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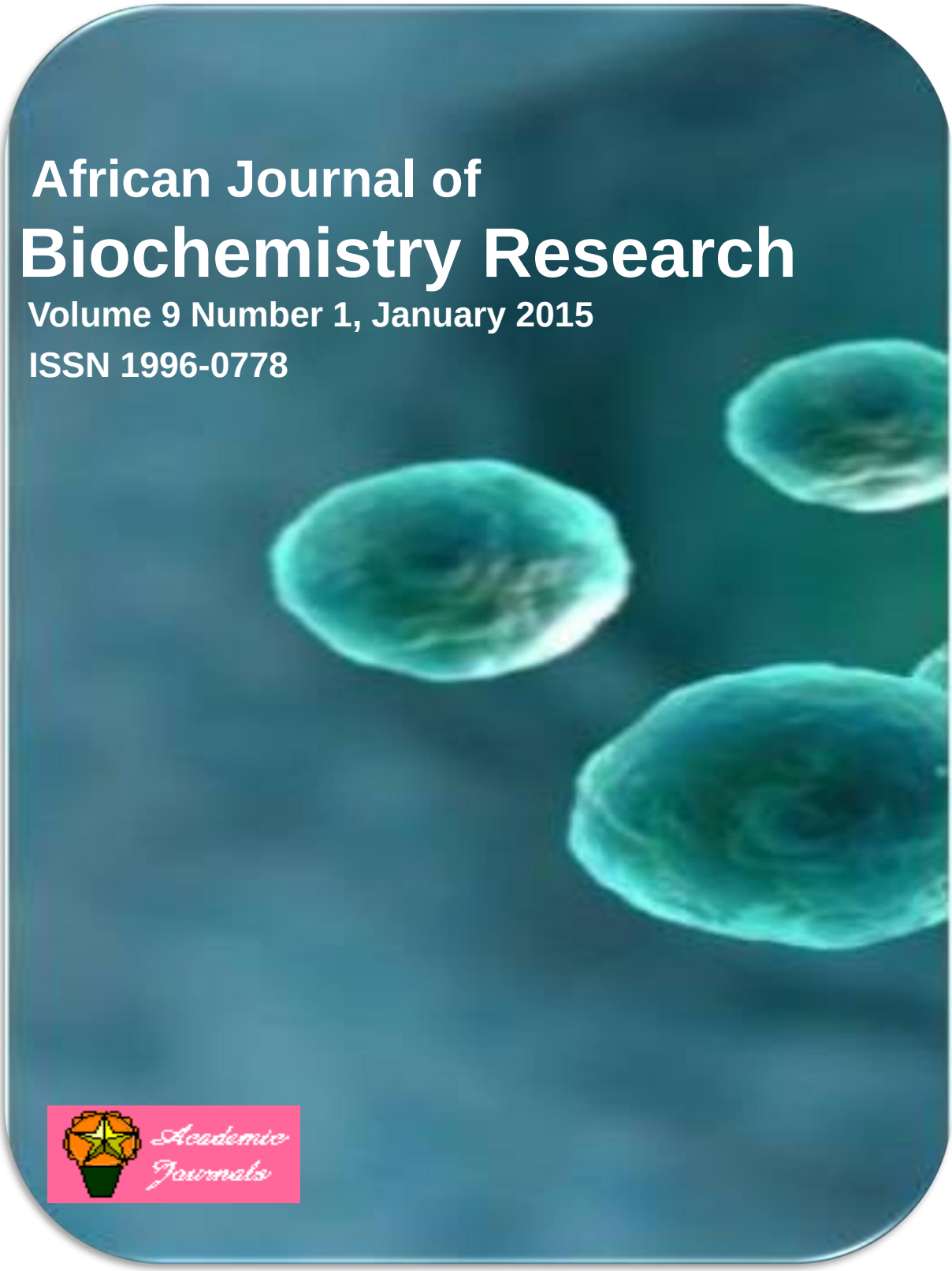

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Changes in enzymes activities of soil samples exposed to electromagnetic radiations (EMR) from mobile phone

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This study aimed at investigating the changes in activities of dehydrogenase, catalase, alkaline phosphatase, acid phosphatase and alkaline protease of soil samples exposed to electromagnetic radiations (EMR) from mobile phone for the periods of 0, 30, 60 and 90 days. EMR-unexposed soil samples served as the source of control enzyme activities. The mean enzyme activities from the EMR-exposed soil were significantly ($p<0.05$) lower than those from the unexposed samples. The overall percentage changes in enzymes activities of the EMR-unexposed and exposed soil samples for dehydrogenase, catalase, alkaline phosphatase, acid phosphatase and alkaline protease relative to their respective starting values were 124.42 and -65.15%, 138.01 and -13.87%, 94.09 and 19.70%, 101.01 and -41.00%, and 162.55 and -21.71% respectively. The results show that EMR from mobile phones elicited significant negative impact on soil enzymes activities.

Key words: Electromagnetic radiations, mobile phones, enzymes.

INTRODUCTION

The use of mobile phones in recent years has raised and continued to raise tremendous safety questions. This is because users are exposed to electromagnetic radiations, the effects of which on the body depend on their frequencies, durations and powers.

The radiations generated by mobile cell phones are non-ionizing radiations (NIR). There are wide ranges of data documenting the ability of non-ionizing radiations (NIR) to affect living cells, including changes in the biochemical and molecular mechanisms of cells both *in vitro* and *in vivo* (Barnes, 1996). Changes also occur in cell metabolism and proliferation, inducing potentially damaging effects in various cell components ranging from the cytoplasmic membrane, where the distribution of proteins is modified (Bersani et al., 1997) to the cytoplasm itself and the nucleus, where the activities of intracellular enzymes and molecules regulating cell growth are altered (Hill, 1998).

Contrary to what the Telecommunication Industries proclaim, there are vast scientific, epidemiological and medical data that affirm that exposure to electromagnetic radiations from mobile phones and their towers, even at low levels, can have profound adverse effects on biological systems.

Experiments have revealed the biological effects that occur in activities of isolated enzymes, cell cultures and

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animals after exposures to low-intensity electromagnetic radiations. Mobile phone radiation exposure can cause cell damage via reactive oxygen species formation, and cell death (Oral et al., 2006; Sokolovic et al., 2008). Exposure of acetylcholinesterase, an important central nervous system enzyme, to mobile phone EMR altered the structural and biochemical characteristics of the enzyme, resulting to a significant change of its activities (Barteri et al., 2005). Three different cell types (rat hepatoma cells, egg cells of the Chinese hamster, human melanoma cells) were exposed for 1 h to a 450 MHz fields with a 16 Hz amplitude modulation and a power flux density of 10 W/m^2. The exposure raised ornithine decarboxylase (ODC) activity by a little more than 50%. The increased ODC activity remained fairly constant for several hours after the exposure. Similar fields with a 60 Hz and a 100 Hz modulation had no effects (Byus et al., 1988).

The mobile phones in their receiving and sending processes use (mostly) microwave frequency of 945 MHz (Bakr, 2004), emitting radiation that may be absorbed by various organs of the body depending on their location (Ozguner et al., 2005; Oktem et al., 2005). Mobile phones also emit low-frequency magnetic field pulse generated by battery currents in the phone that are too weak to produce non-thermal effects (Hyland, 2000). Recent studies have shown that the intense radiations from mobile phone towers adversely impact on every biological organism within 1 square kilometer (Bakr, 2004).

Mobile phone towers are especially dangerous because they emit electromagnetic radiations at a frequency range of 900 to 1900 MHz. Radiofrequency of this range is considered to be within high frequency range (HF-EMF) (Scehihr, 2006). However, the electromagnetic radiations emitted by mobile phones and base stations penetrate the living bodies to a distance that decreases with increasing frequency (Stewart, 2000).

It has been reported that at 900-1900 MHz, EMR does not penetrate deeply into the body; instead, it is absorbed by the skin and the underlying tissues. The heat that is generated in the tissue is then channeled into blood circulatory system (Otto and von Muhlendahl, 2007). However, the same cannot be said for unicellular organisms or even enzymes where it is expected to penetrate deep into the nucleus where DNA is located.

The importance of microorganisms in the soil cannot be over emphasized. For example, the main role of mould and fungi in the soil is to breakdown the remains of plant materials using their appropriate extracellular enzymes such as pectinases, celluloses etc., and these are further broken down through the activities of bacterial enzymes. Through the activities of these microbial enzymes, carbon, nitrogen and other minerals are released to the soil for plants utilization. Therefore, such factors like temperature, pH, substrate concentrations etc that affect the activities of these enzymes cum their host microorganisms in soil, for instance, will negate the availability of these minerals and by extension soil fertility. Also, bacterial and fungal communities occupy overlapping niches in soil. Disturbing these communities, for instance, through the denaturation or reduction of their enzymes activities, may alter the balance existing between them. The resulting imbalance may affect the influence of bacteria and fungi on their niches, and consequently, to the functional ecosystem.

Duration of EMR exposure for instance, seems to be a major determinant of EMR effect on the activities of enzymes. The time of exposure and power density are correlated in a way that decrease in power density (PD) could be compensated by increase in duration of exposure.

MATERIALS AND METHODS

Sampling and sample preparation

Soil samples were collected from the surface to a depth of about 30 cm into thoroughly washed and air-dried plastic containers from five different sites (at a distance of 2m from each other) on a five-year fallowed garden farm of Federal University of Technology, Owerri, Nigeria, in the month of August, 2013 and then homogenized (Kenwood, UK). Five kilograms (5 kg) of the homogenized soil sample were then weighed into each of two experimental tanks, measuring120 × 120 × 100 cm each.

Experimental set up

A commercially available Nokia 2700 mobile phone (90 0MHz band with modulated voice and Specific Absorption Rate of 0.927 W/kg) was used for the generation of EMR for this study. The mobile phone was sandwiched at a depth of 5cm in the 5 kg soil sample of experimental tank one. During exposure, the mobile phone was kept on talking mode for 4 h per day, in vibration and the phone battery was also kept charged. The unexposed sample (with no phone) was placed away (at a distance of 150 m) from the exposed sample (with phone) and at the same atmospheric conditions.

Both the exposed and the unexposed soil samples were irrigated continuously to maintain 75% humidity and a room temperature of 28±2°C for periods of 0, 30, 60 and 90 days. The mobile phone was temporarily removed (2 min) from test experimental tank during every irrigation exercise. The exposed and unexposed sample setups were kept away from any instrument or machine to avoid interference from any form of unwanted EMR. At the end of each period, the soil enzyme activities were assayed.

Soil enzyme activities assay

Dehydrogenases (EC 1.1.1) activity

Soil dehydrogenase activity was assayed based on the estimation of the rate of reduction of triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF) after incubation at 30°C for 24 h. Field-moist soil sample (5 g) was weighed into five test tubes and mixed with 5 ml of TTC solution. The tubes were sealed with rubber stoppers and incubated for 24 h at 30°C. After the incubation, 40 ml acetone was added to each tube, and the tubes were shaken thoroughly and further incubated at room temperature for 2 h in the dark (shaking the tubes at intervals). The soil suspension (15 ml) was then filtered and the optical density of the clear supernatant was measured colorimetrically against the blank at 546 nm (Thalmann, 1968).

Phosphatases

The method used was based on the determination of p-nitro-phenol

released after the incubation of the soil samples with p -nitrophenyl phosphate (ρNP) for 1h at 37°C. Five grams (5 g) of the moist soil sample were placed in Erlenmeyer flasks (50 ml) and treated with 0.25 ml of toluene, 4 ml of buffer solutions (pH 6.5 for the assay of acid phosphatases and pH 11 for the assay of alkaline phosphatases), and 1 ml of p-nitrophenyl phosphate solutions made in the same buffer. After stoppering the flasks, the contents were mixed and incubated for 1 h at 37°C. After the incubation, 1 ml of $CaCl_2$ (0.5 M) and 4 ml of NaOH (0.5 M) were added. The contents were mixed and the soil suspension filtered through a Whatman filter paper grade 2. For the standard, 1ml of pNP solution was added after the addition of $CaCl_2$ (0.5 M) and 4 ml of NaOH (0.5 M) immediately before filtration of the soil suspension. Absorbance of the solutions were read at 400 nm (Eivazi and Tabatabai, 1977).

Proteases

Soil alkaline protease activity was assayed using Folin-Ciocalteu reagent based on the determination of amino acid released after the incubation of the soil samples with sodium caseinate for 30 min. The soil suspension was subjected to centrifugation (5,000 rpm for 10 min), and the supernatant was used for alkaline protease activities determination according to (Nigam and Ayyagari, 2007).1 ml of supernatant was mixed with 1 ml of casein (1%) [prepared in 0.1 M citrate buffer of pH 5 and 0.1 M glycine-NaOH buffer of pH 10] followed by incubation at 50°C for 30 min. Then 5 ml of 5% trichloroacetic acid (TCA) was added to precipitate the undigested protein. The solution was then centrifuged at 10,000 rpm for 10 min, and the supernatant subjected to estimation for the amount of amino acids released by Lowry's method (Lowry et al., 1951). International unit of protease was calculated as:IU= Net amount of amino acid released (µg) × dilution factor/ 181× 30, where 30=incubation period, 181=amount of protein present in the sample.

Catalase (EC1.11.1.6) activity

The method used was based on the volumetric determination of oxygen liberated after incubation of soil sample with hydrogen peroxide for 3 min at room temperature (Isamah et al., 2000; Cohen et al., 1970). Five grams (5 g) of moist soil samples were weighed into five test tubes, mixed in 20 ml of distilled water, and centrifuged at 3500 rpm for 10 min at 4°C. To the supernatant, 5ml of cold 6mM H_2O_2 was added and the reaction stopped after 3 min by adding 0.25 ml of 6 N H_2SO_4 rapidly with thorough mixing. Excess potassium permanganate (10 ml) was added, shaken gently and absorbance read at 480 nm for 30 s. The blank was prepared as the test but with the replacement of supernatant with distilled water.

A standard was prepared by mixing 10 ml of potassium permanganate with 5.5 ml of potassium phosphate buffer (pH 6.5) and 0.25 ml of 6N H_2SO_4 and the absorbance read at 480 nm. Enzyme activity was calculated using the following formula: $^{K}/_{0.00693}$.

Where the first-order rate constant $(K) = \text{Log}\left(\frac{S_o}{S_t}\right) \times \left(\frac{2.3}{t}\right)$.

Where, S_0 (Initial substrate concentration) = Absorbance of standard minus Absorbance of blank; S_t (final substrate concentration) = Absorbance of standard minus Absorbance of sample; t = reaction time (min).

Statistical analysis

All analyses were done in triplicate and the data generated were analyzed using ANOVA and Duncan's test with the aid of Statistical Package for Social Science (SPSS). Values for $p<0.05$ were considered statistically significant; data were presented as mean± standard deviation.

RESULTS AND DISCUSSION

The effects of non-ionizing radiation, emitted by mobile phones, in living cells have been widely reported, however, the mechanism of action has not been fully documented, though it is thought to be through the action of reactive oxygen species. Living tissues are 70-90% water by weight, non-ionizing radiation therefore, would be expected to induce cellular generation of free radicals by excitation of water molecules. The dividing line between radiations that excites electrons and radiation that forms ions is often assumed to be equal to the energy of ionization of water (1216 kJ/mol). Radiation that carries less energy can excite the water molecule and is therefore called non-ionizing radiation. Radiation that carries more energy than 1216 kJ/mol can remove an electron from a water molecule, and is called ionizing radiation (Daniel et al., 2008). The capability of non-ionizing radiation to induce cellular damage has been reported by Barnes (1996) and other scientists; whether these reported effects were as a result of free radical formation, thermal or athermal action, remained an interesting area for further investigation.

EMR is also believed to exert its biological effects through: non-thermal action, thermal action, and/or a combination of the two. EMR emitted by mobile phone is at non-thermal power density level, so far, no common ground exist on non-thermal exposure levels of EMR in literatures. Thermal effect results from the conversion of the EMR energy into heat energy in the living systems. Polar molecules in cells exist in the form of water, DNA and proteins, and these molecules respond to electromagnetic radiations by rotating. This rotation creates an angular momentum which results in friction with neighboring molecules, thereby developing a linear momentum (vibrational energy) (Saifuddin et al., 2009), through this means, radiation energy is converted into thermal energy.

It can therefore be said that the effect resulting from vibrational energy is ther-mal effect which occurs in a biosystem due to penetration of electromagnetic radiation into biological materials and subsequently heating up the intra- and extra- cellular fluids by transfer of vibrational energy (Tahir et al., 2009). It must be noted that EMR thermal effects is different from conventional heating effect. This is because dipolar polarization and rotation of molecules in an attempt to align the dipoles with applied electromgnetic field produces effects which cannot be achieved by conventional heating (Zelentsova et al., 2006).

The non-thermal effect of EMR has been a matter of debate in scientific community. A non-thermal effect has been suggested to result from a direct stabilizing interaction of electric field with specific (polar) molecules in reaction medium with no rise in temperature (Herrero et al., 2008). The interest in non-thermal effect is predicated on the established fact that thermal effects alone

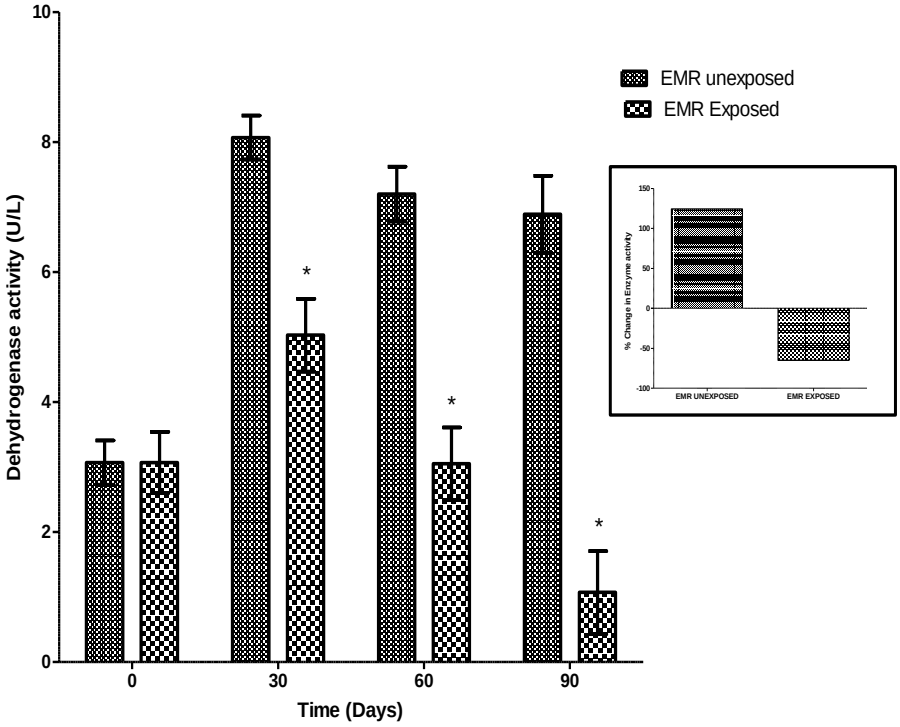

Figure 1. Dehydrogenase activity (U/L) of Electromagnetic radiation (EMR) exposed and unexposed soil samples. %Δ, *Values are significant ($p < 0.05$) in comparison with their respective EMR unexposed control. Insert chart; overall percentage change in dehydrogenase activities of EMR unexposed and exposed soil samples.

cannot explain the manner in which the EMR affects biological systems. As a result of pausity of information on the exact mechanism involved in *'nonthermal* EMR effects' (*athermal* effects), their existence has been a subject of controversy. The influence of EMR on the soil enzymes may also result from its effect on the proteinaceous nature of the enzymes that changes the secondary and tertiary protein structure of enzymes and thus, denaturation of enzymes. These changes in enzyme activities may be correlated with the probability of electroporation (Reina et al., 1998) and reported irreversible cell membrane break down of microbial hosts.

The concept of resonant absorption and resonant interactions has been suggested as another possible explanations for the marked sensitivities of living systems to EMR (Cosic, 1997). In the present study, changes in enzymes activities of soil samples exposed to electromagnetic radiations (EMR) from mobile phone were assessed.

Figure 1 shows the dehydrogenase (DHG) activity of the electromagnetic radiation (EMR) of exposed and unexposed soil samples. It indicates significant ($p < 0.05$) reductions in DHG activity of EMR exposed soil samples at exposure periods of 30, 60 and 90 days, giving an overall percentage change in DHG activity between 0 to 90 days of 124.42% and -65.15% for the EMR unexposed and exposed soil samples respectively. Dehydrogenases are used as an indicator of overall soil microbial activity because they occur intracellularly in all living microbial cells. They are tightly linked with microbial redox processes (Moeskops et al., 2010). Most importantly, dehydrogenases do not accumulate extracellular in the soil. They play vital roles in the biological oxidation of soil organic matters by transfer of hydrogen from organic substrates to inorganic acceptors (Zhang et al., 2010). DHG activity, therefore, reflects metabolic ability of the soil and it is usually proportional to the biomass of the microorganisms in soil (Salazar et al., 2011). Stress on microbial biomass is reflected on dehydrogenase activities.

Our results show that between 0 to 30 days of EMR exposure to the soil samples, the activities of catalase were enhanced (Figure 2). This finding is consistent with the induction of catalase and peroxidase activities following microwave pretreatment of wheat seedlings (Chen et al., 2008). Generally, the catalase activity of the EMR exposed soil samples decreased significantly ($p<0.05$) with increase in duration of exposure, and in comparison with their respective EMR unexposed controls. The overall percentage change in catalase activities between 0 to 90 days of EMR exposed soil sample was -13.87% as compared to 138.01% from the EMR unexposed soil samples, thus indicating the negative impact of the treatment on the enzyme activities. Catalase, also known as hydrogen peroxidase oxido-reductase is an

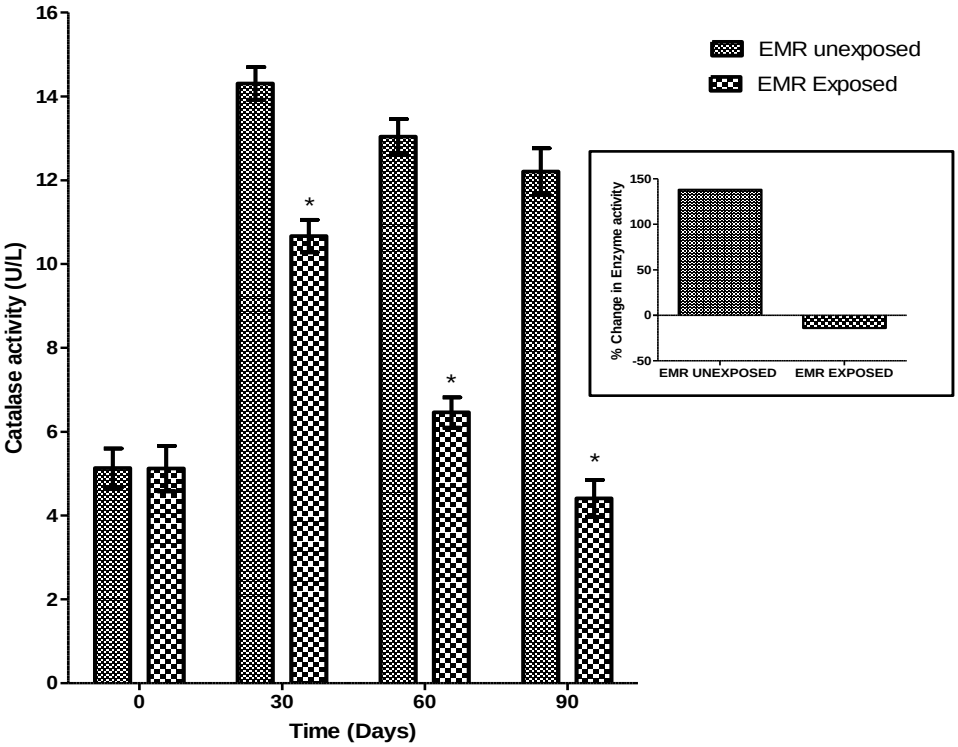

Figure 2. Catalase activity (U/L) of Electromagnetic radiation (EMR) exposed and unexposed soil samples. %Δ, *Values are significant ($p < 0.05$) in comparison with their respective EMR unexposed control. Insert chart; overall percentage change in catalase activities of EMR unexposed and exposed soil samples.

enzyme that has a detoxifying function in cells, catalyzing the reaction: H_2O_2 ------→ H_2O + ½O_2. All aerobic and most facultative anaerobic bacteria, but not obligate anaerobic bacteria, exhibit catalase activity. Catalase activity has been shown to be very stable in soil and significantly correlates with the content of organic carbon and decreases with soil depth (Ladd, 1978). However, no relation has been detected between catalase activity and soil biomass. Furthermore, storage of moist or air-dried soils at room temperature for up to a period 4 months had not been found to have effect on catalase activity.

Figures 3 and 4 show the effect of EMR exposure on alkaline phosphatase (ALP) and acid phosphatase (ACP) activities of soil samples respectively. ALP and ACP are both phosphatases, which are known to catalyse the hydrolysis of organic phosphomonoester to inorganic phosphorus. Based on their optimum pH, the enzymes are classified as acid, neutral and alkaline phosphatases. Acid (pH optimum: 4 – 6.5) and alkaline phosphatase (pH optimum: 9-10) have been found in soils (Speir and Ross, 1978). Acid phosphatase is predominant in acidic soils, while alkaline phosphatase prevails more in alkaline soils (34). Both ALP and ACP activities were significantly ($p<0.05$) lower in the soil samples exposed to the EMR. The observed reductions in the phosphatase activities decreased with increase in duration of exposure giving an overall percentage activity decrease of -19.70 and -41.00% in comparison with the increased activity observed in the unexposed soil samples (94.09% and 101.01%) for ALP and ACP respectively. Generally, ACP recorded minimal activities than ALP in both the EMR exposed and unexposed samples. This is because the soil samples were basic with an average pH value of 9.40.

Alkaline proteases are degradative enzymes that catalyze the partial or the total hydrolysis of proteins. These enzymes are mainly produced by bacteria and fungi. Microbial proteases are predominantly extracellular and can be secreted in the fermentation medium by several species of bacteria, bacillus and fungi for example *notatum* (Ellaiah et al., 2002; Raju et al., 1994; Haq et al., 2006). In this study, EMR inhibited alkaline protease activities by -21.71% and this is consistent with the report of Dholiya et al. (2012). However, between 0-30 days, the activities of the protease were enhanced in the EMR-exposed samples. This agrees with the findings of Afzal and Mansoor (2012) who reported increased activities of proteases following EMR exposure to radicles for 72 h. While the alkaline protease activity remained high in the EMR unexposed samples, that of the exposed samples decreased significantly ($p<0.05$) with increased duration of exposure. Thus, the overall percentage changes

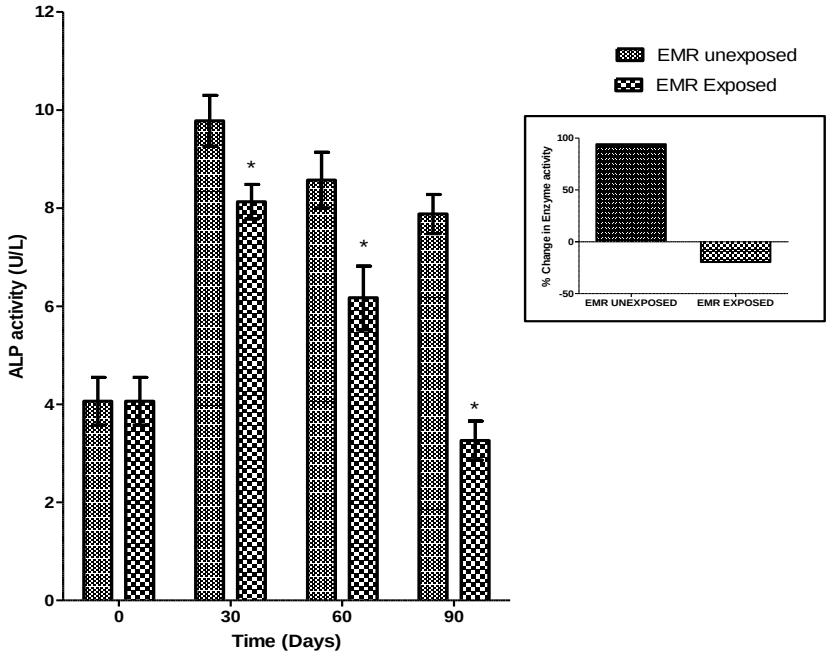

Figure 3. Alkaline phosphatase (ALP) activity (U/L) of Electromagnetic radiation (EMR) exposed and unexposed soil samples. %Δ, *Values are significant ($p < 0.05$) in comparison with their respective EMR unexposed control. Insert chart; overall percentage change in ALP activities of EMR unexposed and exposed soil samples.

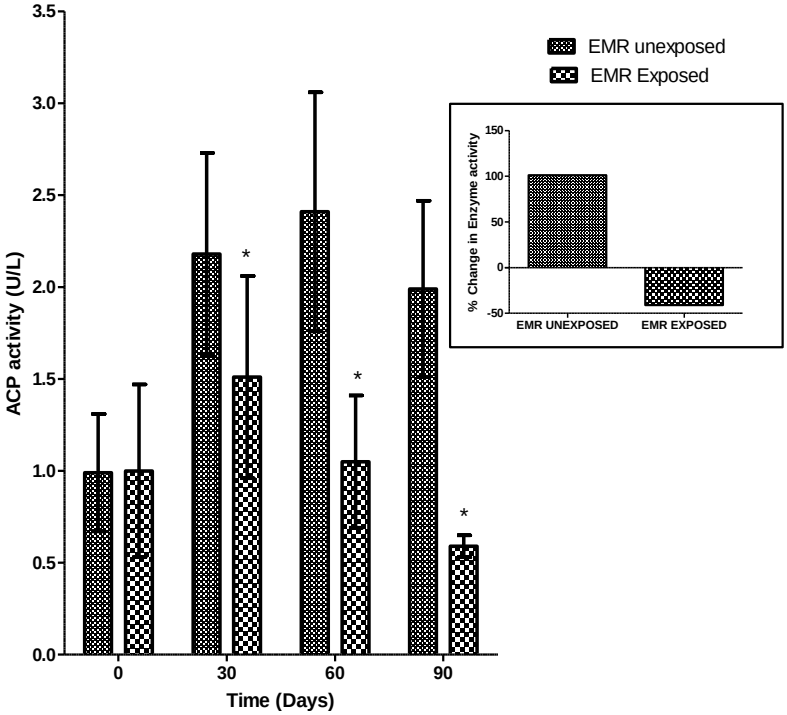

Figure 4. Acid phosphatase (ACP) activity (U/L) of Electromagnetic radiation (EMR) exposed and unexposed soil samples. *Values are significant ($p < 0.05$) in comparison with their respective EMR unexposed control. Insert chart; overall percentage change in ACP activities of EMR unexposed and exposed soil samples.

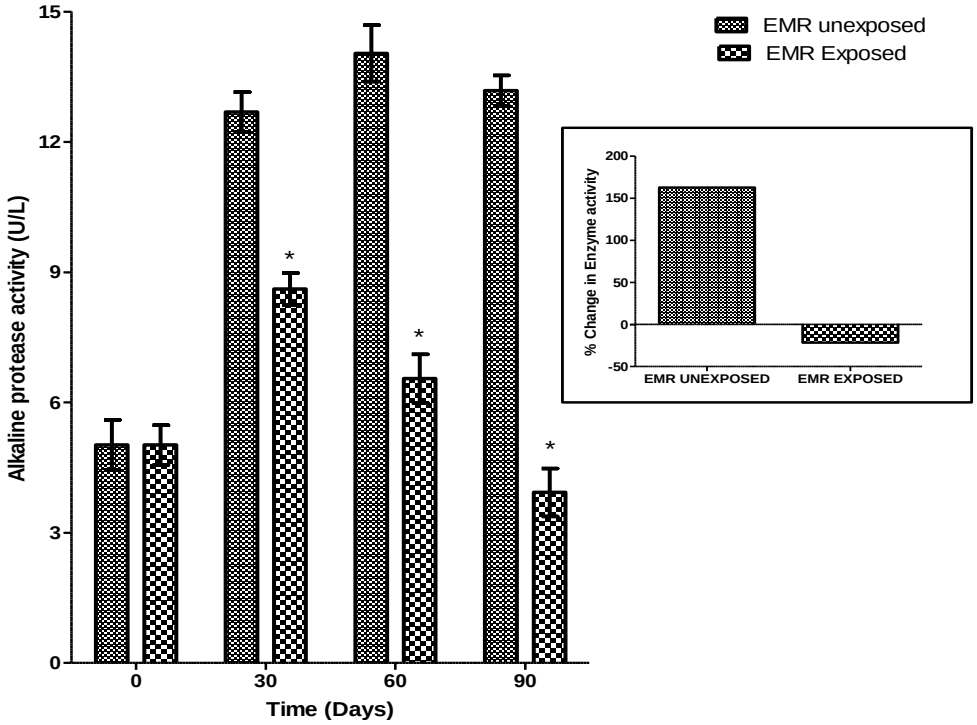

Figure 5. Alkaline protease activity (U/L) of Electromagnetic radiation (EMR) exposed and unexposed soil samples. *Values are significant ($p < 0.05$) in comparison with their respective EMR unexposed control. Insert chart; overall percentage change in alkaline protease activities of EMR unexposed and exposed soil samples.

in activities of the enzymes between 0 to 90 days were found to be -21.71 and 162.55% for the exposed and unexposed samples respectively (Figure 5).

Conclusion

This study indicated that EMR had significant negative impacts on soil enzyme activities at the exposure periods of 30, 60, and 90 days, with dehydrogenase being the most affected and catalase the least affected vis-a-vis their corresponding enzymes activities in the unexposed soil samples. It can be suggested that the EMR effects might be the result of micro-thermal heating that was importantly different from conventional heating.

Based on the observed effects, it is therefore concluded that chronic ex-posure to electromagnetic radiations from mobile phones can inhibit enzyme activities in soil. The findings have further paved ways for studies on the impact of EMR on soil and its biomass around telecommunication masks.

Conflict of interests

The authors do hereby declare that no conflict of interest exist.

ACKNOWLEDGEMENT

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Full Length Research Paper

Higher tolerance of a novel lipase from *Aspergillus flavus* to the presence of free fatty acids at lipid/water interface

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The main objective of this work was to identify novel lipases of industrial interest. In this paper, *Aspergillus flavus* lipase (AFL) was isolated from the traditional tannery of Fez city in Morocco; it kept its stability even in the presence of high concentrations of detergent from 0 to 10 mM sodium deoxycholate (NaDC). Bile salts showed no inhibitory effect on the lipolytic activity, whereas the calcium salts showed a stimulating action on the lipase activity. Unlike most of the lipases which were quickly denatured at the lipid/water interface, the accumulation of free fatty acids at the oil/water interface did not affect the activity of the enzyme which effectively hydrolyzed the emulsified olive oil even in the absence of bile salts. Furthermore, AFL was more active on long chain triacylglycerols than on short chain triacylglycerols. This study allowed us to prove that AFL had the interfacial activation phenomenon. A 3D structure model of AFL was built and we have concluded that the ratio hydrophobic surface/hydrophilic surface was 51% versus 50%; it could be responsible for a higher tolerance to the presence of free fatty acids at lipid/water interface.

Key words: *Aspergillus flavus* lipase (AFL), detergent, interfacial activation, free fatty acids, model.

INTRODUCTION

Triacylglycerol acyl hydrolase (EC.3.1.1.3), lipases belong to the carboxylic ester hydrolases family. The physiological role of lipases is to hydrolyze triglycerides to diglycerides, monoglycerides, glycerol and fatty acids (Mats and Karl, 1994). These enzymes exist in all living organisms; they have the ability to achieve synthesis reactions such as esterification (reaction between an acid and an alcohol), the transesterification (ester andalcohol), interesterification (ester and ester), and in transfer reactions acetyl group of an ester. Lipolytic enzymes are perfectly soluble in water.

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They are responsible for the hydrolysis of lipids which act on insoluble lipid substrates in water (Bénarouche et al., 2014). Lipids are formed by an aliphatic backbone, which are cyclic or polycyclic, that constitute the hydrophobic portion, which can be fixed polar groups and form the hydrophilic portion. Lipids include fats, oils, waxes and certain substances that are related (sterols, steroids, terpenes, etc).

The study of lipases has contributed to the development of the interfacial enzymology, which catalysis occurs in a heterogeneous medium at oil-water interface. The biochemical properties of these enzymes depend much on the quality of the interface and certain conventional parameters such as pH or ionic strength (Bénarouche et al., 2014). Lipases and esterases are important biocatalysts and particularly suitable for industrial applications, as they are very stable and active in organic solvents (Schmidt et al., 2004).

MATERIALS AND METHODS

Chemicals

Tributyrin (99%; puriss) and benzamidine were from Fluka (Buchs, Switzerland); tripropionin (99%, GC) was from Jansen (Pantin, France); phosphatidylcholine, sodium deoxycholic acid (NaDC), Tween 20, yeast extract and ethylene diamine tetraacetic acid (EDTA) were from Sigma Chemical (St. Louis, USA); gum arabic was from Mayaud Baker LTD (Dagenham, United Kingdom); acrylamide and electrophoresis grade were from BDH (Poole, United Kingdom); PVDF membrane was purchased from Applied Biosystems (Roissy, France); casein peptone was from Merck (Darmstadt, Germany); and pH-stat was from Metrohm (Switzerland).

Lipase activity determination

The lipase activity was measured titrimetrically at pH 8.5 and 40°C with a pH-stat under standard conditions using TC_4 (tributyrin) (0.25 ml) in 30 ml of 2.5 mM Tris–HCl pH 8.2, 2 mM $CaCl_2$, 2 mM NaDC (sodium deoxycholate) or olive oil emulsion (10 ml in 20 ml of 2.5 mM Tris–HCl pH 8.2, 2 mM $CaCl_2$, 4 mM NaDC); the olive oil emulsion was obtained by mixing (in a Waring blender) 10 ml of olive oil in 90 ml of 10% GA (gum Arabic) as substrate (Sarda and Desnuelle, 1958). Some lipase assays were performed in the presence of bile salts. Assays were carried out in 30 ml of 2.5 mM Tris–HCl buffer pH 7.0 containing 0.1 M NaCl. Standard conditions for measuring enzyme activity at increasing esters concentrations have been described previously (Stöcklein, 1993). When measuring AFL activity in the absence of $CaCl_2$, we added EDTA to the lipolytic system. Lipolytic activity was expressed as units. One unit corresponds to 1 µmol of fatty acid released per minute.

pH-stat titrimetric assay

Titration method pH-stat of the liberated fatty acids from triacylglycerol continuously stirred, and coupled to an automatic burette, recorder and connected to a circulating water bath. This technique allows for automatical titration of the fatty acids released by addition of 0.1 N sodium hydroxide, maintaining the reaction medium at a constant pH during the hydrolysis of triacylglycerol emulsion by a lipase, and permits in varying the assay conditions, including substrates and bile salts. The pH chosen is generally appropriate in the optimum pH of the enzyme studied (Taylor, 1985). The enzyme activity is expressed in International Units (1U = 1µmole of fatty acid released / min).

Determination of protein concentration

Protein concentration was determined as described by Bradford (1976) using BSA ($E^{1\%}_{1cm}$ = 6.7).

Procedure of AFL purification

1000 ml of culture medium, obtained after 72 h of cultivation, were centrifuged for 15 min at 8500 rpm and filtered to remove the cells. The supernatant containing extracellular lipase was used as the crude enzyme preparation.

Ammonium sulphate precipitation

The cell-free culture supernatant was precipitated using solid ammonium sulphate to 70% saturation. The pellet obtained after centrifugation (30 min at 8500 rpm) was dissolved in 10 ml of buffer A (20 mM sodium acetate pH 5. 4, 20 mM NaCl, and 2 mM benzamidine) and within 5 min, insoluble material was removed by centrifugation at 13,000 rpm.

Heat treatment

The supernatant obtained (10 ml) was incubated for 30 min at 70°C and in 5 min; insoluble material was removed by centrifugation at 13,000 rpm. Filtration on Sephadex G-100: The enzyme solution (10 ml) was applied to a Sephadex G-100 column (3 ×100 cm) previously equilibrated in buffer A at a rate of 30 ml/h. The fractions containing the lipase activity (eluted at a void volume) were pooled.

Cation exchange chromatography

Active fractions eluted from Sephadex G-100 column were poured into a mono-S Sepharose cation exchanger equilibrated in buffer A. The column (2 ×30 cm) was rinsed with 400 ml of the same buffer. No lipase activity was detected in the washing flow. Adsorbed material was eluted with a linear NaCl gradient (300 ml of 20 to 500 mM in buffer A) at a rate of 45 ml/h. AFL activity was eluted between 170 and 220 mM NaCl. The fractions containing the lipase activity were pooled and concentrated.

Effect of free fatty acids

The lipase activity was measured according to various substrate (TC_4, olive oil) in assigned concentrations ranging from 0-40 mM. The Michaelis-Menten (KMapp) and the maximum velocity (Vmax) for the reaction were calculated by Lineweaver-Burk (Taylor, 1985).

Effect of detergents

The lipase activity was measured using tributyrin and olive oil as a substrate in the presence of increasing concentrations of NaDC

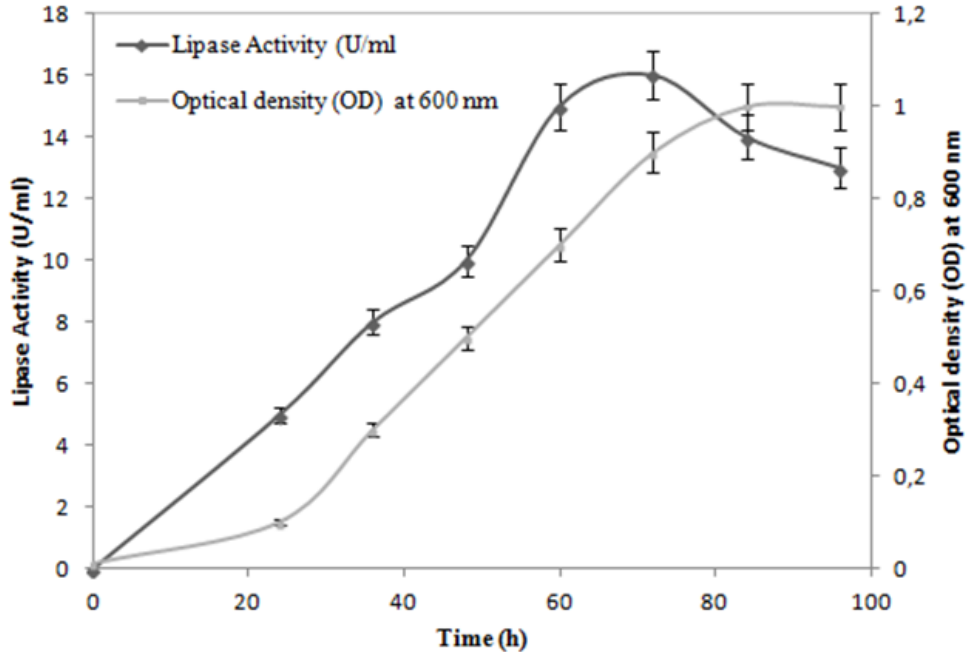


Figure 1. The time courses of lipase production. The culture was carried out at 28°C in shaking at 150 rpm. Bars correspond to standard deviation

ranging from 0 to 10 mM, under optimum conditions of pH and temperature (Taylor, 1985).

Effect of calcium

The lipase activity was measured using tributyrin and olive oil as a substrate in the presence of increasing concentrations of calcium from 0 to 6 mM and under optimum conditions of pH and temperature. In the absence of calcium, the lipase activity is measured in the presence of 10 mM EDTA or EGTA (Taylor, 1985).

3D structure prediction

The AFL structure was modelled using the 3D coordinates of the closed form of the Aspergillus lipases (AL) (PDB code 3DXL_A). The method of minimization (Gromos96) (located in the server Swiss-PDB-Viewer) was used with the version of force field GROMOS43B1. This method enabled the evaluation of minimizing energy of the three dimensional structure and the distortion compensation. The geometry quality of the model was checked using PROCHECK program (Laskowski et al., 1993).

RESULTS AND DISCUSSION

Production of lipase

The maximal production of AF lipase with inoculum size of 3×10^7 cells/ml was 16 U/mL, which was obtained by incubating 1 ml of the enzyme with olive oil emulsion (10 ml in 20 ml of 2.5 mM Tris-HCl pH 8.2, 2 mM $CaCl_2$, 4 mM NaDC) as substrate, The lipase activity was measured titrimetrically at pH 8.5 and 40°C with a pH-stat. The time course of lipase production was followed at 28°C with cell growth (Figure 1). The lipase activity was observed to start after incubation and reached the maximum (16 U/mL) at the end of the exponential phase corresponding to 72 h of cultivation.

Purification of AFL

The AFL was purified according to the procedure described. The protein elution profile which was obtained at the final step of the purification was shown in (Figure 2A).The purified lipase was homogeneous when tested by the Coomassie blue staining in SDS-PAGE (Figure 2). This figure shows that one band was revealed for AFL with a molecular mass of 55 kDa. The specific activity of the pure lipase reached 4300 U/mg using olive oil emulsified with gum Arabic as substrate at pH 8.5 and 40°C. Under the same conditions, a specific activity of 3400 U/mg was obtained when using TC4 as substrate.

Kinetic studies of AFL

Lipase hydrolysis emulsified triglycerides in the presence of bile salts. Some microbial lipases like *Rhizopus oryzae* lipase (Ben Salah et al., 1994) may lose its enzymatic activity, when TC_4 or olive oil is used as substrate in the

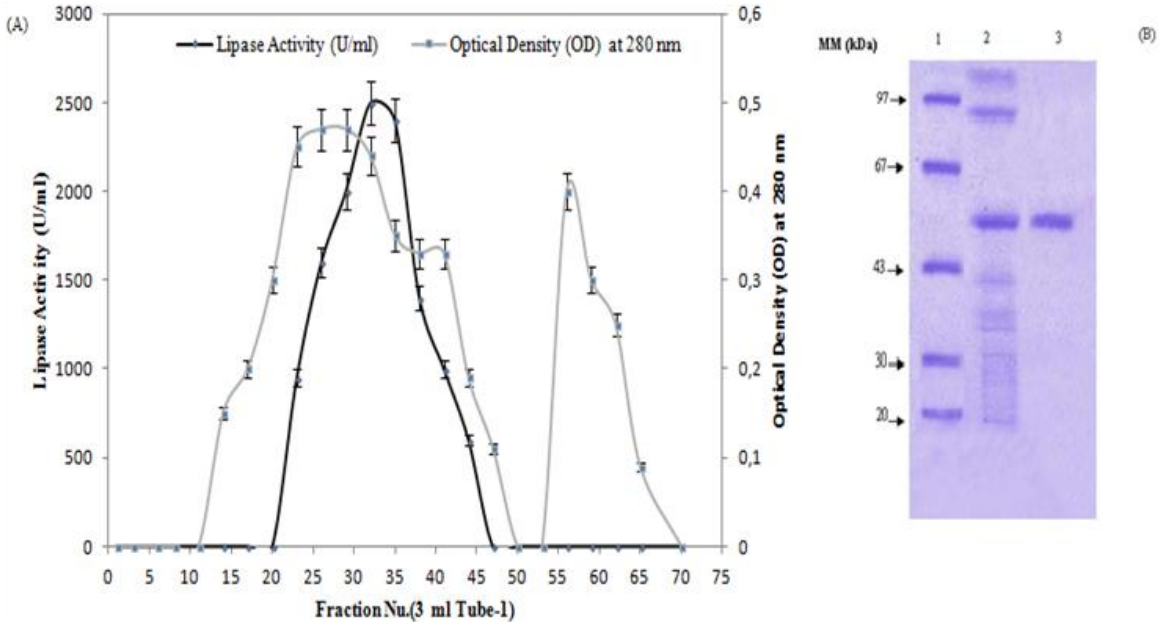


Figure 2. (A) Chromatography of AFL on Sephacryl S-200. The colomn (3 × 100 cm) was equilibrated with buffer A (20 mM sodium acetate pH 5.4, 20 mM NaCl, 1 mM benzamidine). The elution of lipase was performed with the same buffer at a rate of 26 ml/h. Lipolytic activity was measured under standard conditions at pH 8.5 and 40°C using a pH-stat. (B) SDS/PAGE (15%). Lane 1, molecular mass markers (Pharmacia); lane 2, characterisation of the AFL obtained after Mono-S chromatography chromatography; lane 3, 20 µg of purified AFL. Bars correspond to standard deviation.

absence of amphipathic reagent; the high energy existing at lipid/water interface is responsible for their irreversible denaturation. The enzyme denaturation cannot be reflected in the number of disulfide bridges, but behave very differently at interfaces. AFL was able to hydrolyze the TC_4 or the olive oil emulsion alone (Figure 3). The kinetics of substrate hydrolysis remained linear for more than 20 min, accordingly; AFL probably presented a three-dimensional structure allowing it to hydrolyse its substrate efficiently and without any denaturation at high interfacial energy. Also, it tolerated the presence of long-chain free fatty acids, at the olive oil/water interface without any addition of amphipathic reagent (NaDC, Triton X-100); a difference between Aspergillus lipase and other microbial lipases which had a strong preference for short-chain substrates (Simons et al., 1996; Laachari et al., 2013; Sayari et al., 2001).

Effect of calcium on AFL activity

Metal cations, particularly Ca^{2+}, plays important role in influencing the structure and function of lipases (Shangguan et al., 2011). The activity of lipases may depend on the presence of Ca^{2+} ions, like the staphylococcal lipases (Elkhattabi et al., 2003). The effect of various Ca^{2+} concentrations on the rate of hydrolysis of AFL was studied. A specific activity of 4300 U/mg was measured in the presence of 10 mM of chelator such as EDTA or EGTA, when using olive oil emulsion as substrate. In the absence of chelators, the specific activity of AFL reached 4900 U/mg at 2 mM $CaCl_2$ (Figure 4). The enzymatic activity of AFL was stimulated by Ca^{2+}. The lipases from *P. Glumae* and *S. hyicus* (Elkhattabi et al., 2003; Tiesinga et al., 2007) contained a Ca^{2+} binding site which was formed by two conserved aspartic acid residues near the active-site, dramatically enhanced the activities of these enzymes.

Effect of detergents

To verify whether AFL was capable to hydrolyze triglycerides in the presence of surfactants such as bile salts, we measured the rate of hydrolysis of TC_4 and emulsified olive oil in the presence of various NaDC concentrations, from the results as shown in Figure 5, we notice that the NaDC has no inhibitory effect on activity of lipolytic enzymes even at a high concentration (10 mM). This result confirmed that unlike many lipases, such as *Staphylococcus xylosus* lipase, the maximal activity was reached in presence of 2 mM of NaDC using tributyrin as substrate. In presence of 4mM of NaDC, only 40% of residual activity was detected (Mosbah, et al., 2005). AFL

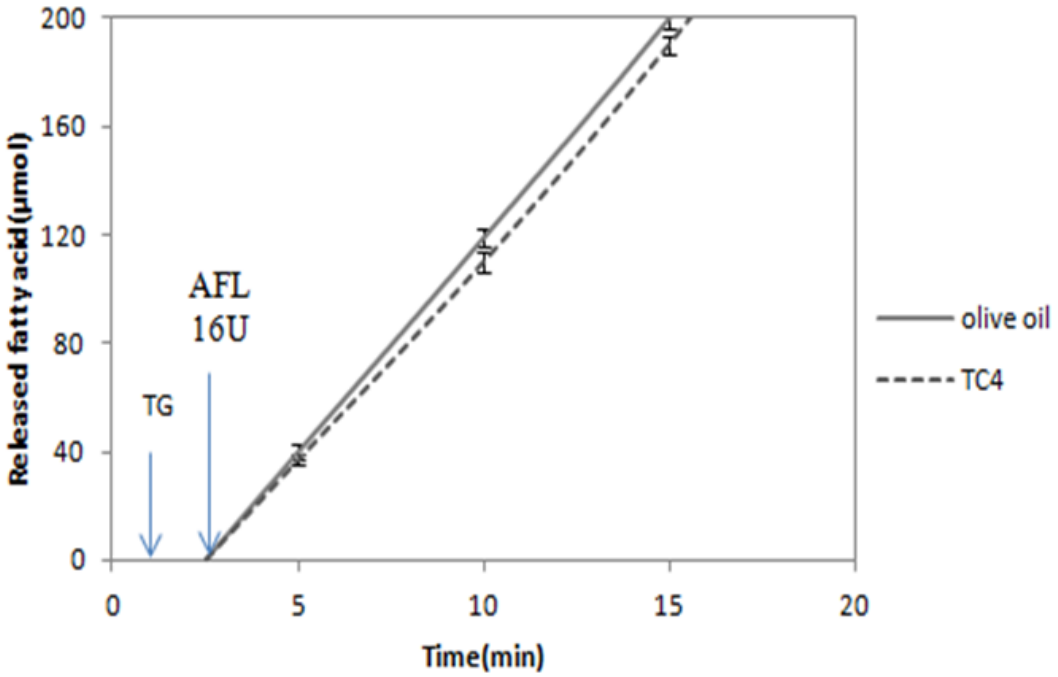


Figure 3. Kinetic of hydrolysis of olive oil emulsions or tributyrin by AFL (16 U). Lipolytic activity was followed at pH 8.5 and 40°C in the absence of bile salts under standard conditions at pH 8.5 and 40°C using a pH-stat.

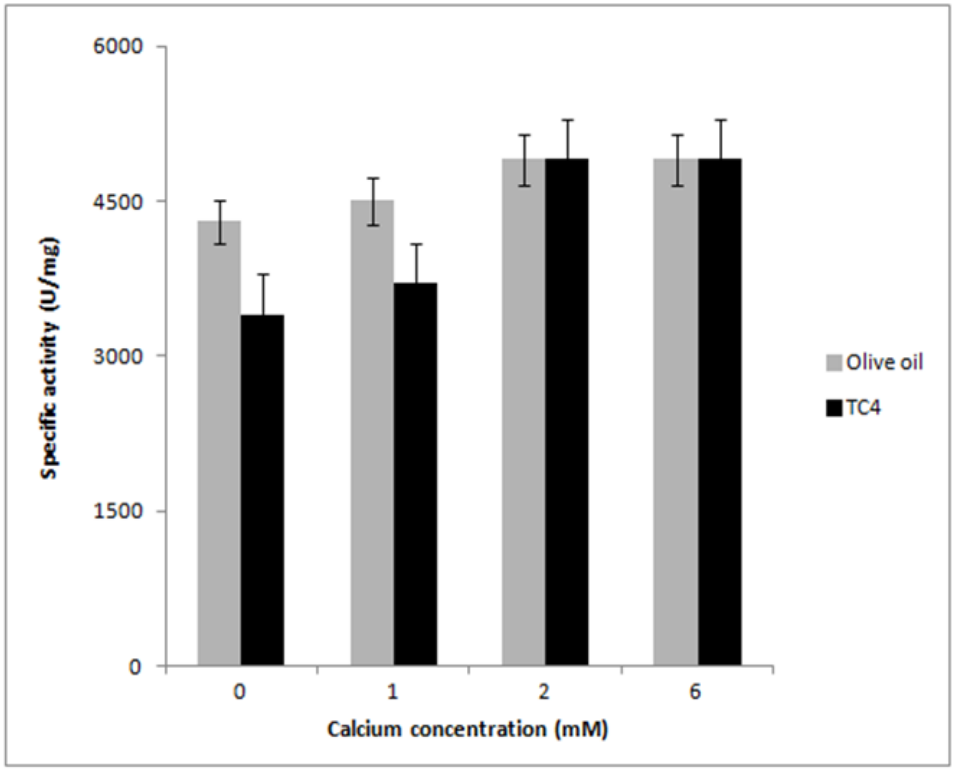


Figure 4. Effect of increasing concentrations of calcium on the rate of hydrolysis of tributyrin and olive oil emulsion by AFL measured in the presence of 10 mM EDTA or EGTA under standard conditions at pH 8.5 and 40°C using a pH-stat.

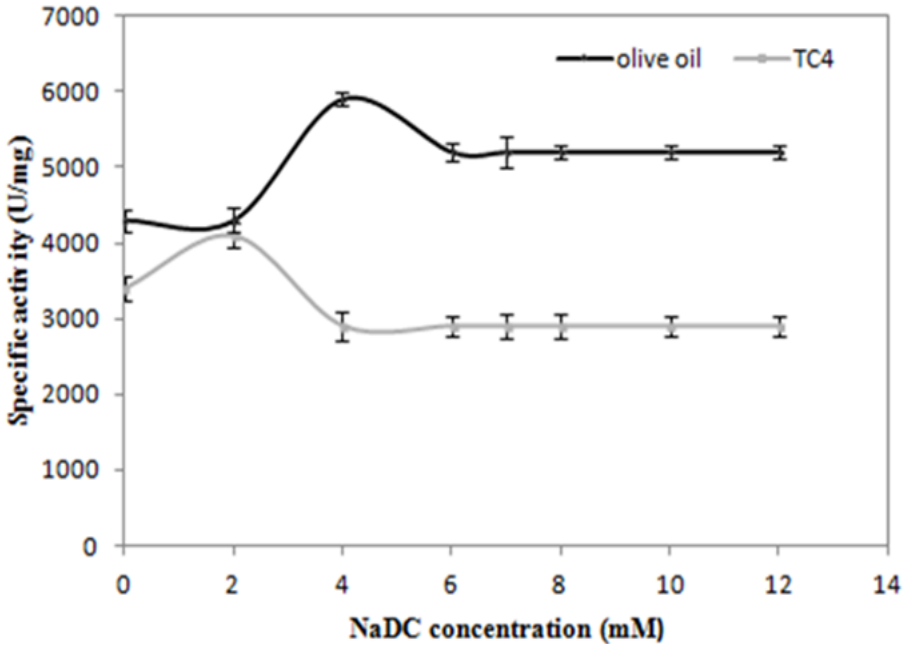
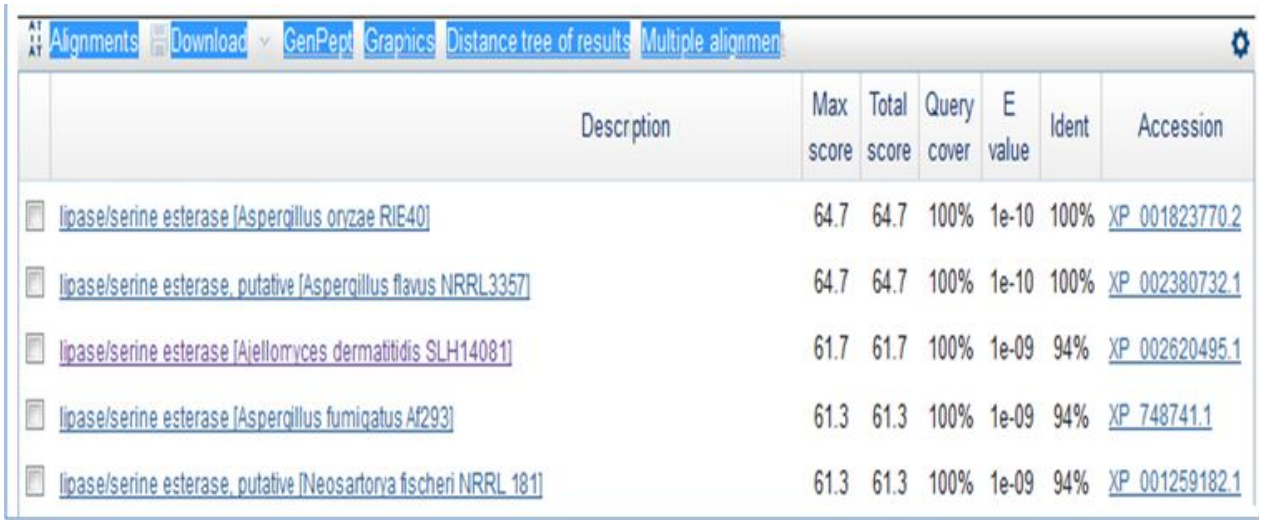


Figure 5. Effect of increasing concentration of NaDC on the rate of hydrolysis of tributyrin and olive oil emulsion by AFL. Lipolytic activity was measured under standard conditions at pH 8.5 and 40°C using a pH-stat.



Description	Max score	Total score	Query cover	E value	Ident	Accession
lipase/serine esterase [Aspergillus oryzae RIE40]	64.7	64.7	100%	1e-10	100%	XP_001823770.2
lipase/serine esterase, putative [Aspergillus flavus NRRL3357]	64.7	64.7	100%	1e-10	100%	XP_002380732.1
lipase/serine esterase [Ajellomyces dermatitidis SLH14081]	61.7	61.7	100%	1e-09	94%	XP_002620495.1
lipase/serine esterase [Aspergillus fumigatus Af293]	61.3	61.3	100%	1e-09	94%	XP_748741.1
lipase/serine esterase, putative [Neosartorya fischeri NRRL 181]	61.3	61.3	100%	1e-09	94%	XP_001259182.1

Figure 6. Alignment of the amino acid sequence of AFL using the program Blast.

was able to reach its substrate even in the presence of certain active agents surface, such as bile salts. Similar results were obtained with SSL (lipase from *Staphylococcus simulans*) (Sayari et al., 2001). Similarly, SSL was not inhibited by anionic detergents such as NaDC (Simons et al., 1997). Therefore, it can be inferred that probably AFL had a higher penetrating power than those of other microbial lipases that enabled it to hydrolyze the olive oil or TC_4 in the presence of bile salts.

3D structure model of AFL

The research of homologous with the AFL sequence was made in the database using the BLASTp (Basic Local Alignment Search Tool protein) program by multiple alignment and the results are shown in Figure 6. Sequence analysis allowed us to reveal a 100% homology with *A. flavus* NRRL3357 (33.5 kDa), *A. oryzae* RIB40 and *Ajellomyces dermatitidis* SLH14081. In order

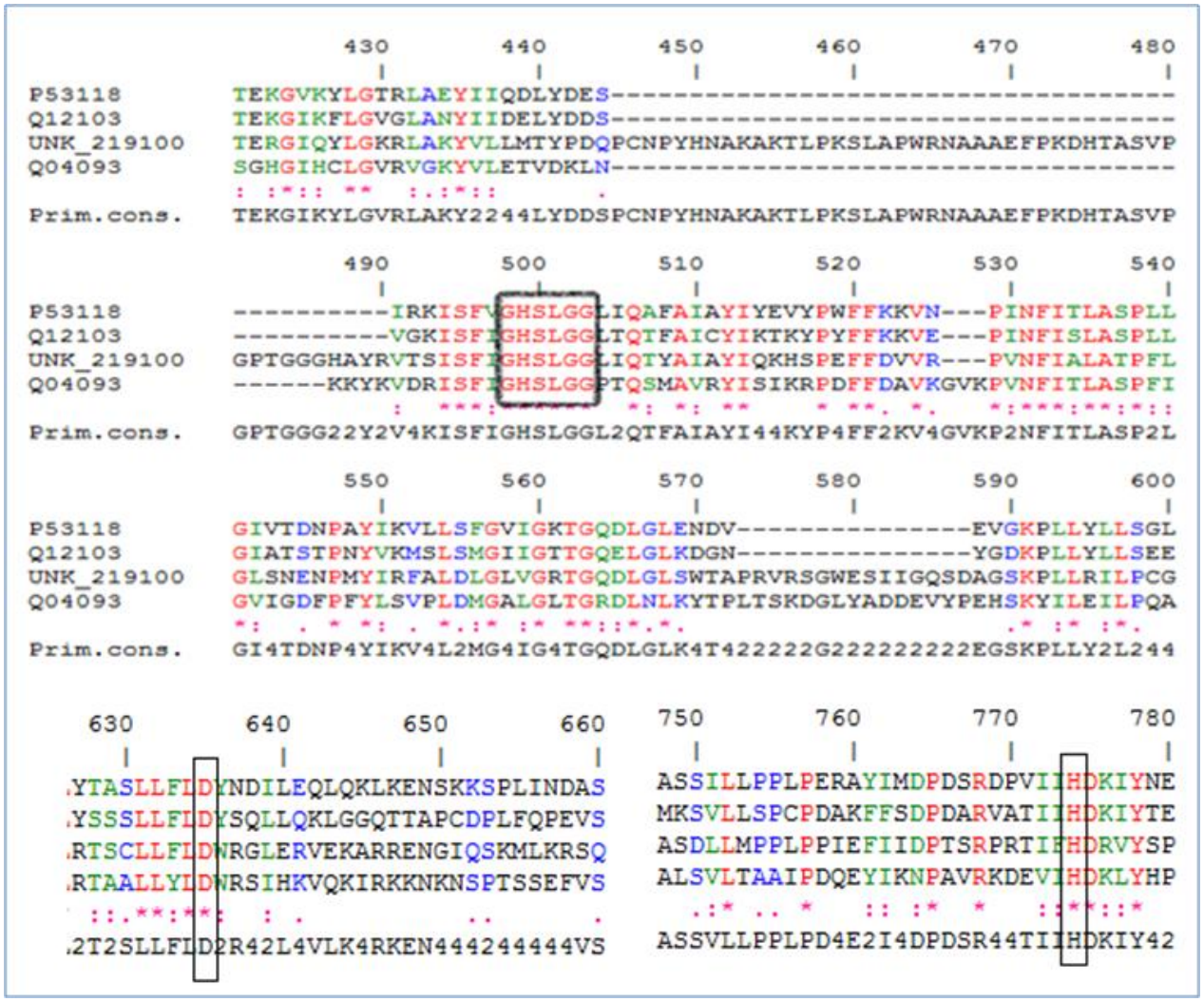


Figure 7. The multiple sequence alignment of AFL (UNK_219100) with lipase sequences of Aspergillus genus: chain (P53118), chain (Q12103) and chain (Q04093) with the additional sequence (cons prim). The catalytic triad is framed and the catalytic serine500 is present in the conserved pentapeptide G-X-S-X-G, Asp635 and His774.

to analyze sequences in databases NPSA (network protein sequence analysis) that allowed the comparison of protein sequences, a sequence alignment of the N-terminal portion of AFL was completed. From Figure 7, we observe that the catalytic triad of AFL constituted Ser500, Asp635 and His774. The three amino acids were located on the C-terminal side of β core lamina. We also noticed that the Ser500 was a part of the pentapeptide Gly-X-Ser-X-Gly and located at the apex of the nucleophilic elbow between strand β5 and helix α5. The alignment of 3 sequences structures with the reference sequence allowed us to identify the location of the catalytic triad of AFL (Figure 7). Indeed, the catalytic triad (Ser-His-Asp) was a characteristic structure of a well known serine proteases (Brumlik and Buckley, 1996). It was also observed in the catalytic sites in several lipases with a carboxylate residue of aspartic acid or glutamic acid. These amino acids were generally determined by chemical modification studies (Ruiz et al., 2007) or by site directed mutagenesis (Hyun-Ju et al., 2000). The model of the *Bacillus stearothermophilus* lipase P1 constructed by the use of the basis of secondary structure predictions, showed an organization in fold and this enzyme α/β-hydrolase was identified by the catalytic triad of Ser-113, Asp-317 and His-358, in close proximity to each other which played a key role in the catalytic mechanism (Sinchaikul et al., 2001).

In order to create a sample of the closed form of the *A. flavus* lipase, we used the automatic modeling by the Swiss-Model server (http://www.expasy.org/spdvb). The model of AFL was a subject of several cycles of energy minimization using Gromos96 installed on the server

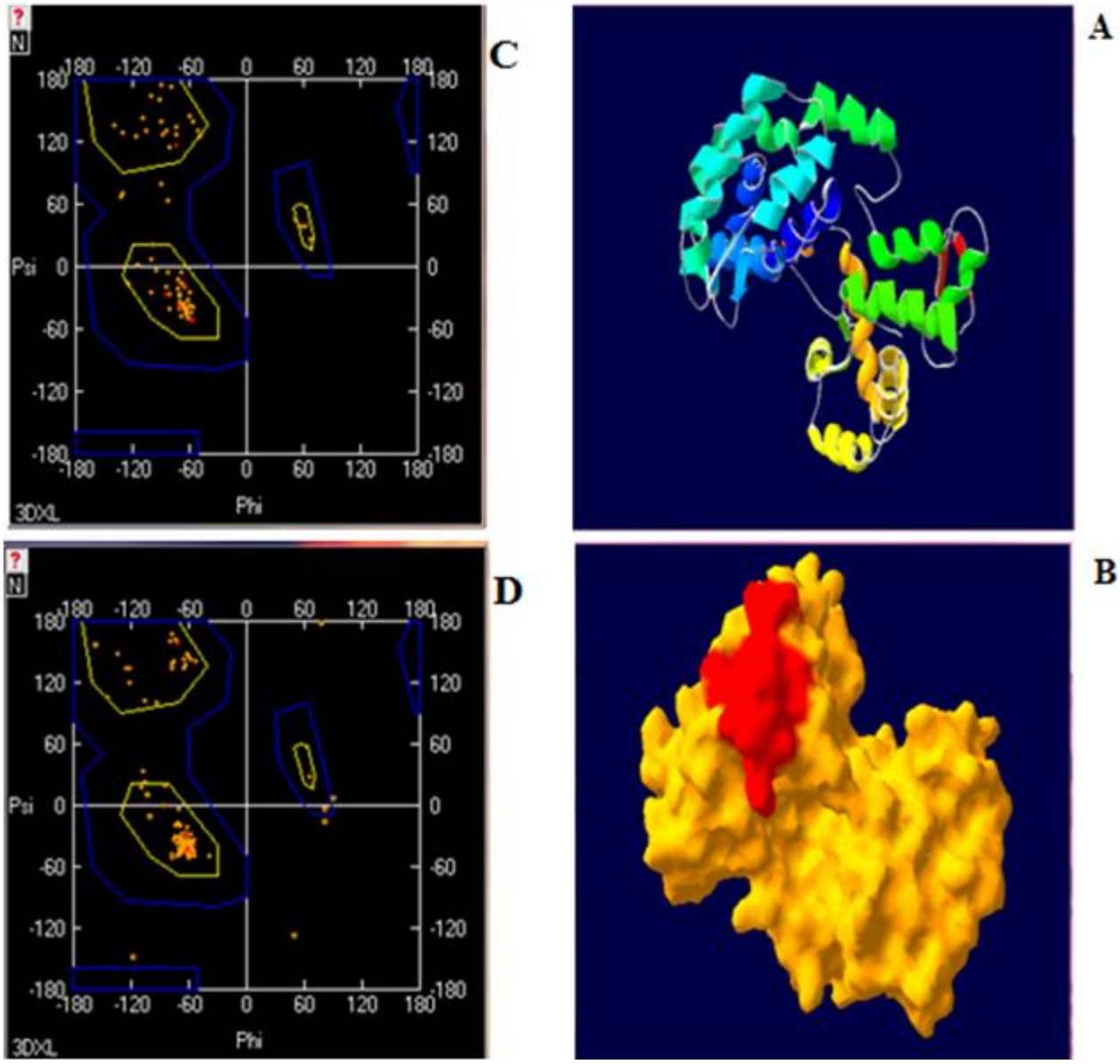


Figure 8. Modeling of a closed model of the AFL (A) and the surface structure (B). The catalytic triad is colored in red (B). Hydrophobic amino acids, belonging to the polar N-terminal domain are located in the Ramachandran plot (C). The hydrophobic residues of the AFL involved in increasing the accessible surface interacting with an interface is indicated (D).

Swiss PDB. The superposition of the two closed structures gave an average standard deviation (rmsd) equal to 1.46 Å. The stereochemical quality of the closed model of AFL and statistical analysis of the distribution of amino acids in the Ramachandran plot were also tested by the program PROCHECK (Laskowski et al., 1993). According to the Ramachandran plot (Figure 8), we found that 99 and 97% of non-polar and polar amino acids respectively, were located in suitable areas (Figure 8).

The crystal structures of *Bacillus sp*. H257 lipase in its free form at 1.2 was found complexed with phenylmethyl-sulfonyl fluoride at 1.8 Å. The catalytic residues were buried at the bottom of a long chain ~ 22 Å, from the surface to the active site, the bottom of the binding pocket of the substrate had more polar character with contributions from catalytic residue Ser97, His226, Met98 and Phe29, built the oxyanion hole, which stabilized the tetrahedral intermediate formed during the hydrolysis reaction (Rengachari et al., 2012; Laachari et al., 2014). Furthermore, the study of the total surface accessible(N-terminal domains) of the closed form of AFL, showed that the ratio hydrophobic surface/hydrophilic surface was

51% versus 50%. This could explain the fact that AFL tolerates accumulation of long chain fatty acids in the lipid/water interface and had a linear kinetics over 15 min, during the hydrolysis of an emulsion of oil olive.

The structure of the lipase from *A. niger* was composed of a core domain (residues 1-82 and 97-269), showing the typical characteristics of the α/β hydrolase and a lid domain (residues 83-96), with a simple pattern of loop-helix-loop. The orientations and the positions of residues of the catalytic triad (Ser145-Asp198-His260) followed the movement of the lid (Liu et al., 2013). These observations allowed us to suggest that the structure of the lipase from *A. flavus* conformed to all the characteristics of the lipases in the open conformation.

Conclusion

The interest of microbial lipases in biotechnological applications has taken a meteoric rise in recent years. Therefore, the industry requires new enzymes, which meet the criteria of use, particularly in terms of thermostability. The *A. flavus* lipase kept their stability even in the presence of high concentrations of detergent (NaDC). Calcium salts showed a stimulating action of the lipase activity. Molecular modeling of the 3D structure was also carried out and the results have allowed us to demonstrate that AFL tolerates the accumulation of long chain fatty acids in the lipid / water interface. These results show that AFL has biochemical properties attractive for various industrial applications.

Conflict of interests

The authors did not declare any conflict of interest.

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