews 1986; Galvanoskis & Sandblom, 1998; Shckorbatov *et al.*, 2000) as well as ATPase activity (Stevens, 2004)

The magnetic fields can interact with atom and molecules of the cell to produce free radicals, which are able to diffuse enough to interact with the cell and cause damage

for the fungal cell which prevent cell to cell communications that leads to cell death. So when we try to explain the decrease and increase in biomass, it will be regarded to the effect of magnetic field on the calcium signal transduction (Blank & Goodman.,2000 , May *et al.*,2009).

The decrease in cell conductivity of exposed fungus culture is an indicator for the inhibition of fungal cells, which explain the mechanism of interaction of ELF-MF with fungi i.e. as a result of exposure time increased the heating effect of ELF-MF could be the result of Faraday induction this means that the electric fields and currents lead to charge movements, which is related to the electrical parameters of the biological cell so living cell changes under the influence of ELF-MF exposure; that affects cell structure and composition which leads to decreased cell conductivity.

Cellini *et al.* (2008) studied the genetic effect of electromagnetic fields on bacterial DNA when exposed to 50 Hz, DNA fingerprinting revealed no obvious differences among the DNA patterns at each conditions of study. Where Potenza *et al.* (2004) when recording DNA changes during RAPD-PCR studies found that exposure to magnetic fields radiation can induce DNA alterations normalized in organisms with cellular protective responses.

### Conclusion

Application of magnetic field induces changes in the metabolism characteristic of *A. niger*. These changes indicate the role of ELF-MF in inhibition of the growth, CMCase productivity of the fungus and made variations in DNA molecular structure. These results open a wide door on alternative and save physical method for fungal growth control.

### Declaration of interest

The authors report no conflicts of interest.

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