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THE PREDICTION OF MUTAGENS AND/OR CARCINOGENS IN THE AQUATIC ENVIRONMENT OF IRAQ

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ABSTRACT

The aquatic environment in Iraq is exposed to a variety of chemical mutagens and/or carcinogens. The main input sources of these agents are related to the three wars during the last three decades. Some of these pollutants may enter the human body through the food chain and drinking water creating serious health hazards. Therefore, monitoring the aquatic environment for such pollutants is very important for future plans in determination of the input sources of these agents and their subsequent prevention.

Three procedures have been developed for such purposes:

First: performing analysis on water samples in order to identify every chemical constituent within them which can then be individually tested for its genetic toxicity. This may be a formidable task because of the very large number of chemical and biological agents.

Second: collecting water samples and concentrating their chemical constituents, then expose the resulted concentrates to a range of biological systems. This approach has two fundamental limitations, the developing a suitable concentration and perhaps more importantly, the difficulty of detecting mutagens in the presence of toxic chemicals.

Third: to reduce the masking effects of toxic chemicals, some aquatic organisms can be used as bio-indicators for detection of the potentially hazardous chemical agents. The technical procedure for the last approach will be discussed where bacteria, yeast and tissue culture can be used. It has been approved that such approach is fast, cheap and reliable in the preliminary assessment for water quality and the determination of the most health hazard pollutants in the aquatic environment.

INTRODUCTION

It is quite clear that the kinds and quantities of the carcinogenic and/or genotoxic materials in the Iraqi environment after the wars are numerous. A quick look to the United Nation Environmental Protection (UNEP) Desk Study (2000) on the Iraqi Environment before the last war may shows how serious is the impact of such pollutants on the human health in Iraq. Therefore, a rapid screening and identification procedures for these hazardous agent are urgently required. That is regardless of the possible argument on the strengths and weaknesses of different short-term tests when compared to the long-term carcinogenicity tests on animals.

A well conducted long-term carcinogenicity study is usually so slow and so expensive that the results will be regarded as definitive, may be because the test would never be repeated. Unfortunately, when they are repeated they do not always give the same result. However, different short-term tests may give different results, and, the more tests that are used, the more likely is that one of them will give a positive result. In this case, there will some sort of confusion which represent a more realistic picture of

carcinogenic risk than the arbitrary simplicity of results from a single test in the long-term assays. Therefore, there will be a little doubt about their impact on the whole field of prediction of carcinogenicity (Green, 1980)

Many scientists are highly convinced that short-term tests must be used quantitatively rather than qualitatively for the prediction of human genetic or carcinogenic risk (Green, 1980; Ames and Hooper, 1978). This is related first to the large number of compounds for which some evidence of possible carcinogenicity exists. And secondly, short-term tests such as Ames test are sensitive and give reasonably low frequency of both 'false' positives and negatives. In addition to that the addition of the liver enzyme fraction (S9) to the *in vitro* tests will bring the gab between the *in vitro* and *in vivo* close to each other.

So that a good effect of short-term tests assays is that they encourage a critical screening of carcinogenicity data. However, there are some difficulties in classifying the short-term bioassay as negative. Nevertheless, a variety of arbitrary criteria have been proposed to validate the short-term bioassys: for instance, a two fold increase in the number of mutant colonies on the treated plates, or a statistically significant increase, a dose-related increase in the number of the mutants in Ames test.

Kinds of Short-Term Bioassays:

In this paper, the most currently used short-term bioassays for induction of DNA base-changes will be described. These are almost fast, cheap and reliable. Hence, they can be used in the rapid screening for environmental pollutants in water air and soil.

Detection of DNA Base-Changes:

This assay system is quite necessary for the preparation of a data-base to be used for assessment of the level of the indigenous pollution. It will be implemented for detecting a wide variety of chemical pollutants and predicting their possible impacts on the aquatic organisms such as fish, phytoplankton and zooplankton as will as human beings who will be exposed to them directly or indirectly through the consumption of these organisms. However the actual evaluation of any possible risk of human health may needs further research. Since the health hazard assessment of studies is usually time-consuming and of high expense, the short-term bioassays will be of considerable importance.

The systems used in the measurement of induced point mutation after chemical exposure are generally based upon the quantification of the frequencies of specific genotypes detectable in the presence of various selective agents (Venitt and Parry,1984). These selective systems include resistance to the effects of toxic drugs as is widely used in mammalian cell culture systems (Cole and Arlett,1984)and growth of prototrophic cells in auxotrophic cell populations, such as those used in Salmonella / mammalian microsome system (Venitt etal.,1984).

These selective systems have provided us with methods for the *in vitro* measurement of mutant frequencies in bacteria and cultured cells. *In vivo,* the availability of point mutational assays is more limited and at present confined to methods such as the mouse spot test (Fahring, 1975). More recently, assays based upon the use of

transgenic animals carrying selective genetic markers particularly those of bacterial origin are in use (Gossen and Vijg, 1990). In terms of environmental monitoring, the fundamental problem with all the above assays is that they are based upon the use of specialised laboratory methodologies that are not applicable to species in the natural environment.

Therefore, a new methodology has been developed by Prof J.M. Parry and his colleagues at the School of Biological sciences, University of Wales, Swansea, U.K; which can be, theoretically, applied to the study of DNA base changes in any gene of any species for which DNA sequence information is available. This methodology is based upon the measurement of base changes which occur in the DNA sequences which code for recognition sites for bacterial restriction enzymes (Parry, et al.,1990 ; Zijlstra et al.,1990).

The Restriction Site Mutation Assay(RSM):

As part of their work on the development of methodologies for detecting genetic damage and genetic changes in aquatic species, they were utilizing the methodology which was termed "The Restriction Site Mutation Assay (RSM)" to study the effects of geno-toxin exposure to the **α** globulin gene XEL HBA1 of *Xenopus laevis* (Partngton and Baralle, 1981).

Briefly, this technique involves polymerase chain reaction (PCR) by alternating cycles of polimerisation and denaturation always the amplification of DNA sequences between two unique reaction primers (amplimers). For the XEL HBA1 gene the utilized region is between base 20 and base 203 to amplify a DNA fragment suitable for mutational analysis. Using matched base primers
5'-ATATTGTCTGAATGAATGAATG-3' and
3'-AGATGTCCTAGAAGTATCAGT 5' a region of 204 bases by 30-35 cycles of incubation with substrates and Taq polymerase in a temperature cycler can be amplified. The resulting amplified product can then be run on a polyacrylamide gel and can readily be identified as a characteristic band.

The basic principle of RSM methodology is the DNA extracted from an organism exposed to a genotoxin which may contain a sequence changes leading to a wild type restriction site or may contain a sequences changes leading to a mutant restriction site. DNA containing wild type sequences, for example, 5'-CTAG-3' is cut by the restriction enzyme Male 1, where as DNA containing a mutant such as C to T transition at the first base of the sequence, i.e. to 5'-TTAG-3' will be resistant to the cutting action of Mac-1. For more details see (Jones and Parry, 1992).

Thus after treatment of DNA with restriction enzyme under optimal condition all wild type sequences are cut where as the 204 base region under study containing a restriction enzyme resistant site will remain intact. The restriction enzyme resistance sequence can then be amplified by PCR treatment to produce a sample of mutant DNA. Thus the basis of the assay is the selective amplification of mutant sequences.

Polymeras Inhibition assay (PI assay):

This technique has been developed by Jenkins et al.(2000) for a rapid screening of a large number of samples with very low concentration of genotoxic compounds; the polymeras inhibition has been shown that it is capable of detecting DNA damaging agents of biological relevance, i.e. know human carcinogens. PI assay is based upon the inhibition of DNA polymerases (including those used in the polymerase chain reaction PCR), in countering damaged DNA bases. Hence DNA-damaging agents can be identified by corresponding reduction in PCR amplification after exposure.

The PI assay exploits the well-reported fact that DNA damage blocks DNA synthesis both in vitro and in vivo (Moore and Strauss, 1979; Villani, etal, 1978). This DNA damage can take the form of DNA adducts or a basic sites and/or strand breaks.

In practice, templates containing DNA damage are known to be poor substrate for the polymerase chain reaction (PCR) (Govan et al. 1990, Jennerwein and Eastman, 1991) whereas non adducted template can be readily amplified. Hence the presence of DNA damage in target DNA sequences can be deduced from the accompanying reduction in PCR amplification. The use of PCR allows us to harness the exponential amplification of this technique to distinguish between undamaged and damaged DNA templates (Mc Carthy et al., 1996).

CONCLUSSION

These are examples of the most widely used techniques for the prediction of environmental carcinogens. However, the classic bioassays may also be implemented as they are also valid for such purpose. These include the detection of prototrophic mutants in auxotrophic cell population. For example, Salmonella/manmalin microsomal (Ames et al., 1973) yeast (Parry and Al-Mossawi, 1979) or Neurospora (Bridges, 1972).

These procedures deal with such huge amount of chemical pollutants, many of which may be carcinogenic, it is essential to chose a quick, cheap and reliable procedure. By these techniques we can assess the water supply resources especially drinking water resources for the presence of the mutagenic/ carcinogenic pollutants. Medicines, food, air quality and even the industrial environment can also be assessed. However, these assays will play an important role in chemical identification of the most known carcinogens. In this case, they chemical analysis and the biological assay will go side by side and the later will be used as a guide in determination of the most active carcinogene.

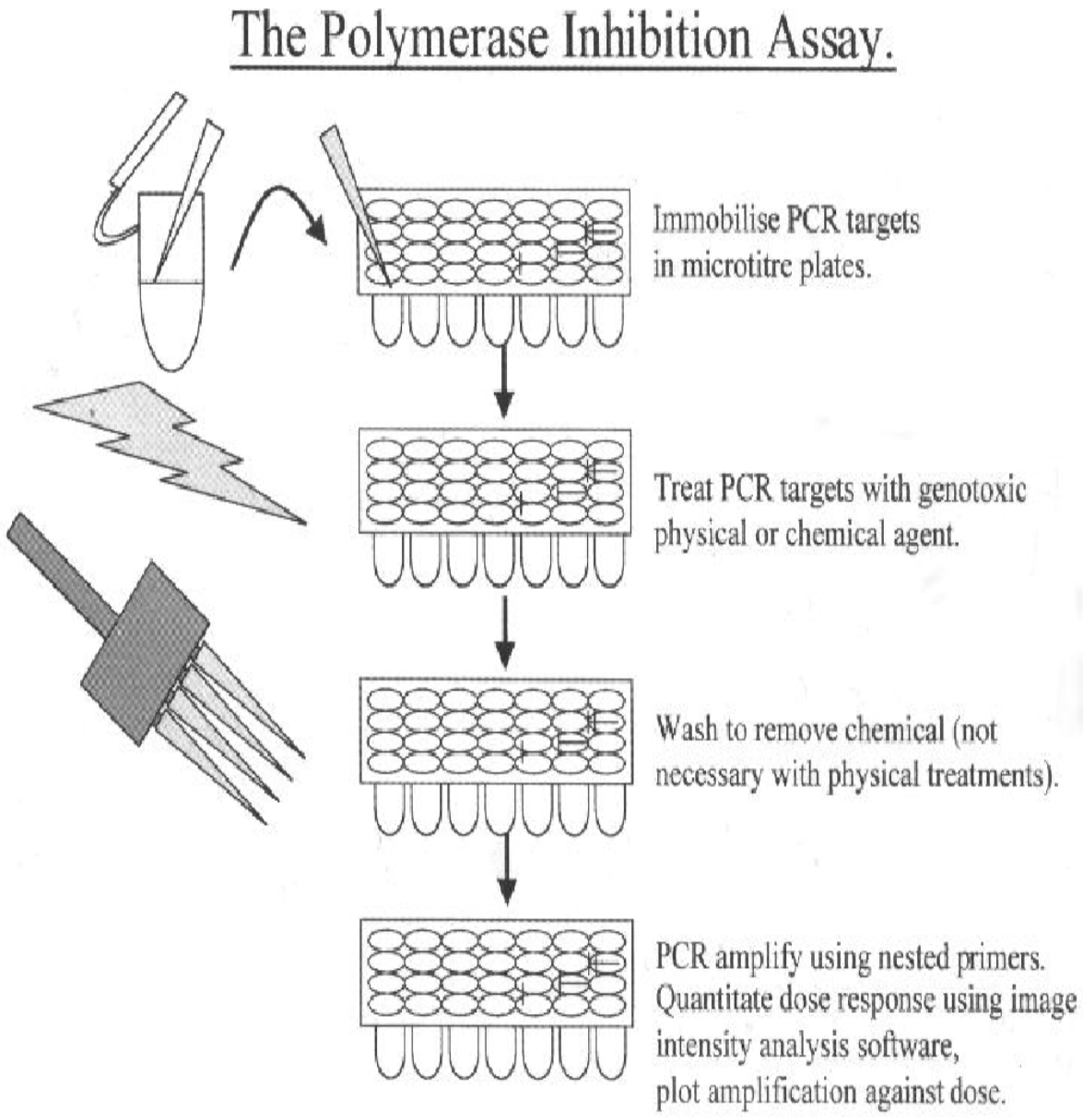

Figure 1. Schematic representation of the methodology involved in the PI assay.

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التبؤ بوجود المواد المطفرة / المسرطنة في البيئة المائية العراقية

محسن عبد جبار / كلية الطب / جامعة كربلاء – العراق

تتضمن البيئة المائية في العراق من انواع مختلفة من المواد المسببة للطفرات الوراثية او المسببة للسرطان . ومصدر تلك المواد هي الحروب التي مر بها العراق خلال العقود الثلاثة الاخيرة من القرن العشرين ويمكن لتلك المواد ان تدخل جسم الانسان مع المواد الغذائية او مياه الشرب مسببة خطراً جسيماً على الصحة . ولذلك فان تحري مصادر تلك الملوثات ضروري جداً للتخطيط العام لمنع إنتشار تلك المواد في البيئة المائية.

وهناك ثلاث وسائل لتحقيق ذلك الهدف :-

اولاً : تحليل العناصر الكيمياوية الموجودة في الماء ومحاولة التعرف على تأثير التطفير على كل منها وبالنسبة للعدد الكبير لمكونات الماء الكيمياوية فان هذه المهمة غير ممكنة .

ثانياً : تركيز المكونات الكيمياوية للماء وتعريض الكائنات الحية الموجودة في نظام الفحص البيولوجي الى ذلك الكون المركز الكيمياوي . وفي ذلك محددان رئيسيان يمنعان من متابعة الفحص بشكل صحيح هما :-

صعوبة تهيئة التركيز المناسب والأهم منه صعوبة تحري المواد المسببة للطفرات الوراثية بوجود المواد الكيميائية السامة .

ثالثاً : استعمال الأحياء المائية للكشف عن المواد المسببة للطفرات والتي يمكن ان يكون مسرطنة وفيها هذا الاسلوب يمكن ان يختصر تأثير المواد السامة إضافة الى احتمال وضوح الآثار الجانبية السمية على انسجة تلك الاحياء وثبت ان هذه الطريقة سهلة وفعالة ويمكن الاعتماد عليها في تقييم الأثر البيئي للملوثات المختلفة خصوصاً تلك التي تسبب الطفرات الوراثية او المسرطنة والمنتشرة في البيئة المائية .

PROCEEDING OF THE SECOND SCIENTFIC ENVIRONMENTAL CONFFER, 2007, ZAGAZIG UNI., 125-141

PALEOENVIRONMENTS OF WADI EL RAYAN EOCENE, SOUTHWEST FAYOUM, EGYPT BY USING COMMUNITY FAUNAL ANALYSES

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ABSTRACT

The geology of Wadi EL-Rayan area attracted the attention of many scientists especially the geologists since the nineteenth century till the present days; particularly it represents the most interested site of vertebrate fossils in Egypt**.** The present work studies the paleoenvironmental parameters controlled the deposition of the Eocene rock units depending on the analyses of the faunal community prevailed in the studied area. Three locations were selected to cover Wadi El-Rayan area stratigraphicaly and geographically. More than sixty macro-faunal (vertebrate and invertebrate) species have been collected and identified dealing with detecting the comprehensive prevailed conditions.

The faunal content of Wadi El-Rayan Formation as well as its lithologies resolute the alternation between two transgressive sedimentary cycles enclosing a regressive phase in-between. The prevalence of the molluskan shells in the Gehannam Formation reveals that they had deposited on a shallow shelf occupied a middle part occurred during an interval of low sea stand that flooded by vertebrate fossil fragments. The upper part of Gehannam Formation and the lower part of Birket Qaroun Formation are rich in Celesite that reflects unusual restricted oceanographic conditions where partial evaporation of the seawater concentrated calcium and strontium sulfates. The lithologies of Birket Qaroun Formation as well as the lack of fossils throughout point to the presence of a barrier bar that emphasizes the idea of a low sea stand which only preserved in the stratigraphic record when buried during subsequent transgression. The recognized lithologies of Qasr El Sagha Formation associated with the characteristic fauna suggest deposition in distinct environments dominated by shallow restricted lagoon environment.

INTRODUCTION

Wadi El-Rayan Depression is located southwest of the Fayoum Town representing the famous low landforms in the eastern side of the Western Desert**.** It is defined by latitudes 29 00' 00" - 29 24' 11 " N and longitudes 30 00' 00" - 30' 34' 00" E.

The geology of Wadi EL-Rayan area attracted the attention of many scientists especially the geologists since the nineteenth century till the present days, especially it represents the most interested site of vertebrate fossils in Egypt. The earlier studies of the geology of the Fayum Depression were carried out by Schweinfurth (1886), Mayer Eymar (1892), Beadnell (1901, 19o5), Dart (1923), Cuvillier (1930, 33), Iskander (1943), Ansary (1955). Regarding the studies which treated the paleontology and biostratigraphy of the Fayum Depression area, those of Abdel-Kareem (1971), Ismail and Abdel-Kareem (1971), Abdou and Abdel-Kareem (1972), Strougo (1977a, b, 1992), Shama (1981), Bassiouni et al. (1984), Stourugo and Haggag (1984), Haggag (1985, 1990, 1992), Samir (1986), Bassiouni

et al. (1987), Bown and Kraus (1988), Abdel Ghany (1990), Allam et al., (1991), Boukhary et al., (1993), Anan (1994), Haggag and Bolli (1995, 1996), Abdallah et. al. (1997), El Bedewy et al. (1998), Omar (1999), Helal (2002), and Uhen (2004) are worthy to mention.

In 1992 Gingrech reviewed the previous geologic studies on the Middle Eocene to Early Oligocene rocks exposed in Wadi El Rayan area, using the measurement of Beadnell 1905, and affirmed that, Wadi EL-Rayan presents four geological formations:

1- The lowest (oldest) is the Wadi EL-Rayan Formation; it consists of compact limestone forming the uppermost members of Rayan series; the upper beds of that limestone, contains among other fossils numerous examples of the large *Nummulites gizahensis* (Beadnell 1905). Iskander (1943) divided this limestone into Muelah, Midawara, Sath El-Hadid and Gharq formations.

2- Gehannam Formation: it dates back to about 40-41 million years ago, and consists of white marly limestone and gypseous clays. This formation yields many skeletons of *Basilosaurus isis* and *Zeuglodon osiris*, beside plenty of the foraminifers *Nummulites frassi*, *N. beaumenti*, and macro invertebrate *Vusella crispate* and *Lucina fajumoni*.

3- Birket Qarun Formation: It consists of sandstones, clays and compact limestone, which, is almost invariably weathered giving rise to peculiar shape of rock. This unit yields also remains of the Eocene whales skeletons

4- The highest (younger) unit is the Qasr EL-Sagha Formation of Late Eocene age: it yields *Turritella carinifera*, *Nicaisolopha clot-beyi*, *Pycnodonte gigantica*, *Turritella pharaonica* and *Ostrea elegans*. This formation consists of limestone reflecting a shallow marine environment that suggests different environment than in northern Fayoum Depression where it exhibits fluvial marine environment.

The present work studies the paleoenvironmental parameters and stratigraphy of the Eocene rock units exposed in Wadi El-Rayan area. More than sixty macrofaunal (vertebrate and invertebrate) species have been collected, identified and used in detecting the comprehensive paleoenvironmental conditions that prevailed during the Eocene times in the studied area.

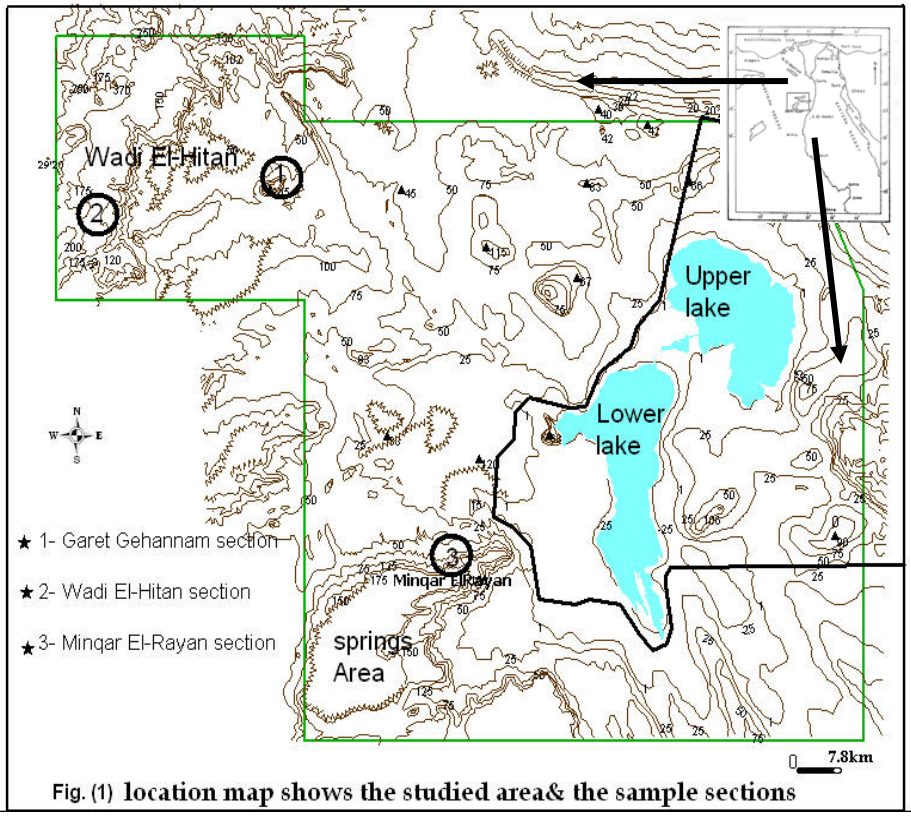

Fig. (1) **location map shows the studied area& the sample sections**

MATERIAL AND METHODS

Three locations were selected to cover the Eocene sediments exposed in Wadi El-Rayan area stratigraphically and geographically. At these locations, three stratigraphic sections have been selected and sampled dealing with both lithological and macropaleontolgoical investigations. These sections released the sedimentary facies of different Eocene strata. Locations and lithologies of these sections are shown in figures (1, 2, 3 and 4).

1 - **Minqar El Rayan escarpment**, it is located in the area of Rayan Springs at the south east of Garet Gehannam, the studied section lies at Lat. 29° 6' 22" N, and Long. 30° 17' 35" E.

2 - **Garet Gehannam escarpment** represents a prominent and well-known high land on the side road of Wadi EL-Hitan area. It is situated at the northwestern part of Fayoum Depression, the examined section was selected at Lat. 29° 19' 2" N and Long. 30° 9' 14 " E.

3 - **Wadi EL-Hitan** or Zeuglodon Valley, is located 12 Km west southwest of Garet Gehannam escarpment. The valley is characterized by many isolated hills, hillocks of peculiar shape sculptured by the weathering action of the wind and rains, earth pillars are found in many places. The studied section in Wadi El-Hitan is exposed at Lat. 29° 17' 50" N and Long. 30° 02' 16" E.

The fieldwork included measuring of the fore mentioned stratigraphic sections and collecting representative samples of the rocks and the available invertebrate macrofossils as can as possible. About 220-rock samples were collected at every change in the lithology associated with recording any filed observations. More than 500 fossil specimens were collected and studied in detail. The laboratory investigation included the determination of carbonate-sand-mud composition of all the studied samples.

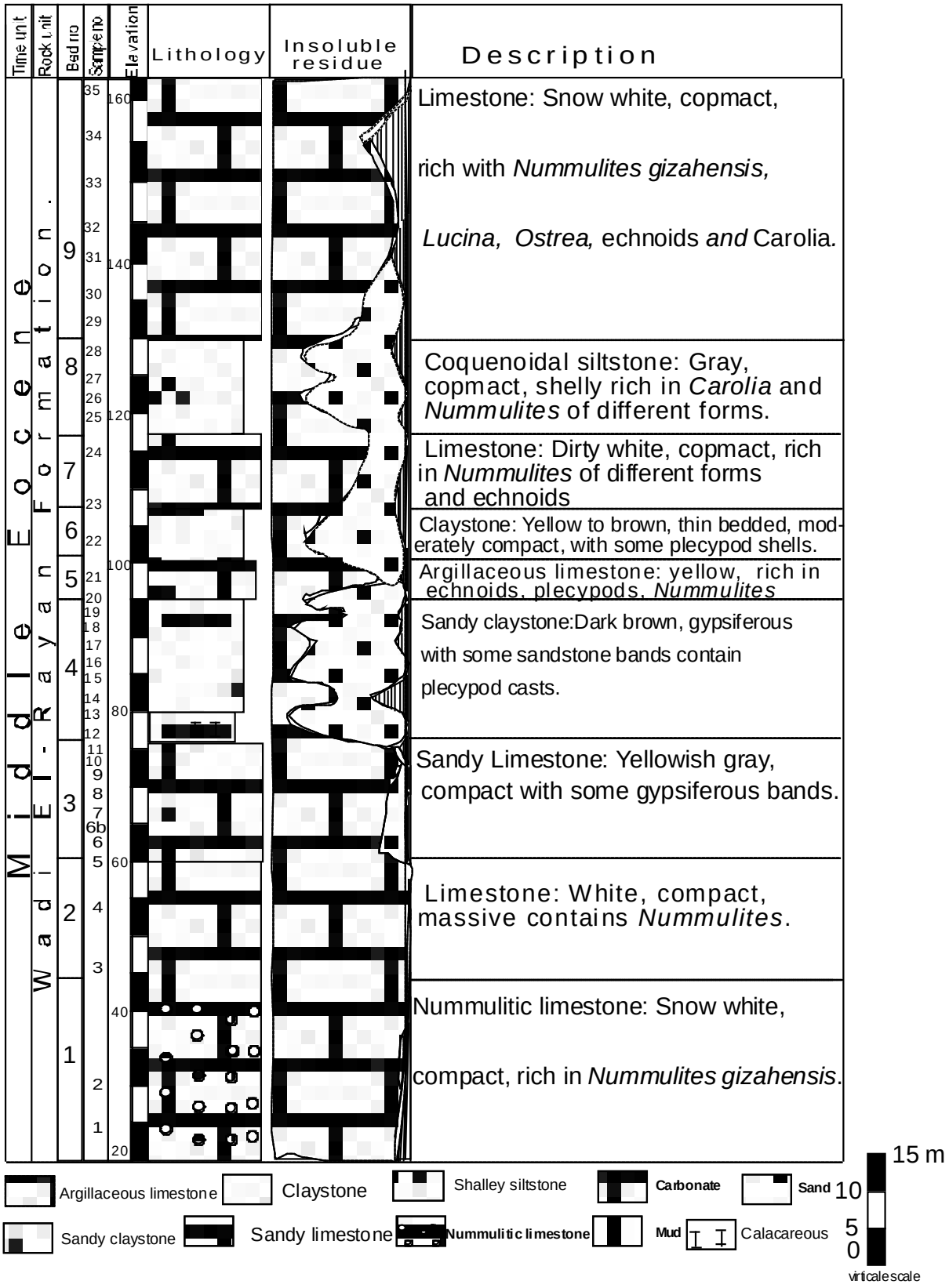

Figure.(2) Lithostratigraphic succession of the Eocene exposure at Minqar El Rayan locality.

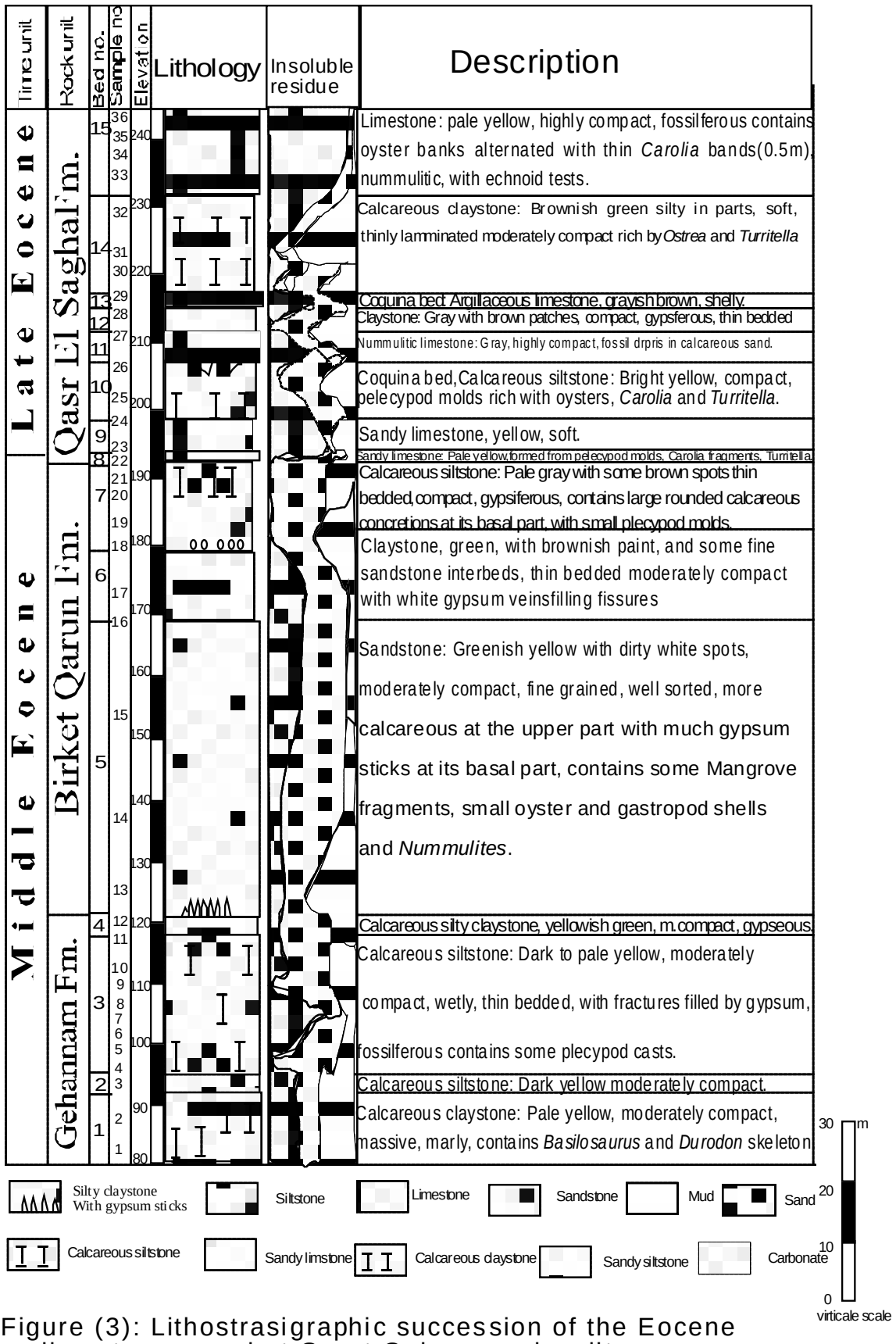

Figure (3): Lithostrasigraphic succession of the Eocene sediments exposed at Garet Gehannam locality

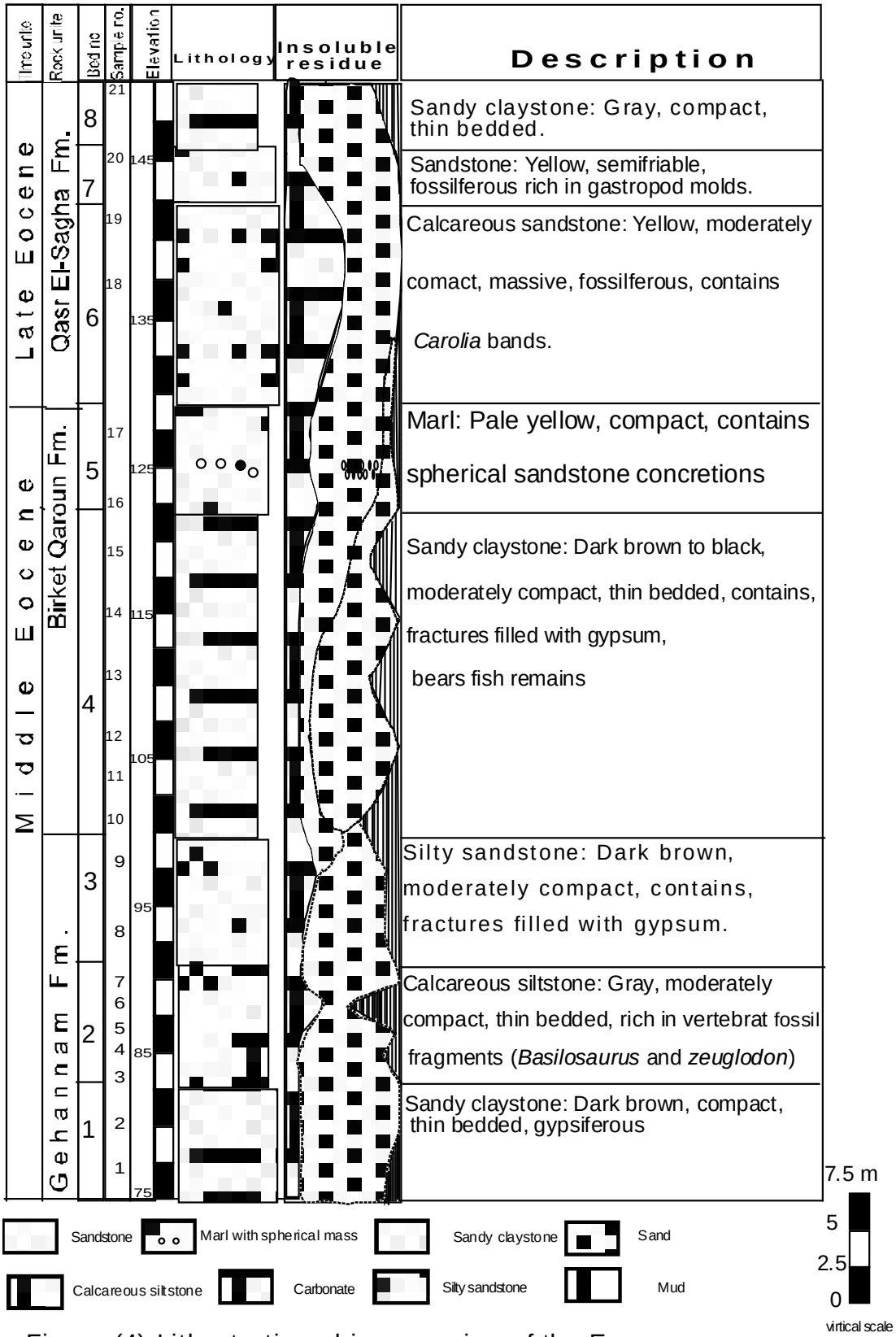

Figure (4) Lithostratigraphic succssion of the Eocene exposure at Wadi El Hitan locality.

FAUNAL COMMUNITY ANALYSES

The basal part of Wadi El Rayan Formation represents the oldest exposed Eocene rocks in Wadi El Rayan area. This formation comprises the Lutetian or possibly Lutetian – Bartonian stages, while the overlying Gehannam, Birket Qaroun and QasrEl Sagha formations represent the Bartonian and Priabonian respectively.

The invertebrate fossil content of these formations includes many mollusks as well as some echinoids with many forms of Nummulites. The richness of this content lends a hand in affirming the paleoenvironmental conditions. Most of these fossil associations are concentrated in calcareous bands embedded mostly in sandstone thick beds especially at the upper part of Gehannam Formation and through the main parts of both Birket Qaron and Qasr El Sagha formations. In addition, Wadi El Rayan area has considered as a superlative paleontological site in Egypt where the Eocene vertebrate Zeuglodon or Basillosaurus whales had discovered. The Wadi Hitan site itself contains about 400 known whale skeletons.

The Minqar El Rayan exposure is considered the most precise sediments representing Wadi El Rayan Formation at the southern part of Wadi El Rayan area, (Figs.1, 2). The lower part of this section consists of "snow white nummulitic limestone rich in ***Nummulites gizahensis"*** and leaks any macro invertebrate fossils; it reveals open marine back reef conditions. The middle part is dominated by argillaceous limestone rich in ***Echinolmpas africana, Cassidulus romani*** **and** ***Amblypygus dilatus***, alternated with calcareous, yellow to brown sandstone with many shells such as ***Wokullina (Hayella) leferei, Vulsella (Vulsella) crispta*** and ***Conoclypeus conoideus***. This faunal assemblage indicates that the area came to be shallower than it was during the deposition of the lower part. This middle part has followed by a distinctive shelly, gray, silty sandstone rich in *Carolia* and *Nummulites*. The uppermost part consists of a thick bed of hard snow-white limestone; it is similar to the lower part and rich in ***Nummulites gizahensis***. The lithologies as well as the faunal content of this formation indicate the repetition of two transgressive sedimentary cycles enclosing a regressive phase in-between. It is worth to mention that, there is no evidence supporting a major break in sedimentation within the Wadi El Rayan Formation. This cyclic repetition enforced some writers to subdivide this formation into three distinctive formations (see Boukhary *et.al* 1993).

The uppermost part represents the floor of most locations of the southwestern areas of the Fayoum Depression and can traced northward. Near the prominent Garet Gehannam exposure, where the wadi floor rises up revealing the deposits of the lower part of Gehannam Formation, the boundary of this last unit with the Wadi El Rayan Formation is almost unexposed.

The exposed sediments of the lower part of Gehannam Formation (Figs. 1, 3) consist of alternating beds of yellowish green calcareous claystones, which became silty upward. The dominance of the molluskan shells in the Gehannam Formation such as "***Pycnodonte gigantica, Acanthocardiac (Schedocardia) mohgoobi, Gastrana ibrahimi, Solen (Solen) mokattamensis*** with many others, reveals that it had deposited on a shallow shelf and emphasizes that the area was subjected to an interval of low sea stand. This conclusion is supported by the presence of the famous ***Zeuglodon isis*** or ***Basillosourus isis*** whales. However, there are some conspicuous evidences of a well-marked low sea stands during the deposition of the middle part of the Gehannam Formation, these include:

1 – The abundant eroded mangrove pneumatophores and anchor roots (Fig 5) at the top of Gehannam Formation over the abroad area of Wadi El Hitan. Pneumatophores have

related to the aerobic respiration in mangroves that grow upward out of waterlogged substrates to reach air (Hutching and Saenger, 1987). The stratigraphic interval-containing mangrove is 1-2 meters thick, suggesting that the time interval of low sea stand was relatively short.

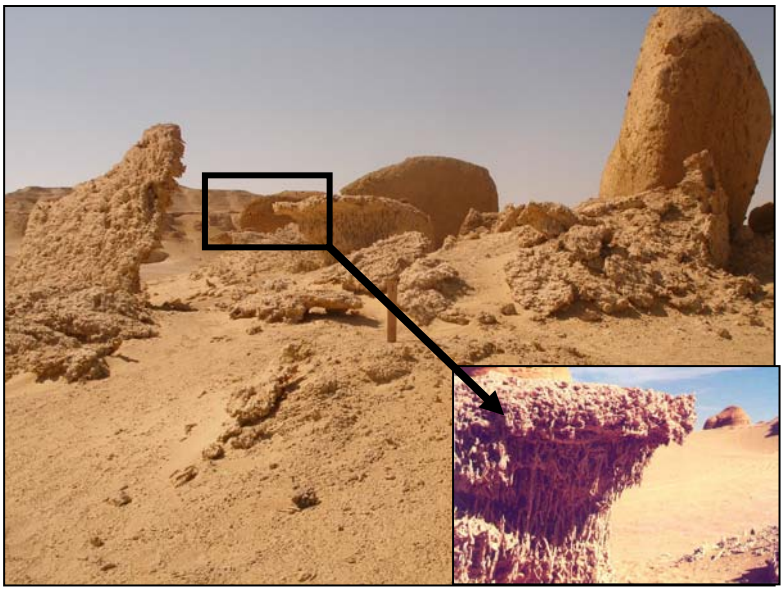

Figure 5 - Broken standing blocks of mangrove roots, about 1.5m high.

2 - Celestite filling many mollusks, including *Nautilus*, *Lucina and Tredolites* shells at the transition between Gehannam and Birket Qaroun formations. There is a large tree trunk 18 m long and about 1 m in diameter resting about 2 m below the top of Gehannam Formation (Fig. 6). It riddles with 'ship worm' (bivalve *Teredo*) burrows, and must have been sunk or beached as driftwood.

3 - Presence of ribs, vertebra, and both pelvis of estuarine proboscidean *Moeritherium* in Wadi El Hitan, that does not found elsewhere in Fayoum except in the upper part of Qasr El Sagha Formation (Gingrich 1992).

All these evidences indicate a marked interval of low sea stand, possibly with limited sub-aerial exposure.

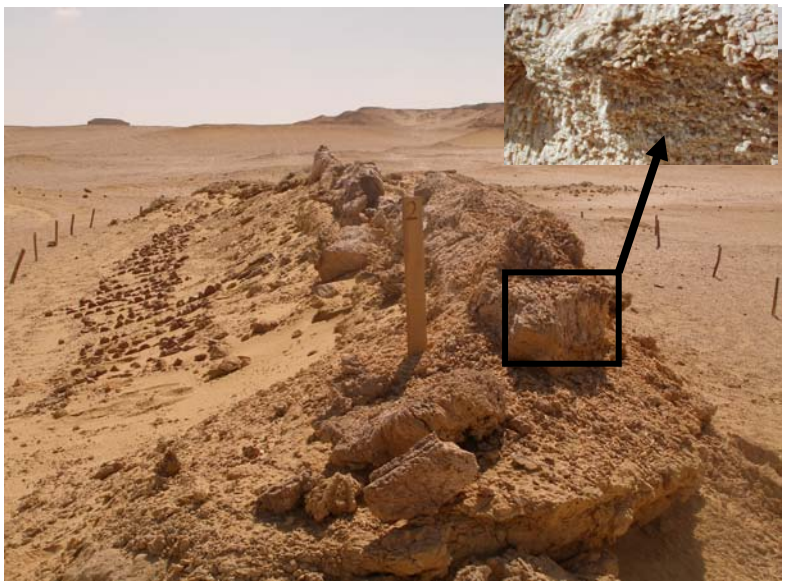

Figure 6 – Trace of *Tredolites* fossil 18 m long.

The middle part of the Gehannam Formation in Garet Gehannam and Wadi El Hitan sections contains many skeletons of the marine archeocetes ***Basilosaurus isis*** and ***Prozeglodon atrox***, in addition to diverse segments and parts of sharks. Also, skeletons of large sea turtles (specialized for swimming; Fig. 7) have preserved with rare crocodile.

This low sea stand condition, during the deposition of the middle part of the Gehannam Formation helps greatly in explaining why so many *Archaeocete* skeletons have preserved in Wadi El Hitan. Some may have beached on shallow shoals by retreating tides (although nothing has known of possible tidal ranges during the Eocene). Alternatively, the decreasing of water depth might attract *Archaeocetes* for other reasons: possibly calving in the case of *Prozeuglodon* and feeding in the case of *Basilosaurus*, Gingrich 1992. The upper part becomes marly and crowded with thin to moderately thick gypsum sheets with mangrove root horizons. The upper most part of the Gehannam Formation is characterized by the presence of thick gypsum stock work. For a long time, the upper part of Gehannam Formation and lower part of Birket Qaroun Formation known to be unusually rich in Celesite (Beadnell, 1905), this is interpreted before as an indicator of unusually restricted oceanographic conditions where partial evaporation of sea water concentrated lime and strontium sulfate.

The lower part of Birket Qaroun Formation, 18m thick, consists of greenish yellow compact, fine grained, well-sorted slightly argillaceous, calcareous sandstone, and contains some Mangrove fragments at the base and small oysters, gastropod shells and small *Nummulites* test, especially in the upper part. The following 15m are made-up of alternating claystone and shale, brownish in paint, with some fine sandstone and white gypsum veins filling the fractures. The upper part consists of soft bright-yellow, with brown spots siltstone that forms vertical cliffs, moderately compact, contains large, yellow, rounded, compact calcareous sandstone concretions at its basal part; few fossils are recorded as gastropod molds ***Scolaria (Acrilla) aegyptiaca*** and bivalves as *Carolia* shell fragments.

The lithologies of the upper part of Birket Qaroun Formation as well as the scarcely nature of fossils throughout as a barrier bar emphasizes the idea of a low sea stand at its base. The barrier bars, as a rule, are only preserved in the stratigraphic record when buried during subsequent transgression. Therefore, the Birket Qaroun Formation could be classified as a 'transgressive system tract' followed by the deposition of the following sediments of Qasr El-Sagha Formation,

Figure 7 - Internal mold for large sea turtle.

The deposits of Qasr El-Sagha Formation have been studied from two localities (Fig. 3, 4). At Garet Gehannam area, where nearly 50m of pale yellow, compact sandy limestones rich in pelecypod and gastropod molds, alternated with yellow, highly fossiliferous, soft, calcareous and sandy siltstones. The recognized lithologies of the QasrEl Sagha Formation and the characteristic fauna suggest deposition in distinct environments. It starts by a *Carolia* bed rich also in the *Turritella*, it contains some skeletons of small whales and sirenians, in addition to parts of land mammals as represented by the recorded proboscidean tooth, (fig 8). Qasr El-Sagha Formation yields large number of Late Eocene bivalves such as ***Carolia blanfordiana***, ***Carolia placunoides placunoides***, ***Ostrea (ostrea) mutabilis***, ***Ostrea (Turkostrea) multicostata strictiplicata, Ostrea (Turkostrea) reili reili***, ***Lucina pharanum****m* and ***Acanthocardia (schedocardia) schweinfurthi***. Many gasteropods have been recorded from this formation such as, *Gastrana barakai*, ***Raetomya schwenfurthi***, ***Turritella lessepsi***, ***Turritella (Ispharina) naimi, Turritella (Torquesia) carinifera, Turritella (Torquesia) pharonica, Mesalia bassiouni*** and ***Mesalia fourtaui.*** Most of this funal assemblage assert the late Eocene age for this rock unit.

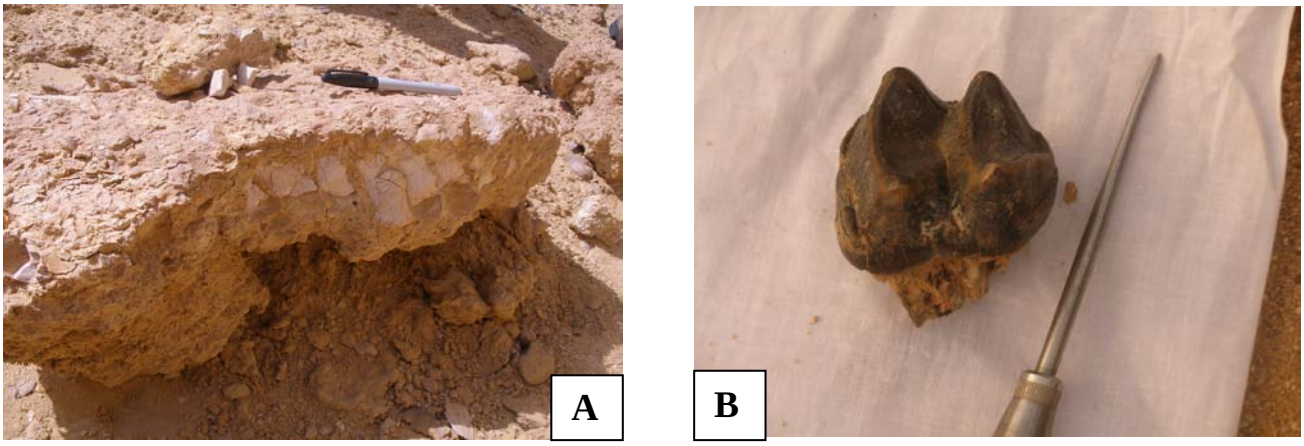

Figure 8 - A - Skeleton of Sirenian B - A molar of proboscidean.

Whales in Wadi El Hitan

Wadi El-Hitan was the first explored and mapped area during the winter of 1902-1903 by the geologists of Egyptian Geological Survey, as a part of a long-term investigation of the western Fayoum for storage of Nile River floodwater; surveys were carried out by Cairo Geological Museum and University of Michigan of Paleontology. Following its discovery, Wadi El-Hitan has more than 400 whales and other fossil vertebrate mapped in an area of about 25 square kilometers (after Gingerich, 1992), 205 were complete enough to be identified with some certainity. Some of these are the large skeleton of *Basilosaurus* that are so conspicuous in Wadi El-Hitan today; while others are more cryptic represent skeletons of *Dorudon* and partial skeletons of smaller sea caws.

The precise reason so many ancient whales are located here is unclear. This site may represent periods of “beach strandings” or drownings in lagoons isolated at low tide. On the other side, some hypothesis affirmed that, this area might have been a relatively narrow embayment in the Eocene seaway that favored sites for birthing new whales. This site, for its

protected nature away from open oceanic waters, became hideaway for many whales that have annually returned for millions of years. Whatever the cause, this site is unique in the world and has the richest collection of ancient whales located in any other paleontological site. Shark's teeth, invertebrate fossils, fish skeletons, mangrove root horizons, and a wide variety of fossil plants, termite and insect burrows as well as other "trace" fossils are abundant.

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Plate1

1-*Nuculana (Jupitaria) phacoides* (Cossmann), right exterior, X 1.1;
Bed No. 9, Qasr EL-Sagha Formation, Garet Gehannam, Fayoum, Late Eocene.

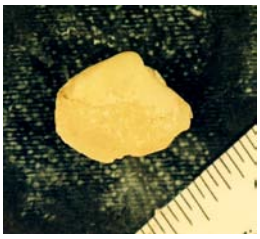
2- *Vulsella caillaudi* Zittel, a- right exterior, b- left exterior, X 0.5; Bed No. 9, Wadi El Rayan Formation, Minqar El Rayan section, Fayoum; Middle Eocene.
3- *Mimaclamys solariolum* Mayer-Eymer, a- right exterior, X 0.8, Bed No. 8, Qasr EL-Sagha Formation, Garet Gehannam section, Fayoum. Late Eocene.
4- *Wokullina (Hayella) leferei* Fischer, a- double valved spiciment, b- right valve, c- left vave, X 1, Bed no 7, Wadi El Rayan Formation, Minqar El Rayan section, Fayoum; Middle Eocene.
5- *Ostrea (Ostrea)elegans* Deshayes, a,b- external and internal view of the right valve, X 0.7; Bed No.8, Qasr EL-Sagha Formation, Wadi El Hitan section, Fayoum; Late Eocene
6- *Ostrea(turkostrea) reili reili* Frass, a, b - external and internal view of the right valve, c- double- valved specimens shows the left valve; X 1; Bed No.8, Qasr EL-Sagha Formation, Wadi El Hitan section, Fayoum; Late Eocene.
7- *Pycnodonte(Pycnodont) gigantica* Deshayes, a- external right valve, b- side vies of double-valved specimen, c- external left valve of double-valved specimen; X 0.3; Bed 10, Qasr EL-Sagha Formation, Garet Gehannam section, Fayoum; Middle Eocene

Plate 2

1- *Lucina innes* Cuvillier, a, b- external views of the surfce of the right and the left valves; X 1.9; Bed No. 8, Qasr El-Sagha Formation, Wadi El Hitan section, Fayoum; Late Eocene
2- *Pegophysema(Rawya) pharaonis* Bellardi, a, b- external view of the left, right valve, c- side view, X 1; Bed No. 5, Birket Qarun Formation, Garet Gehannam, Fayoum; Middle Eocene.
3- *Glyptoactis (Claibornicardia) corpulenta* Stenzel& Crausel, a- external view for the right valve, b- side view of the umbonal area, c- side view of the double-valved specimen; X 0.8, Bed No. 5; Birket Qarun Formation, Wadi El Hitan section, Fayoum; Middle Eocene.
4- *Raetomya schwenfurthi* Mayer-Eymer, a, b- external view of the right and the left valves; X 1; Bed No. 8; Qasr El-Sagha Formation, Wadi El Hitan section, Fayoum; Late Eocene
5- *Aturoidea olssoni* Miller, a- side view, b- aperture view; X 0.3, Bed No. 5; Birket Qaroun Formation, Wadi El Hitan section, Fayoum; Middle Eocene.

6- *Turritella(Torquesia) carinifera* Deshayes, apreture view; X 0.8; Bed No. 8, Qasr EL-Sagha Formation, Wadi El Hitan section, Fayoum; Late Eocene.
7- *Turritella (Torquiesia) abassi* Abass, side view; X 0.7, bed no. 5; Birket Qaroun Formation, Wadi El Hitan section, Fayoum; Middle Eocene.
8- *Mesalia fourtaui* Cuvillier, side view; X 1; bed No. 8, Qasr EL-Sagha Formation, Wadi El Hitan section, Fayoum; Middle Eocene.
9- *Voluta (volutilithes)mokattamensis* Cuvillier, side view; X 1.3; Bed No. 7, Wadi El Rayan Formation, Minqar El Rayan section, Fayoum; Middle Eocene.
10- *Madracies asperula* M.EDW, side view; X 1.3; Bed No. 9, Qasr EL-Sagha Formation, Garet Gehannam, section, Fayoum; Late Eocene
11- *Haimeastraea (Haimeastraea) conferta,* Fig 11, side view; X 1.3; Bed No. 9, Qasr EL-Sagha Formation, Garet Gehannam, section, Fayoum; Late Eocene.

Plate 1



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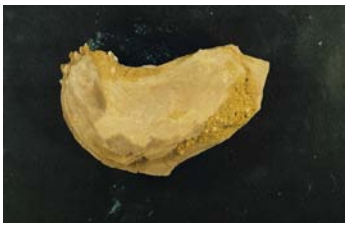

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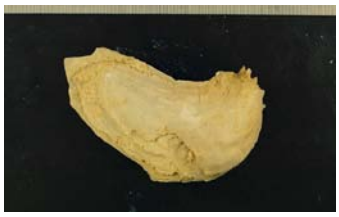

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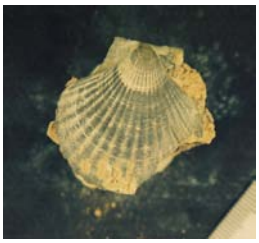

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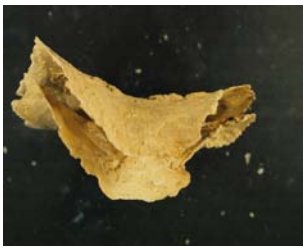

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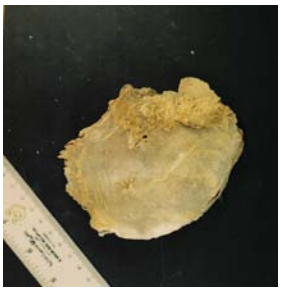

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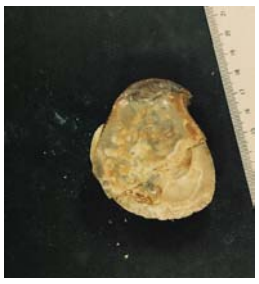

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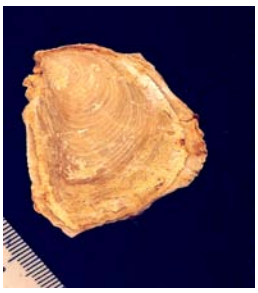

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Plate 2

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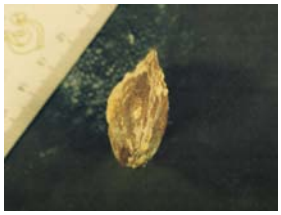

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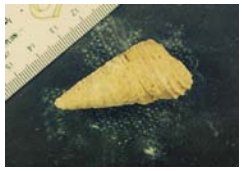

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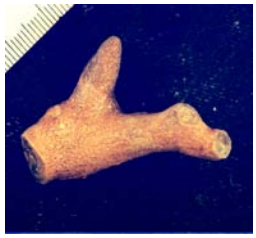

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دراسة البيئات القديمة لصخور الأيوسين في منطقة وادي الريان جنوب غرب الفيوم بمصر باستخدام تحليل التجمع الحفري

عزت عبد الشافي محمد، محمود حاتم متولي، سيد عبد العظيم ومحمد سامح محمد

جذبت منطقة وادي الريان أنظار كثير من المشتغلين بالعلوم المختلفة خاصة في فروع الجيولوجيا منذ القرن الثامن عشر وحتى الآن، وذلك لأنها من أشهر مناطق تواجد حفريات الفقاريات في الإقليم المصري علي الاطلاق 0 ويهدف البحث الحالي إلي دراسة المعاملات البيئية القديمة التي صاحبت تكون رواسب عهد الايوسين في هذه المنطقة اعتمادا علي تحليل محتواها الحفري والذي يمثل ما تبقي من محتواها الحيوي أو اثاره 0 ولهذا الغرض اختيرت ثلاث قطاعات صخرية مثلت منطقة وادي الريان جغرافيا واستراتجرافيا، أمكن تميز صخورها إلي متكونات وادي الريا، جهنم، بركة قارون ثم قصر الصاغة وهو أحدثها0 وقد تم دراسة العينات الصخرية للتعرف علي أنواع بيئات الترسيب المختلفة، كما استخدمت تجمعات الحفريات الكبيرة التي مثلت القطاعات الثلاثة في التعرف علي المعاملات البيئة المختلفة والتي سادت إبان تلك الفترات0

أكدت الدراسة علي أن منكون وادي الريان تكونت في بيئات تمثل فترة تقدم بحري بصفة عامة تخللها فترة انحسار بحري ملموس دام لفترة زمنية قصيرة 0 كما أن سيادة حفريات محاريات البيئات البحرية الضحلة التي وجدت في الرواسب الفتاتية لمتكون جهنم كست بطريقة مؤكدة الظروف البيئية التي سادت هذه الفترة وميزتها0

ومن الجدير بالذكر أن رواسب هذا التكوين أحتوت في منتصفها تقريبا علي كثير من متبقيات فقاريات بحرية (حيتان) جلبت إلي المنطقة لأسباب عدة، بجانب بعض متبقيات فقاريات مناطق قليلة الملوحة (تماسيح) مما أكد أن المنطقة مثلت مناطق مد بحري أقترن باتصال لمد من مياه غير بحرية قدمت الي المنطقة من المناطق الجنوبية لمصر 0 وقد أثبتت الدراسة ان الأجزاء العليا من تكوين جهنم والسفلية من التكوين اللاحق (تكوين بحيرة قارون) احتوت علي كثير من رواسب المتبخرات المتداخلة مع الرواسب الفتاتية والذي أكد علي استمرار الانحسار البحري علي منطقة الدراسة مما حولها إلي بيئة متبخرات0اما رواسب باقي تكوين بحيرة قارون والتي تلت هذه الفترة فإنها تمثلت ببعض الرواسب الفتاتية اقترنت بتواجد حفري ضعيف يؤكد علي غمر طفيف لمياه البحر ميز مناطق شاطئية ضحلة 0 كما أكدت الدراسة أن رواسب متكون قصر الصاغة غنيمت بكثير من الحفريات المتنوعة وبعض رواسب الكربونات التي لم تلاحظ من قبل في التكاوين السابقة (فيما عدا تكوين وادي الريان) مما أكد أن تلك الفترة تمثل تقدما بحريا ساد المنطقة وظهرت معالمه واضحة في الجزء الشمالي من منطقة الدراسة0

PROTECTION OF BROAD BEANS IN KSA AGAINST ADVERSE EFFECTS OF AMBIENT OZONE USING ASCORBIC ACID (VITAMIN C) GROWTH AND YIELD CHARACTERISTICS

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ABSTRACT

The ability of higher plants to scavenge the toxic effects of active oxygen seems to be very important determinant of their tolerance to stresses like ozone (O_3). Antioxidants are the first line of defense against free radical damage. They are critical for maintaining optimum health of plant cells. The primary objectives of this investigation were to examine the interactive effects of air quality and vitamin C on some morphological characteristics of broad beans (*Vicia faba* L. Var. Baladey) during its whole growth. These plants were grown first for two weeks in pots at closed chamber of Botany and Microbiology Dept., Faculty of Science, King Saud University and transported to industrial city until the time of harvesting. Damaging effects of ambient ozone in presence of sulphur dioxide and nitrogen dioxide on growth and productivity of plants were assessed. These plants sprayed with different concentrations of vitamin c (0, 50, 100, 200, 300, 400 mg/L) after the 20-days of plantation. High monthly concentrations of ambient O_3 have ranged between 89-110 nL L^{-1}. The high concentrations of SO_2 and NO_x were ranged between 25-35 nL L^{-1}. Significant differences between soybean plants treated with vitamin c for all measurements were recorded. Effective concentration is 300 mg/L. The total leaf area, plant length, number of damaged leaves, number of seeds and plant biomass were determined. This study concluded that O_3 is only having adverse effects on some crops in industrial areas of KSA in presence of high levels of SO_2 and NO_x. For an evaluation of these results, 300 mg/L concentration of vitamin c may depress the negative effects of O_3 could reach to 31%.

Key words: Ozone, broad beans, vitamin c, growth, productivity, KSA.

INTRODUCTION

Saudi Arabia is one of the most vulnerable countries in the region to climate change. There are many reasons about these vulnerabilities. Human activity (burning fossil fuels and changes in land use) is modifying the global climate with temperatures rises projected for the next 100 years that could affect human welfare and the environment. The climatic record of this region for the past five years showed that there were fluctuations in the temperature and a decrease in rainfall over large portions of Saudi Arabia. In the 21st century, this warming trend, and changes in precipitation patterns are expected to continue along with a rise in sea level and increased frequency of extreme weather events.

One of these negative trends led to habitat loss and fragmentation of plant species-rich ecosystems is atmospheric pollutants such as ozone (O_3) and sulfur dioxide (SO_2). They have been implicated in free radical formation (Asada, 1980, Mehlhorn, 1990) and are considered to be one of the major factors influencing forest decline. The O_3, which originates from a natural photochemical degradation of nitrous oxides (NO_2), seems to be a greater threat to plants than other pollutants (Heagle 1989). Mehlhorn *et al*. (1996) suggested that the phytotoxicity of O_2 is due to its oxidizing potential and the consequent formation of radicals that induce free radical chain reactions.

Ascorbic acid (AA) has been proposed for a long time as a biological antioxidant. It exists in rather high concentrations in many cellular environments, such as the stroma of chloroplasts where its level is 2.3×10^{-3} M. Ascorbate has been demonstrated in many qualitative studies to possess significant antioxidant activity. For example $l0^3$ M ascorbate inhibited the photooxidation of a kampferol by illuminated spinach chloroplasts. Ascorbate reduces two equivalents of O^{-2} produce H_2O_2 and triketo derivative dehydroascorbic acid. Ascorbate also reacts with $^1O_2$ at a relatively fast rate. Shalata and Neumann (2006) reported that stress increased the accumulation in roots, stems and leaves of lipid peroxidation products produced by interactions with damaging active oxygen species. Additional ascorbic acid partially inhibited this response but did not significantly reduce sodium uptake or plasma membrane leakiness.

The primary objectives of this investigation were to examine the interactive effects of air quality and AA on physiological and morphological characteristics of broad beans (*Vicia faba* L. Var. Lara) during its whole growth. Damaging effects of ambient ozone in presence of sulphur dioxide and nitrogen dioxide on growth and productivity of plants were assessed.

2. MATERIALS AND METHODS

2-1 Experimental design and foliar spray

A pot experiment was carried out at the Botany and Microbiology Department, Faculty of Science, King Saud University during winter 2007. Randomized complete block design with three replicates was used. First, eighteen pots were cultivated with *Vicia faba* L. Var. Baladey at the experimental site for 20 days. Second, all pots were transferred to industrial city, Riyadh, KSA. Then, five foliar sprays of vitamin c (ascorbic acid) were imposed to bean plants. Three pots without foliar spray of vitamin c were used as a control.

Pots were watered with tap water as required. When the first leaf was fully expanded, the exposure of vitamin c will start. The foliar applications of vitamin c were given at 10 days intervals for five times. Vitamin c concentrations are 50, 100, 200, 300 and 400 mg L^{-1} solutions, respectively. These solutions were prepared by suspending ascorbic acid in distilled water for 24 h before application, and were applied as a foliar spray to plants.

2-2 Measurements

Air temperature, rain fall, relative humidity and wind speed were recoded in Table 1.

Monthly concentrations of ambient O_3, SO_2 and NO_x were measured using multi-gas analyzer (Gray Wolf, Sweden).

All plant growth measurements were assessed. Plant length, total leaf area, number of damaged leaves, percent of damaged area per leaf, plant biomass and seeds characters were measured during the all growth stages and plant maturity.

2-3 Statistical analysis

Statistical analyses were carried out using the SPSS BASE 10.0 (SPSS Inc., Chicago, IL) packages. Data were tested by ANOVA and F-protected LSD separated means at $p \leq 0.05$ levels.

3. RESULTS AND DISCUSSION

3.1 Variations in gases levels:

Changes in mean concentrations of O_3, SO_2 and NO_2 during the growing of broad bean (*Vicia faba* L. Var. Baladey) at industrial city, Riyadh, KSA are listed in Table 2. Mean monthly concentrations of O_3 gradually increased in summer reaching to 110 nL L^{-1} in May and recording the lowest concentration in January being 40 nL L^{-1}. Also, gradual increase in SO_2 and NO_2 concentrations was observed. High values were recorded during hot months while cool months were vice versa. High SO_2 and NO_2 levels reached 35and 33 nL L^{-1}, respectively while low levels are 15 and 21 nL L^{-1}, respectively.

In a typical urban atmosphere like Riyadh, O_3, SO_2 and NO_2 concentrations increase rapidly between 1200 and 1500 h entire the day light and when the intensity of solar radiation is at a maximum in hot months and when the NO_2: NO ratio is large (Krupa *et al*., 2001). The rate of O_3 formation may then decline, reaching a steady state during the late afternoon to early evening hours. After that period, O_3 concentrations fall as NO_2 breakdown diminishes and as fresh emissions of NO deplete the O_3. This daily pattern is quite different at high elevations (in general, above approximately 1,500 m from the surface or above the so-called mixed layer of the atmosphere), where O_3 concentrations remain relatively steady through day and night. At that altitude, there is an O_3 reservoir, and destruction of that O_3 by the surface is insufficient to produce the type of daily patterns observed at lower elevations (Krupa *et al*., 2001). They also reported that high levels of SO_2 and NO_2 concentrations can't give more damage in presence of high O_3.

3.1 Variations in growth and yield parameters:

Table 3 shows the periodical examination for lengths (LS) of broad bean plant after different foliar spray additions of vitamin c at industrial city, Riyadh, KSA. No much difference in LS between vitamin c treatments after 30 days was observed. On the other hand, at the end of foliar spray the 300 mg L^{-1} treatment had on average a significant increase on shoot system length being 70.2 cm. Under the foliar spray conditions all plant lengths were stabled after 70 days except the 300 mg L^{-1} of vitamin c reaching the highest values after 80 days. In the 400 mg L^{-1} of vitamin c application appeared was significantly lowered LS in compared with other applications.

Table 4 described the periodical examination for leaf area (LA) of broad bean plant after different foliar spray additions of vitamin c at industrial city, Riyadh, KSA. The percent of LA stimulation observed in the all applications of vitamin c after 30 days was not retrieved the leaf area of broad bean. The 300 mg L^{-1} of vitamin reached the maximum increase by the last addition after 80 days being 5.8 cm^{-2}. The LA of bean leaves treated with the rest of vitamin c was not significant in compared to plants without vitamin additions. Also, plants without

vitamin addition can't increase LA, while other plants subjected to all vitamin concentrations can grow LA gradually. All LA stopped grow before the last application except the 300 mg L^{-1} of vitamin.

Periodically mean effects of vitamin c exposures on percent of damaged areas (%) per area of broad bean leaf are summarized in Table 5. At the end of vitamin applications, the 300 mg L^{-1} improved the healthy leaf areas by 80 %. The healthy leaves were decreased under no vitamin addition by 28% at end of experiment. The less response to vitamin c in overall foliar sprays was the 400 mg L^{-1}. The best concentration from vitamin c after 300 mg L^{-1} was 200 mg L^{-1} which recorded 66% of healthy leaf area.

The foliar spray of vitamin c effects on the number of damaged leaves per broad bean plant (NDLP) was illustrated in Table 6. No response to all foliar sprays after 30 days. Significant effect of the vitamin c treatments on the NDLP was noticed after 40 days. The % of stimulation in number of healthy leaves was recorded 62, 56, 70, 92 and 62 for the 50 mg L^{-1}, 100 mg L^{-1}, 200 mg L^{-1}, 300 mg L^{-1} and 400 mg L^{-1} of vitamin c additions, respectively.

Table 7 listed the Mean values for total biomass (gm) and seeds characters of broad bean plant treated with different foliar sprays of vitamin c at industrial city, Riyadh, KSA. Significant effect was observed for the all vitamin c treatments except 400 mg L^{-1} on the plant biomass produced at the end of growing season compared to non-treated plants. On average, the biggest increase in biomass was 15% in the 300 mg L^{-1} application and 0.03% is the lowest increase between treatments (400 mg L^{-1}). Pod number per plant were 33%, 44%, 44%, 55% and 33% higher on plants grown under the 50 mg L^{-1}, 100 mg L^{-1}, 200 mg L^{-1}, 300 mg L^{-1} and 400 mg L^{-1} of vitamin c additions, respectively. Not only the 200 mg L^{-1} conditions enhanced more seed production per plant, but also the 300 mg L^{-1} treatment was effective increase too. Lower increase in pod length in 50 mg L^{-1} conditions was being 0.08% only, while large increase was 33% in 300 mg L^{-1} treatments. Large variations in the yields among plants in the presence and absence of vitamin c applications were observed. Only significant increase was recorded in the 200 mg L^{-1} and 300 mg L^{-1} treatments being 31% and 46%, respectively.

Ozone is reach to the chloroplast but it nevertheless, causes pigment bleaching and lipid peroxidation (Heck *et al.*, 1983; Heath 1987). Stimulation of both synthesis and degradation of the protein occurs in spruce trees following O_3 treatment (Godde and Buchhold, 1992; Lutz *et al.*, 1992) and a decrease in the activity and quantity of rubisco has been found in poplar following exposure to O_3 (Landry and Pell, 1993). Loss in growth parameters caused by inhibition of the activity of light-activated enzymes of the chloroplast (Shimazak and Sugahara, 1980, Tanaka *et al.*, 1982, Convello *et al.*, 1989).

Exposure to SO_2 results in tissue damage and release of stress ethylene from both photosynthetic and non-photosynthetic tissues (Peiser and Yang 1979, 1985). Fumigation with SO_2 causes a shift in cytoplasmic pH. When plant cells are exposed to SO_2 an appreciable acidification of the cytoplasm occur because this gas reacts with water to form sulfurous acid which may then be converted into sulphuric acid (Laisk *el al.*, 1988 a, b ;Veljovic-Jovonovic *et al.*, 1993). The oxidation of sulfite to sulfate in the chloroplast also gives rise to the formation of O^{-2} (Asada, 1980). The oxidation of sulfite is initiated by light and is mediated by photosynthetic electron transport. Navari-Izzo *et al.*, (1992) reported that the degradation of membrane lipid components possibly by de-esterification rather than peroxidation with SO_2. They found no evidence to support the view that free radical attack on polyunsaturated fatty acids occurred at low pollutant concentrations.

Vitamin C is a universal reductant and antioxidant of plants. It is found at concentration of 1-2 Mm in legume nodules (Dalton *et al.*, 1986; Matamoros *et al.*, 1999) and is positively correlated with nodule effectiveness (Dalton, 1995). It is an essential metabolite for the operation of the ascorbate-glutathione (ASC- GSH) pathways, but it also has beneficial effects that do not require the presence of ascorbate peroxidase (APX). ASC can directly scavenge (reactive oxygen species) ROS (Moreau *et al.*, 1995). It is also involved in hydroxylation of proline, regulation of the cell cycle and numerous fundamental processes of plant growth and development (Noctor and Foyer, 1998). Some reports concerning the exogenous application of the vitamins to salinized plants and their role in stimulation of their growth are scarce (Oertii, 1987). These compounds were also scarcely tried to counteract some of the adverse effects of salinity stress (Oncel *et al.*, 2004). Thus exogenous addition of such substances to the test organism could lead to growth stimulation through the activation of some enzymatic reactions (Kefeli 1981 and Makled, 1995).

Ascorbate is a key soluble antioxidant (Smirnoff *et al.*, 2001). Isolation of an Arabidopsis mutant containing 30% of the wild type ascorbate concentration has provided the first genetic evidence for its importance in stress resistance. It was selected by hypersensitivity to ozone, UV-B and sulfur dioxide (Conklin, 2001). The mutation has no pleiotropic effects on other parts of the antioxidant system with the exception of reduced APX activity, possibly because ascorbate is required to stabilize APX (Conklin *et al.*, 1997). Reduction of cytosolic APX activity by expression of APX antisense mRNA also causes increased sensitivity to ozone damage, suggesting that intracellular, as well as extracellular, ozone detoxification by ascorbate is required (Orvar and Ellis 1997). Overexpression of APX in tobacco chloroplasts had no effect on ozone resistance (Torsethaugen *et al.*, 1997); either it is in the wrong subcellular compartment or it is not a limiting factor.

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Table 1: Mean values of meteorological parameters at industrial city, Riyadh, KSA (2007).

Months	Air temperature (^oC)	Humidity (%)	Wind velocity (km/hr)	Rain-fall (mm)
January	18	44	7	18.8
February	30	43	8	12.6
March	37	40	7	13.6
April	40	32	8	1.78
May	43	28	6	0.55
LSD ($p \leq 0.05$)	3.8	12.6	1.2	4.7

Table 2: Mean values of gases concentration (nL/L^{-1}) at industrial city, Riyadh, KSA during the growth period of broad bean (2007).

Months	O_3 concentrations (nL/L^{-1})	SO_2 concentrations (nL/L^{-1})	NO_2 concentrations (nL/L^{-1})
January	40	15	21
February	52	20	24
March	77	25	25
April	89	33	33
May	110	35	33
LSD ($p \leq 0.05$)	26	11	12

Table 3: Periodical examination for lengths (cm) of broad bean plant after different foliar spray additions of vitamin c at industrial city, Riyadh, KSA (2007).

Foliar spray additions	Lengths after 30 days	Lengths after 40 days	Lengths after 50 days	Lengths after 60 days	Lengths after 70 days	Lengths after 80 days
Without vitamin c	36.5	39.7	44.6	56.7	57.8	57.9
+ 50 mg L^{-1} vitamin c	39.9	44.6	56.9	64.7	65.7	65.9
+ 100 mg L^{-1} vitamin c	38.8	45.6	55.7	66.7	67.8	67.9
+ 200 mg L^{-1} vitamin c	38.7	46.8	55.2	65.8	66.6	66.9
+ 300 mg L^{-1} vitamin c	38.9	47.8	57.9	68.4	68.9	70.2
+ 400 mg L^{-1} vitamin c	37.8	45.6	55.5	56.8	63.3	63.8
LSD ($p \leq 0.05$)	1.4	2.8	4.7	3.6	3.3	4.5

Table 4: Periodical examination for leaf area (cm^2) of broad bean plant after different foliar spray additions of vitamin c at industrial city, Riyadh, KSA (2007).

Foliar spray additions	Leaf area after 30 days	Leaf area after 40 days	Leaf area after 50 days	Leaf area after 60 days	Leaf area after 70 days	Leaf area after 80 days
Without vitamin c	3.4	3.5	3.6	3.7	3.7	3.7
+ 50 mg L^{-1} vitamin c	3.5	4.1	4.4	4.5	4.6	4.6
+ 100 mg L^{-1} vitamin c	3.5	4.1	4.4	4.7	4.7	4.8
+ 200 mg L^{-1} vitamin c	3.6	4.3	4.7	4.8	4.8	4.9
+ 300 mg L^{-1} vitamin c	3.6	4.3	4.9	5.1	5.5	5.8
+ 400 mg L^{-1} vitamin c	3.5	4.1	4.3	4.5	4.5	4.5
LSD ($p \leq 0.05$)	1.3	1.1	1.2	1.2	1.3	1.2

Table 5: Periodical examination for percent of damaged areas (%) per area of broad bean leaf after different foliar spray additions of vitamin c at industrial city, Riyadh, KSA (2007).

Foliar spray additions	Damaged area percent after 30 days	Damaged area percent after 40 days	Damaged area percent after 50 days	Damaged area percent after 60 days	Damaged area percent after 70 days	Damaged area percent after 80 days
Without vitamin c	33	36	38	38	41	42
+ 50 mg L^{-1} vitamin c	31	31	29	23	21	14
+ 100 mg L^{-1} vitamin c	29	26	23	16	12	11
+ 200 mg L^{-1} vitamin c	29	25	24	21	15	10

+ 300 mg L^{-1} vitamin c	26	23	14	12	9	3
+ 400 mg L^{-1} vitamin c	33	32	31	31	29	29
LSD ($p \leq 0.05$)						

Table 6: Periodical examination for changes in the number of damaged leaves per broad bean plant after different foliar spray additions of vitamin c at industrial city, Riyadh, KSA (2007).

Foliar spray additions	**Damaged leaves number after 30 days**	**Damaged leaves number after 40 days**	**Damaged leaves number after 50 days**	**Damaged leaves number after 60 days**	**Damaged leaves number after 70 days**	**Damaged leaves number after 80 days**
Without vitamin c	7	8	10	11	12	13
+ 50 mg L^{-1} vitamin c	7	6	6	5	5	5
+ 100 mg L^{-1} vitamin c	7	6	6	6	6	6
+ 200 mg L^{-1} vitamin c	7	5	5	5	4	4
+ 300 mg L^{-1} vitamin c	7	4	4	3	2	1
+ 400 mg L^{-1} vitamin c	7	5	5	5	5	5
LSD ($p \leq 0.05$)	1.1	1.3	2.2	3.4	5.5	7.2

Table 7: Mean values for total biomass (gm) and seeds characters of broad bean plant treated with different foliar sprays of vitamin c at industrial city, Riyadh, KSA (2007).

Foliar spray additions	**Biomass** (gm)	**Number of pods /plant**	**Number of seeds /plant**	**Pod length** (cm)	**Seed yield** (gm)
Without vitamin c	34.66	9	35	7.3	65.1
+ 50 mg L^{-1} vitamin c	37.86	12	49	7.9	75.6
+ 100 mg L^{-1} vitamin c	37.77	13	53	8.6	76.4
+ 200 mg L^{-1} vitamin c	38.01	13	55	8.9	85.6
+ 300 mg L^{-1} vitamin c	39.12	14	55	9.5	94.5
+ 400 mg L^{-1} vitamin c	35.67	12	48	8.3	74.8
LSD ($p \leq 0.05$)	2.2	2.6	8.8	1.4	11.2

حماية الفول في المملكة العربية السعودية ضدّ التأثيراتِ المضادّةِ للأوزونِ الجوي باستعمال حمضِ الأسكوربيك (فيتامين ج)

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الملخّص

إن استعمال المواد الكيماوية مانعِة التأكسد مثل حامضِ الأسكوربيك (فيتامين ج) يُمْكِنُ أَنْ تعطي حمايةَ إلى النباتاتِ ضدّ تأثيراتِ الأوزونِ الضارة. في هذه الدراسة تم قياس التأثيرات الضارّة للأوزونِ البيئيِ على النمو ومعدل إنتاج نباتاتُ الفول المعرض إلى المستويات العاليةِ مِنْ التلوثِ في المدينةِ الصناعِ ية بالرياض- المملكة العربية السعودية . رَشّتْ نباتاتُ الفول بتوكيزاتِ مختلفةِ مِنْ فيتامين سي (0, 50, 100, 200, 300, 400 مجم /لتر) بعد عِشّرَين يوم مِنْ الزراعةِ. أظهرت القياسات الشهرية العالية لغاز الأوزون تَتَراوح بين 90-110 نانولتر/لتر. في حين أن قيم غاز ثاني أكسيد الكبريت وغازات أكاسيد النيتروجين 25-42 نانولتر/لتر. وقد تم إثبات هذه النتيجة علي مساحة الورقة الكليّة وطول النباتِ والهذرةِ والمحصولِ والكتلة الحية للنبات. ومن نَتائِجِ الدراسة أيضاً أنُ التأثيراتُ المضادّةُ للأوزون تزداد فقط على بَعْض المحاصيلِ في المناطق الصناعيةِ للمملكة العربية السعودية في حضورِ المستويات العاليةِ من غاز ثاني أكسيد الكبريت وغازات أكاسيد النيتروجين. أثبتت الدراسة الاختلاف الهامّ بين نبات الفول المعالجَ بفيتامين ج على كُلّ المقاييس وأَنْ الفاعلية لتركيز 300 مجم/لتر من فيتامين سي في حماية نبات الفول من آثار الأوزون تصلَ إلى تحسن في إنتاجية الفول بنسبة 31%.

OPTIMIZATION, CHARACTERIZATION AND PURIFICATION OF PROTEASE PRODUCTION BY SOME ACTINOMYCETES ISOLATED UNDER CERTAIN STRESS CONDITIONS.

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ABSTRACT

Sixty halotolerant actinomycetes were isolated from salt marches and cultivated soil samples from different localities in Egypt. The most halotolerant proteolytic actinomycetes (5 isolates) were identified as; *Streptomyces halstedii* salh-12, *St. olivaceus* salh-17, *St. clavus* Dakh-28 (at 55ºC) and *St. endus* Salh-40 and *Actinomyces aureocirculatus* Dakh-50 (at 35ºC) in the presence of 7% NaCl in the growth medium. The protein finger print technique was carried out for five selected isolates. *St. halstedii* Salh-12 and *St. endus* Salh-40 were chosen as the most potent proteloytic halotolerant thermophilic and mesophilic isolates, respectively. Optimum biomass and protease activity for the two selected isolates were obtained in presence of 1&2 % NaCl, after four and six days at 50ºC and 35ºC in production medium adjusted at pH 8.0 and 9.0 under shaking condition and in presence of 1.0% mannose and casein as carbon and nitrogen sources, respectively. The different chloride metal ions affected the protease production by *St. halstedii* salh-12 and *St. endus* salh-40 isolates, where Ba^{+2}, Mn^{+2}, Mg^{+2} and Ca^{+2} at low concentration (0.05%) has stimulatory effect on protease produced by both isolates. On other hand, the higher concentration of Na^{+} stimulated protease production than lower concentration. Ammonium sulphate (80%) precipitation of crude enzyme in cell free filtrate of *St. halstedii* Salh-12 and *St. endus* Salh-40 increased the specific enzyme activity (SEA) by 2.14 and 2.21 folds, respectively. Further purification by gel filtration using Sephadex G200 increased the SEA by 2.95 and 14.46 folds for *St. halstedii* Salh-12 and *St. endus* Salh-40, respectively. Analysis of the purified enzymes produced by both selected isolates using gel electrophoresis revealed that the molecular weights were 60 & 35 kDa, respectively. Amino acids analysis revealed that the higher amounts were recorded for glycine, alanine and arginine followed by proline and glutamic acid. The maximum protease activity of the purified enzymes were obtained by using 1% casein adjusted at pH 11.0 and 9.0 at 50ºC and 40ºC for 1h. for *St. halstedii* Salh-12 and *St. endus* Salh-40, respectively. The pure enzyme of both isolates *St. halstedii* Salh-12 and *St. endus* Salh-40, were used as additive to detergents and degradation of human hair (dehairing).

INTRODUCTION

Proteases or proteolytic enzymes were degradative enzymes, which catalyze the total hydrolysis of proteins, and specifically act on the interior peptide bonds of proteins and peptides **(Bayoudh *et al.*, 2000).** Alkaline proteases are well known industrial workhorses due to their robustness, increased production capacities, high catalytic activity, high degree of substrate specificity and they are widely used in detergents, tanning, food processing, waste treatment, peptide synthesis and other such industries **(Kumar *et al.*, 1998; Kumar and Takagi, 1999).** Moreover, alkaline proteases specifically account for nearly 25% of the world enzyme market with a predominant share (35%) taken by detergents **(Layman, 1986).** Proteases are industrially important enzyme, used in the detergent, food, pharmaceutical and

leather industries and also have application in silver recovery from photographic plates and in peptide synthesis **(Paliwal *et al.*, 1994, Mala *et al.*, 1998 and Pastor *et al.*, 2001).**

Actinomycetes are abundant in terrestrial soils, the majority of the isolated species have shown to produce bioactive compounds. Halophiles can be loosely classified as slightly, moderately or extremely halophilic, depending on their requirement of NaCl **(DasSarma and Arora, 2001). Weyland and Helmke (1988)** considered actinomycetes to be part of marine microflora. **Ravel *et al.* (1998)** reported the existence and isolation of actinomycetes population from marine deep oceanic sediments. **Pandhare *et al.* (2002)** showed that three actinomycetes strains producing alkaline protease inhibitors API-I, API-Ц and API- ш.

This study was concerned with the isolation and identification of haloalkalophilic and thermophilic actionmycete isolates from different salt marches and cultivated soil sample. The optimum nutritional and environmental factors required for the maximum yield of the alkaline proteinase enzyme produced by the most halotolerant thermophilc and mesophilic isolates. Furthermore, purification and characterization of the purified protease enzyme and certain applications were obtained.

MATERIALS & METHODS

Soil samples were collected from two regions of Salheya (Ismaelia government) (Salt marches S1&S2); one region of Dakhalia (S3) and one region of Berg El Arab. The chemical analysis of soil samples according to **Jackson (1960).**

Isolation of halophilic and proteolytic active actinomycetes:-

The medium used for isolation, cultivation and stock maintenance of isolated strains was starch nitrate agar medium **(Waksman, 1959),** but with different concentrations of NaCl (%) for screening of halophilic actinomycete isolates. It contained (g/l):- Soluble starch, 10; KNO_3, 2; K_2HPO_4, 1; NaCl (0.05-10%); $MgSO_4.7H_2O$, 0.5; $FeSO_4.5H_2O$, 0.01; $CaCO_3$, 2; agar, 20 and distilled water up to 1000ml. The medium used for protease production was starch nitrate agar medium with replacing of KNO_3 (nitrogen source) by gelatin (0.2%).

Purification and identification of halophilic and proteolytic active actinomycetes:-

According to the morphological characteristics of different colonies which showing most halotolerant and proteolytic activity on gelatin agar plates were picked up and streaked on starch nitrate agar plates. Purified isolates were maintained on starch nitrate agar slants for further biochemical tests and morphological characteristics.

Identification of the selected isolates was carried out according to **Gauze *et al.* (1957)**; **Waksman (1961) Shirling and Gottlieb (1966, 1968 a,b, 1969 and 1972); and Williams *et al*. (1984).**

Protein finger print of tested actinomycetes isolates:

Protein finger print was carried out for five selected actinomycetes in Genetic Engineering Services Unit, Agricultural Genetic Engineering Research Institute, Ministry of Agriculture and Land Reclamation, Egypt, according to the method described by **Laemmli (1970).**

Assay of protease activity of identified isolates:-

(i) Semi-quantitative protease assay technique:

The medium used for semi-quantitative protease assay, contained (g/l):- Gelatin, 10; agar-agar, 15; 0.1 M glycine-NaOH buffer at pH 10 was completed up to 1L. This was carried out according to Gelatin Clearing Zone (GCZ) technique **(Ammar *et al.*, 1991).**

(ii) Quantitative protease assay:

Protease activity was determined using gelatin as a substrate as described by **(Gessesse and Gashe, 1997).** The reaction mixture in a total volume of 2 ml was composed of 1% gelatin, 50m M glycine-NaOH buffer pH 10, and appropriately diluted enzyme. After 30 min incubation at 55ºC, the reaction was terminated by adding equal volume of 10% trichloroacetic acid (TCA). After separation of the un-reacted gelatin precipitate by centrifugation, 0.5ml of clear supernatant was mixed with 2.5 ml of 0.5 M Na_2CO_3 and 0.5 ml of 1:1 Folin-Ciocalteau's phenol reagent. After 30 min at 25ºC absorbance of the solution was measured at 660 nm in spectrophotometer against a reagent blank and calculated the amounts of tyrosine released in the reaction from a standard curve plotted from known concentration of tyrosine (0-100). All reading were measured by using the standard curve **(Shumi *et al.*, 2004).** The blank containing substrate without enzyme and similarly treated. One unit of alkaline protease activity was defined as the amount of enzyme that released 1 mg of amino acid equivalent to tyrosine per min under the standard assay conditions.

Estimation of extracellular protein

Protein concentration was determined by **(Lowery *et al.*, 1951)** using bovine serum albumin (Sigma chemical Co.) for standard curve.

Optimization of cultural and environmental conditions for maximum protease production:

The production broth medium was optimized of maximum protease production by addition of 1 and 2% NaCl for ***St. halstedii* Salh-12** and ***St. endus* Salh-40**, respectively; 1% mannose and casein; 0.05% $MgSO_4.7H_2O$ and K_2HPO_4. the broth medium was adjusted at pH 8.0 for 4 days at 50ºC for ***St. halstedii* Salh-12**, at pH 9.0 for 6 days 35ºC ***St. endus* Salh-40** under shaking condition.

Purification of alkaline protease enzymes:

a- Precipitation of enzymes by ammonium sulphate

100 ml of each of the cell free filtrate of ***St. endus* Salh-40** and ***St. halstedii* Salh-12** isolates under investigation was brought different concentrations of ammonium sulphate (20-100%) and the precipitated protein was obtained by centrifugation for 15 minutes at 15000 rpm. The best conc. of ammonium sulphate was produced highest activity was 80%. Both enzyme activity and protein content were determined for each separate fraction **(Khalil and Gupta, 2003).**

b- Anion exchange gel filtration by chromatography using Sephadex G-200

The dialyzed crude enzymes preparations were applied on to column packed with Sephadex G 200. This was equiliberated with 50 mM glycine NaOH buffer pH 10, then eluted with the same buffer. Preparation of the gel column and the fractionation procedure was carried out as mentioned by **Ammar (1975).**

Active peak fractions were collected and tested for their protease activity and protein content to calculate the specific activity and tested some factors on the tested pure enzymes.

c- SDS-protein electrophoresis*:*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was preformed according to method of **Laemmli (1970)** as modified by **Studier (1973)** and performed by Genetic Engineering Services Unit, Agricultural Genetic Engineering Research Institute, Ministry of Agriculture and Land Reclamation, Egypt. This method was used to determine the molecular weight of the partially purified protease enzyme of the two tested isolates ***St. endus* Salh-40** and ***St. halstedii* Salh-12**.

d- Amino acid analysis of the purified proteases*:*

The amino acid composition of the purified protease hydrolysate which produced by ***St. halstedii* Salh-12** (thermophilic) and ***St. endus* Salh-40** (mesophilic) were carried out by Authorities of the Central International Research Laboratory, Cairo Egypt.

The amino acid analyzer (Eppendorf – Germany- LC3000 Amino Acid Analyzer, at flow rate: 0.2 ml/min) was used for the purpose of characterization and determination of amino acids of protease enzymes produced by ***St. endus* Salh-40** and ***St. halstedii* Salh-12**. Amino acids were transported to micro reaction vial and derivatization procedure was carried out according to **Landault and Guiochen (1964)**, using N-butanol and trifloroacetic anhydride.

Factors affecting pure enzymes activities

This experiments were performed to investigate the effect of different concentrations of the previously purified proteases at concentrations of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 ml, in presence of different concentrations of casein (w/v): 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 at different incubation temperatures 25, 30, 35, 40, 45, 50 and 55ºC when the mixture was adjusted at different pH values (6, 7, 8, 9, 10, 11 and 12) using different buffers; phosphate and glycine-NaOH buffers for different time 10, 20, 30, 40, 50, 60 and 80 minutes on the enzyme activity.

Applications of the purified proteases

a- Detergent industry*:-*

This was carried out in comparison with a commercial detergent (Ariel) commonly used in the Egyptian market. This application of the two isolates as a biodetergent additive was carried out on two types of stain:-

a) White cotton cloth pieces stained with blood.
b) White cotton cloth pieces stained with Chocolate.

The experiment summarized in the following steps:

1- Flask contain 100 ml, Glycine-NaOH buffer pH 11.0 for isolate ***St. halstedii* Salh-12** and pH 9.0 for isolate ***Streptomyces endus* Salh-40** + stained cloth without any treatment as a control (a & b).
2- Flask contains 100ml of used buffer + stained cloth+ 1g commercial detergent (Ariel).
3- Flask contain 100 ml of used buffer + stained cloth + 1.0 ml of protease enzyme solutions for the two tested isolates + 1g commercial detergent (Ariel)..
4- Then all of these stained cloth piece were incubated at 40ºC for ***St. endus* Salh-40** and 50ºC for ***St. halstedii* Salh-12** for 60 minutes.
5- After incubation period, cloth pieces were taken out, rinsed with water and dried **(Labena, 2004).**

<u>Degradation of human hair:-</u>

Untreated black a human hair was used in these experiments. Human hair (1g) was suspended in 50ml of 50 mM glycine-NaOH buffer pH 9.0 and 11.0 for ***St. endus* Salh-40** and ***St. halstedii* Salh-12**, respectively. 1ml of two proteases was added to the hair suspension and the reaction mixture was incubated at 40ºC and 50ºC for ***St. endus* Salh-40** and ***St. halstedii* Salh-12,** respectively for 1h. The reaction mixture was immersed in ice water for 5 min and filtered with a fitter paper No. 1 (Whatman, Germany) to stop the enzymatic reaction **(Takami ., 1992).**

RESULTS AND DISCUSSION

Sixty halotolerant actinomycetes isolates were isolated from different salt marches and cultivated soil samples in presence of 7% NaCl in growth medium under mesophilic (at 35ºC)

and thermophilic (at 55ºC) conditions. The highly active proteolytic thermophilic isolates no. 12, 17 and 28 and mesophilic isolates no. 40 and 50 (5 isolates) were chosen depending on diameter of the clear zones (3.5, 2.8, 3.2, 4.2 and 2.9 cm, respectively) assaying by disk method technique. In this connections, they could be isolated actinomycetes population from marine sediments **(Goodfellow and Haynes; 1984; Weyland and Helmke; 1988 and Ravel *et al.*, 1998).** Slight halophiles grow optimally at 0.2-0.85 mol/1 (2-5%) NaCl, moderate halophiles grow optimally at 0.85-3.4 mol/1 (5-20%) NaCl; and extreme halophiles grow optimally above 3.4-5.1 mol/1 (20-30%) NaCl **(Oren, 1999).** In contrast, non-halophiles grow both in high salinity and in the absence of a high concentration of salts. Many halophiles and halotolerant microorganisms grow over wide range of salt concentrations with requirement or tolerance for salts sometimes depending on environmental and nutritional factors **(DasSarma and Arora, 2001). Li *et al.* (2003)** reported that, the optimum growth of novel, moderately halophilic actionomycete, strain YIM 90007(T); was obtained at temperature between 35 and 37ºC in presence of 10% NaCl.

The maximum protease activities (89.7, 81.3 and 87.4 U/ml) produced by thermophilic isolates no. 12, 17 and 28, respectively in presence of 1% NaCl, while, the maximum protease activities (109.7 and 104.5 U/ml) produced by mesophilic isolates no. 40 and 50, respectively in presence of 2% NaCl (Table 1). **Kaur *et al.* (2001)** reported that ***Bacillus sp.*** P-2 can tolerate 20% NaCl concentration and produce little amounts of protease. While, **Kumar *et al. (*2004)** reported 10% NaCl concentration used for protease production by ***B. clausii***. Also, **Setyorini *et al*. (2006)** found that ***B. subtilis*** strain Fp-133 synthesized two novel halotolerant extracellular proteases (expro-I and expro-II), showing activity and stability at concentrations between 0 - 2% (w/v) NaCl.

The five selected and purified isolates were identified according to description of species in articles of ISP reported by **Shirling and Gottlieb (1966; 1968 a,b; 1969 and 1972)** and key of Bergey's Manual of **Williams *et al.* (1984)** depending on chemical properties, colonial, morphological, physiological characters and microscopic examination (using light and electron microscopes). Four species belonged to genus ***Streptomyces*** identified as; ***St. halstedii* Salh-12, *St. clavus* Dakh-28,** and ***St. flaveolus* Salh-17** (thermophilic isolates); ***St. endus* Salh-40** and one was identified as ***Actinomyces aureocirculatus* Dakh-50** (mesophilic isolates).

The protein finger print technique was carried out for the selected isolates to confirm the identification of isolates. Results presented in fig (1) revealed that, the total cellular proteins of the vegetative cells of halotolerant tested isolates no. 12,50,28,17 and 40 were fractionated in deatured gel by electrophoresis (SDS-PAGE) and the protein bands were visualizes by staining with Coomassie blue-GR250. The figure showed the results of protein banding patterns of the five isolates at vegetative phase of growth.

Isolates number 12 and 40 identified as ***St. halstedii* Salh-12** (thermophilic isolate) and ***St. endus* Salh-40** (mesophilic isolate) were the most halotolerant and proteolytic isolates. Thus had been chosen for further studies under optimum cultural and nutritional conditions for maximum growth and protease production; also the effect of different chloride metal ions with different concentrations. The results presented in fig (2&3) revealed that, the proteases production by ***St. halstedii* Salh-12** and ***St. endus* Salh-40** were highly affected by the addition of ions in lower concentration (0.05) than at higher concentrations (1&2%), where, the best metal ion was Ca^{+2} for both isolates (148.4 and 138.8 U/ml, respectively) followed by Mg^{+2} , Ba^{+2} and Mn^{+2} which had stimulatory effects on protease production at while others as Cd^{+2} and Hg^{+2} had an inhibitory effect on protease production at high and low concentrations and only with 1% $FeCl_3$ with the former isolate and not the latter isolate. However, NaCl has stimulatory effect on activity and specific enzyme activity by both halotolerant thermophilic and mesophilic isolates at higher concentration than at lower concentrations. These results

agreed with those of (**Nikaido and Nakae, 1979*)*** who reported that, proteolysis by ***B. fragilis*** was enhanced by Ca^{+}, Mg^{+2} and Mn^{+2}. This can be attributed either to stabilization of the outer membrane or to interaction of the metal ions directly with the proteases **(Millet, 1970; Morihara, 1974).** The Ca^{+2} was also known to play a major role in enzyme stabilization at higher temperatures and in many reports, Ca^{+2} had been shown to increase the activity and thermal stability of alkaline protease at higher temperatures **(Durham *et al.*, 1987; Rahman *et al.*, 1994 and Kumar *et al.*, 1999).** On other hand, the inhibitory effect of metal ions such as iron ions may be bind to the enzyme and inactivate it (oligodynamic effect).

Further work occurred in order to increase the specific enzyme activity was the purification of halotolerant thermophilic ***St. halstedii*** **Salh-12** and halotolerant mesophilic ***St. endus*** **Salh-40** crude enzyme in cell free extracts. Results presented in table (2) revealed that the partial purification using 80% saturated ammonium sulphate solution at 4ºC for an over night increased the specific enzyme activity (SEA) by 2.14 fold with 108.0% yield for ***St. halstedii*** **Salh-12;** and 2.21 fold with 111.9 % yield for ***St. endus*** **Salh-40**.

P4r4eurification using column of Sephadex G-200 and eluted by glycine - NaOH buffer pH 10.0 (50 mM) increased the (SEA) for ***St. halstedii*** **Salh-12** to 136.2 U/mg.protein with yield of 70.4 % and purification fold 2.95; while, for ***St. endus*** **Salh-40** was 791.0 U/mg.protein with yield of 75.5 % and purification fold 14.46 (table 2, and figs 4&5). Many reports had been published on purification of different microbial proteases using ammonium sulphate precipitate and anion exchange chromatography method **(Yamamoto *et al.*, 1987 and Fujiwara *et al.*, 1993; Hutadilok *et al.*, 1999; Bayoudh *et al.*, 2000**; **Nileganokar *et al.*, 2002 and Adinarayana *et al.*, 2003).**

The molecular weight of purified enzyme of ***St. halstedii*** **Salh-12** and ***St. endus*** **Salh-40** were determined by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). The molecular masses of ***St. halstedii*** **Salh-12** and ***St. endus*** **Salh-40** were 60 and 35 kDa (fig.6). Generally, the molecular masses of alkaline protease from microorganisms range between 15 and 36 kDa, with few exceptions of high molecular mass, such as up to 90 kDa from ***B. subtilis*** **(Kato *et al.*, 1992).** Proteases with molecular weights of 22 and 38 kDa were purified and partially characterized from the marine bacterial species strains **(Lee et al., 1996 and Liu and Lee, 1999).** Extracellular alkaline protease from alkalophilic bacterium ***Alcaligenes faecalis*** was purified by combination of ion-exchange, and size-exclusion chromatographic methods the purified enzyme has molecular mass 67 kDa **(Thangam and Rajkumar, 2002)**, and 34 kDa for ***Vibrio harbeyi,*** another minor band of protease activity was found at 40 kDa **(Estrada and Marquez, 2003).**

The amino acid composition of the purified proteases produced by halotolerant thermophilic ***St. halstedii*** **Salh-12** and halotolerant mesophilic ***St. endus*** **Salh-40** were carried out by using Amino Acid Analyzer. Results in figs (7 & 8) revealed that, the higher amino acid amounts were glycine, analine (out of detection in figures) and arginine followed by proline and glutamic acid for ***St. halstedii*** **Salh-12** and ***St. endus*** **Salh-40** proteases. The protease amino acids were balanced by increased concentration of arginine (basic amino acid) under saline stress 1 and 2% NaCl in both isolates respectively associated with either thermophilic and mesophilic which accounted to 79.79% and 76.75% respectively of the total content of amino acids pool with an increase 1.8 fold of the former than the latter. Also, the thermophilic protease exhibited significant increase in accumulation of basic amino acids (NH_4)and oxy amino acid (proline) by 3.04 and 2.67 fold than the mesophilic respectively, whereas the levels of other detected amino acids exhibited a slight or insignificant difference between the two tested isolates. In the other hand, valine and glutamic acids in mesophilic protease were slightly higher compared with thermophilic protease. In these connections, salinity treatment (10 and 18%) resulted in significant accumulation in proline content in halotolerant and moderate halophilic isolates **(Csonka, 1989);** may a role in stress of

adaptation within the cell **(Gilbert *et al.*, 1998 and Soussi *et al.*, 2001).** The accumulation of amino acids during salinity stress suggested that these compounds may play a role in osmotic adjustment and serve as available sources of carbon and nitrogen **(Abdel-Kader and Saleh, 2002).** The cells may had evolved a mechanism to coordinate synthesis, catabolism and transport activities for the accumulation of proline **(Hong *et al.*, 2002).** The accumulated proline and glutamic acid were formed as a result of their synthesis not by being up taken from the medium **(Skjerdal *et al.*, 1996).** Several explanations for the accumulation of free amino acids under stress had been suggested. These include stimulated de novo synthesis inhibited degradation of amino acids, impaired protein synthesis and/or enhanced protein degradation **(Gilbert *et al.*, 1998 and Soussi *et al.*, 2001).**

On other hand, intracellular accumulation of free alanine under salt stress had previously been demonstrated in ***St. griseus*** and ***St. califoricus*** **(Kilham and Firestone, 1984).** Streptomycetes indigenous to saline soils were found to accumulate the neutral amino acids, alanine which was not found before as a compatible solute in streptomycetes **(Kilham and Firestone, 1984).** They also added that the intracellular concentrations of the free amino acid pool increased in response to salt stress and the neutral free amino acids proline, glutamine and alanine accumulated are salinity increased and the concentration of the acidic free amino acids glutamate and aspartate fell down.

Characterization of the purified proteases of ***St. halstedii*** **Salh-12** and ***St. endus*** **Salh-40** were tested. Data presented in figs 9and 10 (a,b,c,d &e) showed that, 50ºC and 40ºC were the optimum temperature for maximum protease activity at pH 11.0 and 9.0 after 1h incubation, respectively, when 1ml of pure enzyme was inoculated in reaction mixture containing 1% casein as substrate. These results agreed with those of **Schmidt *et al.* (1995)** who reported that, 40ºC was the optimum for protease activity of some strains of ***B. cereus*** and 50ºC for ***B. firmus*** N23A at pH 11.0 **(Gamal *et al.*, 1999)**. **Abd-Rahman *et al*. (1998)** reported that alkaline protease isolated from active culture of ***B. stearothermophilus*** retained about 80-100% of its initial activity within pH range 10.5-11.5 at 70ºC for 45 minutes. ***Alcaligenes faecalis*** at pH 9.0 **(Thangam and Rajkumar, 2002).**

Alkaline proteases find extensive applications in the detergent industry and were produced in large quantities from high yielding strains of ***Bacillus*** **(Ward, 1985 and Kalisz, 1988**). The results in plates (1,2,3,4,5 & 6) revealed that, the stained cloth pieces treated with proteases produced by ***St. halstedii*** **Salh-12** and ***St. endus*** **Salh-40**, were found to be cleaner than the cloth pieces treated with the ariel detergent alone, when washed in the 50ºC and 40ºC buffer, respectively for 1h. if compared with untreated. This agreed with of other investigators; **Gerhartz (1990); Ikasari and Mitchell (1994); Banerjee *et al*. (1999)** and **Nilegaonkar *et al. (*2002)**, also, **Gerhartz (1990)** and **Horkoshi (1990)** reported that, alkaline proteases were extensively used in laundry detergents for protease stain removal. The ideal detergent protease should possess broad substrate specificity to facilitate the removal of a large variety of stains due to food, blood and other body secretions. Activity and stability at high pH and temperature and compatibility with other chelating and oxidizing agents added to the detergents were among the major prerequisites for the use of proteases detergents **(Asha *et al.*, 1993).** All proteases were stable in presence of various components of detergents and are active at washing temperatures and pH values **(Gupta *et al.*, 1999).**

Another application for both tested isolates enzyme was degradation of human hair. The present results in plate 10 (a,b &c) showed that, the human hair degraded by about 50% with 1h at 50ºC and 40ºC at pH 11.0 and 9.0 for both tested pure protease, respectively. The effect of both pure enzymes was more or less the same. These were appeared to be potentially useful in washing powder. Alkaline protease speeds up the process of dehairing, because the alkaline conditions enabled the swelling of hair follicle protein and allowed easy removal of the hair. **Horikoshi (1999)** reported that alkaline enzymes had been used in the hide-

dehairing process, where dehairing was carried out at pH values between 8 and 10. These enzymes were commercially available. Proteases are used for selective hydrolysis of non-fibrillar proteins of the skin and for removal of non fibrillar proteins such as albumins and globulins. The purpose of soaking is to swell the hide, and this step was performed with alkali **(Godfrey & West, 1996).**

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Table (1) Identification and screening of halotolerant and proteolytic active actinomycetes:

Coded number of isolates	**Identification**	**Temperature of isolation**	**Diameter of clear zone (cm)**	**Protease activity (U/ml)**	**NaCl (%)**
12	***St. halstedii* Salh-12**	thermophilic isolates	3.5	89.7	
17	***St. flaveolus* Salh-17**	at 55ºC	2.8	81.3	1%
28	***St. clavus* Dakh-28**		3.2	87.4	
40	***St. endus* Salh-40**	mesophilic isolates	4.2	109.7	2%
50	***Actinomyces aureocirculatus* Dakh-50**	at 35ºC	2.9	104.5	

Table (2) Purification profile of protease produced by *St. halstedii* Salh-12 and *St. endus* Salh-40.

	Purification steps	Protease activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg protein) (SEA)	Purification factor (fold)	Yield
***St. halstedii* salh-12**	**1-Culture-filtrate crude extract**	142.1	3.05	46.2	1	100
	2- Precipitation by $(NH_4)_2SO_4$ (80%)	153.5	1.55	99.0	2.14	108.0
	3- Sephadex-G200	100.0	0.734	136.2	2.95	70.4
***St. endus* salh-40**	**1-Culture-filtrate crude extract**	151.9	2.78	54.7	1	100
	2-recipitation by $(NH_4)_2SO_4$ (80%)	170.0	1.40	121.4	2.21	111.9
	3- Sephadex-G200	114.7	0.145	791.0	14.46	75.5

$$\text{Purification factor} = \frac{\text{SEA of purified enzyme}}{\text{SEA of crude enzyme}}$$

$$\text{Yield (\%)} = \frac{\text{Total activity of purified enzyme}}{\text{Total actviity of crude enzyme}} \text{ X } 100$$

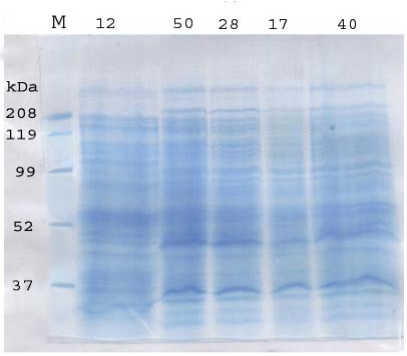


Fig. (1): Coomassie blue R-250 blue SDS-PAGE for the total cellular proteins of the vegetative cells of the five actinomycetes isolates. Lanes from 1-5 banding patterns of the five isolates.

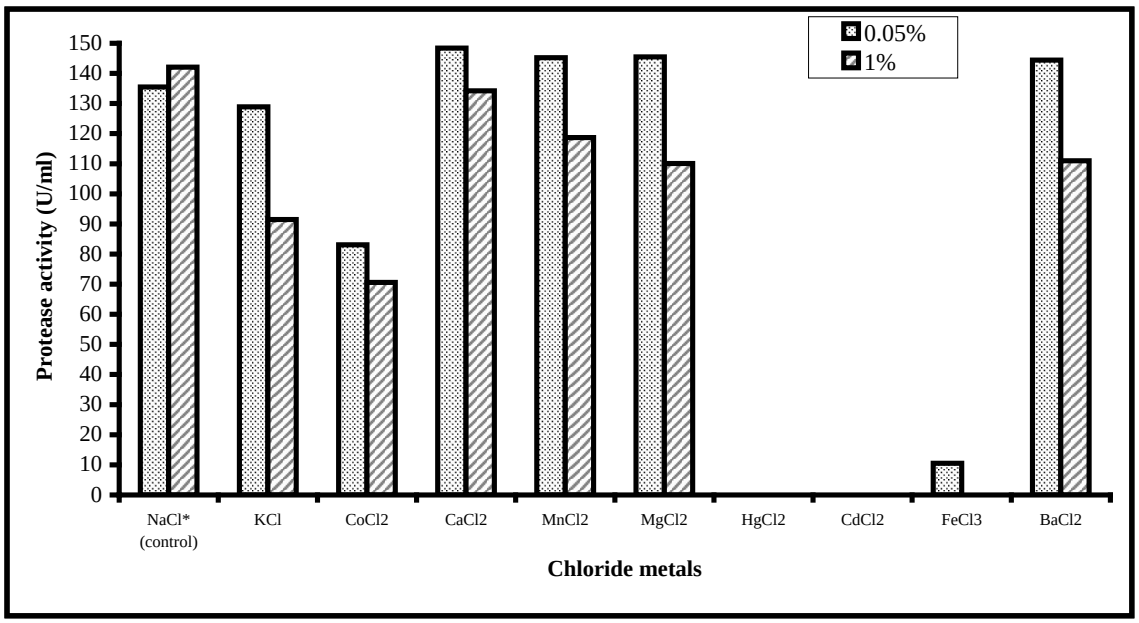


Fig. (2): Effect of different chloride metal ions different conc. (0.05 and 1%)on protease activity (U/ml) by most halotolerant and thermophilic actinomycete isolate *St. halstedii* Salh-12.

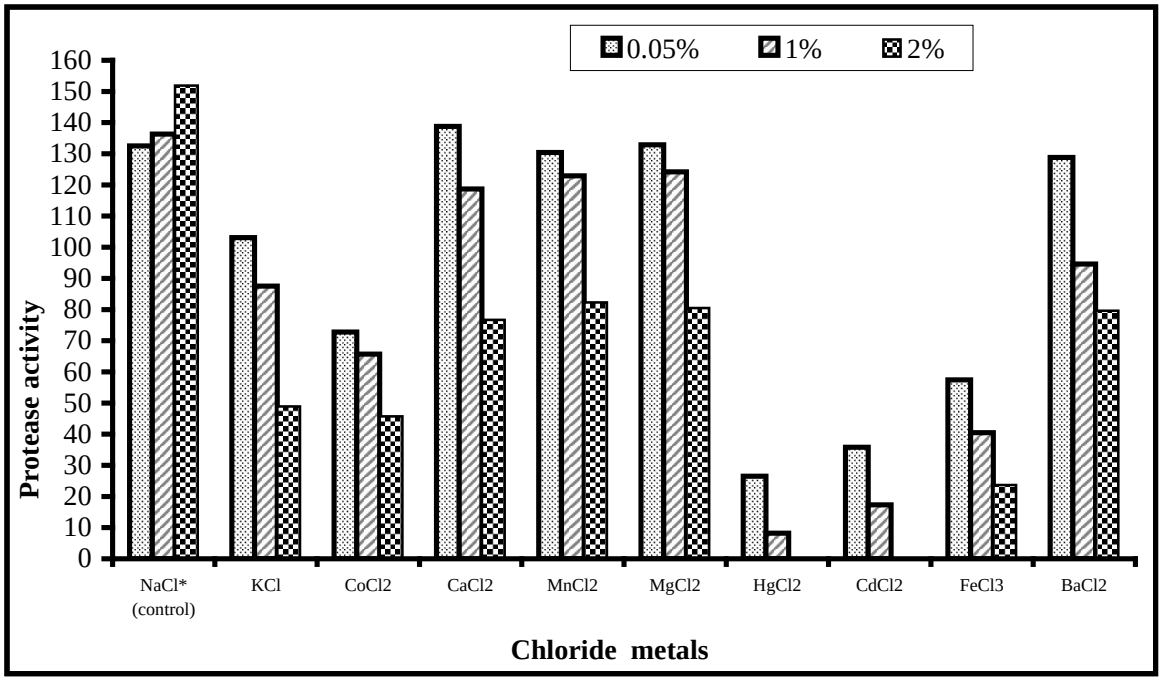


Fig. (3): Effect of different chloride metal ions with different conc. (0.05, 1 and 2%) on protease activity (U/ml) by mesophilic actinomycete isolates *St. endus* Salh-40.

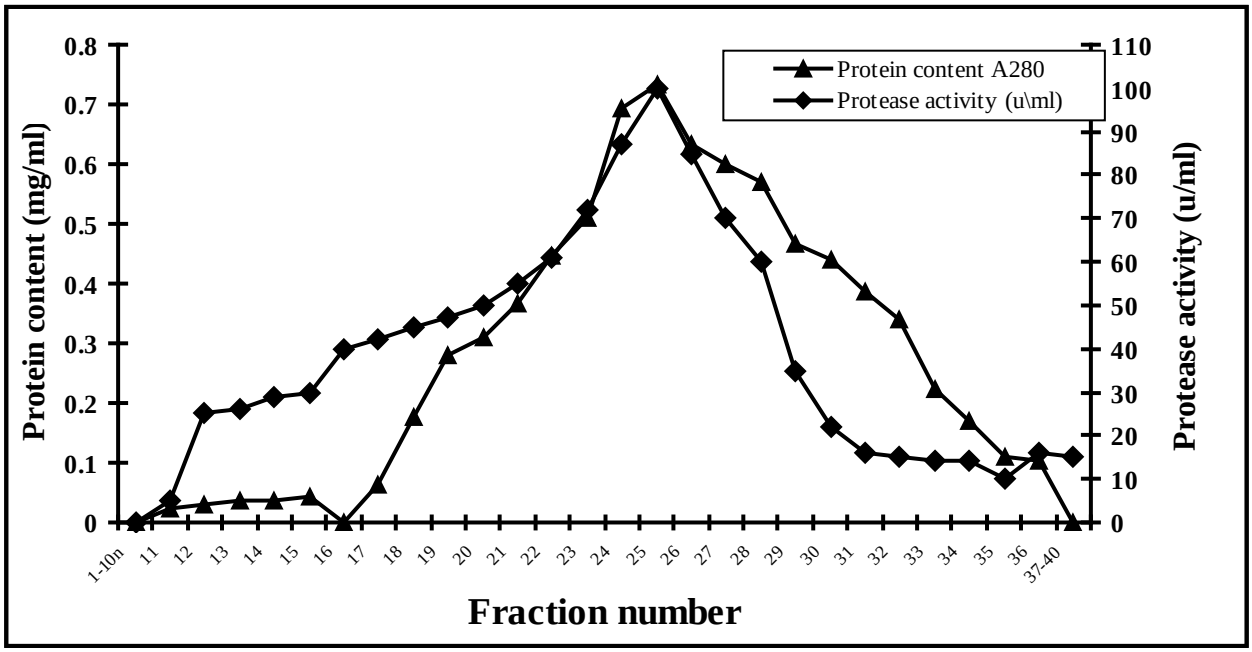


Fig. (4): Fractionations of protease on Sephadex G200 by halotolerant thermophilic *St. halstedii* Salh-12 .

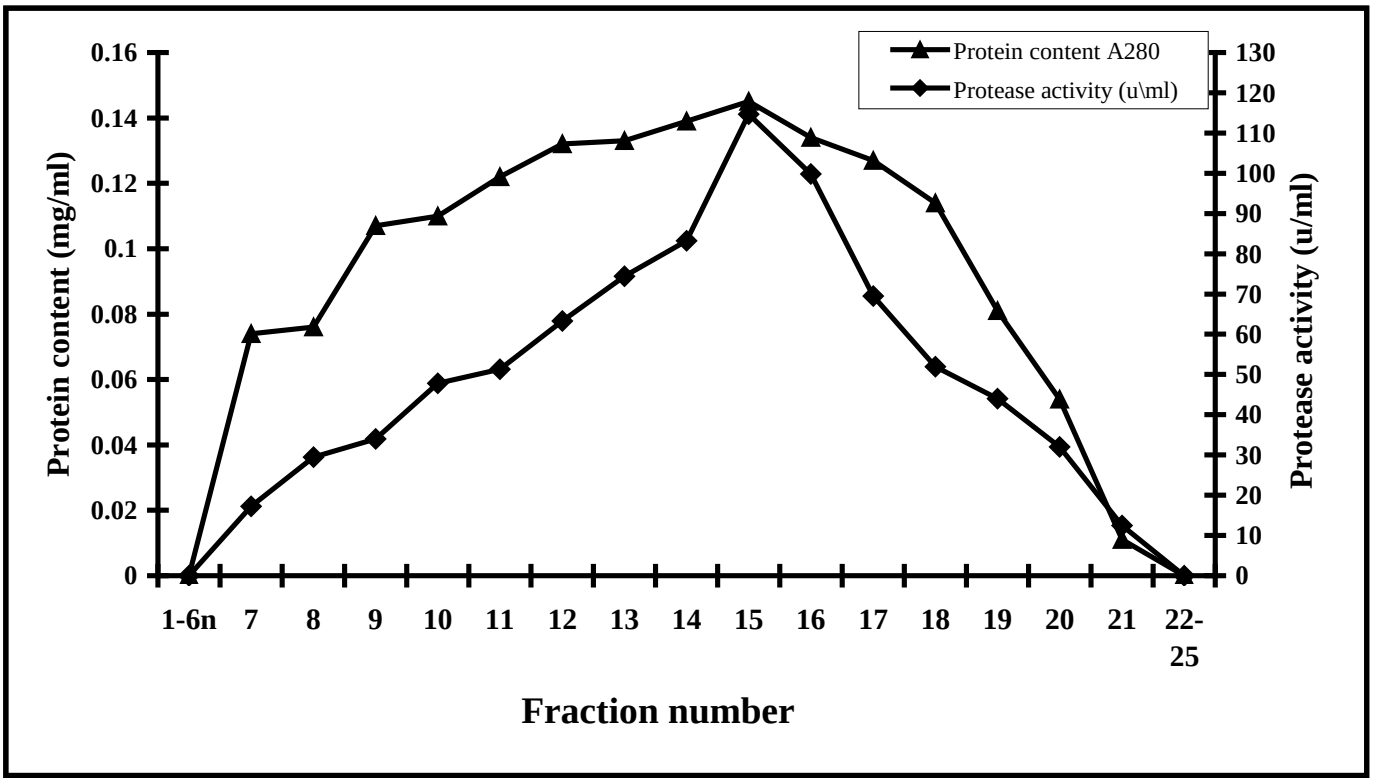
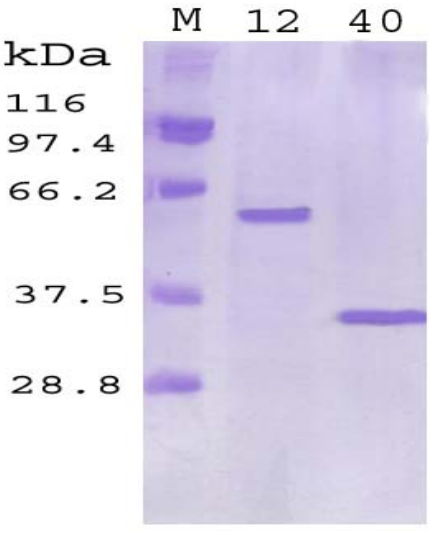


Fig. (5): Fractionations of protease on Sephadex G200 by halotolerant mesophilic *St. endus* Salh-40.

Fig. (6): The molecular weights of pure enzyme produced by ***St. halstedii* Salh-12 (no. 12)** and ***St. endus* Salh-40 (no. 40)**.



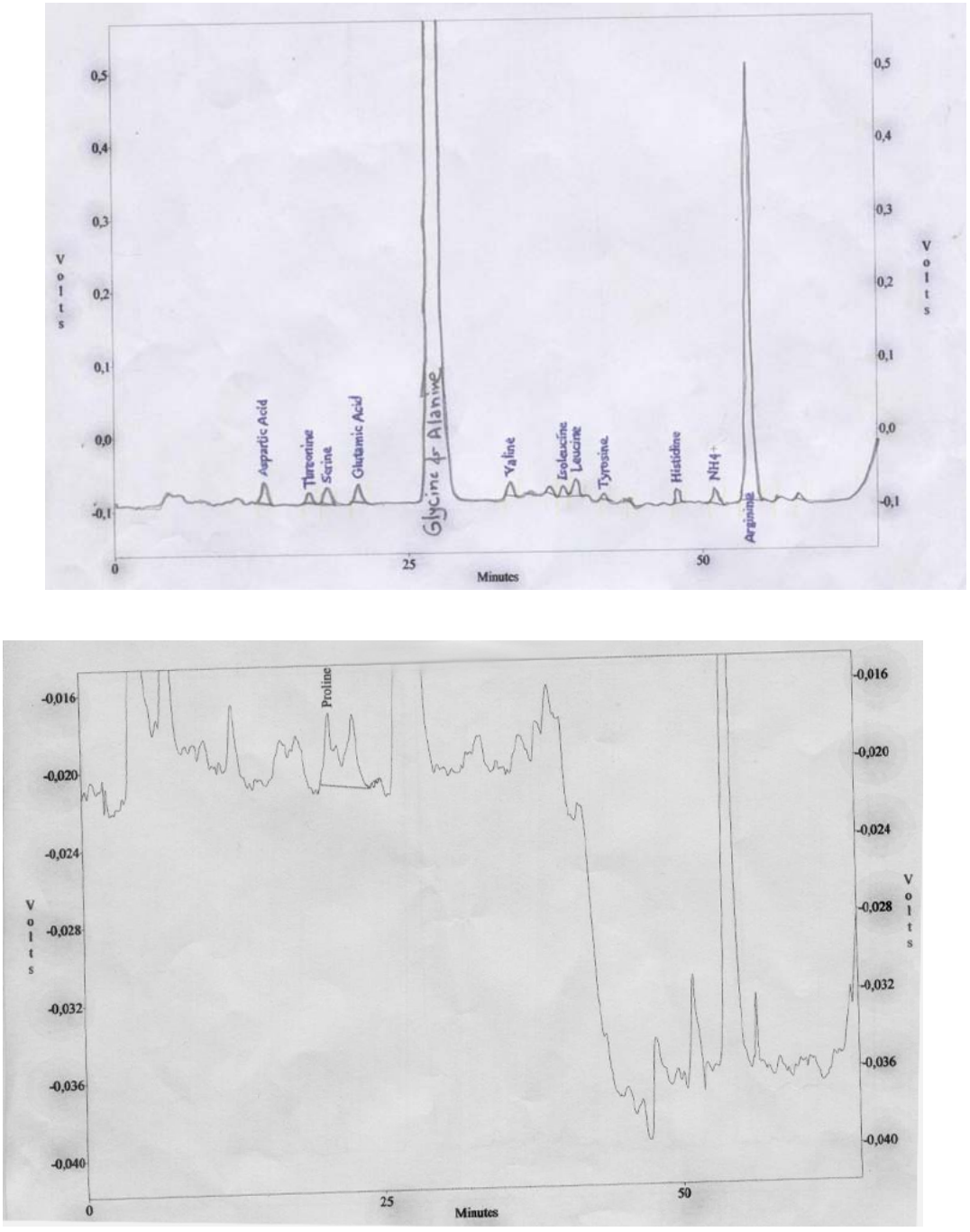


Fig. (7): Amino acids analytical pattern of standard amino acids of the purified protease produced by ***St. halstedii*** **Salh-12.**

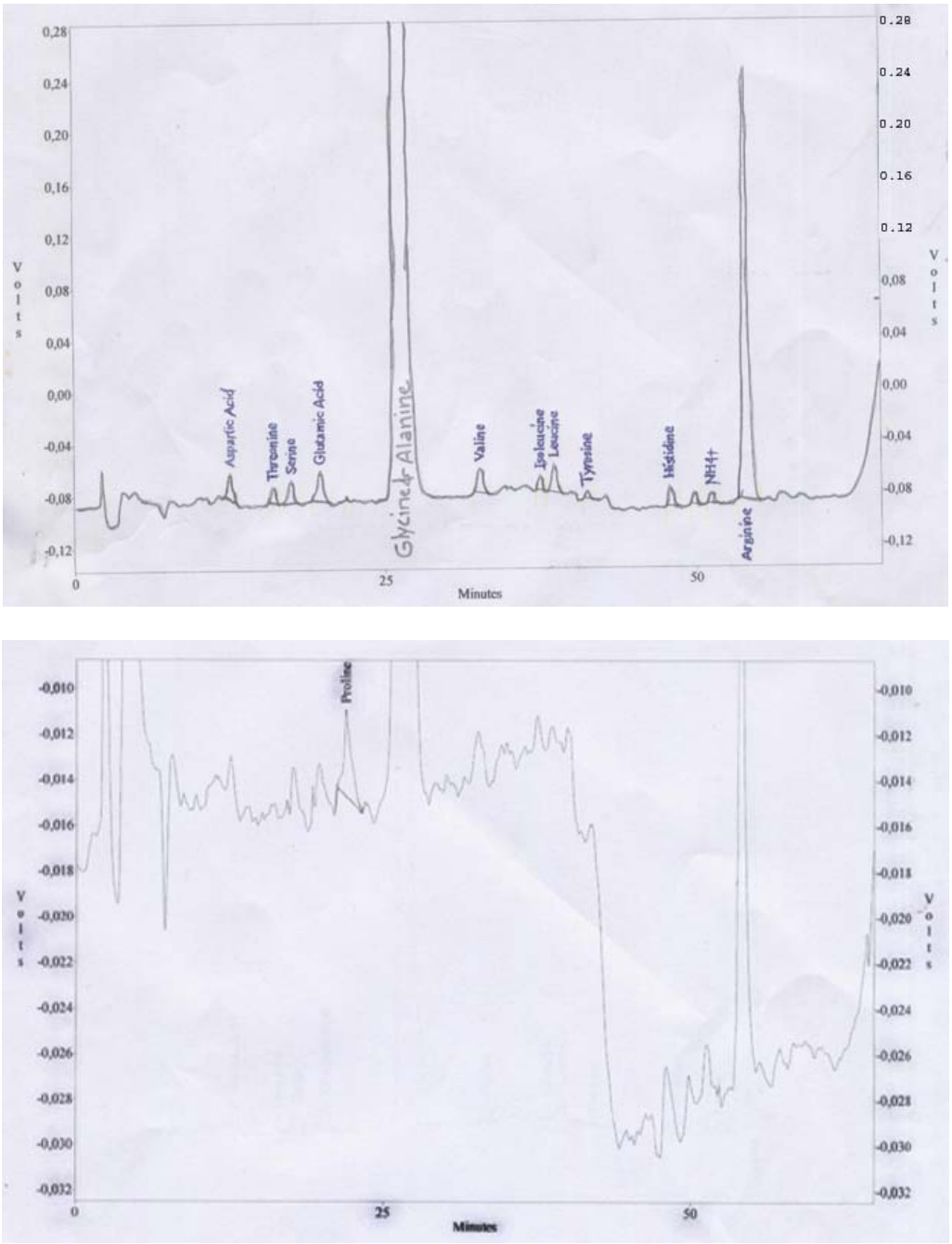


Fig. (8): Amino acids analytical pattern of standard amino acids of the purified protease produced by ***St. endus*** **Salh-40.**

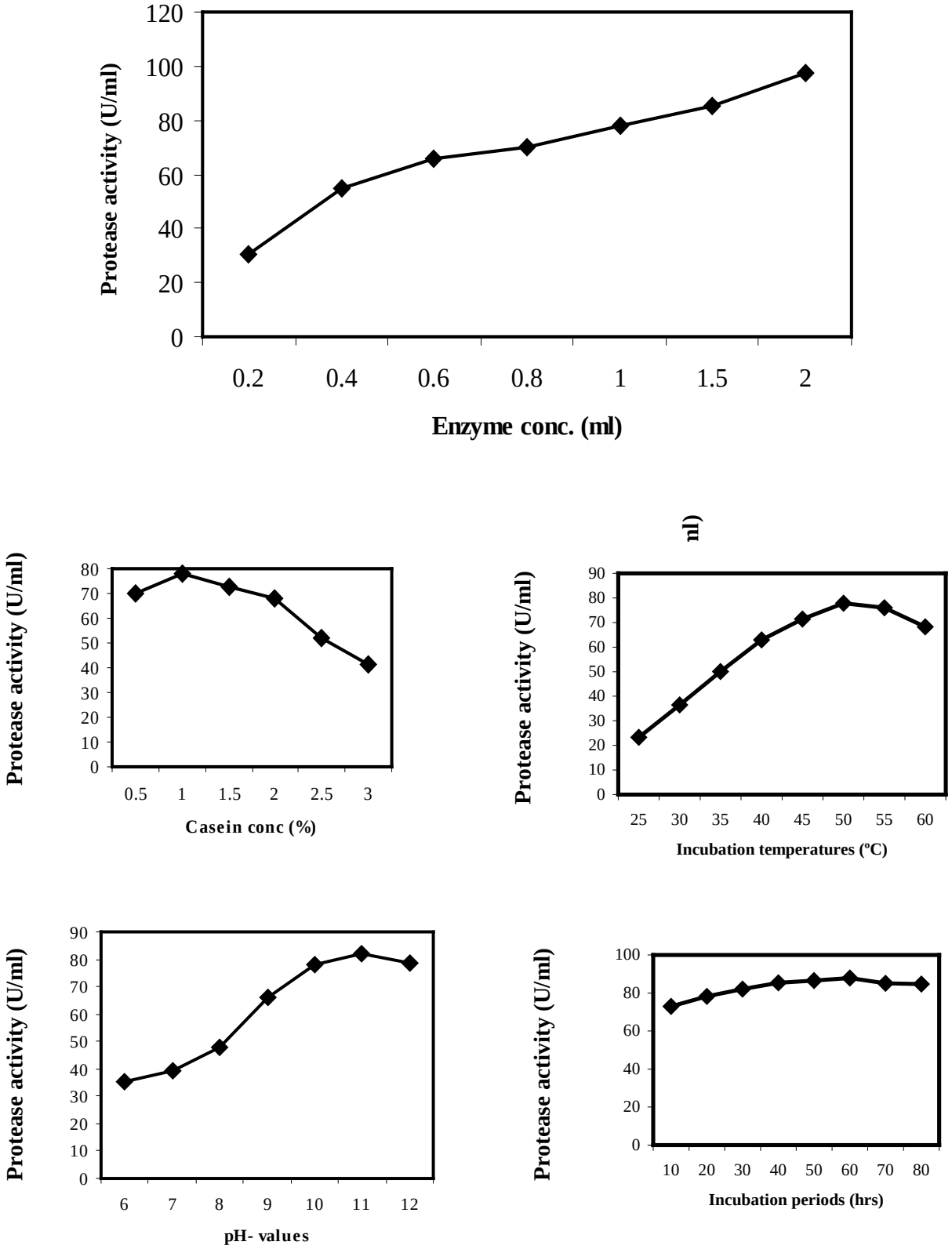


Fig. (9): Effect of (a) enzyme concentration (b) incubation temperature (c) pH- values (d) casein concentrations (e) incubation period on protease activity (U/ml) by *St. halstedii* Salh-12.

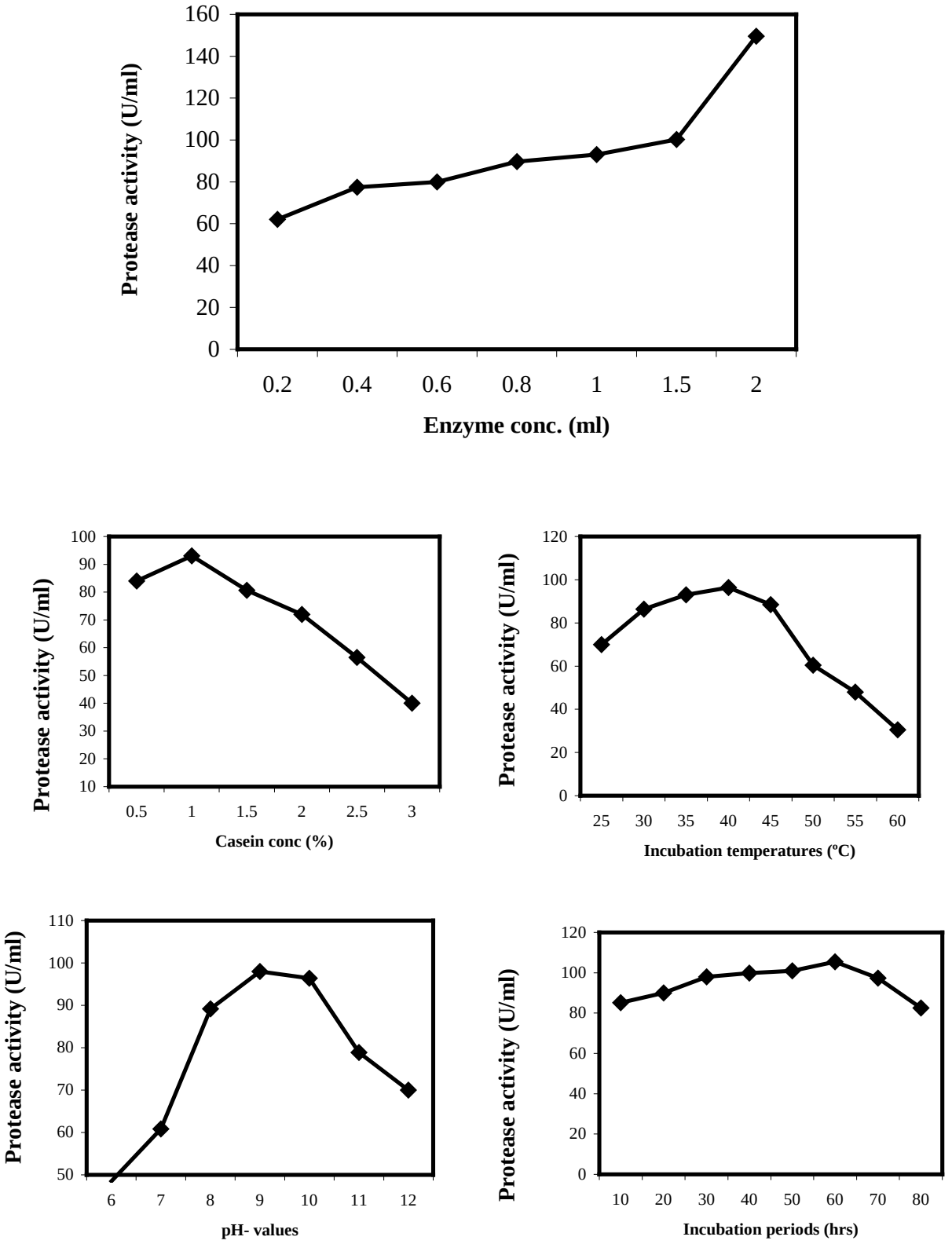
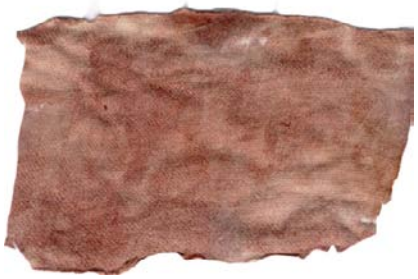


Fig. (10): Effect of (a) enzyme concentration (b) incubation temperature (c) pH- values (d) casein concentrations (e) incubation period on protease activity (U/ml) by *St. endus* Salh-40.



(a)

(b)

Plate (1): Showing stained cloth pieces with (a) blood and (b) chocolate as a control.

Plate (2) showing cloth piece stained with blood and treated with Ariel detergent and ***St. halstedii*** **Salh-12** protease at 50^{o}C.

Plate (3) showing cloth piece stained with blood and treated with Ariel detergent only at 50^{o}C.

Plate (4) showing cloth piece stained with chocolate and treated with Ariel detergent and ***St. halstedii*** **Salh-12** protease at 50^{o}C.

Plate (5) showing cloth piece stained with chocolate and treated with Ariel detergent only at 50^{o}C.

Plate (6) showing cloth piece stained with blood and treated with Ariel detergent and ***St. endus*** **Salh-40** protease at 40^{o}C.

Plate (7) showing cloth piece stained with blood and treated with Ariel detergent only at 40^{o}C.

Plate (8) showing cloth piece stained with chocolate and treated with Ariel detergent and ***St. endus*** **Salh-40** protease at 40^{o}C.

Plate (9) showing cloth piece stained with chocolate and treated with Ariel detergent only at 40^{o}C.

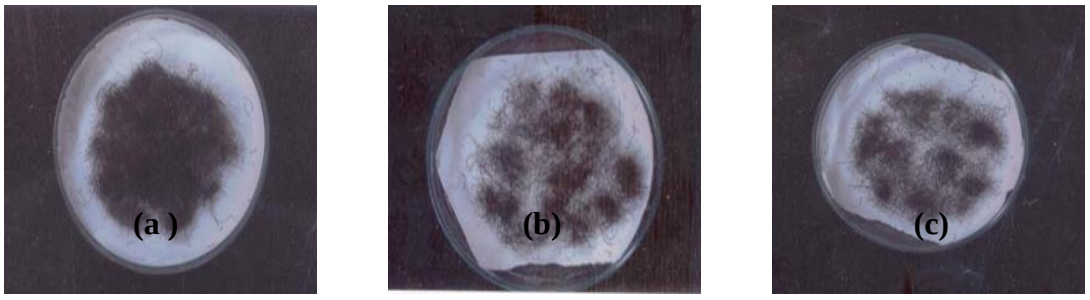


Plate (10)

(a) Untreated human hair incubated at 50 ºC and pH 11.0& at 40ºC and pH 9.0 as a control.

(b) Human hair treated with ***St. halstedii*** **Salh-12** protease and incubated at 50 ºC and pH 11.0.

(c) Human hair treated with ***St. endus*** **Salh-40** protease and incubated at 40 ºC and pH 9.0.

تنقية ودراسة خواص أنزيم البروتيز المفرز بواسطة بعض الأكتينوميستات المعزولة تحت بعض الظروف البيئية الصعبة والظروف المثلى لنشاطه

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تم عزل ستون سلالة من الأكتينوميسيتات المقاومة للملوحة من الأراضى المالحة وأراضى مزروعة في أماكن مختلفة من جمهورية مصر العربية. تم اختيار 5 عزلات الأكثر مقاومة للملوحة والمنتجة لإنزيم البروتيز والتي نمت في وجود 7%من كلوريد الصوديوم تم تعريفهم باسم استربتوميسس هاليستدى، استربتوميسس أوليفشيس واستربتوميسس كلافس (عند 55 °م) استربيوميسس أنديس وأكتينوميسس أيروسيركيولاتس (عند 35 °م). وتم عمل بصمة البروتين للعزلات المختارة. تم اختيار أكثر العزلات نشاطاً للإنزيم وأكثرهم مقاومة للملوحة تحت ظروف درجة الحرارة العالية والمتوسطة هما استربتوميسس هاليستدى واستربتوميسس أندس على التوالى لمزيد من الدراسة. وكان أعلى نمو ونشاط لإنزيم البروتيز المفرز بواسطة الكائنين فى وجود 1و2 % من ملح كلوريد الصوديوم بعد 4 و6 أيام من التحضين ورقم ايدروجينى 8 و 9 فى بيئة سائلة مهتزه تحتوى على 1% من سكر المانوز والكازين كمصدر كربون ونيتروجين على التوالى. وجد أن أيونات أملاح الكلوريدات المختلفة لها تأثير على انتاج الإنزيم حيث كان تركيز أيونات الباريوم والمنجنيز والماغنسيوم والكالسيوم المنخفضة (0.05%) لها تأثير محفز لإنتاج الإنزيم بواسطة الكائنين تحت الاختبار بينهما تأثيراً مثبطاً فى التركيزات العالية(1و2%) فيما عدا أيونات الصوديوم محفزة لإنتاج الأنزيم بواسطة الكائنين فى التركيزات العالية أكثر منها من المنخفضة. أدى ترسيب الأنزيم الخام بواسطة 80% كبريتات الأمونيوم إلى زيادة النشاط النوعى لأنزيم البروتييز إلى 2.14 و 2.21 مرة ، وبعد التقنية باستخدام السيفاديكس ج 200 زاد نشاط الإنزيم إلى 2.95و 14.46 مرة للستربتوميسس هاليستدى واسترميسس اندمس على التوالى. وباستخدام جهاز الفصل الكهروماتوجرافى وجد أن الوزن الجزيئى للأنزيم النقي هو 60و 35 كيلودالتون المنتج من الكائنين استربتوميسس هاليستدى واستربتوميسس اندس على التوالى وبتحليل الأحماض الأمينية للأنزيم وجد ان أعلى تركيز من حمض أمينى كان الألنين والجليسين والأرجنين متبوعاً بالبرولين وحمض الجلوتاميك.تم الحصول على أعلى نشاط من الأنزيم النقى فى بيئة التفاعل التى تحتوى على 1% كازين ورقم ايدروجينى 11 و 9 وتحت درجة حرارة 50و 40 °م للكائن استربتوميسس هاليستدى واستربتوميسس اندس على التوالى. وتم دراسة إمكانية الأنزيم المنتج من استخدام استربتوميسس هاليستدى واستربتوميسس اندس فى المنظفات وتحليل شعر الإنسان.

PROCEEDING OF THE SECOND SCIENTFIC ENVIRONMENTAL CONFFER, 2007, ZAGAZIG UNI., 177- 186

تقدير المواد الصلبة المذابة في بعض العينات من مياه الشرب (المعبأة , المحلية) بالجماهيرية الليبية

TOTAL DISSOLVED SOLIDS DETERMINATION IN SOME SAMPLES OF DRINKING & BOTTLED WATER IN LIBYAN JAMAHIRIYA

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ملخـص

تشهد الجماهيرية تطوراً كبيراً في مجالات عدة كالصناعة والزراعة الأمر الذي ينتج عنه استهلاك كبير في المياه الجوفية والتي تعتبر المصدر الرئيسي للمياه. الأمر الذي يؤدي إلى حدوث خلل في الميزان المائي لبعض الخزانات الجوفية كاختلاط المياه العذبة بمياه البحر أو تغير خواص هذه المياه أو تسرب مياه المجاري أو وصول مخلفات صناعية لمصادر هذه المياه مما يستدعي دراستها بصفة دورية مستمرة خصوصاً أن عدد السكان في زيادة مستمرة.

وفي الآونة الأخيرة انتشرت ظاهرة استعمال المياه المعبأة وتعددت مراكز المعالجة والتعبئة. الجدير بالذكر أن هذه المياه هي عمل جيد وتخدم السكان بشكل كبير ولكن الشائع عند العامة أن هذه المياه صالحة تماماً للشرب والمعروف أن هذه المياه هي مياه جوفية تتم معالجتها بالعديد من العمليات ويتم عمل بعض التحاليل الروتينية عليها بينما الكثير من التحاليل المهمة لا تجرى لعدم توفر الإمكانيات أحيانا" في المختبرات ذات العلاقة وكثيراً ما يكثر الجدل حول مدى صلاحية هذه المياه للشرب ومدى ماتسببه من أضرار للبيئة.

المواد الصلبة تشير للمواد العالقة أو المذابة في الماء ويمكن أن تؤثر عكسيا على جودة المياه إذا زاد تركيزها عن الحد المسموح به . والمياه التي تحتوي على مواد صلبة مذابة عالية عادة تكون غير مفضلة للشرب ويمكن أن تسبب تفاعلات فسيولوجية غير مفضلة ولهذا تم تحديد 500 – 1000 مليجرام/لتر من المواد الصلبة المذابة ((Total dissolved solids)) كحد أقصى. وكذلك تكون غير صالحة لكثير من التطبيقات الصناعية. تحليل المواد الصلبة مهم في التحكم في الخواص الفيزيائية والفسيولوجية لمياه الفضلات أثناء عملية المعالجة . تم في هذا البحث تقدير المواد الصلبة TDS وقد تم شراء عينات مياه من مراكز التوزيع التجارية وهذه العينات هي ((الكفرة, سفاري , النبع , المزن , صافيا)) وأخذنا عينة من مياه الصنبور (المياه المحلية) وعينة من ماء واحة قبرعون وعينة مختلطة من (ماء الصنبور +مياه النبع) وأجرينا عليها بعض التجارب المعملية لتقدير الأملاح الصلبة المذابة(T.D.S) . جميع عينات المياه المعبأة كان لها تراكيز أقل من 500 ملجم/لتر حيث كانت التراكيز (260 ، 65 ، 40 ،305 ،90) ملجم /لتر لعينات مياه الكفرة وسفاري والنبع والمزن وصافينا على التوالي في حين تجاوزت 1500 ملجم/لتر ينما كانت الأملاح الصلبة المذابة لعينة قبرعون اكبر من مياه البحر بحوالي ست مرات.

المقدمــة

نظراً للزيادة السكانية الحاصلة في العالم وتنوع استخدامات المياه في مجالات الحياة التي تعددت وازداد حجمها صناعياً وزراعياً وخدمياً بالإضافة إلى الاستخدام الأساسي وهو الاستهلاك المباشرالأمر الذي سبب في زيادة الطلب على كمية المياه الصالحة للشرب ومن المعروف أن كمية الماء العذب والصالح للشرب وللزراعة يمثل 1% من المياه في العالم والتي تمثل نسبة الماء العذب. وزيادة الطلب على المياه نتيجة للتقدم الزراعي والصناعي وغيره وزيادة استهلاك المياه نتيجة للتطور في عدد السكان وزيادة المشروعات الصناعية وغيرها يتحتم المحافظة على المياه السطحية (أنهار، بحيرات، ينابيع وغيرها) من التلوث. ويميل الاختصاصيون إلى حصر مصادر تلوث المياه في أربعة مجالات، وهي:-

- الفضلات والمجاري: وهما يؤديان إلى تلوث بكتيري بشكل رئيسي.
- الصناعة والزراعة: وهما يؤديان إلى تلوث كيميائي بشكل رئيسي.
- التلوث الحراري.
- التلوث بالإشعاع

لذا فالاهتمام بالمصادر المائية أصبح ضرورة ملحة والإلمام بخصائص المياهالنوعية بشأنها أصبح واجباً وطنياً للمحافظة عليها من التلوث وكذلك من الهدر الجائر الغير مبرر للمياه. وسوف نركز في بحثنا هذا على بعض أنواع المياه التجارية في الجماهيرية.

من المعروف أن شركات المياه المعبأة تحشد كل قدراتها لتسرب للمستهلك اعتقادًا بأن مياه الزجاجات البلاستيكية هو قارب الإنقاذ، وحبل النجاة، له ولأسرته، في مواجهة مياه الصنبور غير الصالحة للشرب، بالرغم من أنَّ الأخيرة تخضع في الوقع وفى معظم الحالات لإجراءات مراقبة لعمليات تنقيتها، وإجراءات تدقيق لجودتها،أشد من تلك التي تخضع لها المياه الفاخرة المعبأة في زجاجات، وقد نجحت الشركات المنتجة بالفعل، في اجتذاب جمهور كبير من المستهلكين للمياه المعبأة، بدليل الرواج المتصاعد لتلك الصناعة والذي يغذيه توجه يسري بين هؤلاء المستهلكين يرى أنَّ استخدام المياه المعبأة دليل على رقي الوضع الاجتماعي بالإضافة إلى ارتباط تلك النوعية من المياه باعتبارات الصحة والسلامة.وعلى مستوى الاستهلاك يأتي الأمريكيون في المرتبة الأولى مع استهلاك 26 مليار لتر من المياه المعبأة في العام 2004 وبعدهم المكسيكيون بمعدل 18 مليار لتر ثم الصينيون والبرازيليون 12 مليار لتر لكل من البلدين[8].

أمَّا على المستوى الاستهلاك الفردي فيأتي الإيطاليون في المقدمة بنحو 184 لترفي العام 2004 ثم المكسيكيون بمعدل 169 لتراً والإماراتيون 164 لترا فالبلجيكيون والفرنسيون 145 لترا. وتشير الإحصاءات إلى أنَّ الدول النامية أصبحت أكثر استهلاكاً للمياه المعبأة بين عامة 1999 و 2004 بحيث ازداد الاستهلاك في الهند ثلاثة أضعاف فيما تضاعف في الصين ونتجت عن هذا الازدياد الكبير في استهلاك المياه المعبأة تكاليف ضخمة أيضاً لجهة تصنيع الزجاجات وغالبيتها من البلاستيك ونقلها البحري أو البري. وفى المقابل يتم تأمين مياه المنازل عبر شبكة توزيع أكثر حماية للبيئة واقل كلفة وفى حين يكفى إغلاق الصنبور بعد الاستهلاك في المنزل فإنَّ مصير القوارير البلاستيكية الفارغة غالبا ما

يكون في المصبات علماً أنه يتم تصنيع الزجاجات عادة من أحد مشتقات النفط الخام البولي إيثيلين/ تيريفتالات.[8]

وبالنسبة للولايات المتحدة فإنَّ تصنيع هذه الزجاجات يمثل أكثر من 1.5مليون برميل نفط سنويا ما يسمح لمائ ة ألف سيارة بالسير لمدة سنة أمَّا على المستوى العالمي فيتطلب تصنيع الزجاجات البلاستيكية من مادة البوليتين تيريفتالات 2.7 مليون طن سنويا من هذا النوع من البلاستيك، و86 بالمائة من الفراغات البلاستيك المستهلكة في الولايات المتحدة تنتهي في المكبات وتكمن خطورة هذا الواقع في أنَّ عملية الطمر تؤدى إلى انبعاث غازات سامة وإلى رماد يحتوى على معادن ثقيلة في حين أنَّ رمي الفراغات البلاستيكية في المكبات يوفر لها استمرارية قد تصل إلى ألف سنة قبل تحللها البيولوجي الكامل. والأسوأ من ذلك هو أنَّ أربعين بالمائة من الفراغات البلاستيكية التي تمَّ تجميعها في الولايات المتحدة عام 2004 ثم نقلها إلى دول بعيدة جداً مثل الصين مما زاد أيضاً من الأضرار التي تلحق بالبيئة وقد أدى إنتاج قوارير المياه المعدنية على النطاق الواسع إلى نقص في مياه الشرب و هذا ما حصل في الهند حيث تراجعت الطبقات المائية بصورة ملحوظة في خمسين قرية في حين كانت شركة كوكاكولا تستخرج منها المياه دون أي رقابة لتنتج مياه داساني التي يتم تصديرها لاحقا، وفى أربعين بالمائة من الحالات تجرى تعبئة القارورة بالمياه التي تصل إلى المنازل ثم يضاف إليها بعض المعادن التي ليست صالحة دائماً لصحة المستهلكين[8].

نتيجة للتطور العلمي الحاصل في العالم في جميع مجالات العلوم الهندسية والكيميائية والرياضيات، والفيزياء، والبيولوجي.. الخ. وكذلك فإن الوعي الصحي قفز قفزات كبيرة خلال الثلاثين عاماً الماضية نتيجة التطور الكبير الحاصل في العالم. إن هذا التطور الكبير جلب الانتباه إلى كافة المجتمعات بحيث تهتم بالموارد المائية ونوعيتها وخصوصاً مياه الشرب ولأجل هذا وضعت الاشتراطات القياسية لكل نوع من أنواع المياه وخصوصاً للاستهلاك البشري فهنالك المياه المعدنية الطبيعية، المياه المعدنية الطبيعية المكربنة، المياه المعدنية الطبيعية غير المكربنة، والمياه المعدنية المكربنة صناعياً، المياه المعدنية الفوارة، المياه الصحية الصالحة للشرب.

والمياه المعدنية هي المياه التي تتميز بما تحتويه من أملاح معدنية بنسب محددة وعناصر هذه الأملاح مصدرها الأساسي هي المصادر الطبيعية كالينابيع والآبار وهي ثابتة التركيز ومستقرة التدفق والحرارة مع الأخذ في الاعتبار دورات الفصول الطبيعية وتجمع تحت شروط نقاءها الأصلي من الناحية الجرثومية وتعبأ في عبوات محكمة مع اتخاذ كافة الاحتياطات الصحية الخاصة، ومن هذه المياه الآتي:-

1 - مياه معدنية طبيعية مكربنة طبيعياً: وهذه المياه تحتوي على غاز ثاني أوكسيد الكربون بالكمية المحددة من قبل المنظمات العالمية بما لا يقل عن 250 ملغم / لتر.
2 - مياه معدنية غير مكربنة: وهذه المياه لا تحتوي على غاز ثاني أوكسيد الكربون الحر بالمقدار الضروري للمحافظة على ذوبان أملاح البيكربونات.
3 - مياه معدنية مكرينة (فوارة) وهذه المياه يضاف إليها غاز ثاني أوكسيد الكربون من مصادر صناعة أخرى.
4 - مياه معدنية مزال منها ثاني أوكسيد الكربون: وهي مياه معدنية لا تحتوي بعد معادلتها وتعبئتها على نفس نسبة ثاني أوكسيد الكربون الموجودة في المنبع أو البئر.

ولأجل إقامة أي مشروع إنتاجي للمياه المعدنية هو إيجاد النبع المثالي أو البئر المثالي الذي يضمن الاشتراطات القياسية والصحية التي وضعت من قبل المنظمات المحلية والإقليمية والدولية مثل تراكيز الأملاح الكلية المذابة Total dissolved solids [T.D.S] ونتيجة للتطور العلمي والتقني أمكن السيطرة على بعض خصائص المياه فمثلا إذا كانت كمية الأملاح عالية يمكن خفضها بالتقنيات الحديثة أما إذا كانت هنالك مشكلة في النقاوة فيمكن السيطرة عليها عن طريق طرق الترشيح المختلفة أما إذا كانت هنالك رائحة ولون فيمكن استخدام طرق الادمصاص. ومن المعروف أن اختيار النبع والبئر هو الأساس عند البدء في إقامة مشروع لإنتاج المياه المعدنية وفق المقاييس والمعايير في تحديد الموقع.

الجزء العملي

الدراسة الاستطلاعية:-

-تمت زيارة بعض مراكز المعالجة والتعبئة و تم الاطلاع على محطات المعالجة والاستماع إلى شروح وافية من المهندسين المشرفين عن آليات المعالجة المستعملة وعن التحاليل الروتينية والدورية.

-تم الاطلاع على المواصفات القياسية الليبية الخاصة بمياه الشرب المعبأة وغير المعبأة.

-تمت زيارة مواقع منظمة الصحة العالمية World Health Organization (WHO)، بشبكة المعلومات (الإنترنت)، حيث تم الاطلاع على المواصفات القياسية لمياه الشرب المعبأة وغير المعبأة

-تم الاطلاع على بعض البحوث السابقة المنشورة والمتوفرة في مجال تحاليل المياه.

أخذ العينات:-

تعتبر عملية أخذ العينات من العمليات الهامة والحساسة جداً ويجب أخذ جميع الاحتياطات اللازمة والضرورية أثناء القيام بها ولا تعود دقة نتائج تحليل العينات إلى المحلل وحده بل يكون لآخذ العينات دوره الكبير في ذلك إذ أن شروط أخذ العينات تتحكم حتماً في النتائج التحليلية وفي التفاسير التي تعطي لها بعد ذلك، ولاشك أن الأمور تسير أحسن عندما يكون المحلل هو نفسه آخذ العينات

ولا يمكن إعطاء وصف عام ومحدد لطريقة جمع عينات الماء بسبب تعدد أنواع العينات واختلاف بعضها عن بعض واختلاف مواقع جمعها وبالمثل فإنه ليس من الممكن توحيد طريقة خاصة لجمع العينات بسبب اختلاف المصدر وتنوع الفحوصات ولكن هناك أساسيات من الواجب اتباعها عند أخذ العينات من أي مصدر مثل

1 - أن تكون العينة متجانسة وتمثل نوعية مياه المصدر المأخوذة منه.

2 - وأن تكون العينات المأخوذة كافية لإتمام التحاليل المطلوبة .

3 - يجب ترقيم العينات وتشخصيها جيداً، ويفضل أن يتم تحليلها مباشرة بعد أخذها .

وقد تم شراء عينات المياه من مراكز التوزيع التجارية وهذه العينات هي ((الكفرة, سفاري , النبع ، المزن , صافيا)) وأخذنا عينة من مياه الصنبور وعينة من ماء واحة قبرعون وعينة مختلطة من (ماء الصنبور +مياه النبع) وأجرينا عليها بعض التجارب المعملية لتقدير الأملاح الصلبة المذابة (T.D.S) .

رقم العينة	اسم العينـة
1	الكفرة (معبأة)
2	سفاري (معبأة)
3	النبع (معبأة)
4	المزن (معبأة)
5	صافيا (معبأة)
6	مياه (صنبور)
7	مختلط مياه صنبور ومياه النبع
8	مياه واحة قبرعون

جدول (1): أسماء وأرقام العينات

ملخص الطريقة:-

تغسل مجموعة كؤوس جيداً بماء مقطر عالي الجودة . ثم تجفف الكؤوس تماماً . توزن الكؤوس بدقه . يوضع في كل كأس 100مل لتر تماماً من مياه العينة ثم تترك على سخان للغليان حتى تمام تبخر الماء. وأخيرا توضع الكؤوس في فرن عند درجة حرارة 180 درجه مئوية لمدة 24ساعة. تخرج الكؤوس من الفرن وتترك لتبرد في مكان جاف ثم توزن بنفس الميزان السابق استخدامه .

الحسابات:- المواد الصلبة الذائبة {ppm}= (وزن الكأس بعد الفرن – وزن الكأس فارغ)x 10000.

النتائــج والمناقشة

مصطلح المواد الصلبة يشير للمواد الصلبة العالقة أو المذابة في الماء ويمكن أن تؤثر عكسيا على جودة المياه إذا زاد تركيزها عن الحد المسموح به . والمياه التي تحتوي على مواد صلبة مذابة عالية عادة تكون غير مفضلة للشراب ويمكن أن تسبب تفاعلات فسيولوجية غير مفضلة ولهذا تم تحديد 1000 ملجم /لتر من المواد الصلبة المذابة ((Total dissolved solids)) كحد أقصى لمياه الشرب والمياه المحتوية على معادن بتراكيز كبيرة تكون غير صالحة لكثير من التطبيقات الصناعية .والمياه ذات المواد العالقة الكثيرة تكون غير مفضلة للإستعمال من الناحية الجمالية كالاستحمام (Bathing) مثلا .وتحليل المواد الصلبة المذابة في المياه مهم في التحكم في الخواص الفيزيائية والفسيولوجية لمياه الفضلات أثناء عملية المعالجة . والمصطلح Total Solid عبارة عن مصطلح يطبق على المواد المتبقية المتروكة في الإناء بعد عملية الغليان للعينة بعد تجفيفها في فرن عند درجة حرارة معينة ، وسابقا كان يستخدم المصطلح Residue مكان المصطلح Solids . [14]

وتتضمن المواد الصلبة الكلية المواد العالقة (Suspended solids) والمواد المذابة الكلية (Total dissolved solids) . والمواد العالقة هي الجزء المتبقي من المواد الصلبة الكلية بعد عملية الترشيح ، وسابقا كان يستخدم المصطلح Nonfilterable بدلا من المصطلح Suspended. والمواد المذابة الكلية هي الجزء الذي يمر خلال ورقة الترشيح ، ويعتبر نموذج المصفى المستعمل ، حجم المسام ، المسامية ، المساحة ، سمك المصفى (thickness) ، الطبيعة الفيزيائية ، حجم الحبيبات ، ومجموع المواد التي تترسب على المصفى ، هي عوامل رئيسة تؤثر على فصل المواد العالقة من المواد المذابة . وسابقا كان يستعمل المصطلح filterable بدل المصطلح dissolved [14]

من الآثار السلبية لزيادة تركيز المواد الصلبة في المياه الطبيعية تراكمها على مصادر غذاء الحيوانات المائية وتجعل الماء غير صالح للاستعمالات المنزلية كما أن تراكمها في الأحواض والأنابيب يستدعي تنظيفها المستمر. إذا كانت المواد الصلبة المذابة (T.D.S) اقل من 1000 ملجم /لتر في الماء فيمكن اعتبار أن المياه عذبة، أما إذا زادت عن ذلك فتعتبر المياه مالحة ، وتزداد الملوحة كلما زادت هذه القيمة وإذا زادت قيمة الأملاح الصلبة المذابة عن 10000 ملجم/لتر يصبح الماء أجاجاً (مالح جداً) .
في هذا البحث تم قياس T.D.S لعينات من المياه التجارية وكذلك لعينات من (مياه الصنبور) ومياه بحيرة قبرعون وذلك لغرض المقارنة . النتائج المتحصل عليها موضحة بالجدول رقم (2) وشكل رقم (1)
وتعرف مياه الشرب المعبأة حسب ما جاء بالمواصفات القياسية الليبية رقم 10 لسنة 1997 ف بأنها مياه شرب طبيعية غير معالجة أو معالجة بإحدى الطرق المتعارف عليها للقضاء على الأحياء الدقيقة . وهناك اشتراطات يجب أن تتوفر في هذا النوع من المياه مثل أن يكون مصدر المياه سطحيا أو جوفيا بعيدا عن مصادر التلوث ومصرحا به من قبل الهيئة العامة للمياه .

وقد حددت المواصفة الحد الأقصى المسموح به من المواد الصلبة T.D.S ب 500 ملجم /لتر ويمكن القول بناء على هذه المواصفة أن جميع عينات الدراسة المعبأة لها تراكيز أقل من 500 ملجم/لتر حيث كانت التراكيز (260 ، 65 ، 40 ،305 ، 90) ملجم /لتر لعينات مياه الكفرة وسفاري والنبع والمزن وصافيا على التوالي ونلاحظ أن بعض العينات لها تراكيز منخفضة جدا مثل عينة النبع والتي بلغ تركيز T.D.S بها حوالي 40 ملجم/ لتر

وبالنظر للمواصفة رقم 82 لسنة 1992 الخاصة بمياه الشرب (المياه المحلية) والتي تعرف مياه الشرب المحلية (البلدية) بأنها المياه الصالحة للاستهلاك البشري ومن ضمن الاشتراطات القياسية لهذا النوع من المياه بأنها يجب أن تكون خالية تماما من التعكر الناتج عن التلوث ولا تحتوي على أي نوع ناتج من التلوث وخالية من المواد الضارة بالصحة سواء كانت كيميائية أو حيوية ولا تحتوي على إلا على المقادير المسموح بها من بقايا المواد الكيميائية المستعملة الناتجة من عمليات التنقية وتشترط المواصفة أن تكون T.D.S من 500ملجم /لتر كحد أمثل إلى 1000 ملجم /لتر كحد أقصى . وبما أن مصدر المياه المعبأة هي مياه جوفية أي يمكن تطبيق مواصفات مياه الشرب المحلية عليها يمكن القول أن العينات التي تحتوي على تراكيز واطئة جدا من T.D.S تعتبر عينات ليست على مستوى عالي من الصلاحية حيث تؤكد كثير من الأبحاث أن الأملاح الكلية الموجودة في المياه ينبغي أن تكون في معدل متوسط بمعنى أن لا تكون عالية جدا فوق 1500 ملجم/لتر أو لأتكون قليلة جدا أقل من 500 ملجم /لتر حيث أن الجسم بحاجة لهذه الأملاح وأن الاعتماد على هذه النوعية من المياه ربما يؤدي بمرور الوقت إلى مضاعفات خطيرة حيث يعتقد أن الأشخاص

الذين يتناولوها أكثر عرضة للإصابة بإمراض الكلى لان الكلى لاتقوم بعملية التصفية للأملاح كما يجب وربما بمرور الوقت تتعود الكلى على هذا النوع من المياه مما يؤدي إلى أمراض خطيرة وهذا ما جعلنا ندرس T.D.Sفي المياه المحلية والتي كثير من الناس لا يفضلونها على أساس أن أملاحها مرتفعة في حين يتم تفضيل المياه المعبأة على أساس أن T.D.S لها حسب المواصفات العالمية ,ويوصى بعض الأطباء بأن يتم الخلط بين هذين النوعين من المياه فنحصل على نتائج وسطية مرضية وبالنظر إلى النتائج يلاحظ إن T.D.S للمياه المحلية 1100 ملجم / لتر وعندما قمنا بخلط هذه المياه مع المياه المعبأة نقصت ال T.D.S إلى حوالي 310 ملجم/ لتر وهذه القيمة مرضية ويمكن الاعتماد عليها .وللمقارنة قمنا بجلب عينات من بحيرة قبرعون وتم حساب ال T.D.S لها وكانت مرتفعة جدا حوالي 6 أضعاف مياه البحر حيث تراوحت قيمة T.D.Sلبحيرة قبرعون حوالي 234500 ملجم / لتر, كما هو موضح بالجدول رقم (3) والشكل رقم (2) في حين كانت مياه البحر المتوسط كما تشير معظم المراجع في حدود 38600 ملجم / لتر.ويمكن تفسير ذالك جزئيا على أساس أن معظم البحيرات التي تشغل أحواضا مفتوحة عادة تحوي مواداً صلبة ذائبة كلية تتراوح بين (1000–2000) ملجم / لتر ويرتفع تركيز المواد الصلبة الذائبة بالبحيرات ذات الأحواض المغلقة بسبب التبخر ليصل فى بعض الأحيان إلى أكثر من 100.000 ملجم / لتر . وتعتبر هذه البحيرات ذات ملوحة اعلي من ملوحة البحر والتي تكون تقريبا 38600 ملجم / لتر.

والجدير بالذكر أن الملوحة تعرف بأنها التركيز الكلى للمكونات الأيونية ويعتبر هذا المصطلح شائعا في علم البحار ولكنه لايستعمل بكثرة بواسطة العاملين في حقل المياه العذبة بسبب التراكيز القليلة للايونات الكلية الموجودة في المياه العذبة ويعبر عن الملوحة في المياه العذبة عادة بالملجم لكل لتر والجدير بالذكر أيضا" أنه يمكن ربط T.D.S بالتوصيل الكهربي حيث يستعمل التوصيل الكهربي في المعمل لإغراض منها تقدير T.D.S فى العينة بواسطة ضرب التوصيل الكهربي (ميكروموز /سم) في عامل تجريبي هذا المعامل يتراوح من 0.55– 0.9 معتمدا على المحتويات المذابة في المياه وعلى درجة حرارة القياس .علما بان T.D.S المحسوبة بهذه الطريقة لأتطابق T.D.S المحسوبة بطريقة التبخير ولقد وجد بالتجربة أن النتيجة المعطاة حسابيا اقل من النتيجة المتحصل عليها بواسطة التبخير وخاصة في المياه ذات التوصيل الكهربي الأكبر من 833 ميكروموز /سم ولذلك تم تحسين الطريقة بإعطاء عدة علاقات لحساب T.D.S بواسطة التوصيل الكهربي. الماء المقطر المحضر أنيا له توصيل كهربي من 0.5–2 ميكروموز/سم تزداد بعد أسابيع قليلة من التخزين من 2– 4 ميكروموز/سم وهذه الزيادة سببها عادة امتصاص CO_2 من الجو والى اقل وجود للامونيا.

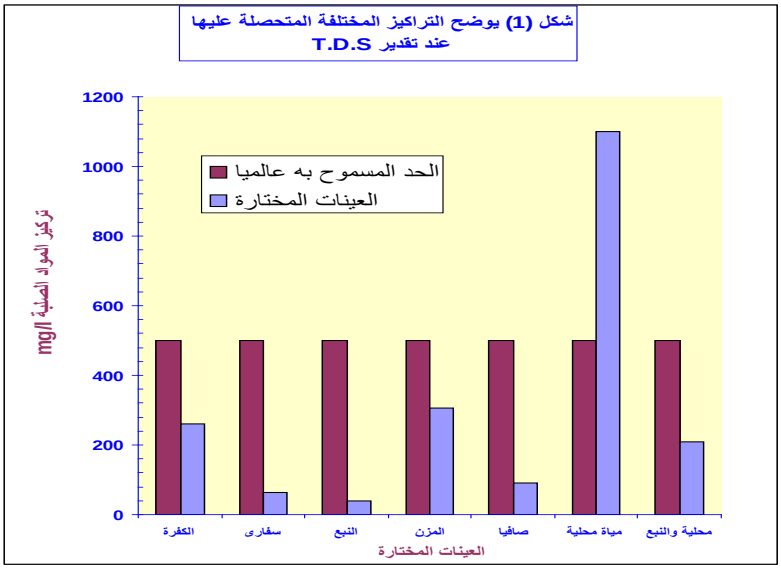
TOTAL DISSOLVED SOLIDS DETERMINATION IN SOME SAMPLES

رقم العينة	أسم العينـــة	التراكيز المتحصل عليها (ملجم/لتر)
1	الكفرة (معبأة)	260
2	سفاري (معبأة)	65
3	النبع (معبأة)	40
4	المزن (معبأة)	305
5	صافيا (معبأة)	90
6	مياه البلدية	1100
7	مياه البلدية ومياه النبع	210

جدول (2) يمثل التراكيز المتحصل عليها عند تقدير(T.D.S)للعينات

رقم العينة	اســـم العينـــة	التراكيز المتحصل عليها ملجم / لتر
1	بحيرة قبرعون	234500
2	البحر المتوسط	38600

جدول(3) يمثل التراكيز المتحصل عليها عند تقدير (T.D.S) لعينة(بحيرة قبرعون)



شكل (1) يوضح التراكيز المتحصل عليها عند تقدير T.D.S

(صورة لبحيرة قبر عون)

(صورة لتقدير T.D.S لملح بحيرة قبر عون)

الاستنتاجات

1 – المياه المعدنية المعبأة في زجاجات بلاستيكية والتي تضاعف استهلاكها العالمي ليبغ 154 مليار لتر عام 2004 تكلف البيئة ثمناً باهظاً إضافة لأنَّ تكلفتها تفوق غالباً تكلفة الوقود. وهي ليست في غالب الأحيان أكثر سلامة من المياه التي تصل إلى المنازل عبر شبكات التوزيع في الدول الصناعية وقد تكلف عشرة آلاف مرة أكثر إذا أخذت بالاعتبار الطاقة المستخدمة وتصنيع وإعادة تدوير الزجاجات، ومع سعر يقارب 2.50 دولار للتر الواحد فإنَّ كلفة المياه المعبأة تفوق تكلفة الوقود[8].

2 – من النتائج التي تم التوصل إليها يتضح أن بعض المياه التجارية المعبأة تحتوي على مواد صلبة مذابة قليلة جدا" أقل بصفة عامة من مياه الصنبور وذلك لأن المياه التجارية تخضع لعمليات

معالجة خاصة وحفظها في أوعية معقمة تضمن عدم تعرضها للملوثات قبل وصولها للمستهلك.

ولكن يجب التأكيد على أن لا تكون .T.D.S قليلية جدا" لان ذلك أيضا" له أضرار صحية.

3 – نوصي بأن يتم الخلط بين المياه ذات الأملاح المذابة المرتفعة والتي يمثلها غالبا مياه الصنبور و المياه ذات الأملاح المذابة المنخفضة والتي يمثلها غالبا المياه التجارية لنتحصل على مياه تكون T.D.S لها حسب المواصفات العالمية.

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التلوث البصري والسمعي لبيئة الشارع الليبي
(دراسة حالة لواقع الشارع في بيئة مدينة الزاوية)

حسين البشير شفشه و فاطمة رمضان صاكال

المقدمة

تشهد مدينة المجتمعات الحديثة كما هائلا من الظواهر والمشكلات والقضايا التي جعلت من الاتجاه نحو البيئة أمرا ملحا وحيويا ولعل مجرد الإشارة إلى تلوث الهواء والبحار والمحيطات وانتشار الأوبئة والأمراض والنشاط الإشعاعي .. وغيرها يكفي دليلا على أن البيئة في خطر ولذلك ظهر في العقود الثلاثة الماضية اهتماما عميق بالبيئة وبالكفاح من اجل حمايتها فغدت قضية عالمية تطرح نفسها بإلحاح في عالم اليوم وصار هناك إجماع على أهمية المحافظة على البيئة وعلى إخطار التراخي في مواجهة مشكلاتها الأمر الذي يؤدي إلى المزيد من ضحايا الموت البطيء بسبب رعونة التعامل معها بلا اهتمام . فقضية البيئة لا تتعلق فقط بالمشاريع الصناعية والتقدم التكنولوجي بل أيضا تتعلق بالمشاكل الاجتماعية والاقتصادية وأنماط الاستهلاك و الإنتاج وأسلوب استمتاع قطاع عريض من الجماهير بالحياة وعدم المساواة في توزيع الثروة والفقر وأعباء الديون والانسياق وراء الأهواء والكسب المادي السريع والعبث بالمقدرات البيئية وأنظمتها ومشكلاتها المستقبلية[1].

من هنا تأتي أهمية انعقاد المؤتمر العلمي الثاني للبيئة والذي يعقد في رحاب كلية العلوم بجامعة الزقازيق الموقرة وتحت شعار " حول بيئة نظيفة خالية من التلوث " . وبالرغم مما أنجزته ثورة الفاتح في مجال الحفاظ على البيئة الليبية وتعظيم مواردها الطبيعية والتصدي لكل مظاهر اختراق الهوية والتراث إلا أن هناك طغيان للعشوائية على الشارع في بيئة المدينة مما أدى إلى زيادة التلوث البصري والسمعي واختراق الهوية والتراث الليبي في ظل غياب أدوات تطبيق اللوائح والقوانين وما لذلك من تأثير مباشر على الحالة النفسية للمواطن الليبي وحالته الصحية وأسلوب تفكيره وعمله وإنتاجه .

وتعد مدينة الزاوية من المدن الهامة غرب طرابلس ويعود تاريخها إلى سنين قديمة نشأة واستقرارا موضعيا أشبه ما كان ثابتا فترة من الزمن حتى نهاية الخمسينيات وفي بداية الستينيات شهدت تطورا ، ولكنه ليس كبيرا مقارنة بمثيلاتها في المناطق القريبة والبعيدة عنها ، وفي أواخر الستينيات وأوائل السبعينيات وحتى أواخر الثمانينيات صار النمو الحقيقي للمدينة بحيث أصبح مخططها يغطى مساحة تقدر بحوالي (880 هكتار) بعد أن كان لا يزيد عن (412.2 هكتار) في أواخر الخمسينيات ، وقفز عدد سكانها فأصبح عام 1995 ما يقارب من (105000 نسمة) ، بعد أن كان عددهم لا يزيد عن (53000 نسمة) عام 1980 لا لشيء إلا بسبب تحسن الأوضاع الاقتصادية والصحية وبسبب الزيادة الطبيعية الثابتة والهجرة الداخلية والخارجية للمدينة ، وهو ما سيؤدي إلى أن تكون الزاوية ذات تركيبة سكنية سكانية خدمية وظيفية متميزة الأمر الذي يجعل مخططها سيصل عام 2025 إلى حوالي (195000 نسمة) إذا ما استمر معدل النمو والتوسع بهذا الشكل آخذا في النمو والتوسع في الاتجاه الجنوبي الشرقي والغربي ومحددا صوب الشمال[2] .

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(1) خيرية سعيد المبروك الفرجاني (2000) : التوعية البيئية في الصحافة اليومية الليبية ، مجلة البحوث الإعلامية ، مركز البحوث والتوثيق الإعلامي – العدد 31 ، ص ص 77 – 78 .

(2) نفيسة رمضان القزيطي (2003) : مورفولوجية مدينة، هيكلية البناء والتركيبة الوظيفية من واقع استعمالات الأراضي، رسالة ماجستير غير منشورة ، قسم الجغرافيا ، كلية الآداب ، جامعة السابع من ابريل ، ص 12 .

وفي ضوء نتائج الدراسة الاستطلاعية التي قام بها الباحثان والتي تضمنت مسح للدراسات التي تناولت بالتوصيف بيئة مدينة الزاوية من حيث مورفولوجية هذه المدينة ، وهيكلية البناء والتركيبة الوظيفية ، واستعمالات الأراضي ، وتقييم مخطط المدينة والسكان . ومن خلال استطلاع رأي مجموعة من السكان القاطنين بالمدينة حول أسباب التلوث البصري والسمعي ومظاهر اختراق الهوية والتراث الليبي . هذا إلى كون الباحثان من أبناء المدينة ، وبالاطلاع على اللوائح والقوانين المنظمة لحماية البيئة الليبية . أمكن تحديد مشكلة البحث .

مشكلة البحث :

تحدد مشكلة البحث في طغيان العشوائية على الشارع في بيئة مدينة الزاوية مما أدى إلى زيادة التلوث البصري والسمعي واختراق للهوية والتراث الليبي في ظل غياب أدوات تطبيق اللوائح والقوانين .

تساؤلات البحث :

1 - ما مظاهر طغيان العشوائية على الشارع في بيئة مدينة الزاوية ؟
2 - ما أسباب زيادة التلوث البصري والسمعي واختراق الهوية والتراث الليبي في الشارع في بيئة مدينة الزاوية ؟
3 - ما التصور المقترح للحد من التلوث البصري والسمعي واختراق الهوية والتراث الليبي في الشارع في بيئة مدينة الزاوية ؟

أهداف البحث :

1 - حصر وتوصيف العشوائيات في الشارع في بيئة مدينة الزاوية .
2 - تحديد أسباب زيادة التلوث البصري والسمعي واختراق الهوية والتراث الليبي في الشارع في بيئة مدينة الزاوية .
3 - تقديم تصور مقترح للحد من التلوث البصري والسمعي واختراق الهوية والتراث الليبي في الشارع في بيئة مدينة الزاوية .

أهمية البحث:

قد تسهم نتائج هذا البحث وما يقدمه من توصيات ومقترحات في التصدي لمظاهر طغيان العشوائية على الشارع في بيئة مدينة الزاوية والتي أدت إلى زيادة التلوث البصري والسمعي واختراق للهوية والتراث الليبي ، ومساعدة أعضاء المؤتمرات الشعبية على سن القوانين واللوائح لمواجهة مظاهر التلوث البيئي ، وكذلك تقديم مقترحات لمساعدة أعضاء اللجـــان الشعبيـــة لتطبيق اللوائح القوانين التي يتم التوصل إليها لمواجهة تلك العشوائيات .

فروض البحث:

1 - حصر مظاهر طغيا ن العشوائية على الشارع في بيئة مدينة الزاوية يمكن من الوقوف على أسباب زيادة التلوث البصري والسمعي واختراق الهوية والتراث الليبي.
2 - التصور المقترح له فاعلية كبيرة في الحد من التلوث البصري والسمعي واختراق الهوية والتراث الليبي في الشارع في بيئة مدينة الزاوية.

حدود البحث :

تقتصر حدود البحث على حصر مظاهر التلوث البصري والسمعي ومظاهر اختراق الهوية والتراث الليبي نظرا لقلة الدراسات التي تناولت هذه المظاهر. كما تقتصر حدود البحث على دراسة حالة واقع شارعي عمر المختار، وجمال عبد الناصر باعتبارهما يشكلان نواة المدينة ومداخلها ، بالإضافة إلى احتوائهما على مجموعة من المراكز التجارية والاقتصادية والإدارية لمدينة الزاوية نظرا للأهمية الاقتصادية والجغرافية والسكانية لها على مستوى مدن الجماهيرية .

مصطلحات البحث :

1 - التلوث البصري :

يأخذ الباحثان بتعريف (محمد احمد عوض، 1994) [1] للتلوث البصري على انه إهدار القيم الجمالية في البيئة ، وهو تشويه للرؤية البصرية التي تقدر جماليات البيئة ، فالعين تألف الأشياء المرتبة والمنظمة في المظهر اللوني والحسي في البيئة . وأن هناك تناسبا طرديا بين القصور في اتزان البيئة العمرانية وتدهور عناصرها الجمالية وتكويناتها ، مما يؤدي إلى اعتياد كل مظاهر التلوث البصري ، والفني والمعماري ، والعمراني . وبالتالي فقد القيم الجمالية والابتكارية .

2 - التلوث السمعي :

يأخذ الباحثان بتعريف (محمد احمد عبد الهادي ، 2003)[2] للتلوث السمعي على انه الخليط المتنافر من الاهتزازات الصوتية الغير مألوفة التي تنتشر في الجو سواء أكانت متقطعة أو مستمرة تقتحم طبلة الأذن وتسبب مضاعفات صحية ونفسية وتذوقية . ويعتبر كل صوت يزيد شدته عن 50 ديسيبل ضوضاء سواء أكانت صناعية أم ناتجة عن ضجيج الأحياء المزدحمة .

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(1) محمد احمد احمد عوض (1994) : دراسة التلوث البصري الناتج من تأثير عامل التلوث الجوي على تماثيل النوافير الرخامية الاثرية ، بحوث المؤتمر العلمي الخامس – " الفن والبيئة " ، كلية التربية – جامعة حلوان ، ص 815 .

(2) محمد احمد عبد الهادي (2003) : التلوث الضوضائي آثاره على صحة الطفل النفسية والجسمانية – دراسة مقارنة بين التعرض للضوضاء الصناعية وضجيج المزدحمة، ايتراك للطباعة والنشر والتوزيع، القاهرة، ص 12.

منهج البحث وخطواته :

اتبع البحث المنهج الوصفي التحليلي في حصر وتوصيف مظاهر العشوائية في الشارع في بيئة مدينة الزاوية وذلك للوقوف على الأسباب التي أدت إلى زيادة التلوث السمعي والبصري واختراق الهوية والتراث الليبي ، والمنهج التاريخي في تتبع مظاهر التلوث في الشارع في بيئة مدينة الزاوية من خلال الدراسات التي تناولتها للتعرف على مسببات تلك الظواهر، وفقا للخطوات الآتية :

1 - إجراء الدراسة الاستطلاعية ، والتي تمت الإشارة إليها فيما سبق .
2 - عرض للدراسات السابقة المرتبطة بموضوع البحث ومحاوره .
3 - مسح تصويري لشارعي عمر المختار ، وجمال عبد الناصر لحصر وتوصيف مظاهر العشوائية والتي أدت إلى زيادة التلوث البصري والسمعي واختراق الهوية والتراث الليبي .
4 - نتائج البحث ، والتوصيات ، والمقترحات .

الدراسات السابقة :

أولا: دراسات تناولت توصيف بيئة مدينة الزاوية من حيث تاريخها ونشأتها ، ومورفولوجية هذه المدينة ، وهيكلية البناء والتركيبة الوظيفية ، واستعمالات الأراضي ، وتقييم مخطط المدينة ، والسكان.

تتخطى مدينة الزاوية اليوم ما كانت عليه من الوظيفة والبناء سابقا ، وصارت مدينة ذات حجم متوسط وربما ستصير مدينة كبيرة على الساحل الليبي في اقل من ربع قرن قادم ، متأثرة بأنماط بنائية مخالفة لما كانت عليه المدينة قديما ، ويؤيد ذلك ظهور كثير من المراكز الخدمية والأنشطة الوظيفية والتغيرات البنائية التي ظهرت في أكثر من موضع بها وخاصة بها على طول الشوارع الرئيسية ، والمحاور المتفرعة منها .

ومدينة الزاوية شانها شأن غيرها من المدن القديمة في التاريخ نوعا ، إذ ترجع بداية الإشارة لهذه المدينة إلى القرن الثاني الهجري عندما ذكر (العجيلي) [1] ، إن هذا القرن وتحديدا في بدايته قد شهد إنشاء العديد من الرباطات الساحلية وخاصة في عهد ابراهيم ابن احمد بن الأغلب الذي أمر ببناء القلاع والحصون لحماية الساحل الممتد من الإسكندرية إلى إفريقيا ، وهي الرباطات الساحلية التي انبثقت عنها الزوايا الدينية في العصر الموحدي بعد أن تحولت مهام الدفاع عن الثغور إلى القلاع ، وفيها اختصت الزاوية بمهمة التعليم ، والتي أصبحت من أهم المراكز العلمية التي أنشأها العرب المسلمون لخدمة الإسلام والدعوة إلى التمسك بمبادئه كما بينها (الكعاك)[2] .

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(1) مختار ابو عجيلة العجيلي (1992): دور ليبيا في النشاط البحري العربي الإسلامي (مجلة البحوث التاريخية) السنة الرابعة عشر ، العدد الأول 1992 ، مركز الجهاد الليبي للدراسات التاريخية ن طرابلس ، ص 96.
(2) عثمـــــان الكعـــــاك (بدون تاريخ): مركز الثقافة في المغرب ، معهد الدراسات العربية العالمية ، القاهرة ، ص 53 .

ولقد ذكر موضع مدينة الزاوية في كتب الرحالة والجغرافيين العرب المسلمين حيث جاء في جملة الحصون التي عددها الشريف الإدريسي في حدود منتصف القرن الخامس الهجري عندما ذكر أن احد الحصون الواقعة بين صبراته وطرابلس يعرف بقصر سنان [1] والذي يعتقد بأنه نسبة إلى أولاد سنان بن عامر ، من بني سليم احد أهم القبائل العربية التي استقرت بطونها بين طرابلس وقابس بعد أن هاجرت من مصر [2] وكان لهذا الاستقرار بداية جديدة لتاريخ هذه المنطقة ، خاصة من الناحية الديموغرافية ، فقد عربت المنطقة ووطد اللسان العربي بعد قرون من عد الاستقرار والاضطرابات .

ومنذ بداية القرن السابع الهجري أخذت المصادر التاريخية تظهر بعض المعلومات عن موضع الزاوية ، حيث مر بها الرحالة التونسي (التيجاني) عام 706 – 708 هـ ، فذكر عنها بعد تجاوزه زاوية أولاد سهل قائلا : " ثم أصبحن وسرنا فاجتزنا بزاوية هي أضخم مالا وأكثر رجالا وبها مباني كثيرة ولها ارض متسعة تعرف بزاوية أولاد سنان أخوة الوشاحين النوائل " مما يعني أنها تأسست قبل ذلك بوقت طويل على يد عرب من بني سليم[3] .

وكانت في المئة السادسة الهجرية ذات شان ، حيث كانت مجمع العرب وسوقهم التي يجلبون إليها أمتعتهم ويجتمعون فيها لبيع وشراء ما يحتاجون لبيعه وشراءه ، وأخذت الزاوية بعد ذلك تتسع وتمارس دورا علميا وثقافيا في المنطقة بحكم موقعها على طريق الحد الموصلة بين بلاد المغرب والمشرق العربي مما سهل عليها عملية الاحتكاك بمختلف الافكار والاتجاهات الدينية التي كان يعيشها العالم الاسلامي [4] .

ومن الدراسات التي تناولت توصيف بيئة مدينة الزاوية :

دراسة (عواطف الأمين محمد عمر ، 1996) [5] بعنوان : " تقييم مخطط مدينة الزاوية لسنة 2000 " والتي هدفت إلى دراسة العوامل المختلفة التي هيأت الظروف لمدينة الزاوية لتصبح على ما هي عليه من خلال توصيف المدينة وظروف نشأتها وتطور النمو السكاني بها والتغيرات السياسية والاقتصادية والاجتماعية التي أثرت على شكل وطبيعة المدينة والتي كان من أهم نتائجها تحديد هوية مدينة الزاوية باعتبارها مدينة خدمية بالدرجة الأولى ، و تؤدي وظائف أخرى متعددة عملت على استقطاب السكان بشكل مباشر، وان المدينة تعاني مشكلات عديدة جراء عدم تطبيق توصيات المخطط فيما يخص بعض الطرق الرئيسية ، والمرافق الترفيهية ، والمناطق الخضراء ، والمناطق الصناعية ، ووجود العديد من الاختناقات في العديد من الأماكن المخططة .

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(1) محمد الهادي شعيرة (1968) : الرباطات الساحلية الليبية ، مؤتمر ليبيا في التاريخ ، الجامعة الليبية ، كلية الآداب ، بنغازي ص 236
(2) عبد الرحمن ابن خلدون(ب.ت): تاريخ ابن خلدون ، دار الفكر ، بيروت ، ج6 ص 111 .
(3) عبد الله بن احمد التيجاني (1981): رحلة التيجاني ، (تحقيق حسن حسني) الدار العربية للكتاب ، ليبيا ، تونس ، ص 213 .
(4) الطاهر احمد الزاوي (ب . ت): معجم البلدان العربية ، مكتبة النور ، ليبيا .
(5) عواطف الامين محمد عمر (1996) : تقييم مخطط مدينة الزاوية لسنة 2000 ، رسالة ماجستير غير منشورة ، كلية التربية قسم الجغرافيا ، جامعة السابع من ابريل .

ودراسة (خيرية سعيد المبروك الفرجاني ، 2000)[1] بعنوان : " التوعية البيئية في الصحافة اليومية الليبية ". وهدفت الدراسة في التعرف على حجم اهتمام الصحف الليبية بالبيئة وبأحداثها وقضاياها وتحديد طبيعة الأداء الصحفي في الصحف الليبية في القيام بنوعية بيئية وقد استخدمت الدراسة المنهج المسحي ، والوصفي ، والمنهج المقارن ، والمنهج الإحصائي بالإضافة إلى تحليل المضمون لجميع أعداد صحف الشمس ، والجماهيرية ، والفجر الجديد وقد توصلت الدراسة إلى مجموعة من النتائج من أهمها ضعف وضآلة حجم المساحة المخصص لمواد وموضوعات البيئية في صحف الدراسة ، غلبت الخبر على التغطية الصحفية لأحداث البيئة .

ودراسة (نفيسة رمضان القزيطي ، 2003) [2] بعنوان " مورفولوجية مدينة الزاوية ، هيكلي البناء والتركيبية الوظيفية من واقع استعمالات الأراضي ، والتي هدفت إلى التعرف على التركيبية البنائية الوظيفية ، وما هو عليه حال استعمالات الأراضي في هذا المجال قصد إظهار قيمة ومستوى الخدمة والوظيفة والاستعمال ، ومعرفة مدى التأثير والتأثر الذي تلعبه المدينة موضعاً وموقعاً على إقليمها المجاور والعكس ، ودور ذلك في عملية النمو والتوسع الحضري الذي شهدته وتشهده المدينة حالياً . واستخدمت الباحثة في ذلك المنهج التاريخي والمنهج الوصفي التحليلي . ومن أهم النتائج التي توصلت الباحثة أن مركز المدينة ما زال يمثل بؤرة رئيسية ، ومنطقة جذب حساسة خدميا ووظيفيا وتجاريا وان بعضا من شوارع المدينة القديمة فشلت في تأدية وظيفتها ، وفي التكيف مع المعايير التخطيطية الجديدة حتى في حالة الهدم وإعادة البناء . ومن أهم توصياتها ضرورة الأخذ بالمعايير التخطيطية ، آخذه في الاعتبار حماية البيئة والمحافظة عليها .

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1) خيرية سعيد المبروك الفرجاني (2005) : التوعية البيئية في الصحافة اليومية الليبية ، مرجع سبق ذكره .

(2) نفيسة رمضـــان القزيطـــي (2003) : مورفولوجية مدينة، هيكلية البناء والتركيبة الوظيفية من واقع استعمالات الأراضي، مرجع سبق ذكره .

ثانيا : دراسات تناولت التلوث السمعي وتأثيراته على الإنسان

تنوعت الدراسات التي توضح الآثار الناتجة عن الضوضاء فمن هذه الدراسات ما أوضح تأثير الضوضاء على الفهم والتحصيل والأخطاء و اثر ذلك على التعليم ومنها ما أوضح علاقة الضوضاء بالاضطرابات والتغيرات النفسية وعلاقتها بالنمو النفسي والاجتماعي وتأثيرها على الانتباه والتركيز وكذلك تأثيرها على الانجاز والتأدية ثم علاقة الضوضاء بالتغيرات الفسيولوجية والصحية. أما تأثير الضوضاء على حاسة السمع فينتج من موجات الصدمات والتغيرات المفاجئة في الضغط الجوي فهذه الموجات تنتشر في أعقاب سماع انفجار أو طلق ناري وتؤدي إلى تغيرات مفاجئة في الضغط من واحد إلى عدة وذات ضغط جوي إذن فسعة هذه الموجات اكبر بكثير من أي صوت معروف وتكون موجة الصدمة مصحوبة بضوضاء ناتجة عن تحرك الجسيمات أو سخونتها أو احتكاكها أو من الصدى وهي ضوضاء مركبة للغاية لها مكونات منخفضة أو أحيانا حادة بصورة بارزة وتكون شدة الضوضاء اضعف من شدة موجة الصدمة وهي لا تتعدى شدة الضوضاء الأكثر قوة وبها أن سرعة موجة الصدمة أعلى بشكل عام من سرعة الصوت فإننا نستطيع أن نلتقط عند مسافة بعيدة من المصدر موجة الصدمة أولا ثم الضوضاء وهذه الأخيرة تنتشر بفعل الصدى ولان عاقبة الإصابات الحادثة مماثلة لتلك الناتجة من الأصوات الشديدة فإنه عادة ما يكون من الصعب التمييز بين ما ينتج عن موجة الصدمة وبين ما ينتج عن الضوضاء المصاحبة وتحدث حالات فقدان السمع عادة في مناطق الترددات الحادة نتيجة لإصابة القوقعة كما انه من الممكن أيضا أن تسبب موجات الصدمة بعض الحوادث على مستوى الإذن الوسطى كتمزيق الطبلة وتصدع سلسلة العظمات واقتلاع عظيمة الركاب من النافذة البيضاوية ونزيف المخ وتظهر حالات فقدان السمع نتيجة موجة الصدمة .

ومن الدراسات التي أكدت ذلك ما يأتي :

دراسة (كريلوف ، 1985) [1] : والتي أشارت إلى أن ضوضاء الشارع المزمنة تسبب عيوبا في السمع .

و دراسة (رالوف ، 1982)[2] : والتي أشارت إلى أن امتصاص النيتبيوتك متزامنا مع التعرض للضوضاء يمكن ان يزيد تأثيرات الضوضاء ويسبب فقد سمعي أكثر من تأثير الدواء بمفرده أو تأثير مستويات الضوضاء بمفردها .

و دراسة (اجرستون ، 1987) [3] : والتي بينت أن الحالات التي شخصت على أنها ضغط دم مرتفع كانت معرضة لضوضاء 105 ديسيبل لمدة 30 دقيقة وكانت قياسات ضغط الدم قد أخذت في فترات الهدوء والضوضاء فأثناء الضوضاء كانت هناك زيادة جوهرية في انبساط وتمدد القلب وضغط الدم قد لوحظت مبدئيا بواسطة زيادة في مقاومة الأوعية الدموية الخارجية كما أن قوة انقباض القلب قد نقصت بالفعل ولهذا فان الضغط الذي يرجع إلى التعرض للضوضاء كان يتزامن مع انقباض الأوعية الدموية ويساهم في زيادة ضغط الدم .

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(1) ت.باكاكس (1985): الابعاد الاصحيحة للتحضر، ترجمة محمد عبد الرحمن الشرنوبي، مطابع الخط، الكويت، ص 187 .

(2) ـــــــــــــــ : الابعاد الاصحيحة للتحضر ، المرجع السابق نفسه ، ص 189.

(3) Paul A - Bell & Jeffrey D.Fisker – hot , Rinckia – T and Winston , INC. Environmental psychology Bell /Fisker / Baum / Grren Think Edition – Fost worth shiage – Sanfrancisco – London – Sydney –Toky . P.136

كما أكدت نتائج بعض الدراسات علاقة الضوضاء بالتغيرات والاضطرابات النفسية ومنها دراسة (كوزارني ، 1992) [1] في بولندا على المدرسين في المدارس التي تزيد فيها نسب الضوضاء فضلا عن الازدحام وما ينتج عن ذلك من زيادة الضوضاء وقد وجد أن ذلك هو العامل الرئيسي لانزعاج المدرسين فالضوضاء تؤثر بالسلب على المدرسين وعلى مشاعرهم فوجد أن مجموعة المدرسين الذين يعيشون في حالات اجتماعية واقتصادية سيئة يزيد عندهم القلق وعدم التركيز والإرهاق والاكتئاب والصداع بالإضافة إلى أعراض الجهاز الدوري والتنفسي والهضمي بالإضافة إلى علامات القلق وبعض هذه المشاكل تزيد بالتدخين أيضا .

ودراسة(محمد احمد عبد الهادي 2003) [2] : عن التلوث الضوضائي وآثاره على صحة الطفل النفسية والجسمانية – دراسة مقارنة بين التعرض الضوضاء الصناعية وضجيج المدن والتي استهدفت دراسة مستوى الحالة الانفعالية لدى عينات من الأطفال المعرضين للضوضاء الصناعية وضجيج الأحياء المزدحمة وكذلك مستوى القلق ، ومستوى العدوانية ، ومستوى التوافق النفسي ، ومستوى الذكاء لدى هؤلاء الأطفال حيث قام الباحث برصد نسب الضوضاء بالديسيبل وعمل كشف سمعي وتصميم مقياس لذلك ومن أهم نتائج التي توصل إليها الباحث وجود زيادة لها دلالة إحصائية التلاميذ للحالة الانفعالية بالنسبة للعينات المعرضة للضوضاء من الأطفال .

ثالثا : دراسات تناولت مواجهة ظاهرة التلوث البصري وحالة العرض بالشارع في بيئة المدينة والارتقاء بها

دراسة (ايناس على الخولي 1999)[3] بعنوان : النظريات العالمية في تخطيط المدن ليست عالمية والتي هدفت الى التحقق من صفة العمومية للنظريات العالمية في تخطيط المدن وذلك من خلال الوصف التحليلي لمجموعة من النظريات العالمية في تخطيط المدن وتوصلت إلى مجموعة من النتائج من أهمها أن النظريات التيتم استعراضها في تخطيط المدن لم تحقق لها صفة العمومية في التطبيق الأمر الذي ينفي عنها صفة العالمية التي تطلق عليها كما ان خصوصية كل مجتمع بل وخصوصية كل بيئة من بيئات المجتمع تفرض على المخطط وضع هذه الخصوصية في الاعتبار عند التصور المناسب لمدن كل بيئة .

ودراسة (جودت نصر بباوي ، 1999) [4] بعنوان : دور العمارة الداخلية في الحفاظ على هوية الشارع المصري والتي هدفت إلى إحياء ما خلفته لنا العمارة من تراث جميل في الشارع المصري للحفاظ على هوية البيئة والمجتمع حيث قام الباحث بدراسة العناصر المكونة للصورة البصرية في الشارع المصري عبر تاريخه الاسلامي مع التركيز علـــــى المشربية والنـــافذه

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(1) Au : Koszarny – Z AD. Zaklady Hijieny Komunal – neg Banstwowego Zakladu Higiney WWarszawie 188 : 0035-7715 Py: 1992 .

(2) محمد احمد عبد الهادي (2003) : التلوث الضوضائي آثاره على صحة الطفل النفسية والجسمانية – دراسة مقارنة بين التعرض للضوضاء الصناعية وضجيج المزدحمة، مرجع سبق ذكره .

(3) ايناس علي الخولـــــي (1999) : النظريات العالمية في تخطيط المدن ليست عالمية ، بحوث المؤتمر العلمي السابع لكلية التربية الفنية ، جامعة حلوان ، ص ص 149-166 .

(4) جودت نصر ببـــــاوي (1999): دور العمارة الداخلية في الحفاظ على هوية الشارع المصري، بحوث المؤتمر العلمي السابع " دور التربية الفنية في خدمة المجتمع العربي " كلية التربية الفنية جامعة حلوان، ص ص 167 – 180.

كحدود للبحث . وكان من أهم النتائج التي توصل إليها الباحث تحديد الوظائف النفعية والجمالية للمشربية والمؤثرات التي عليها ، ومن أهم توصيات الباحث تحقيق الطابع التاريخي للشارع المصري في شكله الجميل له اثر كبير على ازدهار الساحة في مصر والتي يمكن الإفادة منها اقتصاديا وإعلاميا.

دراسة (مها محمود البنوي الشال ، 1999)[1] بعنوان : دور الفن التشكيلي في تجميل مؤسسات المجتمع والبيئة التي هدفت إلى تأكيد اللمسة الجمالية في المبادئ التعليمية من خلال التركيز على أهمية المباني التعليمية ودور التعليم من الناحية الجمالية والمعمارية والتذوقي وتأثيرها على أذواق طلابها وإحساسهم بالجمال والتي أكدت نتائجها على المدرسة بمبانيها ومظهرا ينبغي أن تكون وسيلة للتربية الجمالية مهما يكن مدى اللاشعور في تطبيق ذلك المبدأ .

كما أكدت نتائج وتوصيات بحوث المؤتمر القومي الثاني للدراسات والبحوث البيئية والذي نظمه معهد الدراسات والبحوث البيئية بجمهورية مصر العربية ، 1990) [2] على أن للبيئة الاجتماعية بجميع مكوناتها والتي تشكل خصائصها من (أوضاع سياسية ، وعقائدية ، ومتطلبات اجتماعية ، ومتطلبات حسية ، وأوضاع اقتصادية) دورا كبيرا في نشأة أي تجمع عمراني ، ولها أهمية لا تقل عن أهمية الموقع الجغرافي أو البيئية الطبوغرافية ، ولا يمكن قيام عمران بأي حال من الأحوال دون وجود المجتمع الذي يرغب في التعمير وان يؤخذ في الاعتبار أن التشكيل العمراني للمدينة يؤثر بصورة واضحة على المجتمع الإنساني لذا فالارتقاء بالعمران هو في نفس الوقت ارتقاء بالمجتمع والعكس .. البيئة العمرانية المتدهورة تؤثر على السكان بالسلب . كما انه يجب دراسة التأثيرات النفسية الناتجة عن التخطيط العمراني للمدينة ، حيث أن التخطيط قد يسبب آثارا اجتماعية تظهر بصورة واضحة بمرور الوقت .

نتائج البحث :

أولا : حصر وتوصيف مظاهر التلوث البصري والسمعي في الشارع بيئة مدينة الزاوية :

من خلال قيام الباحثان بالمسح التصويري لكل من شارعي عمر المختار ، وجمال عبد الناصر بعد اخذ موافقات جهات الاختصاص وذلك بهدف حصر وتوصيف مظاهر العشوائية والتي ادت الى زيادة التلوث البصري والسمعي واختراق الهوية والتراث الليبي أمكن حصر وتوصيف تلك المظاهر بكل من الشارعين والذي تظهره مجموعة من الصور التالية :

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(1) مها محمود البتوي الشال (1999) : دور الفن التشكيلي في تجميل مؤسسات المجتمع والبيئة ، بحوث المؤتمر العلمي السابع " دو التربية الفنية في خدمة المجتمع العربي ، الجزء الاول كلية التربية الفنية جامعة حلوان ، ص ص 3 – 18 .

(2) معهد الدراسات والبحوث البيئية (1995) : نتائج وتوصيات المؤتمر القومي الثاني للدراسات والبحوث البيئية – المجلد الثالث ، جمهورية مصر العربية . ص ص 78- 79 .

شكل (1)
استخدام الألوان الصارخة في الواجهات

شكل (2)
مفردات في حالة من عدم التناسق

شكل (3)
عدم مراعاة النسب بين العناصر الوظيفية

شكل (4)
تكدس السيارات والاختناقات المرورية

شكل (5)
أنماط معمارية بعيدة عن مقوماتنا الحضارية

شكل (6)
واجهات تفتقر إلى مبادئ التشكيل الجمالي

وتتحدد مظاهر التلوث البصري والسمعي في بيئة الشارعين (حدود البحث) فيما يأتي :

1) تعلية المساكن بإضافة وحدات إليها بنمط معماري يختلف عن نمط المبنى الأصلي فيصبح المبنى متنافرا مع نفسه ، ومتنافرا مع البيئة المحيطة .
2) تحويل الأدوار السفلية إلى محلات تجارية دون النظر لمدى تلازم واجهاتها وألوانها مع المبنى نفسه ، وكذلك اقتطاع جزء من الفراغات لإقامة محلات تجارية بشكل منفر .
3) التعديلات التي تقام لتغيير شكل الواجهة أو إغلاق أجزاء منها لإضافة فراغ جديد بطريقة تشوه المبنى الأصلي .
4) إضافة أجهزة التكييف بصورة عشوائية دون دراسة للاماكن المثلى لوضعها وكذلك استخدام هوائي الإرسال التلفزيوني وأطباق الدش بطريقة عفوية مما يشوه الواجهات والأسقف بصريا .
5) تفتقر معظم واجهات المباني إلى مراعاة النسب والمقاييس ، مع عدم احترام الانسجام والتجانس مع المباني المجاورة في علاقاتها مع بعضها وبالتالي عدم مراعاة الذوق أو الجمال في اقتراح أشكال الواجهات مما يؤذي شعور الغير ويؤذي العين عند رؤية هذه المباني .
6) استخدام ألوان صارخة في العديد من الواجهات المعمارية للبنايات وكذلك المجال واستخدام مواد تشطيب غريبة وغير منسجمة مع بعضها البعض وبطريقة مفتعلة تخلو من الذوق الفني.
7) استعارة بعض المفردات التراثية بطريقة بدائية دون وعي بها .
8) فقدان الطابع العام والشخصية البصرية المميزة للشارع لمدينة الزاوية .
9) التركيز الشديد على العنصر المادي دون الجوانب الجمالية في تصميم واجهات المباني والمحال المتمثل في استخدام مواد بناء مرتفعة السعر ولكن منعدمة الذوق .
10) ظهور المباني والمحال في حالة من التلوث والإهمال .
11) الضوضاء الناتجة عن الاستخدام السيئ للسيارات وكذلك الضوضاء الناتجة عن الاستخدام السيئ لأجهزة التسجيل وتضخيم الصوت في المحال التجاريق .
12) الاستخدام غير المدروس لأعمدة الإضاءة ولوحات الإعلانات والنافورات وأحواض الزهور وساعات الميادين ، مما يجعلها تعطي نتيجة عكسية فبدلا من تحققها لتجميل المدينة تصبح مصدرا كبيرا لتشويهها بصريا.
13) حالة العرض غير المدروس جماليا للمعروضات بالمحال التجارية وعدم الاستعانة بالمنسق المتخصص في العرض .
14) تكدس السيارات في الشارعين .
15) عدم مراعاة الذوق العام أو مستوى محدد من الجماليات في أشكال الواجهات للعديد من المباني القائمة .

ثانيا : الأسباب التي أدت إلى زيادة مظاهر التلوث البصري والسمعي واختراق الهوية والتراث الليبي في الشارع في بيئة مدينة الزاوية :

يتضح من عرض مظاهر التلوث البصري والسمعي أن أسباب هذا التلوث في سلوكيات الأفراد والتي تتحدد بالمستوى التعليمي والثقافي والاقتصادي والاجتماعي والبيئي لأفراد المجتمع . وكذلك اللجان الشعبية المسئولة والموارد الاقتصادية المتاحة والقوانين والتنظيمات المنظمة .

أولا : سلوك الأفراد :

يؤثر سلوك الافرد بشكل مباشر على تلوث لمدينة بصريا وقد يكون التأثير سلبا او إيجابا وذلك من خلال ما يأتي :

1 – المستوى التعليمي :

- يؤدي انخفاض المستوى الثقافي والتعليمي إلى قلة الوعي لدى الأفراد وإهمالهم النواحي التنسيقية والجمالية ، والى عدم وجود وعي بأهمية الحفاظ على الوجه الحضاري للمدينة والى فقدان التذوق الجمالي .
- يؤدي انخفاض المستوى الثقافي والتعليمي إلى تصرفات غير واعية من شأنها الإساءة إلى الشكل الجمالي للمدينة باعتبار أن مسكنه يخصه وحده (ملكية فردية) بالرغم من الشكل الخارجي يخص المجتمع كله .
- يقوم الإنسان المتعلم أحيانا بالتأثير على المعماري و التدخل في تصميم المبني فتظهر أشكالا غير مدروسة في الواجهات لتلبية رغبات العميل فقط دون ان تتفق مع القيم الجمالية .

2 – المستوى الاقتصادي:

يؤدي انخفاض المستوى الاقتصادي إلى زيادة حدة التلوث البصري خاصة في الأحياء التي يسكنها ذو الدخل المحدود وتنحصر أسبابه في الآتي :

- تعلية المبنى القائم بشكل يتنافي مع المبني دون وجود إمكانيات مادية تسمح بالتشطيب أحيانا ، بالإضافة إلى الضرب بقوانين البناء والمقاييس الجمالية عرض الحائط .
- عدم بياض واجهات المباني ، وعدم الاهتمام بصيانة ونظافة المبنى .
- استغلال الوحدة السكنية بأكثر من أسرة بسبب قلة المعروض من الوحدات السكنية وارتفاع قيمتها الايجارية مع وجود زيادة كثافة سكانية .

- إقامة مناطق الإسكان العشوائي خاصة في ظل الانفجار السكاني والرغبة في تكوين اسر جديدة (الأسرة الممتدة)
- مخالفة تراخيص البناء بمحاولة استغلال الأرض أقصى استغلال وذلك بالبناء في الفراغات التخطيطية المستغلة كمناطق فضاء أو بتحويل الأدوار السفلية والفراغات إلى محلات تجارية .
- قيام ذوي الدخول المرتفعة باستخدام أشكال وخامات مبهرة في التنفيذ بغرض التباهي فتكون النتيجة ظهور أشكال وألوان منفرة .
- قيام ذوي الدخول المرتفعة بتركيب أجهزة التكييف طبقا لاماكن يحددونها بمعرفتهم وبطريقة عشوائية .

3- المستوى الاجتماعي والبيئي :

يعتبر التلوث البصري في المدينة نتيجة مباشرة للهجرة غير الموجهة من الريف إلى الحضر حيث تؤثر الخلفية الاجتماعية والبيئية على الأفراد وعلى تصرفاتهم ، وتأتي تصرفاتهم بطريقة عفوية كالآتي :

- نزوح أهل الريف حاملين معهم أساليب ونمط معيشتهم الريفية مما يجعلهم يقيمون عشش الطيور في البلكونات وعلى أسطح المباني ، ويخزنون المواد التموينية في البلكونات .
- تقفيل البلكونات لزيادة مسطح أو طلبا للخصوصية ، كذلك فتح الشبابيك في الحوائط طلبا للتهوية .
- قيام أهل الريف بدهان مبانيهم وزخرفتها بنفس ألوان الزخارف الشائعة الاستخدام في المساكن الريفية بالرغم من أن وجودها يجعلها تبدو غريبة .

ثانيا : سلوكيات النظام :

1- الموارد الاقتصادية المتاحة :

يؤدي النقص في الاعتمادات المالية إلى زيادة التلوث البصري حيث أن قلة أعمال النظافة وقلة المرتبات دفعت العمال إلى عدم العمل بجدية والى عدم الاهتمام بالعمل كما أدى انخفاض الموارد الاقتصادية المتاحة وخاصة في إقامة مباني بمعرفة الجهات الرسمية إلى انخفاض مستوى تشطيب هذه المباني مما أدى إلى تدهور معايير القيم الجمالية بها .

2- التنظيمات والقوانين :

بالرغم من وجود العديد من القوانين المنظمة لحماية البيئة الا ان هذه القوانين لم تتعرض للطابع الجمالي لبيئة الشارع في مدينة الزاوية هذا بالإضافة إلى أن التسيب واللامبالاة في تنفيذ القوانين المنظمة بالإضافة إلى الثغرات الموجودة فيها وراء مظاهر التلوث البصري والسمعي .

توصيات ومقترحات البحث لمواجهة مظاهر التلوث البصري والسمعي ومظاهر اختراق الهوية والتراث الليبي لبيئة الشارع :

1 - محاولة الوصول إلى طابع معماري مميز وشخصية بصرية موحدة للمدينة .

2 - استخدام عقوبات رادعة لكل أساليب التحايل على القانون .

3 - التوعية الجماهيرية بمشاكل البيئة وطرق الحفاظ عليها ونظافتها .

4 - تنمية حاسة التذوق الفني لدى العامة وإرشادهم إلى الفرق بين الجميل والقبيح والى التناسق اللوني والتذوق الجمالي للقيم المعمارية .

5 - دراسة العلاقة بين عناصر العرض في الشارعين (حدود البحث) بما يتضمن العلاقة بين أعمدة الإضاءة ، وواجهات المحال ، وعرض الأرصفة ، وعرض الشارع ، وارتفاعات المباني ، ومساحات واجهات المباني والمحال ، واللون في الشارع ممثلا في طلاءات المباني ، وإعلانات المحال التجارية واليافطات واللافتات .

6 - توحيد أساليب تشطيب الواجهات لكل تجمع سكني بهدف إعطاء طابع مميز لكل تجمع عن طريق تحديد نوعية المواد المستخدمة في معالجة الواجهات ، وألوانها ، والتشكيلات المعمارية للواجهة ككل.

7 - تعظيم دور خريجي كليات الفنون والإعلام وأساتذتها في تقديم حلول جمالية للشارع في بيئة المدينة في ضوء مؤشرات الأرصاد بها من حيث درجة الحرارة ومعدلاتها على مدار العام وعلى مدار اليوم وكذلك عدد ساعات سطوع الشمس بها على مدار العام وكذلك كمية الأمطار على السنتيمتر المربع ، وكذلك سرعة الرياح ، واختلاف أطوال أيام الليل والنهار . هذا بالإضافة إلى دراسة أذواق المواطنين القاطنين بالمدينة .

8 - تشديد الرقابة على أعمال تنسيق المواقع سواء أحواض الزهور أو لوحات الإعلانات أو واجهات المحلات أو العلامات التجارية أو النافورات بحيث تلائم أماكنها وأشكالها وواجهات المباني .

9 - ضرورة وضع تصميم للمحال التجارية يلتزم به أصحاب المباني خاصة في الأدوار الأرضية المطلة على الشوارع الرئيسية .

10 - ضرورة توفير أماكن انتظار السيارات تخفيفا لحدة التكدس في الشارع ووضع إشارات ضوئية واضحة لتوجيه أصحاب هذه السيارت إلى أماكن الانتظار .

11 - تشجيع عملية تمايز التكتلات الإدارية ، والمراكز التجارية المتخصصة في شوارع المدينة وان كانت قد بدأت في التخصص حيث يظهر من الرصد الإحصائي لكل من شارعي عمر المختار ، وجمال عبد الناصر ميل المحال إلى التركيز على بيع وعرض الملابس يليها الأطعمة يليها المكتبات (القرطاسية) .

OPTIMIZATION AND CHARACTERIZATION OF PURIFIED CHITOSANASE ENZYME FROM *STREPTOMYCES AUREOCIRCULATUS*

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ABSTRACT

Thirty seven actinomycete isolates were isolated from soil samples collected from different localities at Sharkia Destrict, Egypt. Of which, 14 isolates were found to exhibit chitosanolytic activities. The isolate No. 10 was the most potent, and it was identified as ***Streptomyces aureocirculatus. Streptomyces aureocirculatus*** produced an extracellular chitosanase during cultivation in a colloidal chitosan, as a substrate, containing medium under shaken conditions (200 rpm). Precipitation of the crude enzyme with ethanol, methanol, petroleum ether and acetone were carried out. Also, different ammonium sulphate saturations were tested to obtain precipitates of the crude enzyme. The most active precipitate was obtained at 80% saturation. This precipitate was further purified by gel filtration in column with Sephadex G-200 yielding an active major protein peak showing 0.85 mg/µg specific activity. Factors affecting the activity of the purified extracellular chitosanase enzyme were investigated. The optimal pH and temperature for chitosanase activity were 5 and 40°C, respectively. The addition of $ZnSO_4$, $CuSO_4$ or $FeSO_4$ to the purified chitosanase exhibited stimulatory effect in its activity. Ethylenediaminetetra-acetate (EDTA) and $MgSO_4$ had also slightly activated effect, while the addition of KCl, NaCl, $CoCl_2$ or $CaCl_2$ exerted an inhibitory effect. pH and thermal stability of the purified enzyme could be obtained at pH 5 and 50°C for 90 minutes. Meanwhile the enzyme showed a thermal stability (in absence of substrate) to heat treatment within 30 minutes of exposure. Purified enzyme chitosanase showed an antifungal activity against several phytopathogens as *Aspergillus flavus, Trichoderma viride, Fusarium oxysporium, Alternaria alternata, Helminthosporium solani* and *Candida albicans.* Amino acid analysis revealed that pure chitosanase was composed of 12 amino acid residues, and proline residue concentration was found the highest, followed by gulatmic acid.

INTRODUCTION

Chitosan is a polysaccharide comprising copolymers of glucosamine and N-acetyl-glucosamine, is obtained by alkaline deacetylation of the chitin derived from the exoskeletons of crustaceans and arthropods (Li *et al.*, 1997). It has attracted considerable interests due to their biological activities such as antimicrobial, antitumor and immuno-enhancing effects (No *et al.*, 2002; Jumaa *et al.*, 2002; Zheng and Zhu 2003; and Liu *et al.*, 2004). In the last decade, textile application of chitosan has been attracted
many researchers (Kim *et al.*, 1998 and Chung *et al.*, 1998).

Several chitosanases have been isolated from some microorganisms including fungi (Fenton *et al.*, 1978, 1981), bacteria (Ohtakara *et al.*, 1984), and actinomycetes (Brzezinski *et al.*, 1997). Chitosan degrading enzymes can be also isolated also from vegetables sources, latex sap *Carica papaya* contains lysozyme (Laktin *et al.*, 1995). *Streptomyces griseus* HUT 6037 was first of interest as an organism with strong

chitosanolytic activity when grown on a medium containing chitosan (Mitsutony and Ohtakara., 1992). They found that *S. griseus* HUT 6037 produced two chitosanases, I and II, secreting them into the culture medium. The enzymes had both chitosanase and β-1, 4 – glucanase activities (Tanabe *et al.*, 2003).

Chitosanase from individual organisms differ in their hydrolytic action pattern. However, most chitosanases from the isolated microorganisms intend to make dimers, trimers and tetramers rather than oligamers above DPU, so the utility of these enzymes is not good (Chen *et al.*, 2005).

In our research, we screened various *Streptomyces* isolates to select highly active extracellular chitosanase producer and purifying this enzyme and studying the biochemical factors affecting on its activity and using it as antifungal substances against some pathogenic fungi.

MATERIALS & METHODS

Isolation of actinomycetes

Soil samples were collected from different localities of Sharkia Governorate. Serial dilutions were prepared to cover the range of 10^{-1} to 10^{-6} using sterile distilled water. Agar plates containing starch casein agar (Kuster and Williams, 1964) and starch- nitrate agar media (Waksman and Lechevalier, 1961) were seeded with soil suspensions. The plates were then incubated for 7 days at 28°C after which colonies of actinomycetes were isolated, purified by streaking several times on the medium used for isolation, and then sub-cultured on slants of the same medium.

Taxonomical studies of the chitosan degrading actinomycete isolates.

The taxonomical work, carried out in this study, was according to ISP methods (Shirling and Gottlieb, 1966). The main criteria used for characterizing the freshly isolates of *Streptomyces* spp. were the morphology of sporophores, the colour of aerial mycelium and vegetative mycelia, spore surface, melanin production, pigmentation and carbon sources utilization, growth test and antibiotics sensitivity. The strains were identified by using the determinative key of Bergey's Manual of Systematic Bacteriology (1989), Kuster (1972), Nonomura (1972) and Szabo *et al.*, (1975).

Screening of actinomycete isolates for their chitosanase productivity under shaken conditions.

a- Preparation of cell free supernatant:

Tested strains were cultured in a liquid basal medium cotaining (g/l): colloidal chitosan 200 ml (was prepared according to Rodriguez-Kabana *et al.*, 1983); K_2HPO_4, 1.0 g; NaCl, 0.5 g; KNO_3, 2.0 g; $MgSO_4$, 0.5; $CaCO_3$, 3.0 g and distilled water up to 1000 ml. The culture broth was adjusted to pH 5. The media were subdivided into 50 ml portions in 250 ml Erlenmeyer flasks; the flasks were autoclaved at 121°C (1.5 atmospheric pressure) for 20 minutes. After which, each flask was inoculated with 1 ml spore suspension. The flasks were incubated in an electric shaker incubator (200 rpm) for 4 days at 30°C. Then, the culture was centrifuged at 5000 rpm for 10 minutes to obtain the cell free enzyme supernatants.

b- Enzyme assay:

According to the method of Thangam and Rajkumar (2000), which is a modified method of Anson (1938)? The reaction mixture consisted of 1 ml of 1% (w/v) colloidal chitosan and 1 ml cell free filtrate was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 2 ml of o.5 M sodium carbonate containing 0.05% potassium ferricyanide. The amount of reducing sugar liberated was

measured by the modified Schale's method described by Imoto and Yagishita (1971). Then, put in water bath for boiling, then left to cool and measured at 420 nm in a Spectrophotometer

One unit (U) of chitosanase activity was defined as the amount of enzyme that produced reducing sugar corresponding to one µmol of D-glucosamine per minute

Determination of protein

Protein was determined by the method adopted by Lowry *et al.*, (1951) with bovine serum albumen as a standard.

Partial purification of chitosanase

a- Organic solvents:

The cell-free supernatant was partially purified by fractional precipitation with ethanol, methanol, petroleum ether or acetone. These solvents were cooled at 4°C one day before starting precipitation. Several enzyme fractions were obtained at 50, 100, 150 & 200% concentrations for each agent, respectively. The mixtures were left overnight at 4°C until the complete precipitation occurred and then centrifuged at 15000 rpm in a cooled centrifuge for 15 minutes. Thereafter, each precipitate was resuspended in a definite volume of 0.1M citrate phosphate buffer of pH 5 and dialyzed against distilled water in a refrigerator overnight. After dialysis, the protein content and enzyme activity of each precipitate were determined.

b- Salting out with ammonium sulphate:

Ammonium sulphate was added to 100 ml of the culture filtrate at different concentrations to obtain various fractions at 20, 30, 40, 50, 60, 70, 80, 90, & 100% saturation levels. The precipitated protein was obtained by centrifugation for 15 minutes at 15000 rpm under cooling condition.Each fraction was dissolved in a definite volume of 0.1M citrate phosphate buffer of pH 5 and dialyzed against distilled water in a refrigerator overnight. This dissolved fractional precipitate was tested for both chitosanase activity and protein content.

Purification of chitosanase

A glass column (2.5cm x 80cm) was packed with Sephadex G-200 (Sigma) and eluted with 500ml of 0.1M citrate phosphate buffer of pH 5. A flow rate of 60 ml/h was maintained. The precipitate resulting from ammonium sulphate fractionation 80% saturation was eluted to the column, previously eluted with the same buffer. The column was connected to the buffer reservoir and the flow of the buffer was maintained. Enzyme fractions were pooled and activity of the enzyme as well as protein content in each fraction was measured. Fractions which showed highest protein and chitosanolytic activity were collected.

Effect of pH and temperature on the enzyme activity

The effect of pH on the purified enzyme was determined with colloidal chitosan as a substrate. Chitosanolytic activity was studied in the pH range of 2 – 8 at 37°C for 30 minutes, using 0.01M sodium phosphate buffer. The optimum temperature for chitosanolytic activity was determined by varying the incubation temperature between 30 and 60°C for 30 minutes.

pH stability and thermal stability

The partially purified enzyme was incubated at various pH values in 0.02M sodium phosphate buffer at 40°C for 2h, and then the residual activity was determined at the optimum pH (pH 5). The thermal stability of the partially purified enzyme preparation was studied at the optimum pH. Identical enzyme solutions in phosphate buffer (0.02M, pH 5) were preheated separately at different temperatures (40, 45, 50,

55 & 60°C) for 30 minutes. The residual activity was determined by adding the substrate and carrying out the enzyme assay under optimum reaction conditions.

Effect of storage period of chitosanase activity

The purified enzyme preparation stored without substrate for different period of time namely 0, 7, 14, 21, 28, 35, 42 and 49 days, respectively at 0°C to determine the influence of storage period on its activity. Then, the enzyme activity was measured as described before.

Amino acid analysis

The amino acid composition was carried out in National Research Centre, performed by an amino acid analyzer, type LC 300 – Eppendrof, Germany.

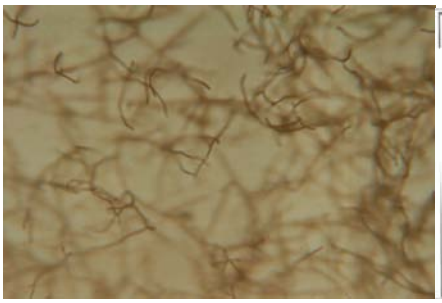
Antifungal activity of chitosanase

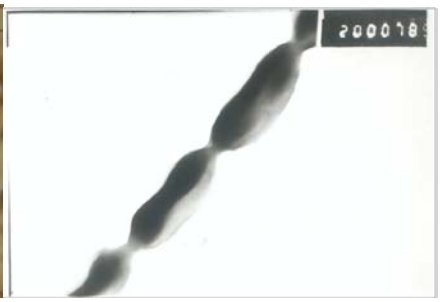
An inoculum of tested fungal strain was suspended into malt agar medium, shaken well, poured into Petri-dishes, and incubated at 28°C for 7 days. Then, diameter of inhibition (mm) was measured for each treatment.

RESULTS

Identification of the most potent isolate of chitosanase enzyme:

The most active isolate was identified to the species level of *Streptomyces* as regarding to the morphological characteristics as follows. Spore chain morphology was straight (Fig.1) with smooth spore surface (Fig.2)





Fig(1):Electron micrograph of spore surface Fig.(2): Micro-morphology of spore chain

Colour of aerial mycelium in the white to yellowish white on starch ammonium sulphate, starch nitrate agar and glycerol-asparagine agar, yellow to whitish yellow on oat meal agar, malt-yeast extract agar and Czapek's solution agar. Reverse side of colony showed yellow to yellowish pigmentation on all media used. Melanoid pigments were not formed on peptone yeast iron agar and tryptone yeast broth, and not formed on tyrosine agar (Table 1

Table (1): Melanoid pigments of the experimental isolate

Melanoid pigments	Results
Peptone-yeast iron agar. yeast broth Tryptone- Tyrosine- agar.	-ve ve- ve-

1-Physiological and biochemical properties

From the result in Table (2), it is evident that liquefaction of gelatin, starch hydrolysis (amylase), reduction of nitrate to nitrite and milk coagulation were positive, but cellulose decomposition, milk peptonization and melanin production were negative

Table (2): physiological and biochemical properties

Test	Results
Gelatin liquefaction	+ve
Reduction of nitrate	+ve
Cellulose decomposition	-ve
Milk coagulation	+ve
Milk peptonization	-ve
Starch hydrolysis	+ve
Melanin production	-ve

2- Utilization of carbon sources:

Data recorded in Table (3) showed that, the tested organism succeeded to grow on D-Fructose, Sucrose, D-mannitol, D-mannose and D-glucose. Weak growth on D-xylose and D-lactose associated with no growth on D-maltose, L-arabinose and L-inositol.

Table (3): utilization of carbon sources by the isolate No. 10

Carbon sources	Results
No carbon source	-
D-fructose	+++
Sucrose	+++
D-mannitol	++
D-xylose	±
L-arabinose	-
D-mannose	++
D-maltose	-
L-inositol	-
D-lactose	±
D-glucose	++

3- Growth test on NaCl:

Data on Table (4) showed that the experimental isolate grew at 2-4% NaCl concentration but the growth was complete inhibited with increasing the concentration us to 12% (w/v) of sodium chloride.

Table (4): Growth rate of isolate in the presence of different concentrations NaCl

NaCl concentrations (%)	Results
2	+++
4	+
6	-
8	-
10	-
12	-

Comparing the previous morphological and physiological characteristics with those of *Streptomyces* species included in International Streptomycetes Project (I.S.P.) (Shirling and Gottlieb 1968a, 1968b, 1969, 1972) and Bergey (1979 and 1994) and Szabo *et al.*, (1975). These keys lead to the identification of the experimental organism as a strain of *Streptomyces aureocirculatus*.

Purification of chitosanase

The crude enzyme produced by *Streptomyces aureocirculatus* was partially purified by organic solvents as ethanol, methanol, petroleum ether or acetone (Table 5) and by fractional precipitation with ammonium sulphate (Table 6). Among the precipitated organic agents, acetone with 200% concentration yielded higher enzyme activity (6.68), soluble protein (16.04) and higher specific activity (0.42) higher than that of ethanol, methanol or petroleum ether. The 80% ammonium sulphate concentration showed the highest chitosanolytic activity (9.00), soluble protein (12.30) and specific activity (0.49), respectively.

Table (5): Effect of different concentration of ethanol, methanol, petroleum ether, or acetone on the enzyme activity and soluble protein produced from *Streptomyces aureocirculatus*.

Different concentrations (%)	Ethanol			Methanol			Petroleum ether			Acetone		
	EA	SP	SA	EA	SP	SA	EA	SP	SA	EA	SP	SA
50	3.13	11.54	0.27	4.88	14.20	0.34	2.31	11.02	0.21	4.56	12.85	0.35
100	4.50	13.01	0.35	6.64	16.72	0.40	5.75	14.96	0.38	5.31	14.00	0.38
150	6.63	18.52	0.36	5.90	15.05	0.39	6.63	16.00	0.41	6.29	15.00	0.41
200	5.50	16.30	0.34	5.41	14.14	0.38	4.50	14.54	0.31	6.68	16.04	0.42

EA: enzyme activity (mg/ml/h), SP: soluble protein (µg/ml), SA: specific activity (U/mg).

Table (6): Effect of different ammonium sulphate saturations on the enzyme activity and soluble protein produced from *Streptomyces aureocirculatus*

Ammonium sulphate saturations (%)	Enzyme activity (mg/ml/h)	Soluble protein (µg/ml)	Specific activity (U/mg)
20	4.00	10.50	0.38
30	5.03	11.90	0.42
40	6.14	14.40	0.43
50	6.99	15.50	0.45
60	7.55	16.20	0.47
70	8.81	16.11	0.50
80	9.00	17.20	0.52
90	7.73	15.50	0.50
100	6.00	12.30	0.49

The partially purified chitosanase which was precipitated with 80% saturation of ammonium sulphate was dialyzed and subjected to gel filtration on a Sephadex G-200 column. The elution profiles for chitosanase and protein content from the column are shown in (Fig.3) and indicate that there are two peaks obtained. The first peak contains the highest activity and protein content. The most active fractions (numbers 15 - 25) from the Sephadex column were pooled for further investigation. A summary

of the purification steps of chitosanase from the culture medium of *Streptomyces aureocirculatus* is presented in Table (7).

Table (7): Purification steps of chitosanase enzyme produced by *Streptomyces aureocirculatus*

Purification steps	Volume (ml)	Enzyme activity (mg/ml/ hr)	Protein content (µ/ml)	Total activity	Total protein	Specific activity (mg/µg)	Recovery (%)	Purification (fold)
Crude enzyme	3.727	6.50	15.20	24225.5	56650.4	0.43	100	1
$NH4)_2SO_4($	150	9.00	16.01	1350	2401.5	0.56	5.57	1.30
Dialysis	49	12.30	18.15	602.7	889.35	0.68	2.49	1.58
Sephadex G-200	25	16.71	19.76	417.75	494	0.85	1.72	1.98

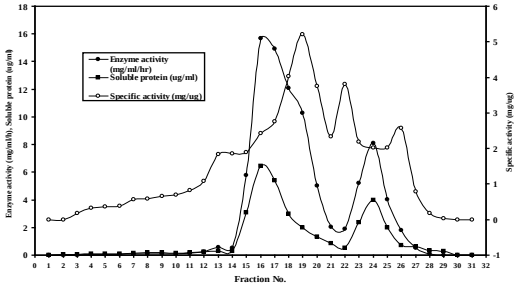
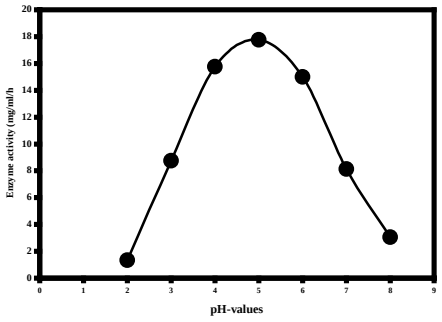
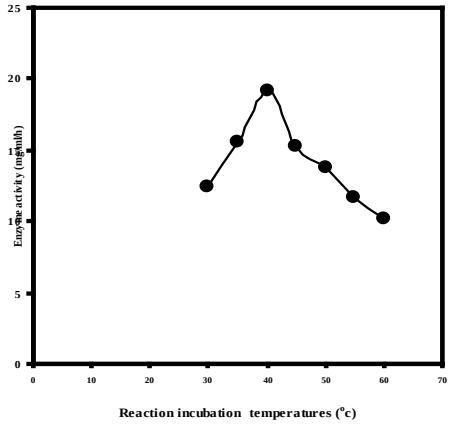


Fig (4): Effect of different pH-values on the enzyme activity



Fig(3): Gel-filtration of crude chitosanase



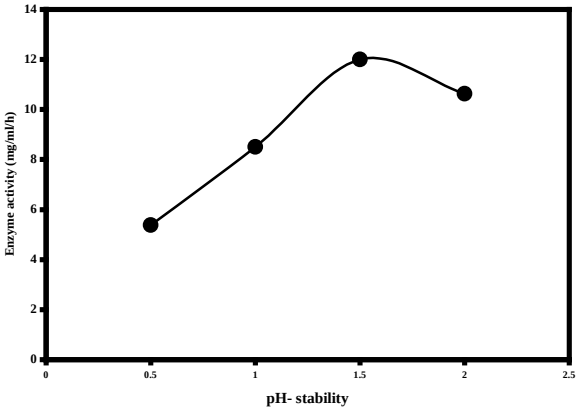


Fig (6): Effect of pH-values on the stability of purified chitosanase

Fig (5): Effect of different temperatures on the enzyme activity

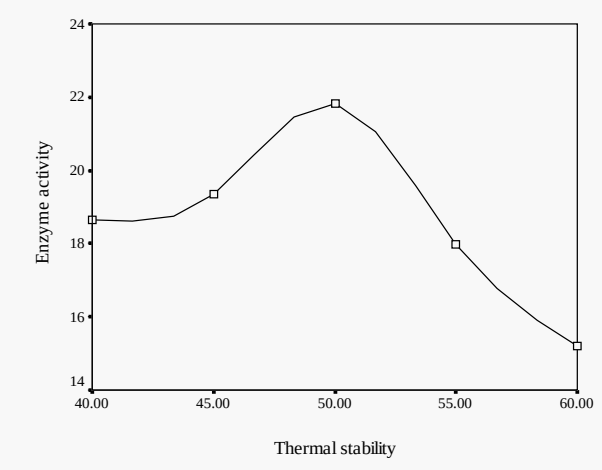


Fig.(7): Effect of temperature on stability of purified enzyme

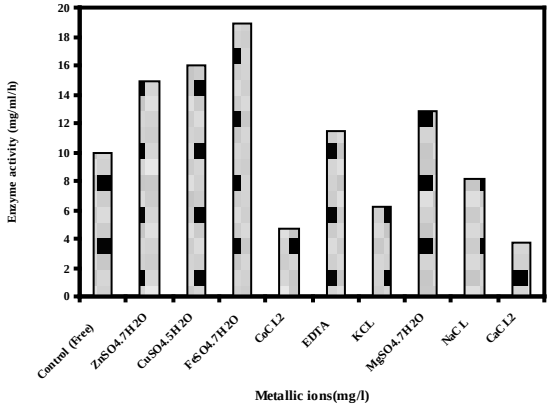


Fig (8): Effect of some metallic ions on the enzyme activity

Effect of pH and temperature on the enzyme activity.

Maximum chitosanolytic activity was observed between pH 4 and 6 with an optimum at pH 5.0 (Fig. 4). The optimum temperature for the partially purified enzyme preparations was recorded 40°C and the range of enzyme ranging between 35°C and 45°C (Fig. 5).Lower or higher temperature leads to inhibition of enzyme action.

pH stability and thermal stability.

The results of the present investigation revealed that the purified chitosanase was stable within 90 minutes with a maximum activity at pH 5 (Fig. 6). Also, the enzyme was stable to heat treatment in absence of substrate within 30 minutes of exposure. The highest activity was obtained at 50°C, but at 55°C and 60°C the enzyme retained most of its activity (17.99 & 15.22, respectively) after the same time of exposure Fig.(7).

Effect of some metallic ions on enzyme activity

The effect of different metallic ions on the activity of the purified chitosanase from *Streptomyces aureocirculatus* was illustrated in (Fig 8). The enzyme was highly activated by $FeSO_4$, $CuSO_4$ or $ZnSO_4$ and slightly activated by EDTA or $MgSO_4$. On the other hand, the enzyme was completely inhibited by $CoCl_2$ or $CaCl_2$ and slightly inhibited by KCl or NaCl.

Amino acid analysis

The amino acid composition of the purified enzyme obtained from *Streptomyces aureocirculatus* showed that it contained a high proportion of proline (355.24 µg/ml) followed by glutamic acid (70.52 µg/ml) (Table 8).

Table (8): Amino acid composition of the purified chitosanase enzyme.

Amino acid	Concentration (µg/ml)
Aspartic acid	23.94
Threonine	6.82
Serine	11.94
Glutamic acid	70.52
Glycine	30.61
Alanine	20.62
Cystin	22.60
Isoleucine	5.56
Leucine	14.38
Histidine	5.97
Lysine	7.34
Proline	355.24

Antifungal activity of chitosanase

Nowadays, great efforts have been performed to use chitosanase enzyme in the biocontrol of pathogenic fungi that destroy crops. Thus, the purified chitosanase preparation of *Streptomyces aureocirculatus* was tested against some pathogenic fungi. The obtained results (table 9) clearly indicated that *Streptomyces aureocirculatus* exerts a marked antifungal action against all the tested fungi.

Table (9): Antifungal activity of chitosanase

Tested organisms	Inhibition zone(mm)
Aspergillus flavus	17.5
Trichoderma viride	30.0
Fusarium oxysporium	25.0
Alternaria alternata	22.5
solaniHelminthosporium	25.0
Candida albicans	35.0

DISCUSSION

Production of chitosanases from actinomycetes was reported by many workers (Imoto and Yagishita 1971; Mitsutony and Ohtakara, 1992; Marcotte *et al.*, 1993 & 1996; Brzezinski *et al.*, 1997; Yamasaki *et al.*, 1994; Tanabe *et al.*, 2003). In the present investigation, chitosanolytic activity and characterization of the identified strain of *Streptomyces aureocirculatus* was studied. *Streptomyces aureocirculatus* proved to have a high potentiality to synthesize an extracellular chitosanolytic enzyme when cultivated in a medium with colloidal chitosan under shaken conditions. Steps of purification program for *S. aureocirculatus* chitosanolytic enzyme were carried out. Fractional precipitations were tested on the resultant supernatants to obtain the crude protein. Initially with different solvents viz., ethanol, methanol, petroleum ether or acetone.Then, salting out with ammonium sulphate yielding the highest enzyme activity and protein content at 80% saturation. Fractional precipitation of crude

chitosanase enzyme with ammonium sulphate saturation levels as well as chitosanase specific activity was carried out. Increasing ammonium sulphate concentration increase the pH of the medium so the enzyme precipitated gradually till reaches the pH that represent the isoelectric point of enzyme at which all enzyme precipitated (at 80% ammonium sulphate saturation), at 100% ammonium sulphate the pH of the medium changed and this may cause mis-ligation to the precipitation enzyme and decreasing its activity. The chitosanase enzyme precipitation was completely achieved at 80% saturation level of ammonium sulphate. This similar to that obtained with *Bacillus* sp. (Imoto and Yagishita, 1971). On the other hand, many investigators reported results conflicted with the present record, where 30% saturation level of ammonium sulphate (Yoon *et al.*, 2001), 90% ammonium sulphate was the optimal level for chitosanase precipitation from *Acinetobacter* sp. C-17 (Zhu *et al.*, 2003).Still, subsequent steps of purification program for *S. aureocirculatus* have been carried out, where the resultant precipitate was dissolved in a least amount of 0.1M citrate phosphate buffer pH 5, then it was dialyzed against distilled water to exclude the sulphate ions. Furthermore it was eluted to G-200 column chromatography. Active fractions of the sharp peaks of fractional purification curve were collected and concentrated by dialysis against the same buffer for having a concentrated preparation of the purified- chitosanase.

Correspondingly, the kinetic properties of the purified- chitosanase were investigated. The optimum incubation temperature for the purified- chitosanase was recorded at 40ºC. This result is more or less similar to incubation temperature for the purified- chitosanase from *Acinetobacter* sp. C-17 (Zhu *et al.*, 2003). Inspection of findings of other investigators, 60ºC was optimal for chitosanase activity from *S. griseus* HUT 6037 (Tanaba *et al.*, 2003).

In case of pH effect on chitosanase activity it was found that optimum pH value of *S. aureocirculatus* chitosanase activity was at 5. Comparable results were recorded by Jo et al., (2003) who found that pH 5.5 was optimum for maximal activity from *Bacillus* sp. P16. On the other hand many investigators reported results conflicted with the present finding.Thus, Tanaba *et al.*, (2003) showed that chitosanase enzyme of *S. aureocirculatus* exhibits its maximal activity at pH 6 . Zhu *et al.*, (2003) decleared that pH 7 was optimum for chitosanase activity of *Acinetobacter* sp.C17.

Results indicated that the stability of pH and heat on the activity of the enzyme was 5 and 50°C after 90 and 30 minutes of incubation, respectively. However, The enzyme from *Bacillus* sp. Strain CK4 was stable after heat treatment at 80°C for 30 minutes or 70°C for 60 minutes (Yoon *et al.*, 2001), while, the chitosanolytic activity from *Acinetobacter* sp.C17 was stable in the pH range of 5-8 and temperature range of 30-40°C (Zhu *et al.*, 2003

One of the most important data concerning the purified- chitosanase under investigation that the activity could be inhibited in the presence of $CaCl_2$, $CoCl_2$, KCl and NaCl . However EDTA, ZnSO4.7H2O, CuSO4.5H2O and FeSO4.7H2O exerted highly stimulatory effect. In outlook of the findings of other investigators, Yoon *et al.*, (2001) found that, the chitosanase enzyme from *B.* sp. strain CK4 was increased about 2.5-fold by the addition of 10mμ Co^{+2} and 1.4 fold by Mn^{+2}. However, Cu^{+2}ion strongly inhibited the enzyme.

Amino acid analyzer for chitosanase protein revealed 12 amino acids with different proportions. Proline showed the highest concentration followed by glutamic acid.

The applied part of this investigation was carried out on several pathogenic fungal strains, all the results were positive. This result was in agreement with that obtained from Chang *et al.*, (2007) who concluded that the growth of the plant pathogenic

fungi *Fusarium oxysporum, F. solani and Pythium ultimum* were considerably affected by the presence of the QQ308 culture supernatant. The supernatant inhibited spore germination and germ tube elongation of *F. oxysporum, F. solani and* Pythium ultimum. Prapagdee *et al.*, (2007) studied the role of chitosan in protection of soybean from a sudden death syndrome (SDS). Chitosan inhibited the submerged growth of *F. solani* f. sp. glycines indicating antifungal property; also, it was able to induce the level of chitinase activity in soybean resulting in the retardation of SDS development in soybean leaves

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الظروف المثلى ودراسة خواص انزيم الكيتوزانيز النقى المنتج من سلالة ستربتوميسيس أوريوسيركيوليتوس

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فى هذه الدراسة تم عزل 37 عزلة من الأكتينوميسيتات من عينات تربة من أماكن مختلفة بمحافظة الشرقية بمصر. تم اختيار 14 عزلة هى أنشط العزلات من حيث قدرتها على افراز انزيم الكيتوزانيز. ووجد أن العزلة رقم 10 هى أنشط العزلات على افراز الانزيم. تم تعريف هذه العزلة وتسميتها ستربتوميسيس أوريوسيركيوليتوس.

تم زراعة ستربتوميسيس أوريوسيركيوليتوس على وسط غذائى محتوى على الكيتوزان الغروى فى ظروف مزرعية اهتزازية (200 لفة فى الدقيقة) لانتاج انزيم الكيتوزانيز. وقد أجريت تجارب لترسيب هذا الانزيم بالمذيبات العضوية مثل الايثانول، الميثانول، بترواليم ايثر والأسيتون. وقد تم أيضا ترسيبه بتركيزات مختلفة من كبريتات الأمونيوم، وقد حصلنا على أنسب راسب عند اضافة تركيز 80% من كبريتات الأمونيوم.

عند تنقية هذا الراسب باستخدام عمود الفصل الكروماتوجرافى المحتوى على السيفادكس ج-200 أنتج قمة رئيسية بروتينية قدر نشاطها النوعى بمقدار 0,85ملليجرام/ ميكروجرام.

وعند دراسة العوامل المؤثرة على نشاط انزيم الكيتوزانيز وجد أن أنسب درجة حموضة لنشاط الانزيم هى 5 وأنسب درجة حرارة هى 40°م. وعند دراسة تأثير العناصر المنشطة والمثبطة للانزيم وجد أن اضافة كبريتات الزنك أو النحاس أوالحديد للانزيم النقى يزيد من نشاطه زيادة ملحوظة وعند اضافة ادتا أو كبريتات الماغنسيوم يزيد من نشاطه زيادة طفيفة. أما عند اضافة كلوريد البوتاسيوم أو الصوديوم أو الكوبلت أو الكالسيوم يثبط من نشاط الانزيم. ووجد أن ثباتية الرقم الايدروجينى كان 5 بعد 90 دقيقة من التحضين أما الثبات الحرارى كان 50°م للانزيم النقى بعد 30 دقيقة من التحضين.

وتم دراسة نشاط انزيم الكيتوزانيز الضد فطرى لبعض الفطريات الممرضة مثل أسبرجلس فلافس، ترايكودرما فيريدى، فيوزاريوم أوكسيسبورم، ألترناريا ألترناتا، هلمنثوسبوريوم سولانى، كانديدا ألبيكانس، وأظهرت الدراسة نتائج ملحوظة.

وبتحليل الأحماض الأمينية لانزيم الكيتوزانيز وجد أنه يتكون من 12 حمض أمينى، كان البرولين أعلاهم تركيزا ويليه حمض الجلوتاميك.

STIMULATION AND CONTROL OF THE MICROORGANISM *E. COLI BY* USING EXTREMELY LOW FREQUENCY MAGNETIC FIELD

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ABSTRACT

The effect of 50 Hz magnetic field of strength 2 m T of E. coli bacteria cells has been studied. Equal volumes of E. coli cells were exposed to the magnetic field for different periods. In this work, the two most effetive periods, namely, 6 hr. and 16 hr. were chosen for all our experimental studies.

Studying the structure of the E-coil was accomplished through investigating the changes in the molecular structure of the extracted water soluble protein (WSP) extract from E-coil exposed. The changes in the molecular structure of (WSP) were investigated through measuring each of the dielectric relaxation in the frequency range 1 → 4 kHz at 4 ± 0.05Cand the molecular weight distribution using SDS polyacrelemid gel electrophoresis. Pathogenecity tests were also estimated and morphological properties. Also the water soluble protein content was studied for (direct&late) effects. The results indicated that the dielectric properties of WSP extract from E-coil exposed showed considerable changes in both permittivity and conductivity values relative to the control. However, the dielectric properties for the 16hr. showed higher values than 6hr. Also, results showed remarkable changes in the molecular structure of the extracted protein molecules such as the molecular weight, radii, shape, relaxation time and the dielectric increment. Also, there were sharp reductions in the number of protein bands and in the protein amount in each band of the exposed samples as well as changes in the molecular weight of this protein. On the other hand the number of E. coli cells decreased after the exposure 6hr process and E-coli cells increase after exposure 16hr process.

Fluctuation in the electrophoretic mobility and appearance of new protein species were observed. Moreover, the data reveal remarkable increase in the water soluble protein content resulted from 16hr. remarkably higher than those resulted from the other 6hr., the effect of Electromagnetic field at 16hr.causes Stimulation.

INTRODUCTION

During the past few decades, due to the increasing consumption of electric energy in industry medicine, research, communication systems and house hold electric appliances, the level of exposure of biological systems to electromagnetic fields has grown by orders of magnitude over a wide frequency range extending from 0 to 100 of GHz. For example hair dryers, electric shavers and electric hand tools may expose the user to magnetic fields of several times above the background.

The extremely low-frequency (ELF) electromagnetic field (EMF) exists in all occupational and residential environments. Some scientists allege that exposure to magnetic fields generated by power delivery systems is responsible for certain cancers, reproductive dysfunction, birth defects, neurological disorders, and Alzheimer's disease (Wartenberg 1996). However, until now, the available date has been inadequate to conclude that electromagnetic field exposures alone are the reason for the observed increase in

carcinogensis effects. Therefore, it is both appropriate and important to evaluate the possible effects of man-made electromagnetic field on living organisms.

Ma-Haile et al., 2003 studied the effect of pulsed magnetic field intensity and pulse number (PMF) on bactericidal property of PMF in sterilization of fresh watermelon juice. Their results showed that the overall bactericidal effect was strengthened as the magnetic field intensity and pulse number increased with the best effect observed when the magnetic field intensity was 2.53 T and pulse number was 20.

Piatti-E et al. (2002) found that exposure of *Serrstia marcescens* to static magnetic field of 80 ± 20 Gauss resulted in the inhibition of the *S. marcescens* growth. **Dacosta Y (1997) and Barnickal-M (1998)** used new non-destructive decontamination technique for reduce the bacteria in milk, orange juice and also in cheeses. Pulsed electric field, pulsed magnetic field and pulsed light were used.

Hui-YH et al (2004) used new technology such oscillating magnetic field, pulsed x-ray, pulsed electric field processing, ohmic and inductive heating for reducing and inactivating pathogens bacteria. **Fajt et al (2004)** found that E-coli, bacteria adecarboxy and staphyloccus aureus viability affected with the magnetic field (10 mT, f = 50 Hz) also they found that the decrease of the colony forming units (CFU) starts immediately after the magnetic field was switched on. **Mei et al (2004)** studied the inactivation of microorganisms by pulsed magnetic field. It was reported that the application of electromagnetic pulses evidently causes a lethal effect on *E. coli* cells suspended in buffer solution.

Ye-Shengying et al (2003) studied the non-thermal sterilization by using the self-designed generator of magnetic field. The results showed that the magnetic flux density which had the greater effect on *E. coli* was 1 T. The greater destruction rate of *E. coli* was 78% under 8 hours of magnetic field (1T) treatment.

Electromagnetic fields are also used in therapy to enhance the transdermal drug delivery ***(Parasrampuria, D. and Parasrampuria, J. 1991)***. In certain dairy industry to manipulate growth characteristics of yogurt culture, where the change in culture metabolism rather than an elevation in the over all bacterial population, was induced by a 60 Hz, 4.3 G EMF ***(Michael, B.D. 1992)***. In soil studies the Electromagnetic fields used in inactivation of indicator bacteria of cattle slurry by exposure of 400 – 700 g for 60 seconds to magnetic field (380w, 50 Hz) ***(Klachkova Yu, F. and Kvitkina, M.E. 1993)***. In some food preservation to control certain pathogenic bacteria as *Salmonella* and *E. coli* contaminated meat samples ***(Borovkov et al, 1994)***. In agriculture it also used to improve soil fertility by increasing the nodulation process by exposure of *Rhizobium* sp. to a low strength (5×10^{-3}T) EMF before inoculation to mycorrhizal chick-pea seedlings ***(Bajwa, et al, 1995)***.

Finaly, EMF has been used either to inhibit or to stimulate the growth rate of microorganisms under appropriate conditions ***(Jaffe, 1983).***

This work is concerned with the study of the biological effect of magnetic fields, as a component of the non-ionizing electromagnetic fields on unicellular system. Pathogenic microorganisms, especially Escherichia Coli- are chosen to be our experimental model for many reasons, it is widely distributed in environment such as soil, water and air. *E. coli* is a member of the normal intestinal flora of human. It causes several diseases such as urinary tract infection, wound infection, traveler's diarrhea. It reaches blood stream and cause sepsis and meningitis ***(Parts et al, 2000)***. *E. coli* are rapidly growing, Gram-negative, rod-shaped

cells measuring approximately 0.5 × 2 μm length ***(Neidhardt et al., 1990)***.Because of the bathogenic effects of these bacteria, we aim, here, a certain exposure period to amagnetic field sufficient to kill all of them .sush a process would , namely, supply an easy and elegant method to get rid of those bacterial, especially for sterilizing medical instruments.

Moreover, we intend to take two exposure periods for investigating the effect of magnetic field (20 G, 50 Hz) on the growth rate, the antibiotic sensitivity and the ultra structure of the exposed cells.

MATERIALS AND METHODS

1- Bacterial strain:

Escherichia coli ATCC ≠ 25992 was cultivated over night on Nutrient broth at 37°C, each ml of bacterial suspension contain 13×10^3 cfu/ml.

2- *Survival curve:*

To study the bacterial growth, a standard survival curve was plotted between the absorbance of volume A (unexposed cells) at 600 nm and the concentration of cells (number of cells / mL). For cell counting the plate count technique was used ***(Stanier et al., 1986)***. Appropriate dilutions of the bacterial cells were used to inoculate nutrient agar plates. Inoculated plates then incubated at 35° C for 24 hr. by counting the number of colonies developed after incubation and multiplying it with the dilution factor the number of cells in the initial population is determined.

3- Magnetic field exposure facility :

Bacteria volumes were exposed to a homogenous magnetic field generated by a solenoid consisting of 320 turns from electrically insulated 8 mm cupper wire wound in a homogenous way around a cupper cylinder 2 mm thick, 40 cm diameter and 25 cm length. The cylinder wall was earthed to eliminate electric field components effects. The magnetic field generator was temperature controlled during the exposure period by using a water pump as shown in fig (1). The temperature during the exposure period was 37° C. The tubes of the exposed bacteria were put in the middle of the coil by using supports inside it to get a homogenous and higher magnetic fields strength. The ends of coil are connected to variac fed from the mains (220v, 50Hz). The field strength was adjusted by changing the voltage through the coil.

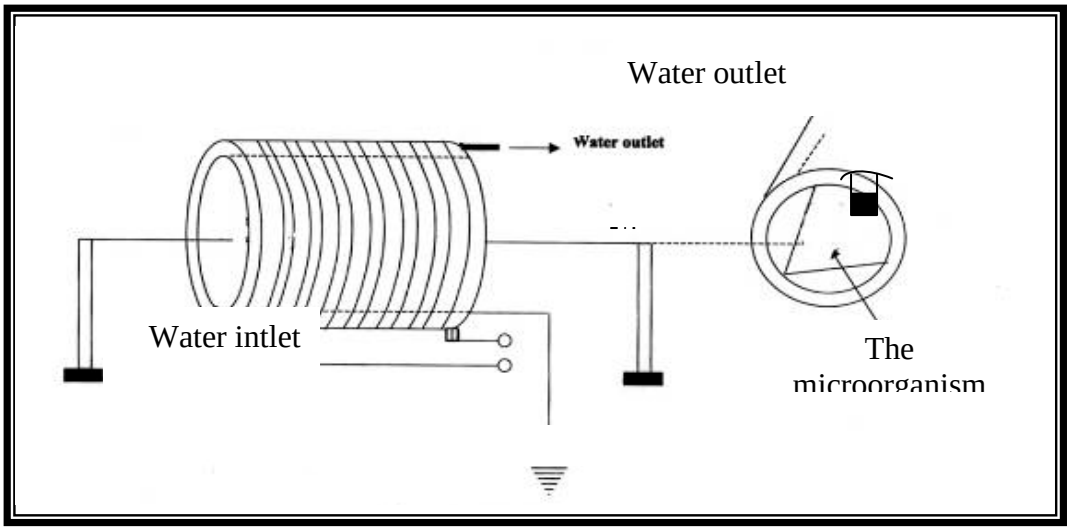


Fig. (1) Magnetic Field Exposure Facility

4- Growth characteristics of exposed and unexposed cells

10 volumes from the strain were incubated to 18 hr. and then exposed to different exposure periods. The first volume exposed to two hour. The second volume exposed to four hours. The third volume exposed to six hours and so on. **For each exposure volume, there was a coresponding control volume, .**Due to their high effect, the two volumes exposed to the two periods, 6 hr. and 16 hr. were chosen for additional investigations.Four volumes were used in this studey A,B,C andD. Volume A is the control of volume B exposed to of 6hr.magnetic field , volume C is the control of volume D exposed to 16hr.magnetic field .

5-The dielectric measurements of (WSP):

The concentrated protein solution was, then diluted with bi-distilled water at a ratio 1:20. Loffer et al. (1970), calculated the dielectric properties of protein and its solvent (comparison). They found that the coupling between the dielectric relaxation of the peptide and that of the water-component is particularly important for correctly describing the dielectric constant of the peptide.

Dielectric measurements for the protein extracted from the gains were carried out at fixed temperature of $4°C$ through the use of an incubator type 2771, Kattermann, Germany and a cell types (PW 950/60 manufactured by Philips). The cell has two parallel square platinum black electrodes of 0.8cm side each, and area 0.64 cm^2, A, with an inter-electrode distance, d, of 1 cm. The dielectric relaxation was measured in the frequency range 10 kHz – 0.5 MHz through the use of RLC bridge 3235 Hioki – Japan. The measured values of capacitance, C, and resistance, R, were used to calculate real ε' (dielectric constant), and imaginary parts ε'' (dielectric loss) of the complex permittivity (PolK and Postow 1996):

$$\varepsilon^* = \varepsilon' - J\varepsilon''$$

using the following equation:

$$\varepsilon' = \frac{Cd}{\varepsilon_0 A} \qquad (1)$$

where ε_0 is the permittivity of free space.

$$\varepsilon'' = \varepsilon' Tan\,\delta \qquad (2)$$

$$\tan\delta = \frac{1}{2\pi fRC} = \frac{\varepsilon''}{\varepsilon'} \qquad (3)$$

The a. c. conductivity S "s^{-1}" was calculated from the equation.

$$S = \frac{\sigma}{\varepsilon_0} = \omega\varepsilon'' = 2\pi f\ \varepsilon'' s^{-1} \qquad (4)$$

and

$$\sigma = 2\pi f\ \varepsilon''\varepsilon_0 \qquad \text{S/m} \qquad (5)$$

where $\omega and\ \sigma$ are, respectively, the angular frequency and the actual conductivity.

The molecular radius (R) of the protein molecules was estimated through the use of equation 6, (Polk and Postow 1996).

$$R^3 = \frac{kT\tau}{4\pi\eta} \qquad (6)$$

where k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the protein solution and τ being the relaxation time, namely, the time at which the dielectric molecule has the ability to relax under the effect of the applied field and be calculated from the relation.

$$\tau = \frac{1}{2\pi f_c} \qquad (7)$$

fc is the critical frequency corresponding to mid point of the dispersion curve in Figure (1).

The difference between the values of ε' s and $\varepsilon' \infty$ at low and high frequencies is called "the dielectric dispersion $\Delta\varepsilon' = (\varepsilon' S - \varepsilon' \infty)$. This quantity is a measure for the shape and volume of the non polar solution consisting of protein and bound water (Hasted, 1973); the dielectric spectrum of a biological macromolecule such as protein at high frequency.

(Hundreds of Kilohertz) are known as β dispersion which came from the polarization of protein and organic macromolecules (Gabriel et al., 1996).

In case of biomolecules, such as proteins, the dielectric relaxation shows broader dispersion curves and lower maxima than those predicated by Deby model, and the $\varepsilon'' versus \varepsilon'$ curves fall inside the semicircle, so Cole-Cole (1941) introduced a new parameter, α, and modified Deby equation. It was shown by Cole and Cole that the angle $c\varepsilon'_{\infty}\ \varepsilon_0 = \theta = \alpha\pi/2$. This in turn enables one to estimate the Cole parameter α, experimentally, so $\alpha = 20/\pi$.

Protein concentration in the different samples was determined according to the method of (Broadford, 1976) with coamassie Brilliant Blue G-250 a rapid-dye binding assay and using bovine serum albumin as a satandard protein.

6- Qualitative analysis of WSP by (SDS) polyacrylamide gel electrophoresis:

The water soluble protein (WSP) was extracted in the form of concentrated solution from rice gains by the method of Irvin et al 1980. The molecular weights of the components of such WSP of the mother gains M and the first generation M_1 were estimated through the use of SDS polyacrylamide gel electrophoresis according to the technique of Lammli, (1970). The gel was stained with coomassie brilliant blue R-250. Six markers of known molecular weights were used as a standard protein, phosphoylas B 97 kDa, Bovine serum 66 kDa ovalbumin 45 kDa, Trypsin 29 kDa, lysozyme 14.4kDa and aprotinin bovine lung 6.5 kDa.

The data were identified and analyzed by using gel pro analyzer version 3Media Cybernetice imaging experts software, which compare the absorbance of each sample in each band, molecular weight and the rate of mobility of each bands for the samples with the standard markers.

7- The morphological and physiological measurements:

The morphological character of the unexposed and expoused form E-coil (direct& late) effect were estimated by using the light microscope methods of ( )according to

8-Statistical evaluation

The statistical analysis of the biological data was performed using the methods of Milton and Tsokos 1983 to asses the significance of the differences between mean of control and the treated grains using t-test.

RESULTS AND DISCUSSION

The results obtained in this work concerning the induced changes in the structure and the characteristic behavior of the E.coli resulting from the exposure to the demonstrated magnetic field. These results may be of a great importance for evaluating the benefits a well as the hazards from the exposure to the low frequency low level magnetic field.

Also the importance of this work lays in the fact that E.coli as a microorganism is a unit cell behaving as a complete alive biological system

1- Survival curve:

Fig. (2) Shows the variation of the number of microorganisms in cfu / ml as a function of the sample absorbance measured at 600 nm. The results show linear dependence of the absorbance on the number of microorganisms in cfu / ml. By using this relation we can calculate the number of the microorganisms / ml from the measured value of its absorbance (A). The liner dependence can be easily expressed by the relation

$$C = 9.7 \times 10^9 A \qquad (8)$$

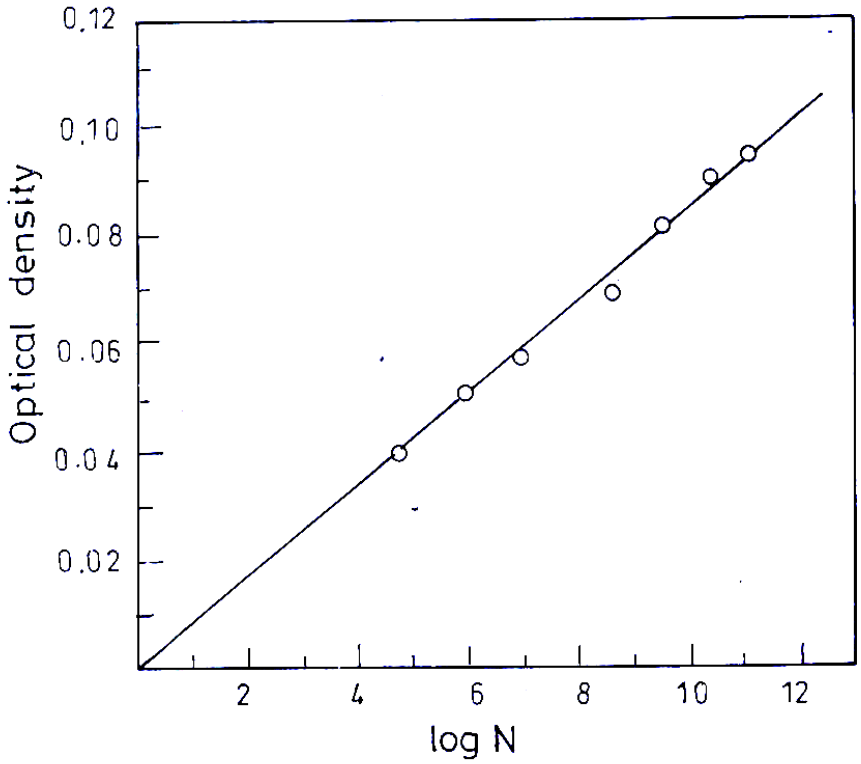


Fig. (**2**) : Calibration curve between the log number of bacteria cells / ml and absorbance at 600 nm.

2- Growth characteristics curve:

Fig. (3) Shows changes in the absorbance of bacterial strain as a function of the time of exposure to the magnetic field.

It is clear from the figure that after exposure period 2, 4, 6, 8, 10 and 12hrs decreased the absorbance and in accordance with equation (1), indicating a decrease in the cells number and consequently an inhibition case for the bacteria. However the exposure periods

14, 16, 18 and 20 hr. the absorbance increased relative to their control indicating an increase in the cells number and a stimulation case. These results are in a good agreement with Mohammed et al. (1997) where is the number of cells of S-typhi microorganism exposed to 20G magnetic fields for 2 hours increased relative to its unexposed.

Also Jaffe (1983) reported that the electromagnetic field has been used either to inhibit or to stimulate the growth of the microorganism under a appropriate conditions.

For this reason we used the exposure period 6 hr. (volume B) as an inhibition case where the number of cells was 10^8 and became 10^7 cells/ml also the exposure period 16 hours (volume D) as stimulation case where the number of cells was 3.5×10^2 and became 3.5×10^4 cells/ml. Moreover, we intend to take the two exposure periods for investigation the effect of magnetic field (20 G, 50Hz) on the growth rate, the antibiotic sensitivity and the ultra structure of the exposed cells.

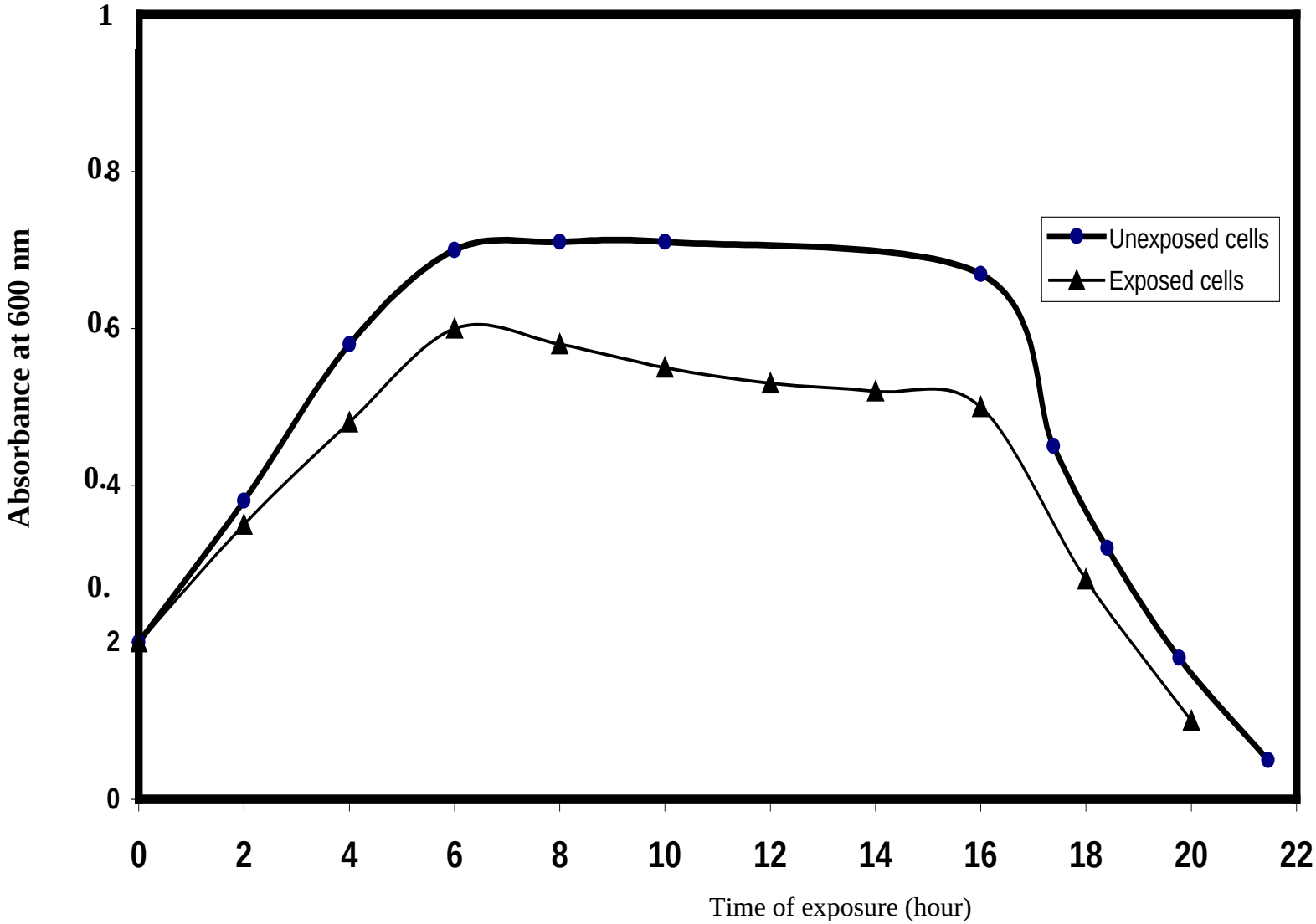


Fig (3) : Biological response of the E. coli to electromagnetic field

Figures 4 → 6(a) illustrate the variation of the permittivity ε′ and dielectric loss ε″ plotted on the left Y axis and the conductivity (S) on the right Y axis as a function of the applied frequency for WSP extract from *E*-coli cells (Direct effect).

Figures 4 → 6(b) illustrate the Cole-Cole plots (ε′ versus ε″) for WSP extract from *E*-coli cells (Direct effect).

Figures 7 → 9(a) illustrate the variation of the permittivity ε′ and dielectric loss ε″ plotted on the left Y axis and the conductivity (S) on the right Y axis as a function of the applied frequency for WSP extract from *E*-coli cells (Late effect).

Figures 7→ 9(b) illustrate the Cole-Cole plots (ε′ versus ε″) for WSP extract from *E*-coli cells (Late effect).

It is clear from the figures that the permittivity ε′ passed through a dielectric dispersion. Grant (1983) measured the same phenomena for other types of protein. The decrease in the value of ε′ was accompanied by increase in the value of conductivity (S) which we considered as indicating confidence in the measurements Fadel et al (2002).

From the figures, it is also, clear that the conductivity (S) of all groups are frequency dependent, and show the conductivity dispersion which are due to the interfacial polarization Bordi et al (1997). In addition, the values of the conductivity and the permitivity for the 16hr were higher than the 6hr.

From the data obtained from the figures and through using equations 1, 2, 3 and 4, the values of each of relaxation time τ, the dielectric increment Δ ε′, the average molecular radii R, the Cole-Cole parameters (α) and the conductivity (S) for (direct effect) were calculated and given in Table 1 and we calculate (late effect) by table (2).

The changes in the dielectric increment $\Delta\varepsilon = (\varepsilon_s - \varepsilon_\infty)$ for the WSP molecules resulting from exposure cells indicate that there are changes in their dipole moments and, consequently, in their shape and volume (Hasted, 1973).

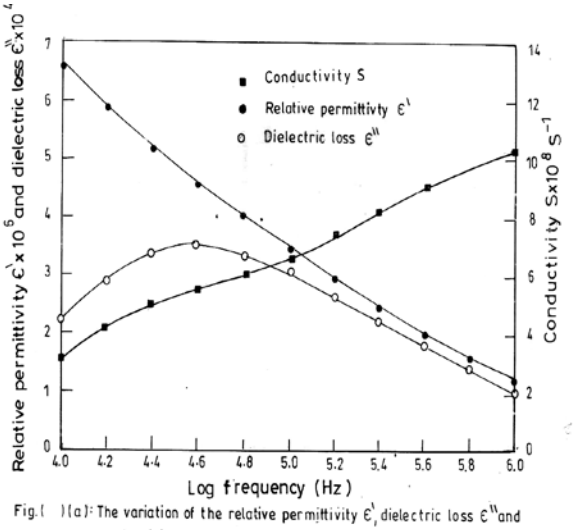


Fig.( )(a): The variation of the relative permittivity ε', dielectric loss ε'' and conductivity S as a function of the applied frequency (Direct effect) control.

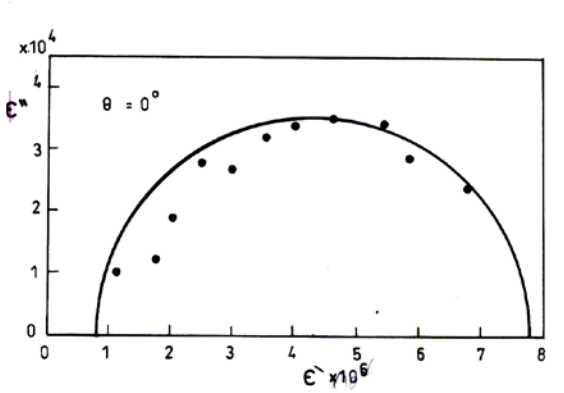


Fig.( ) (b): The Cole–Cole plot (ε' versus ε'') for (Direct effect) control

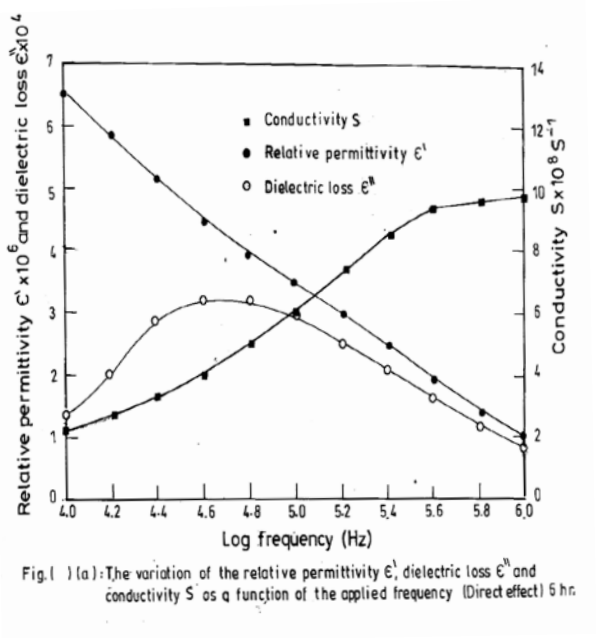


Fig. ( ) (a): The variation of the relative permittivity ϵ', dielectric loss ϵ'' and conductivity S as a function of the applied frequency (Direct effect) 6 hr.

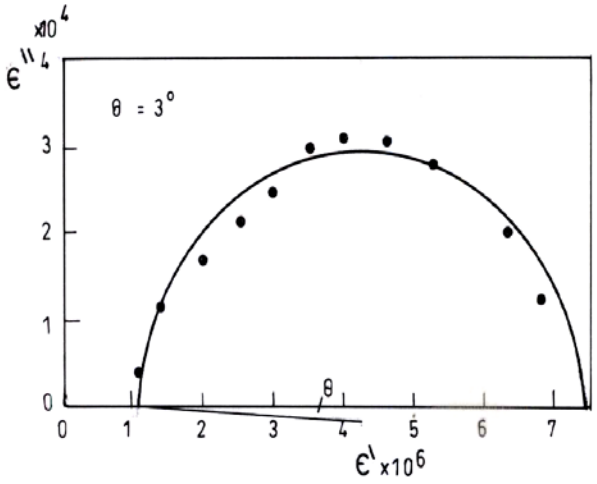


Fig. ( ) (b): The Cole–Cole plot (ϵ' versus ϵ'') for (Direct effect) 6 hr.

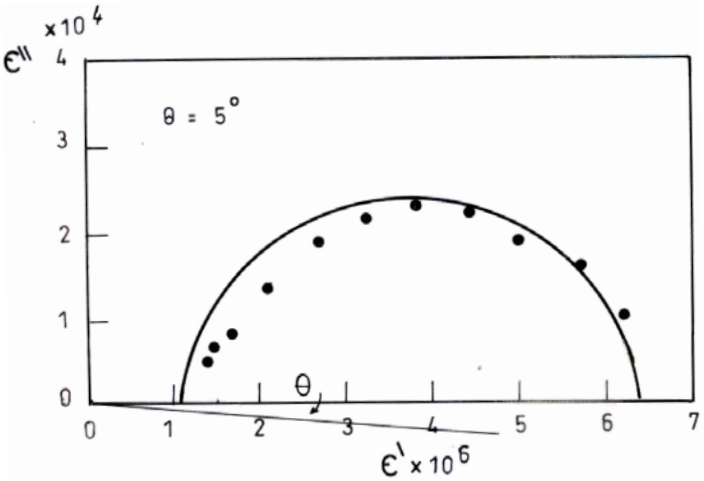


Fig. ( ) (b): The Cole - Cole plot (ϵ' versus ϵ'') for (Direct effect) 16 hr.

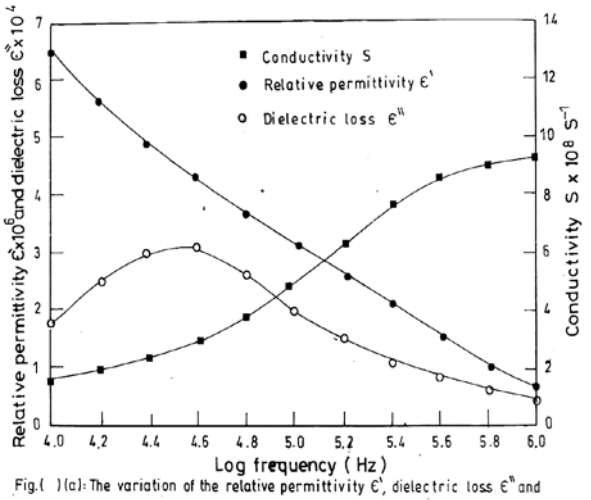


Fig.( )(a): The variation of the relative permittivity ε', dielectric loss ε'' and conductivity S as a function of the applied frequency (late effect) control.

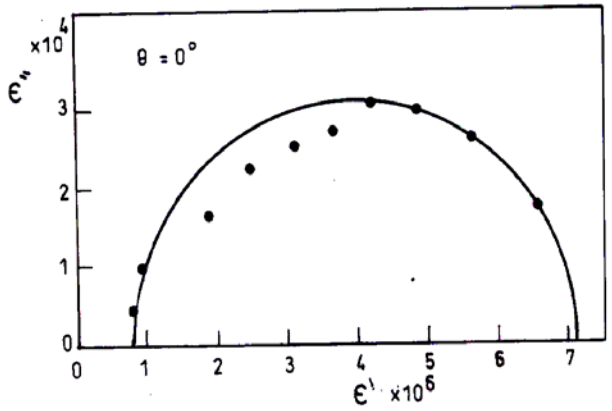


Fig.( )(b): The Cole–Cole plot (ε' versus ε'') for (Late effect) control.

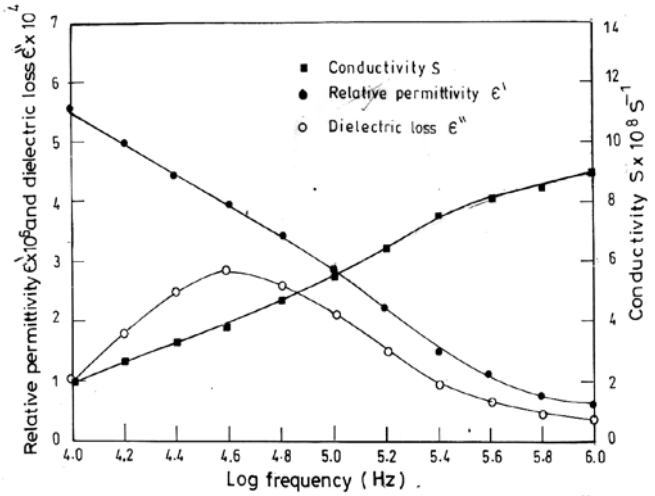


Fig.( )(a): The variation of the relative permittivity ε', dielectric loss ε'' and conductivity S as a function of the applied frequncy (Late effect) 6 hr.

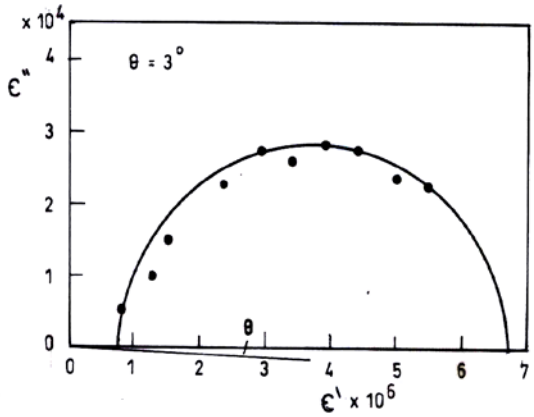


Fig.( )(b): The Cole–Cole plot (ε' versus ε'') for (Late effect) 6 hr.

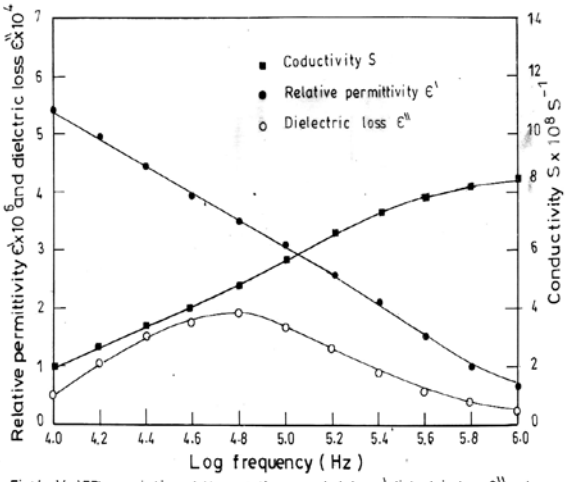


Fig.( )(a): The variation of the relative permittivity ε', dielectric loss ε'' and conductivity S as a function of the applied frequency (Late effect) 16 hr.

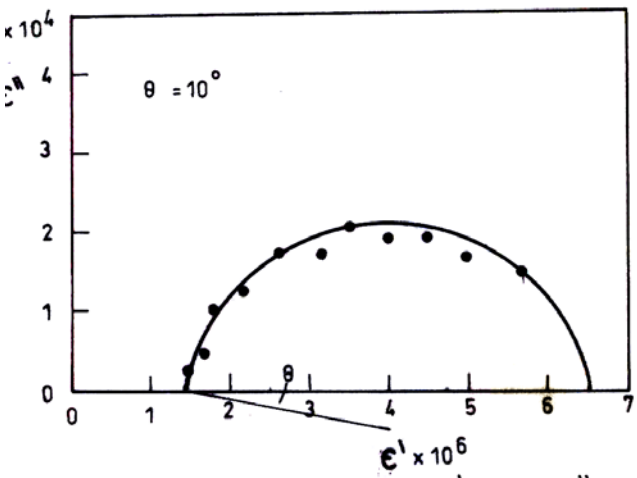


Fig.( )(b): The Cole–Cole plot (ε' versus ε'') for (Late effect) 16 hr.

Table (1) Values of dielectric increment $\Delta\varepsilon$, conductivity S, relaxation time (τ) viscosity η average molecular radii (R) and *E. cole* parameter (α) for the WSP as a function of the time of exposure (Direct effect):

Time exposure	Conductivity S × 10^8	Dielectric increment $\Delta\varepsilon$ × 10^6	τ (μ sec.)	η (Poise)	R_{nm}	α
Control	10.4 ± .1	5.6 ± .1	5.55	0.133	2.24	0
6 hr	9.8 ± .1	5.7 ± .1	3.88	0.124	2.17	0.03
16 hr	9.5 ± .1	5.6 ± .1	3.54	0.134	2.05	0.05

Table (2) Values of dielectric increment $\Delta\varepsilon$, conductivity S, relaxation time (τ) viscosity η average molecular radii (R) and cole cole parameter (α) for the WSP as a function of the time of exposure (Late effect):

Time exposure	Conductivity S × 10^8	Dielectric increment $\Delta\varepsilon$ × 10^6	τ (μ sec)	η (Poise)	R_{nm}	α
Control	9.4 ± .1	5.5 ± .1	5.31	0.125	2.4	0
6 hr	9 ± .1	5.1 ± .1	4.55	0.118	2.33	0.03
16 hr	8.5 ± .1	4.9 ± .1	3.98	0.127	2.17	0.10

It is seen from both of Cole-Cole plots and table 1. How the Cole-Cole parameters α are greater than unity for the WSP extract from *E-coli* cells. This indicates that the protein molecules became semi-circle in shape.

Also, it is clear from the table that there are changes in each of the relaxation time and the average molecular radii for the WSP molecules as a result of exposure from *E-coli*.

The disc electrophoretic pattern and the molecular weight distribution of the WSP extracted from *E-coli* cells are shown in figure 10 (a & b) from (direct effect) and (late effect) are shown in Fig. 11 (a & b) and table 3 respectively.

The scanning profiles of the electrophoretic separation indicate that WSP extract from *E-coli* exposure separated into 23 fractions having molecular weight in the range 160→ 20 kDalton after the 16hr the number of fractions became 23 fractions and 6hr became 6 fractions.

Also, there are changes in the molecular weights of the fractions where they tend towards higher molecular weights for 16hr to changes in its mobility rate relative to the control.

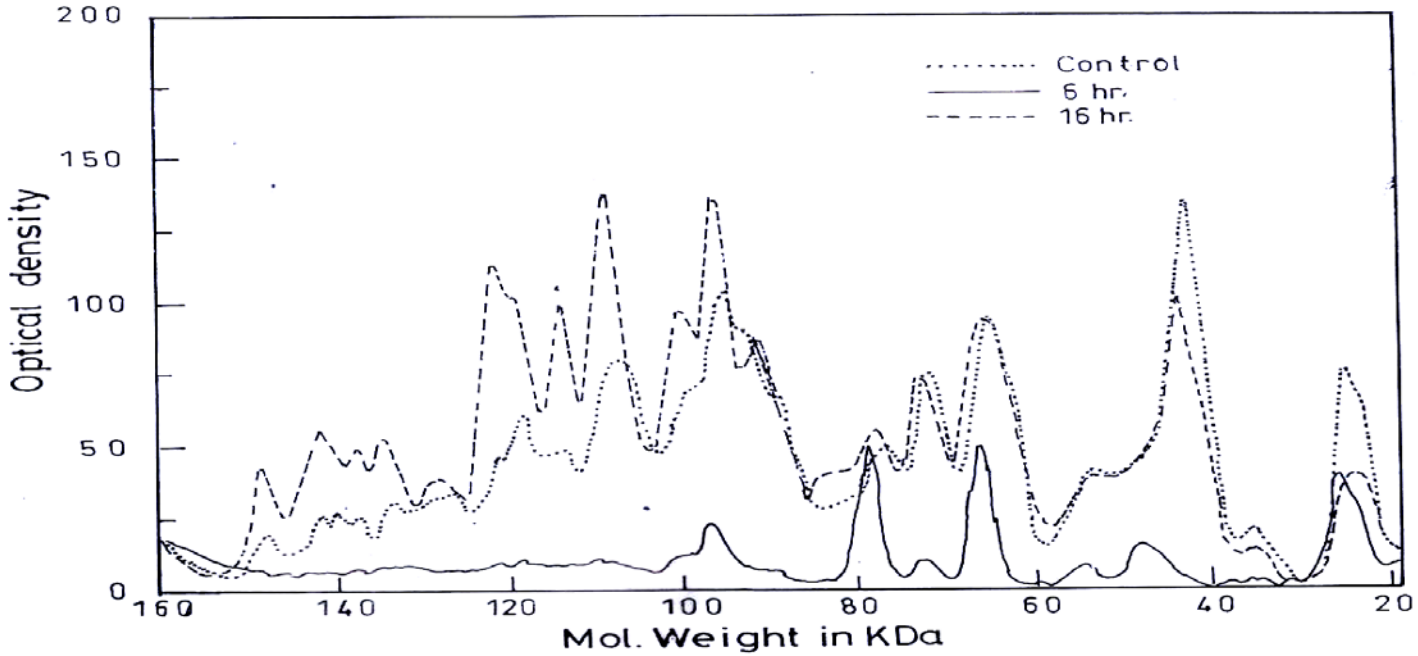


Fig. ( ): The molecular weight distribution of WSP extract from E-Coli (direct effert).

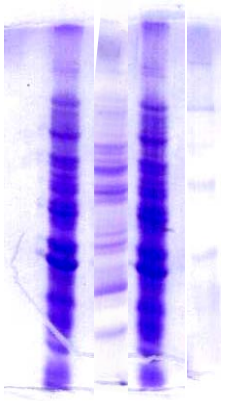


Fig. 11(b): The electrophoretic pattern of WSP extract *E-coli* (Direct effect)

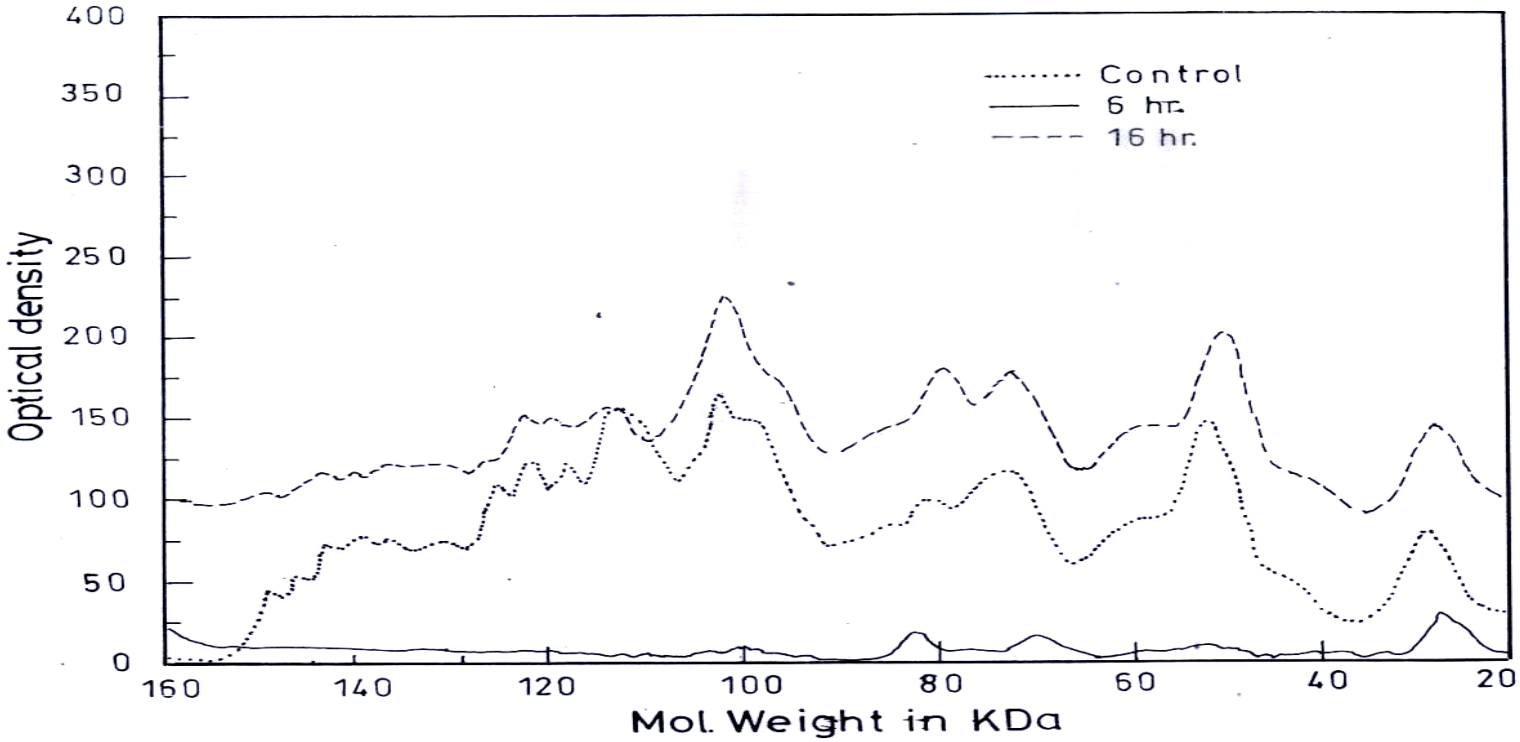


Fig. ( ) : The molecular weight distribution of WSP extraxt from E – Coli (Late effect).

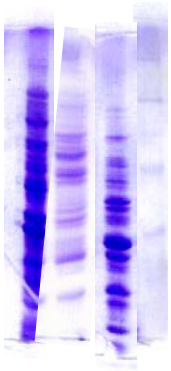


Fig. 10 (b): The electrophoretic pattern of WSP extract *E-coli* (late effect)

Table (3): The values in the molecular weight (M.W.) and the optical density (O.D) of the (WSP) as a function of the (direct effect):

Number of bands	Control		6 hr		16 hr	
	M.W.	O.D	M.W.	O.D	M.W.	O.D
1	159.7	48.5				
2	74.8	20.9			74.11	47.2
3	61.5	28.1	43.7	23.9	61.2	59.5
4	58.2	32.7			59.9	52.2
5	55.3	36.6	36.8	51.3	58.8	54.2
6	52.5	62.96	34.1	16.3	55.9	42.5
7	50.9	49.4	31.7	51.2	54.4	117.1
8	48.7	81.8			52.6	103.6
9	45.5	71.6	26.3	16.3	50.8	102.5
10	43.8	105.9	20.9	41.9	49.1	138.1
11	40.9	68.9			44.8	99.6
12	35.9	52.2			42.9	142.4
13	34.1	77.7			40.6	89.8
14	31.8	97.8			37.9	42.4
15	31.1	75.1			36.8	59.6
16	25.2	137.1			34.7	76.5
17	23.3	22.3			31.6	96.2
18	20.7	77.6			27.6	40.6
19	59.6	26.1			25.2	100.4
20	60.6	29.6			20.5	42.0
21	53.5	45.5			156.3	34.5
22	27.9	42.1			30.8	81.9
23	26.4	49.4				

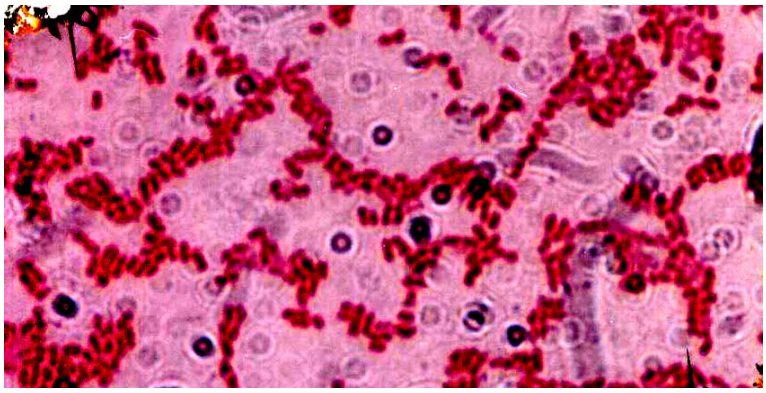
Table (4): The values in the molecular weight (M.W.) and the optical density (O.D) of the (WSP) as a function of the (late effect):

Number of bands	Control		6 hr		16 hr	
	M.W.	O.D	M.W.	O.D	M.W.	O.D
1	159.8	38.7				
2	74.9	43.9	43.69	9.1	74.8	106.28
3	66.6	55.6	37.15	18.4	61.8	119.9
4	61.5	75.2	31.8	17.4	58.2	122.3
5	60.6	78.6	26.4	8.9	55.5	124.4
6	59.8	78.8	20.6	29.8	53.5	153.9
7	58.1	75.8			52.4	151.3
8	55.3	114.4			50.3	158.7
9	53.5	128.3			43.8	225.8
10	52.1	126.1			36.7	154.1
11	50.1	157.2			35.9	181.7
12	43.8	169.2			32.1	178.6
13	40.9	151.2			27.1	144.1
14	36.8	84.7			25.1	204.8
15	35.3	101.7			23.8	117.1
16	31.1	118.1			20.6	147.5
17	28.1	78.9			37.9	144.3
18	25.5	148.8			43.8	225.8
19	20.7	79.8			40.9	178.6
20	45.5	126.7			45.5	181.1
21	48.7	123.7			60.6	119.4
22	26.9	87.8			59.8	124.3
23	32.4	118.4			64.6	107.4
24	37.9	76.2			27.9	142.4
25					30.68	161.3

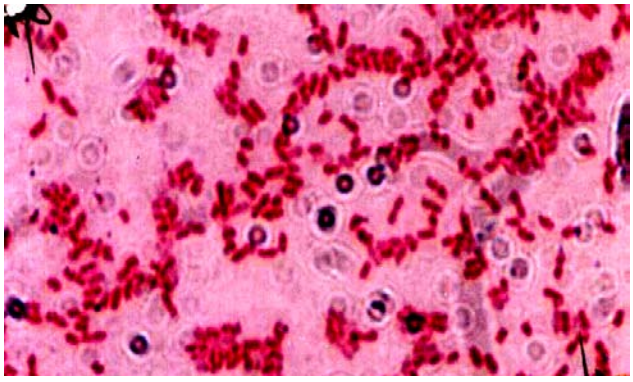
To get better understanding of the interaction mechanism of magnetic field with biological systems, an understanding of the bioelectrical signals resulting from biological system during metabolic activity is required. Mohamed et al. (1997) reported that the bioelectrical signals from the microrganism normally were carried out through bending of their cellular membranes which generate electric impulse through phenomena known as flexoelectricity. The amplitude and the frequency of these impulses depend on the amount and frequency of bending. These impulses travel through the medium separating the microorganisms and received by the signal receptors at the surface and that impeded in the cell membrane .Therefore the flexibility of the membrane is the most important parameters for generation of these signals. Also mentioned that the biomagnetic field from the biological system associating to the bioeiectrical signals from the membrane of the cells through its metabolic function is very week in nanogauss rang (20x 1 0 G). When the biological systems exposed to an external magnetic field whose strength is very large relative to the biomagnetic field of the cells a disturbance in their metabolic function will, be expected and lead to death of the cells or increasing their cell division Fadel et al. (2003) and Shin-Ichiro H. et al. (2002). From the present data it is easily deduced that the cellular membrane of the microorganism had been affected by the external magnetic field in a good agreement with Fadel et al. (2003). Then we can expect the disturbance of cell division and hence, a change in the number of the cells per ml or the measured changed in the membrane sensitivity to antibiotic demonstrated also the change in the internal structure of the cells.

7- The morphological and physiological measurements:

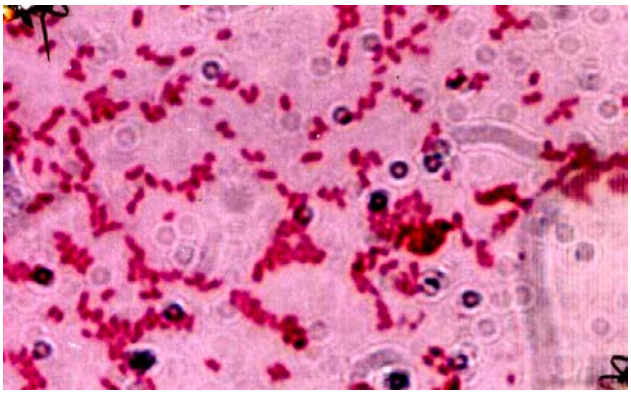
Control



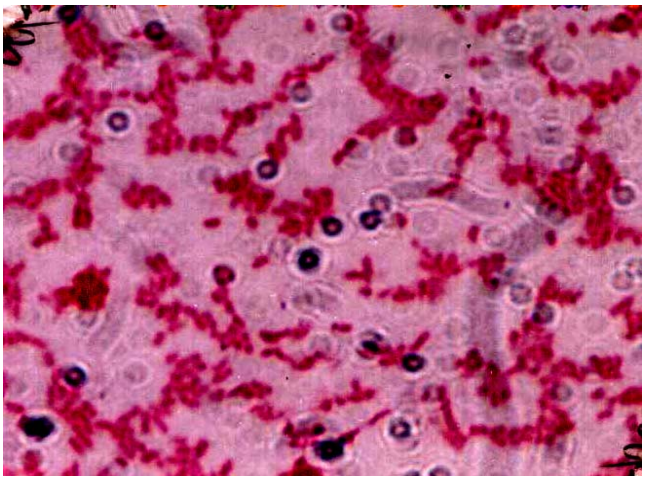
6hr direct



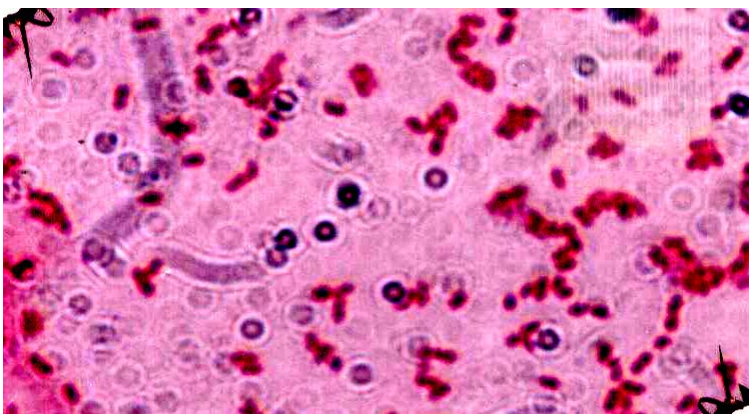
16 hr Direct



6 HR LATE



16 hr Late



CONCLUSION

From this work, it is concluded that the electromagnetic field (20 G) affected considerably the virulence of the *E. coil* cells. 6hr. exposure time was found to cause an inhibition case whereas 16hr. exposure time enhanced the virulence.

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ENHANCEMENT OF PLANT GROWTH THROUGH IMPLEMENTATION OF DIFFERENT *TRICHODERMA* SPECIES

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ABSTRACT

Fifty *Trichoderma* isolates were screened in pot experiment to evaluate their ability to promote growth of pepper plants. Different effects were recorded, but in general *Trichoderma* increased plant vigor. Various modes of application were tested such as drench, seed coating, seedling soaking and spray to shoot system. Drench gave the highest effect (45%) than control, Tomato plant showed the highest response, increase in shoot length reach to (56%) more than its control. Canola plant was less responding to biofertilization with *Trichoderma*. This effect could be due to the secretion of plant hormones by *Trichoderma* as indicated by laboratory test where the production of IAA, in culture filtrate and in plant extract was detected.

Key words: *Trichoderma,* plant growth-promoting, IAA, tomato, kidney bean, peanut

INTRODUCTION

Due to tremendous increase of human population as well as the great green revolution, global demand of healthy food and crops of different types was incredibly increased. This led to extensive utilization of agriculture land. To get colorful flowers and delicious fruits as well as profitable crops we have to add precious nutrients to the field where plants are grown, in order to substitute the depletion of essential elements need by plants. (Loneragan, 1968). Plants need about sixteen elements for optimum growth. Most of these elements are obtained from the soil to promote plant growth. Plants consume different amounts of these elements depending on its own needs. Farmers and plant growers rely deeply on chemical fertilizers to promote their plant and to offset for decrease of soil elements due to plant uptake. However these elements showed, be supplied in balanced amount as excess amount of an element do not substitute the less of another. But even may prevent absorption of a third element. Chemical fertilizers (C.F.) could increase the imbalance of soil elements resulting from intensive agriculture. In addition, C.F. may lead to further loss in soil quality, possibility of water contamination and unsustainable burden of the fiscal system. The adverse effect of C.F.became manifested due to their excessive imbalanced uses. This led to the seek for harmless inputs such as compost and biofertilizers. Biofertilizers are living organisms that have the capability to enrich the nutrient status of the soil and augment the availability of nutrients. They represent renewable inputs which can maximize the ecological benefits and minimize the environmental hazards. The most used organisms are Mycorrhiza, Rhizobium, blue green algae and some higher plants such as legumenose plants. Filamentous fungi and yeast are also used but at less extent.

Microorganisms used in biological control, including bacterial and certain fungal biocontrol agents, can be associated with enhanced plant growth. In some cases, increased growth of the host plant is due to a reduction of viable inoculum of undetected pathogens, such as root infecting *Pythium* species, which cause only slight reductions in vigor or yield. In other cases, enhanced plant growth, particularly in the absence of pathogens, may be due to plant-growth-promoting compounds of microbial origin (Chet, 1993).

Beneficial effects of the introduced microorganisms on plant growth have been reported for numerous crops, including tomato (*Lycopersicon esculentum* Mill.) grown under field (Kokalis-Burelle *et al*., 2002;Guo *et al*., 2004) or greenhouse conditions in organic media (Gagne´ *et a*l., 1993). Such beneficial microorganisms are referred to as plant-growth promoting rhizobacteria (PGPR) plant-growth promoting fungi (PGPF) they enhance plant growth through numerous mechanisms including the protection of roots against infection by minor and major pathogens (Whipps, 1997, 2001), enhancing the availability of nutrients to the host plant, lowering the ethylene level within the plant or by the enhanced production of stimulatory compounds, such as plant growth regulators (Antoun and Prevost, 2005). Among plant growth regulators, indole-3-acetic acid (IAA) is the most common natural auxin found in plants and its positive effect on root growth and morphology is believed to increase the access to more nutrients in the soil (Vessey, 2003). Despite the identification of many filamentous fungi that produce phytohormones, such as indol acetic acid (IAA) and ethylene, whose metabolic pathways have been identified, *Trichoderma* strains that produce cytokinin-like molecules, e.g. zeatyn and gibberellin GA3 or GA3-related have been recently detected (Harman *et al.,* 2004), the controlled production of these compounds could improve biofertilization.

In addition to their biocontrol activities, *Trichoderma* spp. has been reported to promote plant growth. *Trichoderma* strains are always associated with plant roots and root ecosystems. Some authors have defined *Trichoderma* strains as plant opportunistic symbiont, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi and to produce compounds that stimulate growth and plant defense mechanisms (Harman *et al.,* 2004).

The aim of this study was to examine the ability of different *Trichoderma* spp. to enhance plant growth through the production of growth promoting phytohormones externally or inducing production in treated plants.

MATERIALS AND METHODS

Screening ability of *Trichoderma* and *Gliocladium* for growth promotion in pepper plant

Fifty *Trichodema* isolates were recovered from various habitate including agriculture and reclaimed soils, dung and compost**.** The inocula for the pot trial were prepared by growing 50 isolates of *Trichodema* spp. in flasks contain 50 ml potato dextrose broth (PDB) at 28^0C. Mixture of metabolites and spore suspension was diluted twice and mixed with soil and seeds of pepper were soaked in *Trichoderma* spore suspension for about 5 min. (Yedidia *et al.,* 2001). Sterilized diluted broth and water work as control. Six inoculated seeds of pepper were sown in pots containing 5Kg /pot sandy soil; three pots for each isolate are arranged stratified in Botanical Garden under the same condition. After six weeks different parameters were measured such as (shoot length, root length, fresh and dry weight).

Application methods of *Trichoderma* on Tomato

The pot trial was prepared by coating seeds of tomato by spores of *T.harzianum 9* + C.M.C

(Windels., 1981). In another groups application of the same isolate by spore suspension drench, five pot replicates for each group beside control, percentage of excess in shoot and root length in two groups were calculated

Effect of *Trichoderma* on flowering and fruiting of Tomato

Five tomato plants developed from seed coating plant were soaked in spore suspension of *T.harzianum 9* for one hour and control untreated plants were soaked in water for the same time then cultivated in field and this data were recorded (No. of flowers, No. of fruits, weight of fruits fresh &weight and photosynthetic pigments).

Response of different plants to *Trichoderma*

The field experiment was conducted at the Botanical Garden of Botany Department, Faculty of Science. Ismalia, using seeds of peanut cultivar Caltro Apparently healthy seeds were sown in farm sandy soil, 5 seeds per each row with 30 cm intervals. The field was divided into two plots contain the same number of seeds and rows, one plot was drenched with 500 ml *T.viride* spore suspensions (6×10^6spores ml^{-1}) while the other plot was irrigated with normal water as control .The experiment was observed daily to record any changes in growth pattern of treated and control peanut plants. After 110 days ten plants from each plot were randomly selected to measure the plant vigor parameters such as canopy radius (cm), root lengths (cm), number of branches, leaves, leaf area pods, and plant fresh &dry weigh (g) as well as pods fresh weight (g) per each plant representative by weight of five random pods.
Yield was calculated by total fresh weight of pods / total fresh weight of plants. Few random leaves were taken to determine the concentration of pigments (chlorophyll a, chlorophyll b and carotenoids) as mg/g plant fresh weight according to Metzner *et al.*, (1965).

This experiment was repeated with other crops such as potato and kidney bean plants. Different parameters were measured such as (shoot length, root length, canopy radius, No. of fruits and weight of fruits).

***Trichoderma harzianum* as foliar fertilizers**

Metabolites of *T.harzianum 5* were collected from flasks contain 50 ml PDB at 28^0C. in shaker 100 rpm the filtration of metabolites were sprayed on to leaves till runoff, and Different parameters were measured such as (shoot length, No. of flowers and leaf area).

IAA Production

Indole acetic acid produced by ten different *Trichoderma* isolates was assayed colorimetrically using Salkowski reagent (Gordon and Weber 1951). This method estimated the quantities of indole compounds produced by *Trichoderma* in medium containing precursor L-tryptophan. The ten *Trichoderma* isolates were grown in Sterilized broth (25 ml) of Czapex (Sucrose, 30g, $NaNO_3$, 3g, K_2H PO4, 1g, KCL, 0.5g, $MgSO_4$ $.7H_2O$, 0.5g, $FeSO_4$, 0.01 g, Distilled Water, 1,000 ml).on a ratory shaker (15 rpm) at room temperature as seed culture. Was put into glass flasks and inoculated with 100 µl of *Trichoderma* broth culture (10^7spore/ ml) incubated at 28^0C for 72-96h in shaker 100 rpm. The contents of flasks were filtered through Whatman filter paper No.2 before measuring auxin production as indol acetic acid (IAA) equivalents. In measuring the IAA equivalents, 3 ml of the filtrate were pipetted into test tubes and 2ml of Salkowski reagent (2ml 0.5 M $FeCl_3$ + 98ml 35% $HCLO_4$) were added to it. The tubes containing the mixture were left for 30 min for color development. Intensity of this color was measured spectrophotometrically at 535 nm. Similarly, color was also developed in standard solution of IAA and standard curve was established by measuring the intensity of this color (Sarwar *et al.* 1992). The same procedure, except for amended of broth with 5ml 0.5% L- TRP solution, sterilized by filtration 0.22 filter was repeated for L- TRP- dependant auxin producing *Trichodema* .

Extraction and estimation of plant hormones by GLC

The leaves of Tomato plants treated by *T.viride* , *T.harzianum 9* and mixture of both beside control plant were used for plant hormones estimation. The extraction of Indole acetic acid (IAA), gibberillic acid (GA3) and abscisic acid (ABA) in the plant tissue was done by using (GLC), according to method of (Du, and Xu, 2000). The fresh weight 5 g was ground with the extracting solvent (methanol- BHT- ascorbate) in a mortar. After extraction in an ice both, samples were transferred to flasks with 30 ml fresh extracting solvent and stirred gently at 4 C for 12 h. samples were then filtered with suction through Whatman No.1 paper. The residue was rinsed 4 times with 10 ml of the extraction solvent. Methanol was removed by rotatory flash evaporation (RFE) at 35 C. The pH of the aqueous residue was then adjusted at 8 with K_2HPO_4 and the samples were centrifuged for 10 min at 12000 rpm. The supernatant fraction was partitioned twice against 10 ml of washed ethyl acetate – BHT to remove phenolics. The ethyl acetate fraction was then discarded. The residual ethyl acetate was removed from aqueous fraction with 10 ml of hexane. The pH of aqueous fraction was adjusted to 2.8 with H_3PO_4 and the acidified solutions passed through C18 cartridge (Sep- pakpreconditioned with methanol followed by 1mM HCL) to trap IAA, GA3 and ABA. The samples were then rinsed with 10 ml of 1mM HCL IAA, GA3 and ABA. Were then eluted with 0.02 N NH_4OH and the pH quickly adjusted to 2.8. Each hormone was extracted with 10 ml washed diethyl – BHT. Each of 10 ml of ether was partitioned, in turn against the 10 ml portion of 1mM HCL to remove residual polar contaminants. The ether was evaporated by RFEC. The flasks were cooled, by dipping in ice bath, before releasing the vacuum. The residue was immediately dissolved in methanol and samples were ready for injection into GLC. (UNICAM PRO-GC) column 3% OV – 17 (Methyl phenyl silicone) on chromosorb-WHP dimension 1.5 × 4 mm , gases flow rate N_2 30 ml / min, H_2 33 ml / min. Air 330 ml / min. The proper concentration of endogenous plant hormones (IAA, GA3 and ABA) were obtained by comparing the respective peak areas in the plant extracts with their corresponding areas obtained with authentic samples.

RESULTS

Screening ability of *Trichoderma* and *Gliocladium* for growth promotion in pepper plant

According to the obtained results, the fifty isolates of *Trichoderma* could be categorized into four groups. Group (A) comprise *Trichoderma* isolates that increase the length of plant more than 50 % (fig.1) in comparing with control plants irrigated with broth media. This isolates were (*T.viride*, *T.harzianum 5* and *T.harzianum 9*). Group (B) accomdated isolates that increase growth up to ranging more than 10% to less than 50%(fig.2) among this group were isolates (T.16, T.28). The third group(C) includes isolates that increase growth but less than 10 % (fig.3) The last group (D) comprise isolates that suppress the growth of peper plant (fig.4) among this group (T.12, T.40). With regard to dry & fresh weight the previous categorization was almost the same but with some few differences. Such as isolate T.46 although it increased the shoot and root length it decreased the dry and fresh weight of pepper plant (table.1)

Application methods of *Trichoderma* on Tomato

From previous screening *T.harzianum 9* were choosen to determine the appropriately way of application. When comparing seed coating and drench method it was found that drench with the spore suspension was the effective delivery method and percentage of increase above

control in shoot length, root length reach 56% and 44.2% respectively while in seed coating 42.8% in shoot length and 32.7% in root length.(fig.5 & fig.6)

Effect of *Trichoderma* on flowering and fruiting of Tomato

Tomato seedling plant rdeveloped from seeds coated with *T.harzianum 9* are treated again with soaking in spore suspension before transplant to open field. Number of flowers were counted after 10 days of transplant and show increase in treated plant reach to 83.3% more than control (fig.7) It is worth to mention that the content of photosynthetic pigment in treated tomato was higher than that of untreated (fig.8). Number of fruits increased significantly more than control (fig.9). Also weight of fruits, plant fresh and dry weight are increased significantly more than control plant (fig.10).

Response of different plants to *Trichoderma*

Comparison between response of different crops after treatment with *T.viride* spore suspensions were recorded as the increase percentage in peanut canopy radius reach 35 % while in potato 28.5 % (fig.11) with regard to root length of peanut, potato and kidney bean, root length of potato show highest response 47% followed by kidney bean 27.2% (fig.12).Also the No. of leaves and No. of fruits were the highest response in potato 17.8% , 33.3% respectively (fig.13). Percentage of productivity in potato crops reach 46.7% followed by peanut 40% (fig.14).Other parameters were measured in peanut as leaf area by using planimeter (fig.15.a) and photosynthetic pigments (fig.15.b).

***Trichoderma harzianum* as foliar fertilizers**

Application of *T. harzianum 5* to cucarbita shoot system by spraying show significant increase in shoot hight, (fig.16.a) No. of flowers (fig.16.b)and leaf area(fig.16.c)

IAA Production

Measuring of IAA production, colorimetrically, by ten isolates of *Trichoderma* in presence and in absence of L-tryptophan in broth medium showed that *T.harzianum 9* gave the highest production in presence of precursor 13.1µg/ml while T.19 show the lowest production 2.1µg/ml (table.2)

Extraction and estimation of plant hormones by GLC

Internal plant hormones IAA, GA3 and ABA were extracted from the tissue of tomato plant irrigated with *T.viride* , *T.harzianum 9* and mixture of both *Trichoderma* sp . Concentration of IAA was increased in tomato plant treated by *T.viride* (9.29 mg/100g)While GA3 was increased in tomato plant treated by *T.harzianum 9* (14.27mg/100g). Finaally ABA were highly decreased in case of mixture (3.14 µg/100g) as showen in (Table.3).

DISCUSSION

Trichoderma sp are well known as biocontrol agent against several plant pathogenic fungi, they have been reported to promote plant growth on numerous cultivated plants (Kleifeld and Chet, 1992; Ousley *et al*., 11994b; Altomare *et al*., 1999; Harman, 2000; Yedidia *et al.,* 2001). During screening experiment of 50 isolates on pepper plant, four categories were recognized. First one promotes the growth of plant up to 50%. The second group showed moderate increasing growth from 10 to less than 50%, the third group either has no effect less than 10%, the fourth group inhibit the plant growth. This could be attributed to control of minor pathogens leading to stronger root growth and nutrient uptake (Ousley *et al.* 1993), secretion of plant growth regulatory factors such as phytohormones (Windham *et*

al. 1986; Chang *et al*. 1986; Baker, 1988 and Harman *et al*., 2004) and release of soil nutrients and minerals by increased saprophytic activity of *Trichoderma* in the soil (Ousley *et al*. 1994a).

Application of *Trichoderma* as drench by spore suspension on tomato plant gave high yield than seed coating. This could be attributed to the fact that seed coating could provide benefit effect to root in nursery but on transplanting the plant subjected to some deleterious effect to root. Drench can overcome some of this deleterious effect as it provides beneficial effect on whole root after transplanting. (Abdul Wahid, 2007)

Trichoderma increase the number of flowers and fruits as well as the weight of tomato fruits. This could be due to increasing the rate of growth and induction of some plant hormones this finding are in agreement with (Chet *et al.*, 1997) Who record crop productivity in fields can increase up to 300% after the addition of *Trichoderma hamatum* or *Trichoderma koningii*. In experiments carried out in greenhouses, there was also a considerable yield increase when plant seeds were previously treated with spores from *Trichoderma*. The same increase was observed when seeds were separated from *Trichoderma* by a cellophane membrane, which indicates that *Trichoderma* produces growth factors that increased the rate of seed germination (Benítez *et al*. 1998).

Various plants respond differently to application of *Trichoderma* in field as drench, some plants like potato respond by increasing the length of root system more than kidney bean and peanut plant. While the canopy of peanut was greater than potato canopy on other hand productivity of potato was greater than other plants this could be different sensitivity of crops toward various phytohormones.

When applying of *Trichoderma* as foliar fertilizers increased number of flowers and leaf area of cucurbit plant this could be on the basis that metabolites oh *T. harzianum 5* could contain some hormones that would absorb by leaf and interact directly in flowering and leaf expansion mechanism. When filtrates of *Trichoderma* was investigated for the presence of IAA it revealed that different amount produced by different isolates of *Trichoderma, T. harzianum 9* was the highest producers of IAA followed by *T.viride* this hormone could be considered constitutive hormone it was produced in absence of L- tryptophan which is precursor of IAA hormone.

Trichoderma also induce the production of IAA, GA3 and ABA in treated plant at different extent. While *T. harzianum 9* increases the production of GA3 in treated tomato plant *T.viride 6* increase the IAA but not GA3 in some plants. However a mixture of both *Trichoderma* decreases greatly the amount of GA3 and increase slightly the amount of IAA. But the surprising result was the high decreasing in ABA which could be the reason of the high increasing in the plant growth treated with this mixture.

According to the data of this investigation has 2 or 3 different mechanisms to promote plant growth, first of all is the production of external growth regulators (IAA) secondly is the induction and increasing of growth regulators in treated plants, the third possible mechanism is the lowering of ABA content in treated plants this hormone is known to inhibit and regulate plant growth under normal conditions. Under this condition lowering of ABA content of treated plants maximize and increase the effect of other growth regulators such as IAA and GA3.

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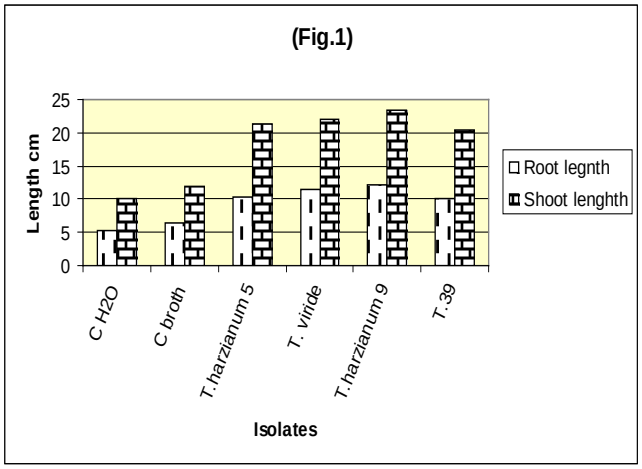
Table 1. Effect of different groups of *Trichoderma* spp on fresh and dry weight of pepper plant on comparing with control. mean of 6 replicates (SE).

Group	Isolates	Fressh weight Mean (SE)	Dry weight Mean (SE)
Control	***C H2O***	**2.7 (0.06)**	**0.8 (0.036)**
	C broth	**3 (0.08)**	**0.9 (0.033)**
A	***T.harzianum 5***	**4.7(0.1)**	**1.1 (0.09)**
	T. viride	**4.9 (0.14)**	**1.3(0.1)**
	T.harzianum 9	**5.3 (0.19)**	**1.5 (0.12)**
	T.39	**4.3 (0.14)**	**1.3 (0.1)**
B	***T.1***	**3.3 (0.17)**	**1.3 (0.03)**
	T.4	**3.2 (0.08)**	**1.2 (0.05)**
	T.13	**3 (0.07)**	**1 (0.04)**
	T.16	**3.6 (0.14)**	**1.4(0.08)**
	T.22	**3 (0.09)**	**0.9(0.03)**
	T.25	**3.1 (0.1)**	**1 (0.05)**
	T.28	**3.8 (0.11)**	**1.3 (0.12)**
	T.46	**2.8 (0.05)**	**0.8 (0.07)**
C	***T.2***	**3.1 (0.15)**	**1 (0.1)**
	T.3	**3 (0.2)**	**0.95 (0.13)**
	T.8	**3.1 (0.13)**	**1.1 (0.12)**
	T.11	**3 (0.1)**	**1 (0.05)**
	T.18	**2.9 (0.19)**	**0.85 (0.04)**
	T.15	**3.9 (0.2)**	**1.2 (0.03)**
	T.19	**2.8 (0.1)**	**0.88 (0.05)**
	T.23	**2.7 (0.07)**	**0.8 (0.02)**
	T.24	**2.9 (0.05)**	**1.1 (0.07)**
	T.26	**3 (0.1)**	**0.9 (0.05)**
	T.27	**3 (0.12)**	**0.9 (0.02)**
	T.29	**3.2 (0.18)**	**1.3 (0.11)**
	T.32	**3.3 (0.2)**	**1.1 (0.05)**
	T.31	**3 (0.09)**	**1 (0.02)**
	T.33	**3.8 (0.22)**	**1.2 (0.05)**
	T.35	**3 (0.12)**	**0.88 (0.06)**
	T.37	**2.7 (0.09)**	**0.8 (0.06)**
	T.logibrachiatum 38	**2.9 (0.21)**	**1.1 (0.09)**
	T.42	**3.2 (0.19)**	**1 (0.11)**
	T.aureoviride 41	**3 (0.14)**	**0.9 (0.07)**
	T.43	**2.9 (0.11)**	**0.8 (0.12)**
	T.44	**3.2 (0.08)**	**0.9 (0.04)**
	T.17	**3.1 (0.07)**	**0.8 (0.03)**
	T.45	**3 (0.15)**	**0.9(0.03)**
	Gliocladium roseum 49	**3.1 (0.2)**	**1 (0.04)**
	Gliocladium roseum 50	**3 (0.1)**	**0.88 (0.03)**
D	***T.7***	**2.4 (0.05)**	**0.7 (0.08)**
	T.12	**2.5 (0.09)**	**0.6 (0.04)**
	T.14	**2.5 (0.1)**	**0.7 (0.05)**
	T.20	**2.7 (0.07)**	**0.8(0.03)**
	T.30	**2.4 (0.09)**	**0.7 (0.09)**
	T.34	**2.9 (0.14)**	**0.88 (0.03)**
	T.36	**2.6 (0.09)**	**0.75 (0.03)**
	T.40	**2.4 (0.11)**	**0.68 (0.02)**
	T.47	**2.6 (0.08)**	**0.7 (0.06)**
	T.48	**2.4 (0.08)**	**0.9 (0.03)**

Table. 2. Measurment of IAA produced by *Trichoderma* sp.spectrophotometrically at 535 nm.		
Isolates	IAA equivalents µg/ml (p.p.m)	
	With out L-tryptophan	**With L-tryptophan**
T.harzianum5	**1.1**	**8.3**
T.viride	**1.4**	**10.2**
T. harzianum 9	**1.8**	**13.1**
T.39	**0.8**	**4.4**
T.26	**0.3**	**3.9**
T.28	**0.22**	**3**
T.19	**0.11**	**2.1**
T.aureoviride 41	**0.21**	**2.8**
T.logibrachiatum 38	**0.33**	**3.5**
T.44	**0.45**	**3.3**

Table 3. Estimation of Internal plant hormones IAA, GA3 and ABA from treated plants by GLC			
Treatment	**IAA (mg/ 100 g F.W)**	**GA3 (mg/ 100 g F.W)**	**ABA (µg/ 100 g F.W)**
Control	**6.17**	**5.53**	**12.93**
T.harzianum	**5.43**	**14.27**	**8.55**
T.viride	**9.29**	**4.41**	**9.12**
Mixture	**7.04**	**1.19**	**3.14**



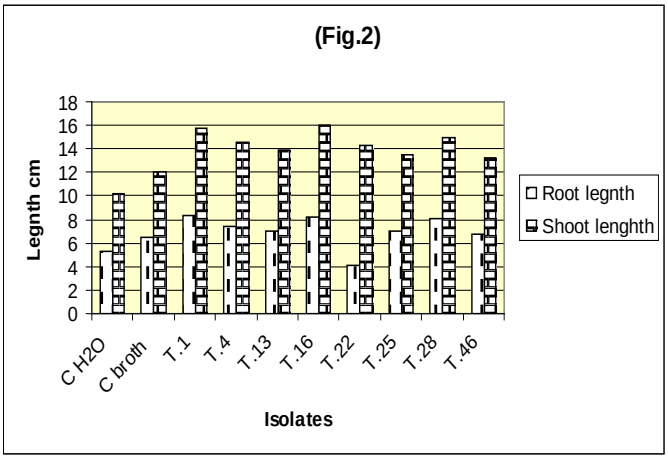


Fig. 1. Effect of group (A) of *Trichoderma* spp on pepper plant on comparing with control. Different letters above a column indicate statistical significance according to LSD (P = 0.05).
Fig. 2. Effect of group (B) of *Trichoderma* spp on pepper plant on comparing with control. Different letters above a column indicate statistical significance according to LSD (P = 0.05).

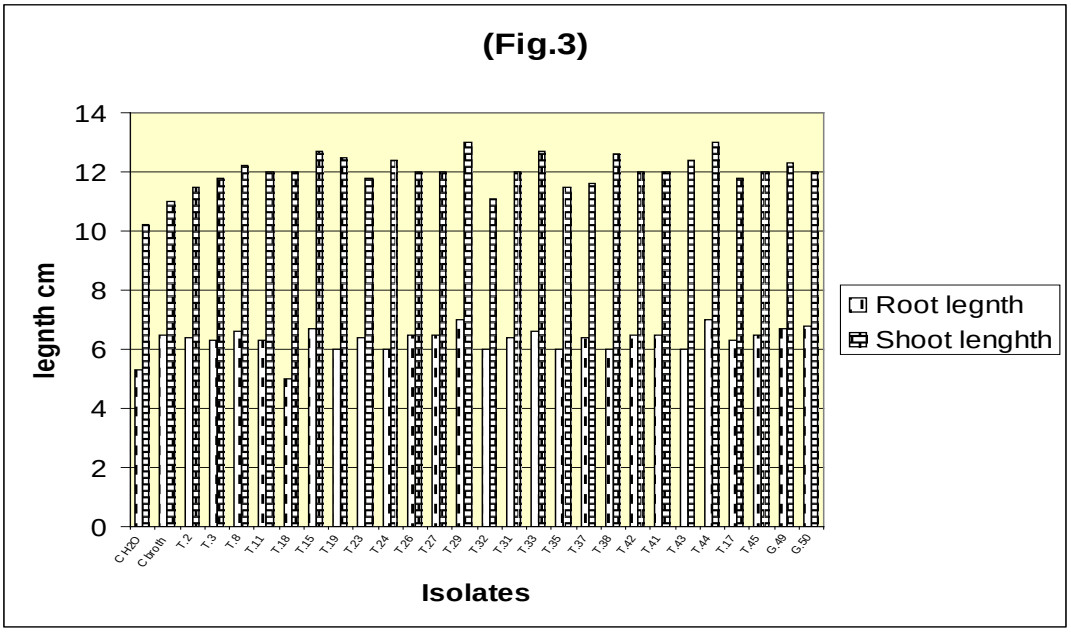


Fig. 3. Effect of group (C) of *Trichoderma* spp on pepper plant on comparing with control. Different letters above a column indicate statistical significance according to LSD (P = 0.05).

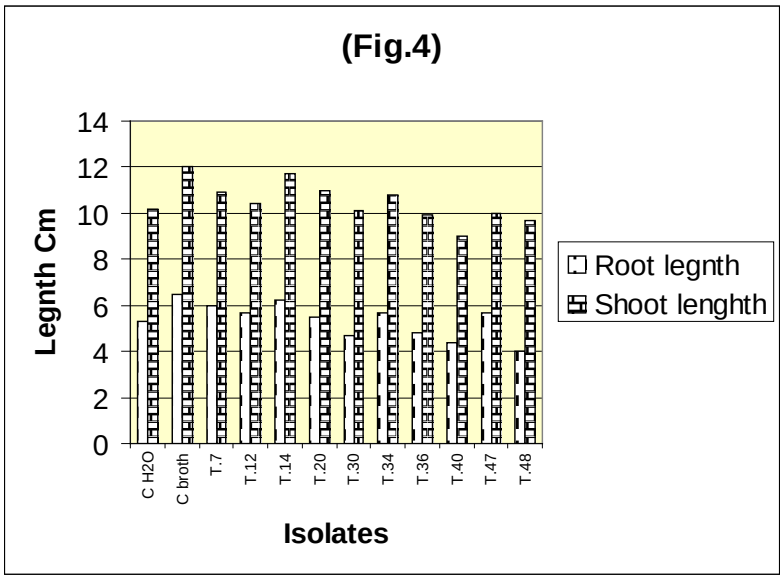


Fig. 4. of group (D) of *Trichoderma* spp on pepper plant on comparing with control. Different letters above a column indicate statistical significance according to LSD (P = 0.05).

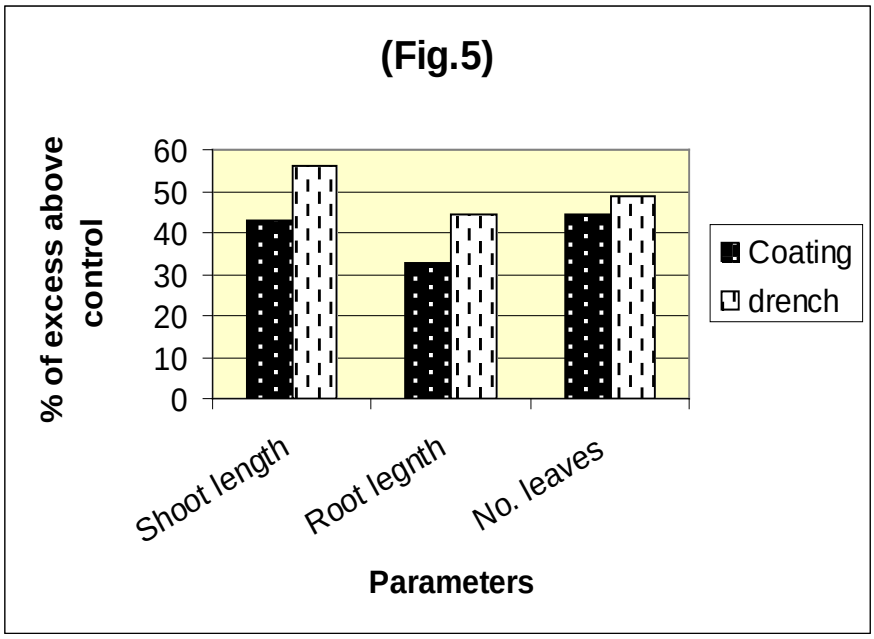


Fig. 5.Comparison between application method of *T.h 9* on tomato plant

Fig. 6. Image show difference between control and *T.h 9* treated tomato plant.

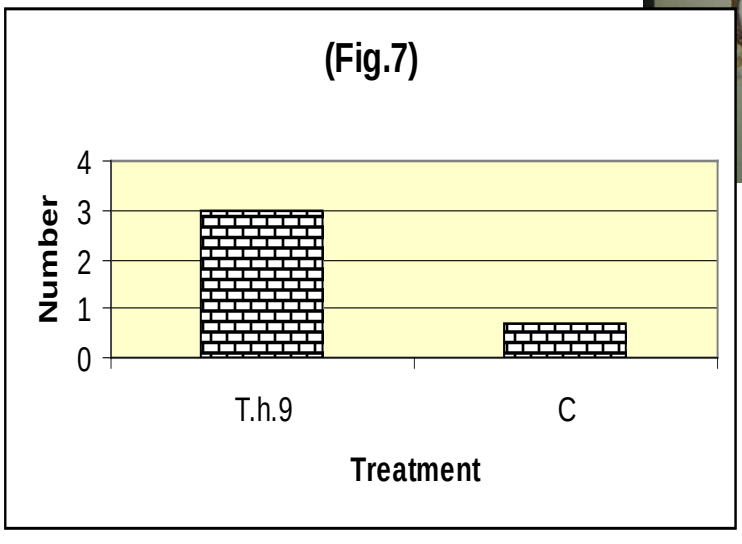
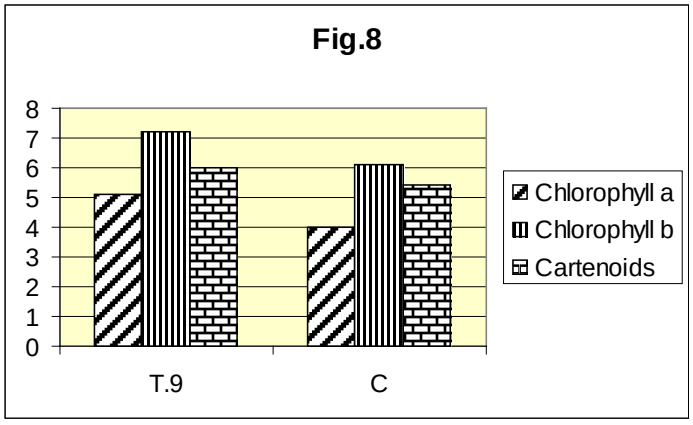


Fig. 7. Effect of *T.h 9* on flowering in tomato plant on comparing with control. Different letters above a column indicate statistical significance according to LSD (P = 0.05).



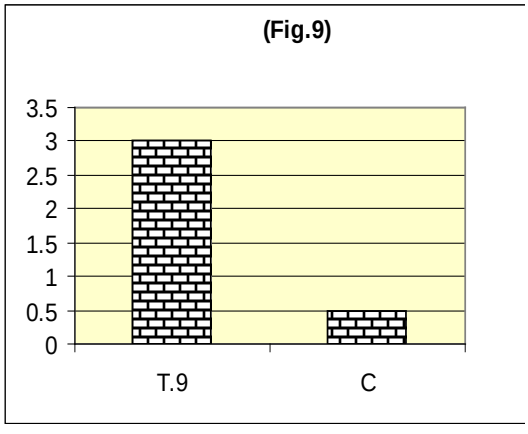


Fig. 8. Effect of *T.h 9* on photosynthetic pigments in in tomato plant in comparing with control.
Fig.9. Effect of *T.h 9* on fruiting in tomato plant on comparing with control. Different letters above a column indicate statistical significance according to LSD (P = 0.05).

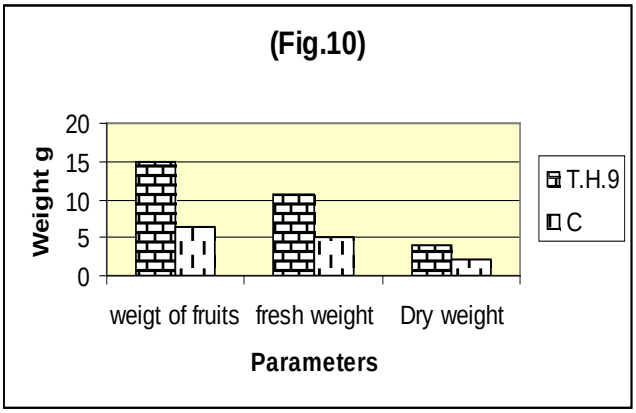
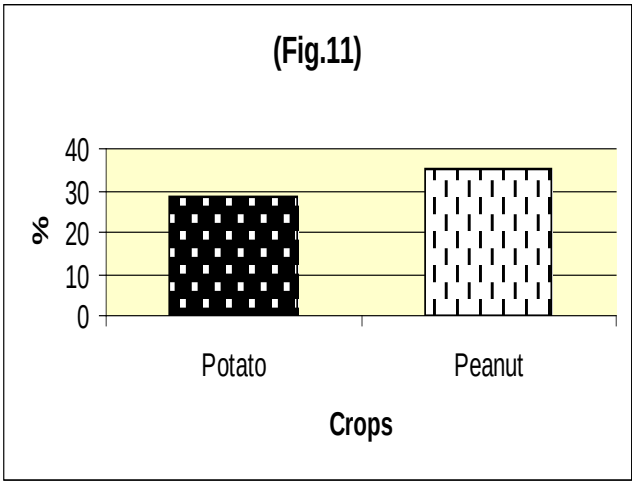


Fig.10. Effect of *T.h 9* on fruit weight, fresh and dry weight in tomato plant on comparing with control. Different letters above a column indicate statistical significance according to LSD (P = 0.05).



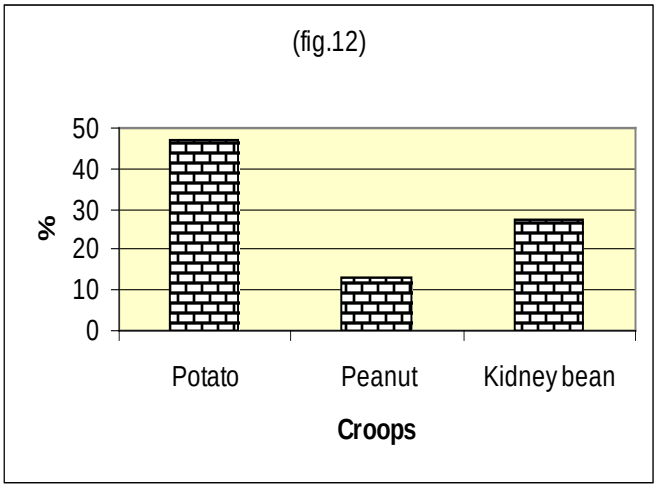
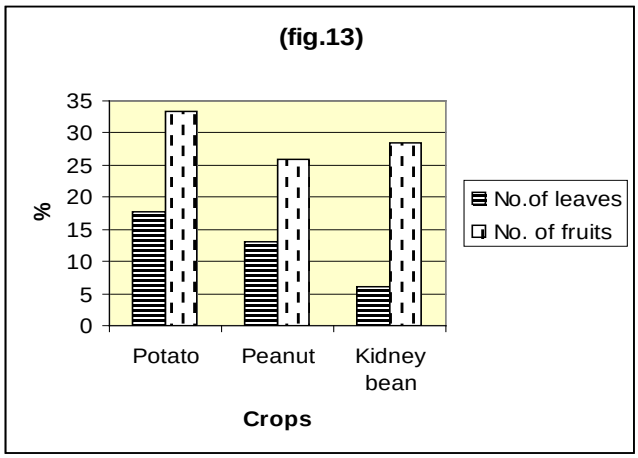


Fig. 11. percentage of increasing in canopy radius of different plants in response to *T.viride* application

Fig. 12. percentage of increasing in root system of different plants in response to *T.viride* application



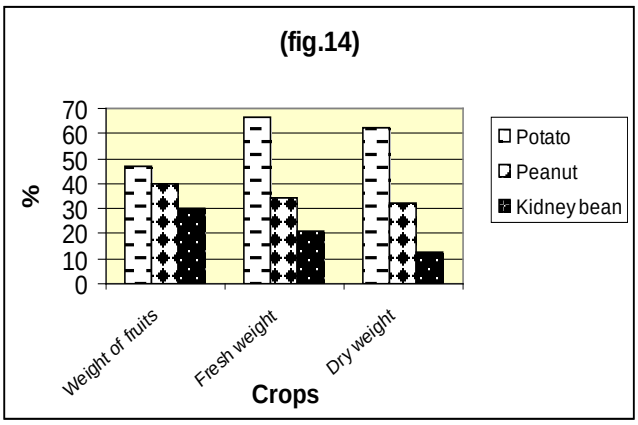
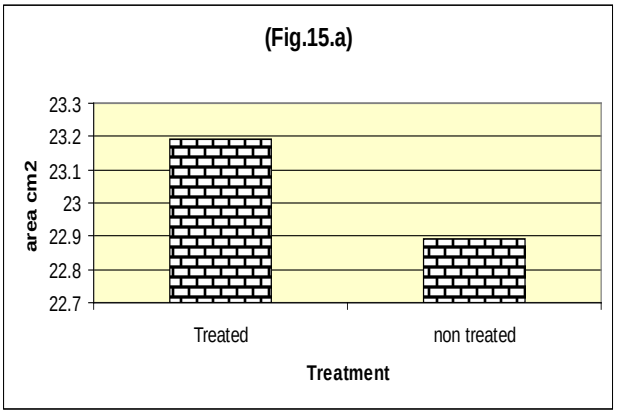


Fig. 13. percentage of increasing in No. of leaves and No. of fruits of different plants in response

to *T.viride* application

Fig.14. percentage of increasing in weight of different plants in response to *T.viride* application



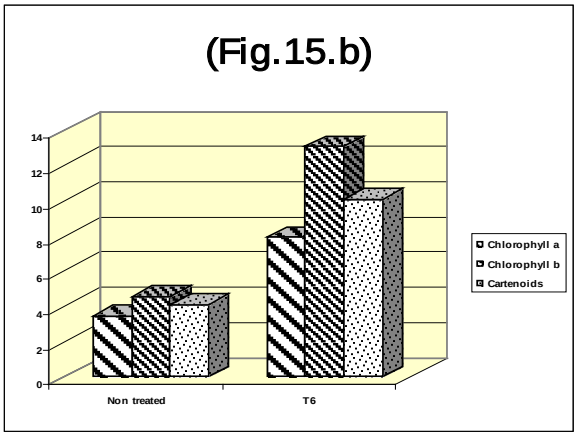
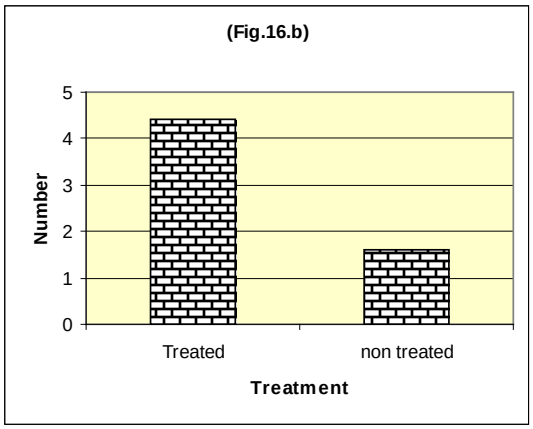
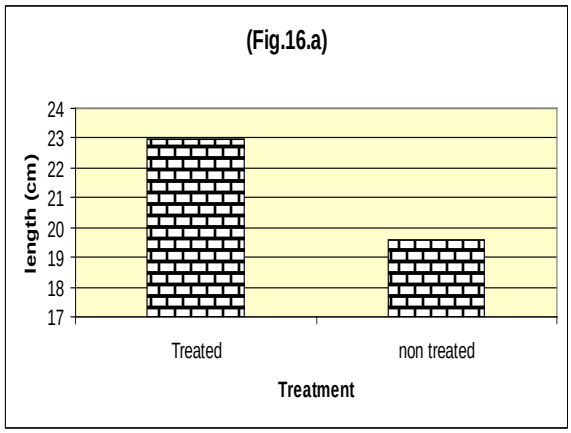


Fig.15.a. Effect of *Trichoderma viride* on leaf area of peanut in comparing with control.

Fig. 15.b.Effect of *Trichoderma viride* on photosynthetic pigments in peanut leaves in comparing with control.





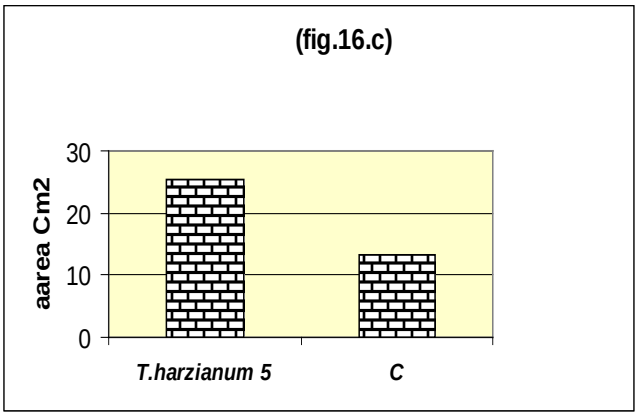


Fig. 16.a. Effect of *T.h 5* on shoot length of cucarbita plant in comparing with control. Different letters above a column indicate statistical significance according to LSD (P = 0.05).
Fig. 16.b. Effect of *T.h 5* on number of flowers of cucarbita plant in comparing with control. Different letters above a column indicate statistical significance according to LSD (P = 0.05).
Fig. 16.c. Effect of *T.h 5* on leaf area of cucarbita plant in comparing with control. Different letters above a column indicate statistical significance according to LSD (P = 0.05).

تحسين نمو النبات عن طريق أنواع مختلفة من فطر التريكودارما

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قسم النبات – كلية العلوم – جامعة قناة السويس- إسماعيلية 41522- مصر

نظرا لأهمية الحصول على غذاء نباتي أمن صحيا خالي من المبيدات والمخصبات الكيميائية كان الاتجاه العالمي لتطبيق مخصبات حيوية منتجة من بعض الكائنات الدقيقة يستخدمها المزارعون كبدائل طبيعية صديقة للبيئة وآمنة على صحة الإنسان في عملية الزراعة. كما تستخدم الفطريات في مجال التخصيب الحيوي لبعض المحاصيل وأمثلة ذلك فطريات الجذور (Mycorrhiza) وكذلك بعض الفطريات الخيطية مثل فطر التريكودارما (*Trichoderma* sp) الذي يمتاز بقدرته العالية على مقاومة بعض الآفات التي تهدد المحاصيل المختلفة بجانب ذلك يساعد النبات في الحصول على بعض العناصر الأساسية من التربة مما يؤدى إلى تحسين نمو النبات وأيضا يساهم في تحفيز النمو عن طريق إفراز بعض منظمات النمو .

وتهدف هذه الدراسة على التركيز على دور فطر التريكودارما في زيادة نمو النباتات تحت الظروف الحقلية واختبار قدرته معمليا على إفراز بعض منظمات النمو مثل (IAA) و كذلك قياس الهرمونات النباتية في النباتات المعاملة بالفطر
ولتحقيق هذا الهدف تم تجميع عينات تربة من أماكن مختلفة وعزل فطريات التريكودارما وكان الناتج خمسون عزلة تم عمل مسح لاختبار قدره هذه العزلات على زيادة نمو نبات الفلفل ونتج عن هذا اختيار أربع عزلات فعالة لتطبيقهم على محاصيل مختلفة مثل (الطماطم – الكانولا- الفول السوداني –الكوسة و الفاصوليا) وأختلف أسلوب تطبيق هذه الفطريات مثل (تعفير البذور- الخلط بمياه الري - نقع البادرات والرش على المجموع الخضري) وكان نبات الطماطم أكثرهم استجابة 56% زيادة فى نمو المجموع الخضري وأفضل أسلوب تطبيق كان عند خلط جراثيم *Trichoderma viride* بمياه الري الذي أنعكس إيجابيا على نمو النبات من خلال زيادة معنوية في المجموع الخضري ووزن النبات وكذلك زيادة في الإنتاجية بشكل عام نتيجة تطبيق التريكودارما على محصول الفول السوداني. وقد تم اختيار عشر عزلات تم قياس قدرتها معمليا على إفراز بعض منظمات النمو مثل (IAA) في بيئة النمو و كذلك قياس الهرمونات النباتية عن طريق جهاز (GLC) في نبات الطماطم مقارنة بنبات غير معامل

ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF HALO AND THERMO TOLERANT BACTERIA FROM COSTAL RIDGE OF MEDITERRANEAN IN EGYPT

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***Microbial Molecular Biology Dept., Genetic Engineering Res. Ins., ARC, Giza**
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ABSTRACT

Costal ridge of Mediterranean and the regions near by it, like Mallahat Maryut, Bourg El-Arab and El-Amiriya in the north of Egypt are present perfect places for the isolation of halo and thermo-tolerant microorganisms. This study presents isolation, identification and molecular characterization of halophilic and thermophilic bacteria. Ten bacterial isolates were isolated using enrichment techniques, up to 30% (W/V) NaCl and 75 º C incubation temperatures. All the isolates showed positive Gram's reaction and identified according to Bergey's Manual as *Bacillus* species, 6 isolates were more identified as *Bacillus thuringiensis* and the other 4 isolates were *B. polymyxa.* Protein profiling of the *Bt* isolates and the PCR detection of *cry* genes showed the presence of lepidopteran *cry*1C gene. On the protein synthesis level, the protein profiling of cells grown in 5.2 M NaCl showed or/ disappeared a set of high-salt related proteins. One isolate (S2) was subjected to cloning and expression of carboxy-terminal truncated 2.2 kb DNA fragment of *cry*1C and the preliminary study showed that it was different from the published *cry*1C genes. On the other hand, the 4 isolates of *B. polymyxa* were able to grow at 75ºC. Protein profiling and the PCR detection of *gln*B & *nif*D genes revealed the presence of these genes. Thus *B. polymyxa* isolates could be used to fix nitrogen on the crops cultivated in hot regions.

INTRODUCTION

Extremophiles, the microbes dwelling in unusual habitates, can potentially serve in a variety of industrial applications. As a result of adaptation to extreme environments, extremophiles have evolved unique properties that make them of biotechnological and commercial significance (Margesin and Schinner 2001; Dodia *et al.*, 2006). Extremophiles include halophiles, alkali philes, acidophiles, thermophiles and haloalkaliphiles. Hypersaline lakes are mostly populated with halophilic neutrophilic organisms. The halophiles were described (Horikoshi 1999) as a subgroup that requires high salinity (up to 33% W/V NaCl). The organisms living in such extreme environment possess special adaptation strategies that make them interesting not only for fundamental research but also towards exploration for applications. In the present study, one natural saline habitat along the Mediterranean cost in north Egypt was selected for the isolation followed by the identification and molecular characterization of halophilic and thermophilic bacteria.

MATERIALS AND METHODS

Sampling: Soil samples were collected from the hyper saline environments, Costal ridge, Mallahat Maryut, Bourg El-Arab and El-Amiriya that located along the Mediterranean in the north of Egypt. Soil samples were taken from depths of about 15 cm. Soil samples were serially diluted from the stock concentration 10 g soil/100 ml sterile saline solution, then 0.1 ml were inoculated on a medium containing (g / Liter): Peptone 5 g, Yeast extract 3 g, Beef extract 1 g and Sodium chloride at different concentrations (0.5 M, 1 M, 2 M, 3 M, 4 M, 4.5 M and 5.2 M) and incubated at different temperature degrees, 30, 37, 55, 75 and 80°C for 2-14 days. Isolated bacteria were counted as (colony forming unit) CFU / g soil (Gebreel 1999).

Morphology and Cytology: Morphological characters of the colony, as appearance, elevation and production of pigments were examined on nutrient agar (Peptone 5 g, Yeast extract 3 g, Beef extract 1 g and Sodium chloride 5 g, adjust pH to 7 and Agar was added 15 g / L) (Ventosa *et al*., 1982). Motility was observed under microscope using the hanging drop technique; log phase of bacterial growth was examined by a high-power dry objective reduced illumination. Motility was confirmed by stabbing the organism in semisolid medium and after 7 days of incubation, diffusion of growth was recorded as positive results. Cell morphology was examined after Gram reaction (Dussault 1955). The purified colonies were subjected to spore stain for microscopic examination; endospore shape and position were characteristic of *Bacillus* species. Formation of insecticidal crystal proteins (ICP) were the gold standard, since it directly identifies *Bacillus thuringiensis* from closely related Bacilli as *B. cereus* and *B. anthracis*.

***Biochemical and Physiological properties*:** Physiological and biochemical tests were performed as previously described in Bergey's manual of systematic bacteriology (Claus and Berkeley 1986). Bacterial isolates were tested for **catalase production** when it was reacted with 3% hydrogen peroxide. Oxygen was released from catalase producer microorganisms (Cowen and Steel 1974). For **acetylmethylcarbinol production**, methyl red and Vogues-proskauer (MR-VP) tests were done. A drop of methyl red solution was added to a 7 day-old bacterial culture. Bright red color refers to positive MR. 0.6 ml of 5% α-naphthol solution and 0.2 ml of 40% potassium hydroxide (Berret's reagent) were added onto 1 ml of that bacterial culture. The development of pink crimson color indicates a positive VP. The ability to **reduce nitrate** was examined as in (Cowan and Steel 1974). To test for **sugar fermentation**, 0.1% of glucose or other source of carbohydrates were added to a medium containing peptone water (Peptone 2 g, D-Glucose 1 g, Sodium Chloride 5 g) and the indicator Bromothymol blue with derhum tube were used (Hugh and Leifson 1953).

Total Protein analysis Total cellular protein of bacterial isolates were grown on LB media (Trypton 10 gm, yeast 5 gm, sodium chloride 5gm) å̊C 30 over night compared to that grown on 30% NaCl. SDS-PAGE was carried out as described by (Laemmli, 1970). The bacterial cells were collected by centrifugation and treated with sample buffer composed of (50 mM Tris-HCl, pH 6.8, 2% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.0025 % (w/v) bromophenol blue) and boiled at 100̊C for 5 min. Samples were applied to a 10 % polyacrylamid gels and run in mini protein Biorad cell. Protein bands on gels were visualized with Coomassie brilliant blue R-250. Total cellular proteins of sporulated bacterial cells from *Bt* isolates were also prepared. Those bacterial cells were grown on T3 medium (tryptone 3 gm, tryptose 2 gm, yeast extract 1.5 gm, sodium phosphate buffer 50 mM, Mn Cl_2 0.005 gm, in 1L dH_2O) for 72 h in shaking incubator at 200 rpm and 30°C. Samples were applied to a 10% polyacrylamid gels as mentioned.

Polymerase Chain Reaction(PCR) and Oligonucleotide primers

Two pairs of specific primers for *cry*1C were used, CJ10 & CJ11, 5' AAAGATCTGGAACACCTTT3' & 5'CAAACTCTAAATCCTTTCAC3' that amplifies a 130 bp fragment of DNA and CJ1-1 & CJ1-2, 5' TGTAGAAGAGGAAGTCTATCCA3' & 5' TATCGGTTTCTGGGAAGTA3' that amplifies a 284 bp fragment of DNA (Ceron *et al*., 1995) and 273 bp in case of *cry* 1Ac. Another two pair of specific primers to *cry*1C gene was also used to test the presence of *cry*1C in the *B. thuringiensis* isolates. The two primers IAF and IAR (Regev *et al*., 1996). IAF, 5' ACGGAGGATCCATATGGAGGAAAATAATCAAAATC3' and IAR, 5'CTCTTGGATCCTAACGGGTATAAGCTTTTAATTTC3',that give 2.2 k bp PCR product. The reaction conditions were performed according (Regev *et al*., 1996), where the PCR mixture was in a total volume of 25 µl contained 1 µg of total DNA, 50 pmol of each primer, 0.2 mM deoxy nucleoside triphosphates (dNTP), 2.5 µl of the Taq polymerase enzyme, 2.5 µl of 10 X enzyme buffer and 2.5 µl Mg Cl_2. The amplification reaction was carried out using 35 cycles of 94 °C denaturation (45 sec), 48 °C annealing (45 sec) and 72 °C extension (120 sec) and then a 7- min termination at 72 °C. The same reaction conditions were used with the second pair of primers IAF and ICR, 5'TTATTCCTCCATAAGGAGTAATTCC3' (Nahed, 2001), that give 3.7 k bp PCR product. The *gln* B specific pair of primers that define the nitrogen regulatory gene were used, *nif*D up & do specific pair of primers were also used to detect both the two genes *gln* B and *nif* D in all the four isolates (S7, 8, 9 and 10). *Gln* B up 5'GCCATCATTAAGCCGTTCAA3' and *gln* B do 5'AAGATCTTGCCGTCGCCGAT3' and *nif*D up 5'ATCATCGGTGACTACAAC3' & *nif*D do 5'ATCCATGTCGCGGCGAA3'. The reaction conditions were as described by (Potrich *et al*., 2001), 250 bp PCR products are amplified by *gln*B pair of primers and 710 bp by *nif*D primers.

Bioassay *B. thuringiensis* bacterial isolates were grown until sporulation in liquid T3 media for 72 h. Cultures were centrifuged and the pellets were washed once with Tris-HCl pH 8.00 containing 1 M NaCl, and lyophilized. The dried cells were used directly for bioassay. A stock concentration of 1000 ppm was made by dissolving 1 g of lyophilized cells in 1000 ml H_2O (Dulmage, 1971). 500 ppm, 400 ppm, 250 ppm, 100 ppm, 75 ppm, 50 ppm, 25 ppm, and 10 ppm different concentrations were added to the surface of solidified artificial medium (dry powdered Lima beans 150 gm, dry yeast 15 g, Ascorbic acid 3 g, Nipagin 3g, agar-agar 6 g and 600 ml dd H2O) (Loutfy 1973) and kept for 2 h at room temperature. 10 neonate larvae of *Spodoptera littoralis* were added to each cup, the mortality was recorded every 24 h until 72 h.

Cloning and Transformation

Samples with amplified fragments of the expected size 2.2 kb were cloned by using pGEM –T easy vector systems as described in (manual of Promega, USA). The ligation mixture was used to transform competent JM109 cells as described by (Cohen *et al*., 1972). Transformed bacteria were grown on IPTG / X-Gal agar plates supplemented with 100 µg/ml ampicillin. Screening of recombinants was performed according (Sambrook *et al*., 1989).

Preparation and partial purification of ICP from recombinant E. coli 25 ml LB media containing 100 µg/ml ampicillin was inoculated with the recombinant *E. coli* overnight at 37ºC. 500 ml culture containing 100 µg / ml ampicillin were inoculated from overnight broth to be started at OD_{600} 0.1-0.2 and were grown to OD_{600} ~ 0.5.

Then cells were induced with IPTG at a concentration of 0.1 mM and incubated overnight on shaking incubator (200 rpm) at 37ºC. Cells were centrifuged and the pellet was resuspended in 25 ml TES buffer (Tris, pH 8 20 mM, EDTA 50 mM, Sucrose 15%). Lysozyme (Final concentration 0.5 mg / ml) and PMSF (25 µl of 100 mM stock concentration) were added at room temperature. Sonication was applied on ice 3 times 1 minute each. The suspension was centrifuged at 10,000 rpm for 20 min at 4ºC and the pellet was resuspended in 20 ml TTN buffer (Tris, pH 8 20 mM, Triton X-100 2%, NaCl 0.5 M) and this step was repeated for 4 cycles. The pellet from previous step was washed twice with 20 ml PBS: acetone (5:1) and washed with PBS (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24g /L) only. The final pellet was resuspended in sodium carbonate buffer (20 ml) and shaked for 4 hours at 37ºC to dissolve the protoxin. The dissolved protoxin was trypsinized with TPCK trypsin (Final concentration of 0.5 mg / ml) for a trypsin : protoxin ratio of 1: 20 and shaken at room temperature for 30 min (Lee *et al*., 1995).

Western Blot The presence of Cry1C delta-endotoxin was detected in crude extracts of transformed cells by a western blot (immunoblot) analysis (Lampel *et al*., 1994). Total cellular proteins were prepared and solubilized by boiling in sample buffer and separated by electrophoresis on 10% polyacrylamid gels. The gels were electrophoretically blotted onto pre wet PVDF membrane (using mini protein Biorad cell at 30V / overnight). The membranes were blocked in blocking buffer containing 1% bovine serum albumin (BSA), then membranes were incubated in blocking buffer contained the toxin (Cry1C) for 2 h. Anti-truncated 65 kDa from *Bt kur*- HD-1 serum (1-1000 dilution) was used as primary antibody and was incubated with membranes in the blocking buffer O/N at 4°C. The membranes were incubated with alkaline phosphates conjugated secondary antibody (1-1000 dilution). CDP-chemiluminescent substrate was used and the emitted light was captured on X-ray film.

RESULTS AND DISCUSSION

Halotolerant and thermotolerant bacterial isolates were counted on plates containing nutrient agar medium that contained different concentrations of sodium chloride (0.5 – 24%). Mesophilic and halotolerant bacteria were incubated at 30ºC while the thermophilic and halotolerant incubated at 55ºC and the thermotolerant isolates were incubated at 75ºC in presence of 10% sodium chloride. The results showed that there was great variation in the number of the viable bacteria per soil in different locations. The highest population of bacteria was obtained in Costal ridge which reached $3.0X10^5$ CFU / g soil at 24% sodium chloride concentration and incubation temperature 30ºC. Ten isolates were selected for further studies which were the most halotolerant (able to grow at 30% NaCl) and thermotolerant (able to grow at 75 º C incubation temperatures) ones. The isolates did not display much diversity as regards to their colony characteristic (Table 1). However, the cell morphology and arrangement did vary significantly among these isolates from the same location. The gram positive character was abundant among these isolates and their colony pigmentation and texture had limited variations. All the isolates characterized with endospore formation and the position of those endospores varied significantly (Table1). Six isolates were able to grow over a wide range of salt (up to 30% NaCl "5.2 M"), the other 4 isolates could not grow over this high range but 10% NaCl was optimum to its growth and they were characterized with the ability to grow on high temperature up to 75ºC. The biochemical and the physiological tests (Table 2) revealed that all the isolates belong to the bacillus species which showed positive

gram reaction and ellipsoidal endospore production. The isolates were facultatively aerobic, they produced catalase, reduced nitrate to nitrite and produced acid from D-glucose during fermentation. Six isolates produced acetylmethylcarbinol, did not produce indol, utilized citrate and they could hydrolyze; gelatin, starch and urea. Mature spores were central in position for 6 isolates while they were terminal in the other 4 isolates. The 6 isolates (S1, 2, 3, 4, 5 and 6) produced characteristic crystals for the insecticidal toxin protein in *B. thuringiensis* and the microscopic examination was clearly showed that (Fig 4, A). The other 4 isolates were identified as *paenibacillus polymyxa* according the biochemical tests of the systematic classification of Bergey's. Identification of our isolates was agreed with (Seija Elo *et al*., 2001).

Protein analysis Total protein analysis of sporulated cells from the *B. thuringiensis* isolates revealed the presence of major bands belonging to the insecticidal crystal protein (ICPs), S1 showed main band near the 116 kDa, S2 showed major band at ~135 kDa that typically related to the lepidopteran Cry protein and also showed other main band at ~ 68 kDa that typically related to coleopteran Cry protein. S3 also showed expressed major protein band at ~ 135 kDa, S4 expressed main protein bands at 116, 97 and 66 kDa. S5 was characterized with the presence of main band under 116 kDa and S6 showed very interesting major band slightly over that was expressed by S3 "above 135 kDa". It was very interesting that the main bands of that *B. thuringiensis* isolates appeared at the same concentration when the isolates were grown on LB liquid media overnight. **On the protein synthesis level**, as in (Mojica *et al*., 1997) when bacterial cells of *Halomonas elangata* were grown at different salinities, there were long-term response, also they showed induction of protein synthesis after a shift of high-salt conditions. Salt adaptation could be indicated by a gradually higher accumulation of certain proteins in cells growing under gradually increasing salinities. Mojica *et a*l., (1997) found that among such proteins were 39, 24 and 15.5 kDa not detected at low salt concentration (0.3% NaCl) and all of these proteins related to the long-term response to high salt concentrations. Our data revealed high concentration of two protein bands at ~ 60 kDa and 30 kDa in isolate S2 (Fig 2, B), two bands at~ 95 kDa and ~ 28 kDa in the isolate S3 (Fig 2, A) and disappearance of a band above 116 KDa, Also in S1 high concentration of a protein band at ~ 100 kDa. In S4 at ~ 65 kDa, ~ 37 kDa and 28 kDa. Thus, our results agreed with Mojica *et al*., (1997) who found that the proteins that were synthesized at high salt concentrations were on long-term response, up to 7 days on shaker. Proteins homologous to those previously described as having a role in osmoregulation in bacteria such *E. coli* as mentioned by (Csonka and Hanson 1991) are expected to be found in this study.

Protein analysis for 4 *B. polymyxa* bacterial isolates were detected and compared with 3 strains identified as *B. polymyxa*. The protein banding pattern of our isolates was similar to that of reference strains in some protein bands, like the band that found under 37 kDa and the protein bands that found at molecular masses between 116 and 70 kDa. Our isolates characterized with main bands; S7 showed characteristic major band under 66 kDa, S8 and S10 showed major different band above that band of S7 (Fig 3), S9 also showed a characteristic band; further studies on these isolates may lead to identification of protein related to the high temperature which these isolates were able to grow on 75°**C. Microscopic examination** of bacterial cells of our isolates that were grown on T3 medium for 72 h, revealed the presence of the insecticidal crystal protein (ICP) in the 6 *B. thuringiensis* isolates and the shape of crystals were different from isolate to other while it was typically bipyramidal as that

characterized Cry 1C crystal (Nahed and Omar 2005) in S2, S3 and S6. In the remaining isolates the crystal shapes were different (Fig 4, A). On the other hand, the 4 *B. polymyxa* isolates showed similar shape of spores (Fig 4, B).

PCR *B. thuringiensis* crystal producing isolates have been isolated that are insecticidal against Lepidopterans, Dipterans and Coleopterans. Nucleotide sequences reported for *B. thuringiensis* crystal protein genes represent 14 distinct genes (Hofte and Whiteley 1989). Cry 1A crystal protein genes exhibiting insecticidal activity against lepidopterans share extensive DNA sequence homology (Carozzi *et al*., 1991). Thus PCR technology and primers specific for *B. thuringiensis* delta-endotoxin genes to develop a rapid screen of new *B. thuringiensis* isolates that can predict their insecticidal activities were used. In our work 6 primers were chosen to give characteristic product profiles from the genes encoding major class (lepidopterans) of ***B. thuringiensis*** crystal proteins. *Cry* 1C – specific primers CJ10 & CJ11 give a 130 bp product and CJ1-1 & CJ1-2 give a 284 bp product with *cry* 1C (Ceron *et al*., 1994 and 1995). IAF & IAR give 2.2 kb (Regev *et al*., 1996). Isolates S2, S3, S5 and S6 gave the expected products with the 1st and 2nd pair of primers (Fig 5, A) while S2 and S3 also gave the expected 2.2 kb product with the 3rd pair of primers but they didn't give the expected 3.7 kb product that represent the full length of the most published *cry* 1C with the primer pair IAF & ICR and this is a preliminary indication that *cry* 1C of S2 and S3 may differ from other *cry* 1C (Fig 5, B). On the other hand a set of primers was designed from previously published sequences of *gln* B gene (the nitrogen regulatory gene) and *nif* D gene (Potrich *et al*., 2001) and used with other 4 isolates that supposed to be *B. polymyxa*. All the 4 isolates gave the 250 bp product with *gln* B specific primers (Fig 5, C) and the 710 bp product with the *nif* D specific primers (Fig 5, D). These results put these *B. polymyxa* isolates in the diazotrophic *paenibacillus polymyxa*.

Insecicidal activity of the *B. thuringiensis* isolates was determined against the lepidopteran cotton leaf worm *Spodoptera littoralis*. One isolate (S1) was convenient and promising when bioassay against neonatal larvae was performed, it displayed LC50 ~ 200 ppm.

Cloning and transformation 2.2 kb PCR product resulted from PCR reaction with the primer pair IAF & IAR with DNA of the isolates S1 & S2. This *cry* 1C fragment starting from the translation start site and containing the sequence of the 1st 756 amino acids and a translation stop codon that was found in the reverse primer IAR (Regev *et al.*, 1996). This 2.2 kb DNA fragment of *cry* 1C gene was cloned into pGEM-Teasy plasmid and was transformed and expressed in E. coli JM109. The expressed Cry 1C toxin at molecular mass of 86 kDa was analyzed on SDS-PAGE (Fig 6, A). Partial purification of protein from transformed JM109 producing Cry 1C toxin was done, solubilized and trypsinized with TPCK trypsin to produce the activated toxin (~65 kDa) and the rest of the 86 kDa was appeared at ~ 22 kDa (Fig 6, B). Moreover, western blotting showed that *E. coli* JM 109 produced a protein of approximately 86 kDa (Regev *et al*., 1996) that cross reacted with poly clonal antisera raised against 60 kDa toxin from *B. thuringiensis kur*-HD-1 (Fig 6, C). These confirmatory tests revealed the expression of the Cry 1C protein that contained the active domain which is very interesting to obtain because of its importance in the biocontrol technology.

In summary, in this work ten bacterial isolates dwell in extreme habitats (high salt, high temperature, low oxygen) were isolated and selected from a huge number of isolates. Six of these isolates were *Bacillus thuringiensis* that tolerate high salt concentrations of NaCl up to 30% and temperature up to 55°C, most of these *Bt* isolates were characterized by the lepidopteran insecticidal crystal proteins. They

differ in molecular masses and shape of crystals from the common Cry 1C. The presence of osmoregulatory proteins that were synthesized at high salt concentrations may convoy us to reveal that we isolated strong isolates of *Bt* and may be novel and could be used in biological control of insect pests. In this work also 4 isolates of *B. polymyxa* were isolated which were able to grow on high temperature 75°C and contained the nitrogen fixing genes *gln* B and *nif* D. These isolates seemed of great importance for the biofertilizers of crops cultivated in hot areas.

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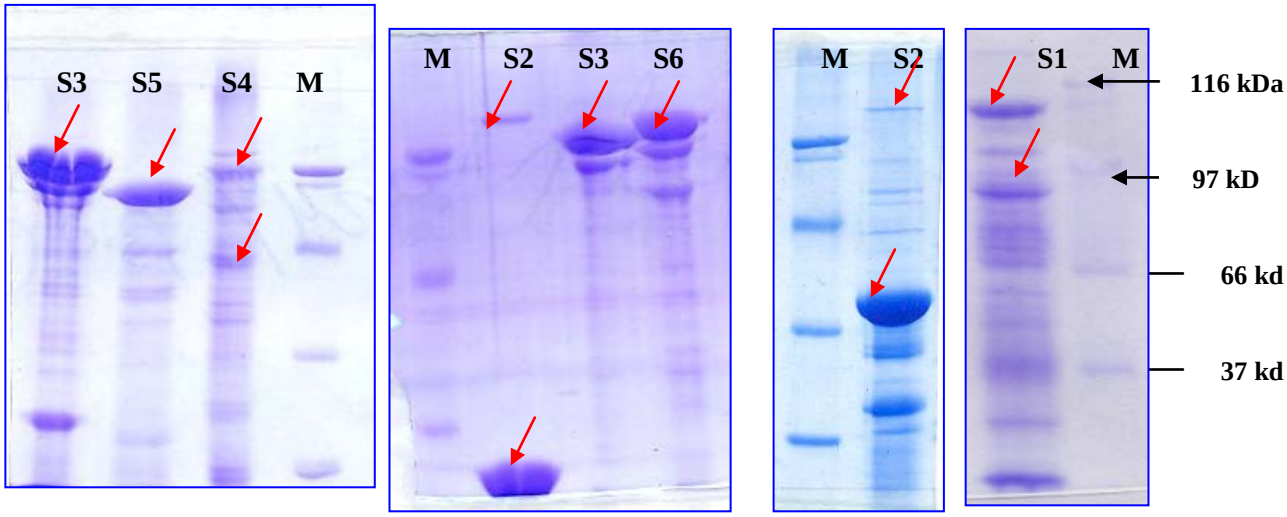


Fig (1): SDS-PAGE analysis of total cellular proteins of sporulated bacterial cells of the 6 *B.thuringiensis* isolates. 25 µl of total cellular proteins were run on 10% poly acrylamide gels. The gels were stained with Coomassie blue.
M: is a protein marker of 116, 97, 66, 37 and 28 KDa and was used with all the gels presented here. The name of each isolate is written on its lane. The red arrows refer to the major bands of the insecticidal crystal protein (ICP) that characterized each isolate.

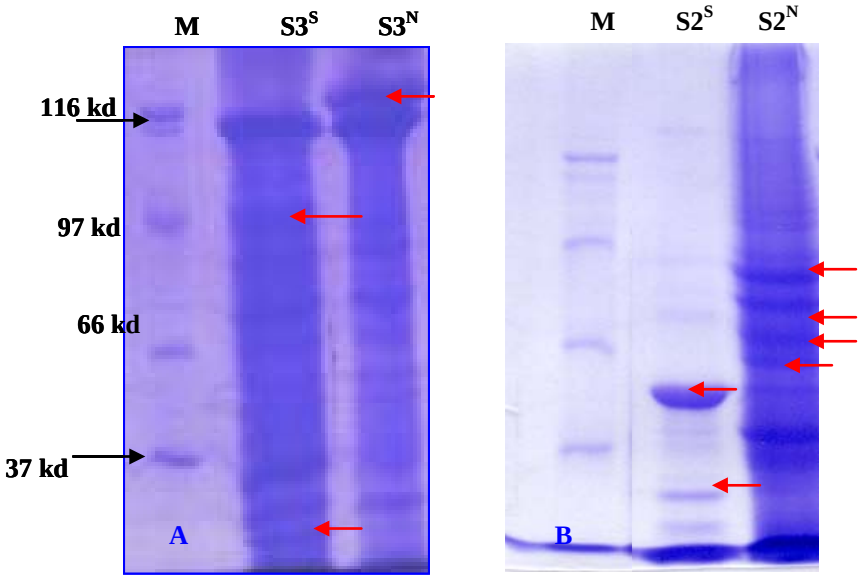


Fig (2): Effects of variations of salinity on protein synthesis in *B. thuringiensis* isolates. Sporulated bacterial cells of 4 *Bt* isolates (S1, S2, S3 and S4) that were grown under high salt concentrations (30% NaCl) compared to that grown under normal salt concentrations (0.1 NaCl). 25 µl of total cellular proteins were run on 10% poly acrylamide gels. The gels were stained with Coomassie blue.
M: is a protein marker of 116, 97, 66, 37 and 28 KDa and was used with all the gels presented here (A, B and E). The name of each isolate is written on its lane (^{N} means normal & ^{S} means high salt conc.). The red arrows refer to protein bands that were found in case of normal salt (A:$S3^{N}$, B: $S2^{N}$, C: $S4^{N}$, D: $S1^{N}$) and become faint or disappeared in case of high salt concentration or refer to unique bands that appeared in case of high salt concentrations (A: $S3^{S}$, B: $S2^{S}$, C: $S4^{S}$, D: $S1^{S}$). E: contain protein marker "M" beside $S1^{S}$ and $S4^{S}$.

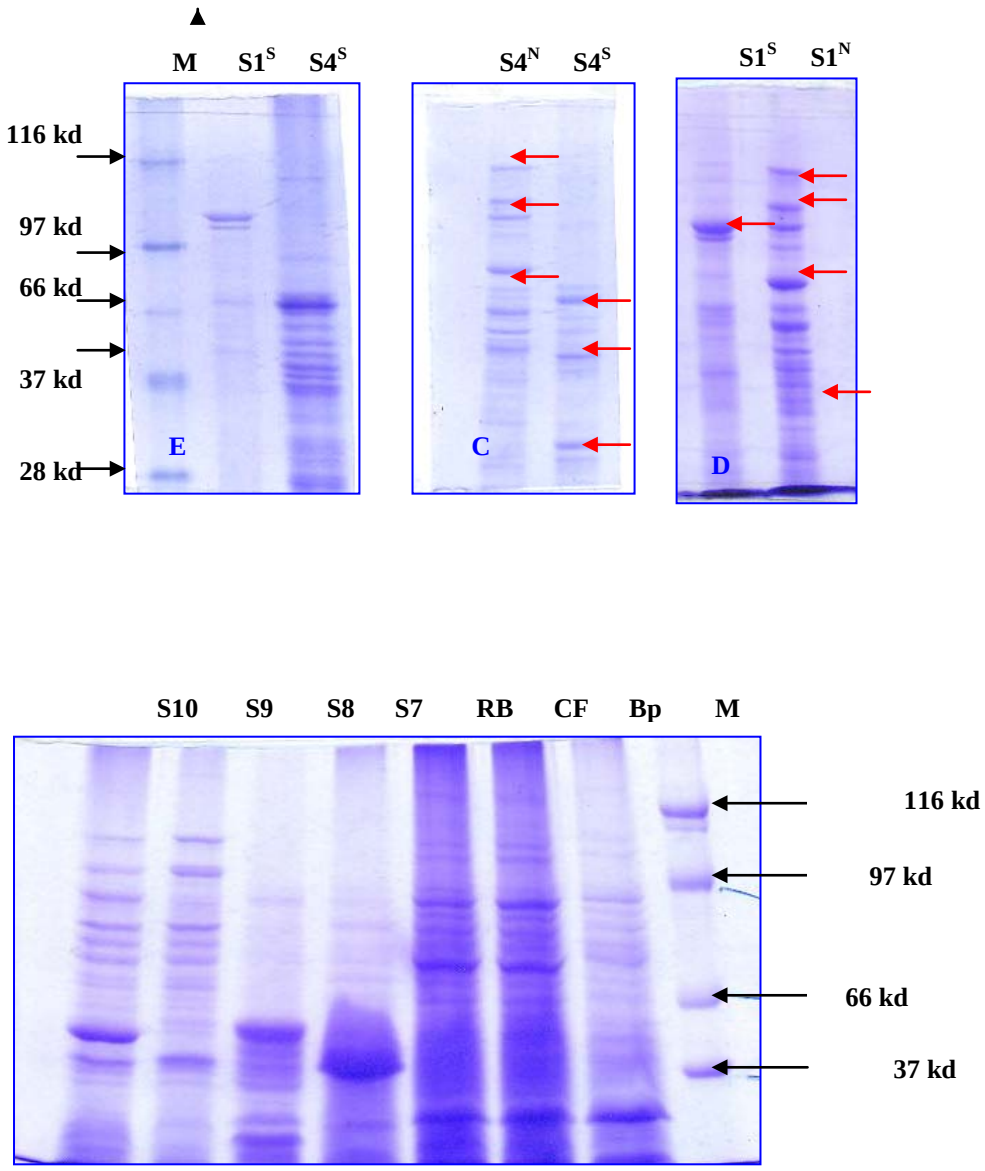
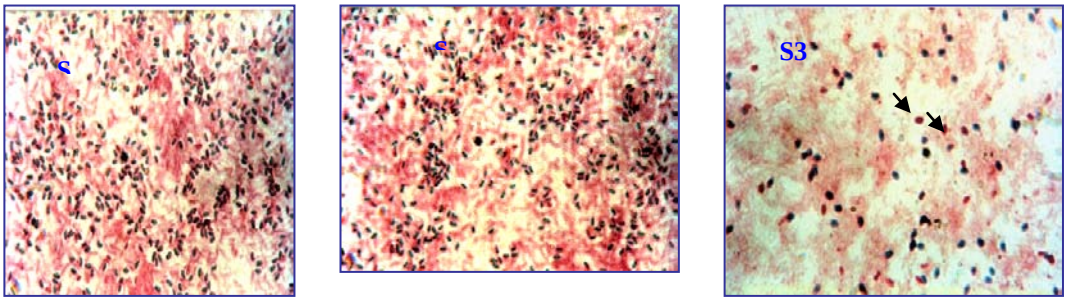


Fig (3): SDS-PAGE analysis of total cellular proteins of bacterial cells of 4 isolates S7, S8, S9 and S10 which are expected to be *B. polymyxa* compared to the protein pattern of 3 strains of bacteria *B. polymyxa*; RB, CF and Bp. The name of each isolate or strain is written on its lane. M: protein marker and its molecular kilo-daltons are written beside it.



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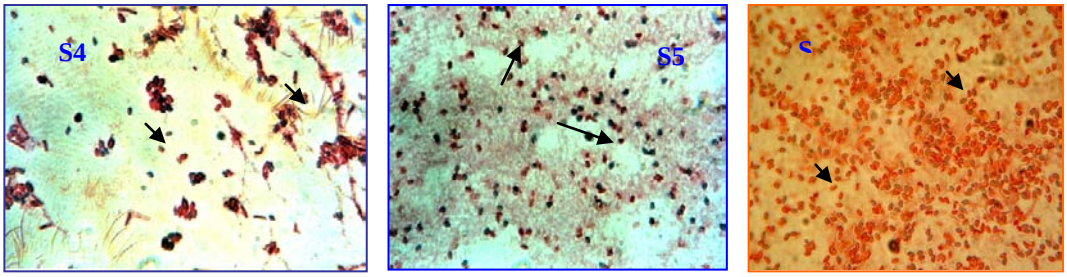

Fig (4) A: The microscopic examination of sporulating cells of the 6 *B. thuringiensis* isolates. Bacterial cells were grown on T3 medium for 72 h. The arrows refer to the crystal protein.

B

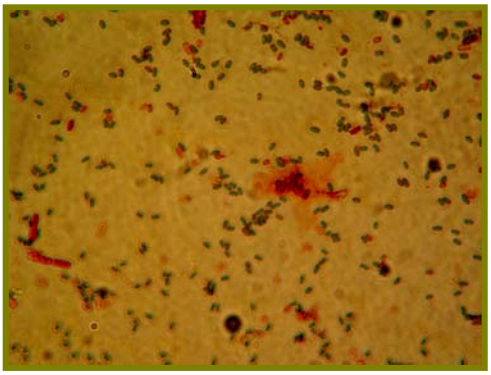

Fig (4) B: The microscopic examination of sporulating cells of the *B. polymyxa* isolate.

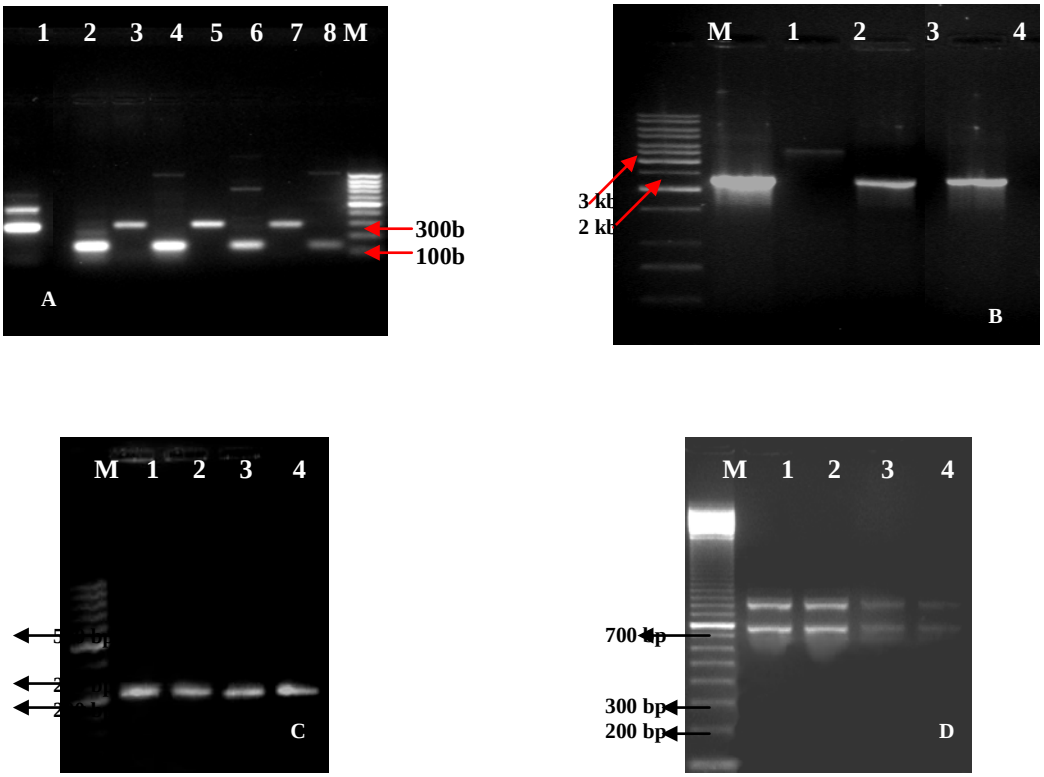

Fig (5): Agarose gel electrophoresis for amplified PCR products from DNA of bacterial isolates.
A: PCR detection of lepidopteran *cry* gene, the primers CJ10 & CJ11 (give 130 bp PCR product with *cry* 1C) and CJ1-1 & CJ1-2 (give 284 bp PCR product with *cry* 1C and 272 bp with *cry* 1Ac) were used. Lane1: S6 with CJ1-1 & CJ1-2 (give 284 bp PCR product). Lane2: S6 with CJ10 & CJ11 (130 bp PCR product). Lane3: S5 with CJ1-1 & CJ1-2. Lane4: S5 with CJ10 & CJ11. Lane5: S3 with CJ1-1 & CJ1-2. Lane 6: S3 with CJ10 & CJ11. Lane7: S2 with CJ1-1 & CJ1-2. Lane8: S2 with CJ10 & CJ11. M: 100 bp DNA molecular marker.
B: The primer sets IAF & IAR (give 2.2 kb with *cry* 1C) and IAF & ICR (give 3.7 kb with *cry* 1C) were used.
M: 1 kb ladder DNA molecular marker. Lane1: Positive control with IAF&IAR (2.2 kb PCR product). Lane2: Positive control with IAF&ICR (3.7 kb PCR product). Lane3: S2 with IAF&IAR. Lane4: S3 with IAF&IAR.
C: The primers set *gln*B up & *gln*B do that give 250 bp PCR products with *gln*B gene were used. M: 50 bp DNA molecular marker. Lane1: S7 with glnB up&do (250 bp products). Lanes 2, 3 and 4 are S8, S9 and S10 with *gln*B up&do.
D: The primer set *nif*D up&do that give 710 bp PCR products with *nif*D gene were used. M: 100 bp ladder DNA molecular marker. Lanes 1, 2, 3 and 4 are S7, S8, S9 and S10 with *nif*D up&do

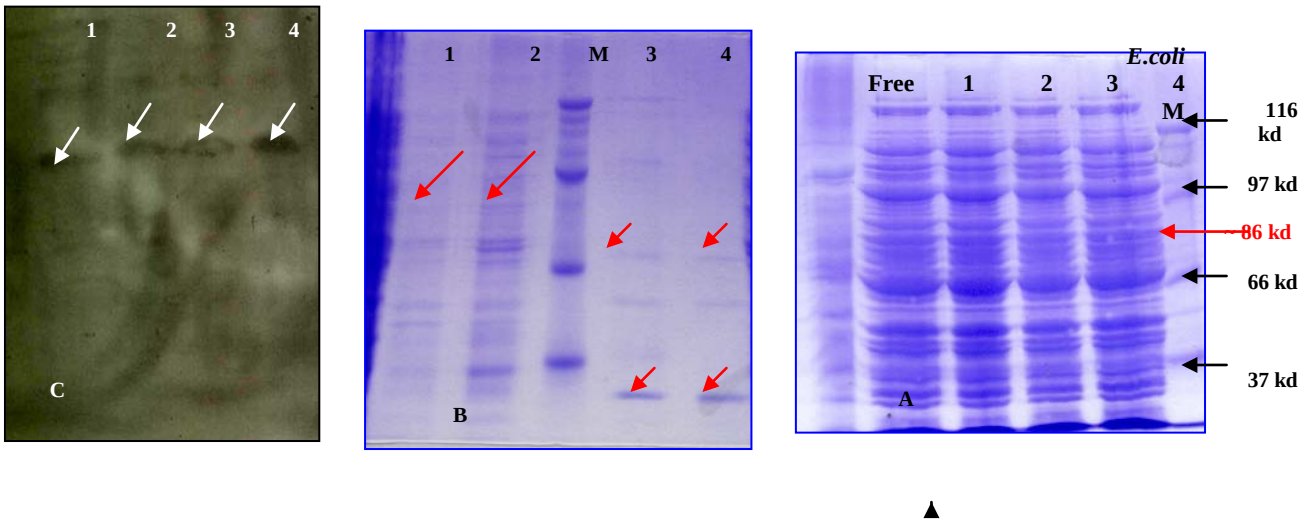

Fig (6): SDS-PAGE analysis, partial purification, trypsinization and immunodetection of the expressed protein of carboxy-terminally truncated Cry 1C in *E. coli* JM109.
A: Expression of 2.2 kb of *cry* 1C in *E. coli* JM109. A Coomassie-stained SDS-PAGE showing the expressed ~ 86 kDa (the red arrow pointed to it) of Cry 1C toxin. Lanes1-4 are 4 clones from both S2 and S3 that expressed the Cry 1C toxin. M: protein marker
B: Partial purification and trypsinization of the expressed protein of Cry 1C. Lanes 1&2: partial purification of protein from 2 clones of the transformed *E. coli*. M: Protein marker. Lanes 3&4: Trypsinized protein with TPCK trypsin to produce the activated Cry 1C ~ 65 kDa (the arrows refer to the expressed band before and after digestion with trypsin).
C:Western blot analysis of the proteins synthesized by the transformed *E. coli* JM109 with polyclonal antibodies for the *B. thuringiensis* crystal protein after SDS-PAGE. Lanes 1, 2, 3 and 4: 4 clones contained the expressed protein of Cry 1C.

عزل و تعريف و توصيف جزيئى لبكتيريا مقاومة للحرارة و الملوحة من على امتداد شواطىء المتوسط فى مصر

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الملخص العربى

يعتبر امتداد شواطىء المتوسط و المناطق القريبة منه مثل ملاحات مريوط, برج العرب و العامرية فى شمال مصر من الاماكن الصالحة لعزل الكائنات المقاومة للملوحة و درجات الحرارة العالية. هذه الدراسة تعرض عزل و تعريف و توصيف جزيئى لبكتيريا مقاومة للملوحة و درجات الحرارة العالية. قد تم عزل عشرة عزلات بكتيرية مستخدما طرق البيئات الغنية التى وصلت الى 30 % (وزن / حجم) من كلوريد الصوديوم و ايضا الى التحضين عند درجات حرارة 75° م. كل العزلات أوضحت التفاعل الايجابى لصبغة جرام و قد تم تعريفهم تبعا الى كتاب التعريفات (برجى') على انهم جنس *باسيلس* ؛ حيث ستة عزلات عرفوا نهائيا على انهم *باسيلس ثورينجينسس* و الاربع عزلات الاخرى كانوا *باسيلس بوليميكسا*.

الطرز البروتينى لعزلات *الباسيلس ثورينجينسس* و تفاعل البلمرة التسلسلى للكشف عن الجينات المسئولة عن تصنيع بللورات البروتين السامة, أظهرت وجود الجينات السامة ضد طائفة الحشرات حرشفية الاجنحة و خصوصا الجين *cry* 1C. و على مستوى تصنيع البروتين؛ فان الطرز البروتينى للخلايا البكتيرية النامية فى 5,2 مولار كلوريد الصوديوم قد أظهرت و فى بعض الاحيان أخفت مجموعة من البروتينات المصاحبة للملوحة العالية. عزلة بكتيرية (S2) من العزلات الستة أخضعت لعزل جزء من الجين *cry* 1C الذى يبلغ حوالى 2,2 كيلو دالتون و هذا الجزء عبارة عن الجين و مقطوع منه جزء مجموعة الكربوكسيل. و قد تم كلونة هذا الجزء من الجين و نقله الى بكتيريا *ايشيرشيا كولى E. coli* حيث ترجم الى البروتين الخاص به. و الدرسات المبدئية أوضحت اختلاف هذا الجين من العزلة المصرية عن الجينات المنشورة ضمن *cry* 1C genes. و على الجانب الآخر, فان الاربع عزلات المعرفة على انها *باسيلس بوليميكسا* كانت لها القدرة على النمو فى درجات حرارة تصل الى 75° م. الطرز البروتينى و تفاعل البلمرة التسلسلى للكشف عن الجينات *nif* D & *gln* B اظهرت وجود هذه الجينات في الاربع عزلات. هكذا, فان عزلات *الباسيلس بوليميكسا* يمكن ان تستخدم فى تثبيت النيترجين على المحاصيل المنزرعة فى المناطق الحارة.

ECOLOGICAL STUDIES ON MITES INFESTING HONEY-BEE COLONIES AND THEIR PHYSICAL TREATMENT

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ABSTRACT

Sharkia governorate was characterized by five locations of agriculture lands and honey-bee colonies which were chosen to study the parasitic mites on the honey-bee. These five areas were: Zagazig, Belbeis, Diarb-Negm, Abo-Kaber, Alhosienea

50 honey-bee colonies in the representative areas in Sharkia governorate were monthly examined and surveyed during one whole year, during March 2002 till February 2003, 1405 mites Varroa Jacobsoni Audeman(Acaridea: Varroidae) were collected. They were ectoparasitic and blood sucking mites in the honey-bee brood and adults. The infestation rate of varroa mites was (21%) /50 hive workers bee. 264.7 another mites only detected through using a dissecting microscope, it was a honey-bee parasitic tracheal mite (HBTM) Acarapis Woodi(Ronnie), these mites hived inside trachea of adult bees. The infestation rate of HBTM was (5%)/50 hive adults.

The mean infestation rate of Varroa mites in the honey-bee worker evaluated monthly from March 2002 to February 2003, reached maximum rate in the middle of September, but in the middle of February the HBTM reached the maximum infestation rate. In seasonal distribution the rate of Varroa mite infestation increased in spring and reached a peak in autumn.

The infestation rate of HBTM increased in spring, reached peak in winter. Electromagnetic field was used to control Varroa mites and HBTM. 10 honey-bee hives were exposed to magnetic field, about 4450 gauss, for 20 minutes every morning and exposed again at the end of the day. These two steps were repeated daily through one month, the infestation of mites completely reduced.

INTRODUCTION

Honey bees are familiar insects to most people. Bees live in colonies dominated by eggs, lying by queen bee, worker bees are all females, daughters of the queen.

Male bees (drones) are produced in the colony only when needed to mate with new virgin queens. Honey bee workers collect pollen and plants nectar. An estimated one third of the human diet is derived directly or indirectly from insect pollinated plant.

Honey bees (hymenoptera apidae) are the world's most important special insect pollinator of fat and vegetable crops. Honey bee feed exclusively on plant nectar and plant pollen. Plant nectar is transformed into honey for storage which is the

colony's main food reserve. Pollen is the colony protein food reserve. Honey bees are the source of honey and bee wax, a fine wax with unique qualities. Honey bees also produce propalies a gummy substance made from tree sap, which has antibacterial properties, and royal jelly and pollen for human consumption.

Honey bee venom is extracted for the production of antivenom therapy and is being investigated as a treatment for several serious diseases of the muscles, connective tissue, and immune system, including multiple sclerosis and arthritis. (Doug 2006)

Honey bees must contend a number of parasites both inside and outside of their natural environment; mites are of particular importance to bees and beekeepers, because of their potential to do great damage to both feral and managed honey bee colonies. The two species of mites that most responsible of the damage to the bee colonies are the Varroa mite (Varroa Jacobson) and the honey bee trachea mites (Acarapis woodi), these mites have killed twenty of thousands of honey bees' colonies. In North America during the past ten years, scientific breeding programs are attempting to develop tolerant strain of domestic honey bee to replace the mites' susceptible ones currently used (Sanford 2001).

The Varroa mite, an ectoparasite of honey bee adult and honey bee brood, it weakness, kills honey bee colonies and can also transmit several honey bee viruses. It was first described by Oudemons (1904) from genus on Apis canard.

In 1951, it was found in Singapore. In 1962/63 the mite was found on Apes mellifera in Hongkong and Philippines and spread rapidly from there, (Definado 1963). The importation of queen from infested area, and the movement of infested colonies of bee for pollination lead to the rapid spread of these mites. (Delaplan and hood 1997, martin 1999)

The honey bee tracheal mites, Acarapis woodi is an internal parasite of adult honey bees. Tracheal mites live in the breathing or tracheal tubes of adult honey bee and only move outside the host to infest another bees. Honey bee tracheal mites preferentially disperse to adult worker honey bees younger than three days of age (Gary et al, 1989), tracheal mites are associated with the death of honey bee colonies in the winter when greater than 30% of the bees within a colony are infested (Pettis & Wilson 1996).

To control the mite, beekeepers have bees using pesticides in their bee colonies', however, that approach has generated problems, including the mite developing resistance.

The aim from this study is to explore the basic ecological importance of the honey bee mites' best controlling method which is a physical method by means of electromagnetic field. This physical method protects human health and may prevent the risk of contaminating honey and hive products to the usage of chemical products such as the anti-acaroids and insecticides.

MATERIAL AND METHODS

1. Field and Laboratory Studies

A. Selection and description of studied area

Sharkia governorate is considered as the third city in Egypt according the population density. Five different zones in it were selected for the ecological study of the honey bees' colonies and survey the distribution, identification and description (Delfinado-baker 1984) of the mite infestation in Sharkia. The five studied areas were: Zagazig, Belbeis, Diarb-negum, Abo-Keber and Alhosaienea. (Map (1))

B. Collection and Isolation of Mites

B.1. Varroa jacobsoni

Method (1)

Brush or shake approximately 50 to 100 worker bees sampled from near the middle of the hive into the wide-mouth mason jar, replace the lid on jar of captured bees and spray a short burst (about one second) of engine starter fluid (approx. two tea spoons of alcohol) into the closed container. After about one minute, gently roll the jar from side to side to coat all the bees with ether (alcohol). If Varroa mites are present, they will full off the bees and adhere to sides of the jar where they can be examined and counted in the laboratory. (Burgett et al 1987).

Method (2)

Brush or shake approximately 50 to 100 worker bees sampled from near the middle of the hive into the wide-mouth mason jar, replace the modified lid and add a heaping table-spoon of powdered sugar through the mesh screen, roll the jar from side to side to distribute the sugar all over the bees. Wait a few minutes and roll the jar again. Pour the sugar and dislodged mites through the screen into chess-cloth which separate the mites from the sugar, examine and count the bee mite by sifting the sugar through the cloth leaving the mites on the cloth surface. (Ellis 2000, Macedos & Ellis 2001)

B.2. Acarapis Woodi

Pin the bee on its back and remove the head and first pair of the legs by pushing them off with scalpel or razor blued in downward and forward motion, using a dissecting microscope, remove the first ring of the thorax (tergites of prothorax) with forceps. This exposes the tracheal trunk in the mesothorax, when the infestation is light, it is necessary to remove the trachea, place it in a drop of lactic acid and glass slide for cleaning and cover glass with cover for examination at x40--100 on a compound microscope. (Delfinad & Baker1984)

2. Treatment

10 honey bee's hives were exposed daily to small doses of electromagnetic field. The source of the electromagnetic field was from a small machine manufactured by the maintenance center of Cairo University, placed in the entrance of the bees' colony.

Examine 100 workers from every hive weekly and count the dead Varroa to calculate the mortality of Varroa.(El-basheir et al 1994/96)

3. Statistical Analysis

All the statistical results were analyzed according to Duncan (1955), Anon (1985).

RESULTS

1. Description:

1. Varroa Mites

Varroa mites are blood sucking ectoparasits that attack young and adult honey bee. Female Varroa are brown to dark brown, shaped like a rib, measuring 1.0 to 1.77 mm long and 1.50 to 1.99 mm wide. Their curved bodies fit into abdominal folds of the adult bee and are held there by shape and arrangement of ventral setae.

Adult male are yellowish with light terminal legs and spherical body shape measuring 0.75 to 0.98 mm long and 0.70 to 0.88 wide. The male chelicerae are modified for transferring sperm. (Fig. 1)

2. Honey Bee Tracheal Mite (HBTM)

Acarapis woodi are endoparasite, blood sucking which attacks tracheal honey bees. Females are (0.005 to 0.008 inches) 145-176 microns in length and 78-82 microns in width. Males are (0.004-0.006 inches) 126-137 microns in length and 61-78 microns in width.

The mites have an oval body shape and posses also a hardened of mandible that is used to feed the blood of its host. (Fig. 2)

2. Mite Survey

The locations and some information about the infested colonies, in the Sharkia governorate, during March 2002 till February 2003, were mentioned in table (1).

The prevalence rate of varroa mite infestation among fifty honey bee colonies in five representative areas during one year (March 2002- February 2003) were analyzed in table(2). So as the Monthly distribution of the Tracheal mites were analyzed in table (3). It was found that the highest collection rate was in Alhosaienea, 25.5% of varro mites and 25.1% of tracheal mites, While the lowest collection rate was found in Zagazig, 13.4% of varroa mites and 14.5% of tracheal mites.

3. Seasonal abundance

The mite population was cyclical observed and recorded in table (5, 4) and shown in fig (1). Varroa mites' population rate was increasing and reached its peak in autumn and had a minimum population rate in summer. While HBTM's population rate was build up in autumn, reached its peak in winter and declined in summer, fig. (3).

4. Treatment

A small machine which produces an electromagnetic field was placed in the entrance of the bees' colony. It was noticed that the effect of small doses of magnetic field (4450 gauss) resulted from a voltage of 5volts on infestation mites, for 5 to 20 minutes, twice a day for a whole month results in a great decrease after the 3rd week. And after four weeks of that treatment there was no infestation in the colony.

DISCUSSION

According to this study, the rate of honey bee mites' infestation in Sharkia governorate was about 21% of 50 hives for *Varroa jacobsoni*, And 5% of 50 hives for *Acarapies woodi*. The highest record of mite infestation was in Alhosaienea (25.5%) while the lowest one was in Zagazig (13.4%).

Parasitic mites of honey bee were considered to be very harmful which cause serious problem especially the varroa mites in Sharkia. Heavily infested colonies usually have large number of unseald brood cells and dead newly emerged bees. Bees' mites infested the colony always take only a few months to destroy the whole colony.

These results were similar to the results of Maria Helena Correa-Marques (2000) in Brazil, Sanford (2000). Rice et al (2000) study HBTM in 80 bee colonies and the data resulted didn't explain why HBTM population rate in these colonies remained suppressed. Rice (2006) reported *Varroa* mites had become widely established in Kentucky and had continued spreading till it became a major pest honey bee in many states.

In this Study, the mite population rate was cyclic observed. *Varroa* mites' population rate was Build up in winter, increased and reached its peak in autumn, fall in spring and had a minimum population rate in summer. While HBTM's population rate was Build up in autumn, increase in spring, reached its peak in winter and declined in summer. This fact was also published by Kim (1990), ED Allein (1998) and Wilson (1990). Diana (1990) was the one who found the *Varroa* population rate had a peak in winter. This was opposite to the normal honey bee population rate cycle in which there was a spring build up, a summer peak and a winter decline.

A good queen bee stops the producing of the mites in spring and summer, by getting rid of them and easing the mite's problem for the colony. Spring re-queening helps boost the bee population. The greatest lose of both individual bees and colonies were in late autumn and early winter. This wasn't surprising if we put in consideration that the mites build up in winter.

The bees' normal difficulties were trying to survive winter in Sharkia with the shortness of life and the large number of bees' death rate in this season time.

As a result of this study, the honey bee mites infestation decreased by exposing a small electromagnetic wave to the colony. After two weeks from the treatment, it showed a high reduction in the infestation and after 4 weeks the infestation was totally eliminated.

The results were similar to the results published by El-bachier (1994 -1996). It was noticed that the mortality rate increased among mites by increasing the period of exposure. Maximum mortality rate was recorded after 20 minutes at 7378 gauss as a result of the magnetic field effect.

CONCLUSION

The *Varroa* mite is a reddish-brown, external honey bee parasite about the size of the head of a pin. *Varroa* mites attach to adults and developing brood, where they

feed on their blood. If left untreated, *Varroa* mites can deform bees and eventually kill the colony.

The honey bee mite, *Acarapsis woodi*, is a microscopic mite only detectable through dissection. They are whitish in color, with oval bodies and have a shiny cuticle with a few long fine hairs on the body and legs. This small mite is an internal parasite of honey bees. It infests and lives entirely within the tracheal (respiratory) system of honey bees.

In Sharkia governorate, the infestation rate of *Varroa* mite increase in spring and reach maximum in autumn while the infestation rate of HBTM increase in spring and reach maximum in winter.

Electromagnetic field (4540 gauss) needs to be in contact with the brood nest for about 20 minutes to be effective.

It is safer to use small doses of electromagnetic field for the bee-keeper, bee and honey bee products.

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Table (1): The locations and some information about the infested colonies, in the Sharkia governorate, during March 2002 till February 2003.

Area	Number of the colony	Number of HB workers	Varroa.M infestation rate (%)	Tracheal.M infestation rate (%)
Zagazig	1	100	25.5	5.4
Belbis	2	100	26.3	7.1
Diarb-negum	3	100	27.2	8.2
Abo-Kabeer	4	100	28.5	8.9
Alhosaienea	5	100	29.12	9.1

Table (2): Monthly distribution of the Varroa mites at five areas in Al-Sharkia governorate, during March 2002 till February 2003.

Month	Zagazig	Belbies	Diarb-Negum	Abo-Kabeer	Alhosaienea
March	14.7 ± 5	16 ± 1.5	16.5 ± 8.5	15 ± 2.5	19 ± 3.5
April	16.3 ± 1.5	22 ± 2	24.3 ± 3.5	29 ± 3.5	32.7 ± 4.5
May	17.7 ± 2.5	19 ± 1	25 ± 0.5	32 ± 8	33 ± 4
June	19 ± 3.5	21.3 ± 5	29.3 ± 7	38.2 ± 2.5	45 ± 4.5
July	19.7 ± 2.5	25 ± 2.5	33.7 ± 10.5	41 ± 1	46 ± 1
August	21.7 ± 2	29 ± 7	35.3± 9.5	43±1	48 ± 1.5
September	22.3±1	33±1.5	36.3±2.5	43.3±3	49.3±5
October	21±3	30.3±0.5	33.7±3	40.3±0.5	45±2.5
November	19±5	29±1.5	31.7±1.5	36.7±6	43±2
December	18.3±1.5	26±3.5	29.7±2	33.3±0.5	40.3±5
January	17±1	21±1	25.3±1.5	29±2	38.3±9
February	15.7±1	19.7±4.5	19.3±8.5	23±8.5	32±22
Total	222.4	291.3	340.1	403.6	471.6
Percentage (%)	13.4	17.5	19.4	24.3	25.5

Table (3): Monthly distribution of the Tracheal mites at five areas in Al-Sharkia governorate, during March 2002 till February 2003.

Month	Zagazig	Belbies	Diarb-Negum	Abo-Kabeer	Alhosaienea
March	2.3 ± 0.5	2.7 ± 0.3	3 ± 1	3.3 ± 0.5	4 ± 2.5
April	3.7 ± 1	4.3 ± 0.5	4.7 ± 1	5 ± 2	5.3 ± 1
May	4 ± 1	5.7 ± 1	6.3 ± 1.5	7.7 ± 2	8.3 ± 3
June	3.3 ± 0.5	4 ± 1	5.7 ± 1	6 ± 2	7.3 ± 1.5
July	-	-	-	-	-
August	-	-	-	-	-
September	-	-	-	-	-
October	-	-	-	-	-
November	-	-	-	-	-
December	5.3 ± 1	5.7 ± 1	6.7 ± 2	8.3 ± 2.5	9 ± 1
January	7 ± 5.2	8.3 ± 5	9.3 ± 1	10.7 ± 1	11.3 ± 0.5
February	9.3 ± 1	12.3 ± 2.5	12.7 ± 1	13.3 ± 2	15.3 ± 1
Total	34.9	43	48.4	54.3	60.5
Percentage (%)	14.5	17.8	20	22.5	25.5

Table (4): Seasonal abundance of Varroa at five areas in Al-Sharkia governorate, during March 2002 till February 2003.

Season	Zagazig	Belbies	Diarb-Negum	Abo-Kabeer	Alhosaienea	Total
Spring	48.7	57	65.8	76	84	331.5
Summer	60.4	75.3	98.3	122	139	495
Autumn	62.3	92.3	101	120.3	137.3	513.2
Winter	51	66.7	75	85.3	110.6	388.67
Total	222.4	291.3	340.1	403.6	470.9	1728.37

Table (5): Seasonal abundance of HBTM at five areas in Al-Sharkia governorate, during March 2002 till February 2003.

Season	Zagazig	Belbies	Diarb-Negum	Abo-Kabeer	Alhosaienea	Total
Spring	10	16.7	19.7	22	24.9	93.3
Summer	3.3	4	5.7	6	7.3	26.3
Autumn	-	-	-	-	-	-
Winter	21.6	26.3	28.7	32.3	35.6	144.5
Total	34.9	47	54.1	60.3	67.8	264.1

Table (6): The effect of a magnetic field which results from a 5 volts voltage taken in variable times on the honey bee mites during a month.

Week	Number of bee exposed to EMFs	Voltage (*Volts*)	Time (*minutes*)	Mortality rate in Varroa	Mortality rate in HBTM
1st	100	5	5	30.6	25.2
2nd	100	5	10	70.0	31.3
3rd	100	5	15	85.4	40.2
4th	100	5	20	90.1	80.9

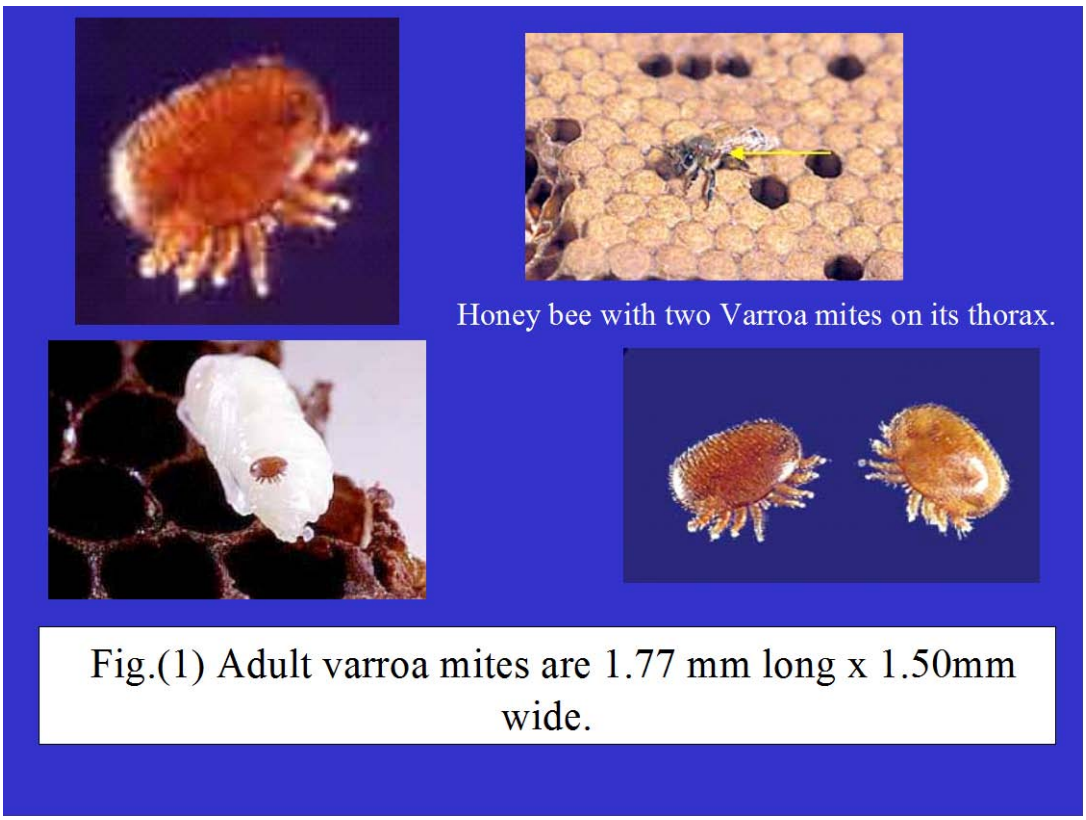

Fig.(1) Adult varroa mites are 1.77 mm long x 1.50mm wide.

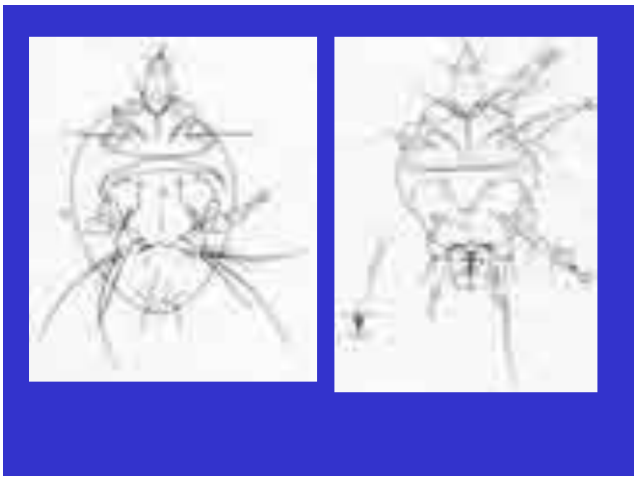

Fig.(2) Adult *Acarapis woodi*, Female(145 long X 78 width microns) and Male(126 long X 61 Width microns)

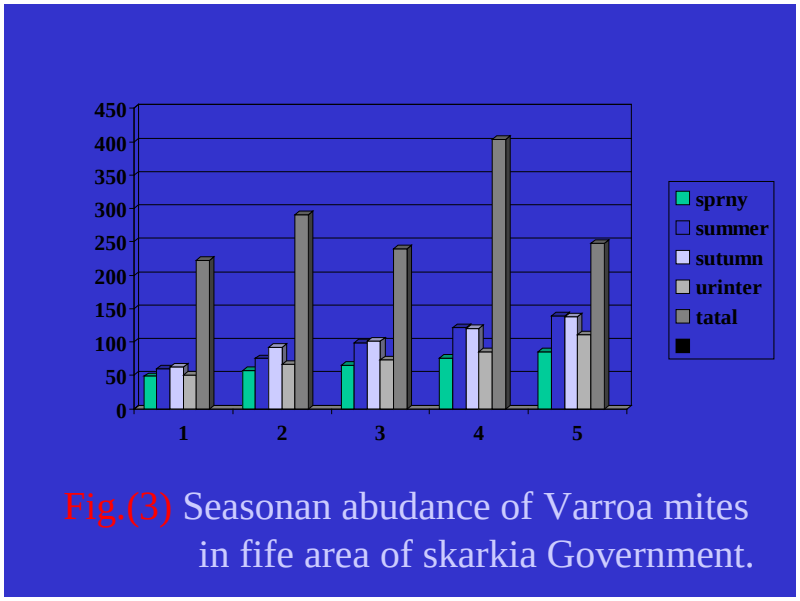

Fig.(3) Seasonan abudance of Varroa mites in fife area of skarkia Government.

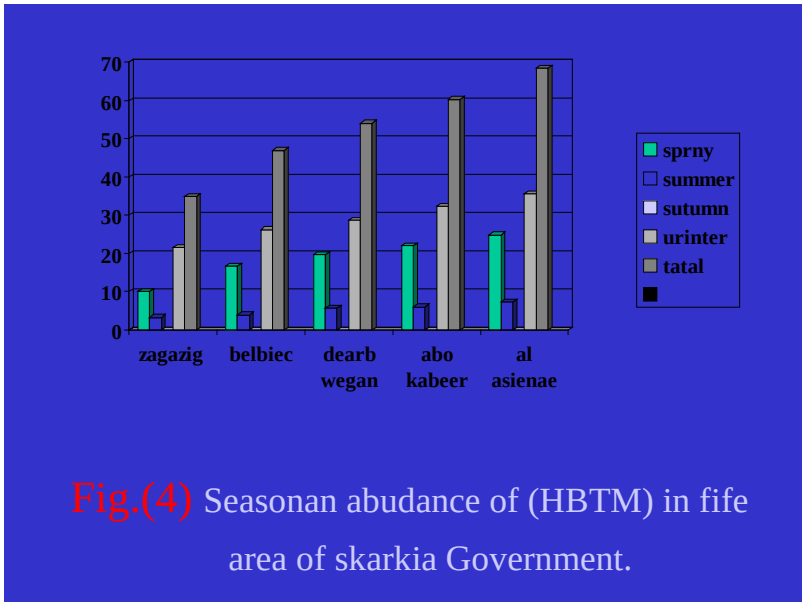

Fig.(4) Seasonan abudance of (HBTM) in fife area of skarkia Government.

دراسات بيئية على الحلم المتطفل على خلايا النحل و طرق علاجة فيزيائيا

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النحل من الحشرات النافعة و هى حشرات مألوفة للإنسان، و إستطاع أن يقوم بتربيتها و الإستفاده من إنتاجها. فهى تمده بالعسل، و هو الغذاء الكامل الذى يحتوى على معظم الأملاح و الفيتامينات و المواد الغذائية التى يحتاج إليها الإنسان فى نشاطه اليومى، و تنتج الشمع الذى يستخدم فى صناعات كثيرة، و كذلك الصمغ الذى يعتبر أحد المضادات الحيوية و يستخدم فى كثير من الطرق العلاجية بل و يستخدم فى مقاومة بعض الطفيليات الضارة للإنسان و الحيوان. و يجمع أيضا النحل حبوب اللقاح ذو القيمة الغذائية العالية للنحل و للإنسان بالإضافة إلى ذلك يقوم النحل بنقل حبوب اللقاح من الأزهار. فهو المسئول عن 70% من تخصيب النباتات و إزدهارها.

أمـــا طفيـــل الحـــلم و هو من أخـطـر الطفيـلـيـــات التـى تـضر خلايـا النحل و تسبـب خسائر فـادحة للـمنـاحـــل.

فى دراسة بيئية عملية تم تقسيم محافظة الشرقية إلى خمس مناطق هى :

1. الزقازيق
2. بلبيس
3. ديارب نجم
4. أبو كبير
5. الحسينية

وهى مناطق زراعية بها مزارع لخلايا النحل، و قد تم فحص عشر خلايا من كل منطقة على مدار سنة كاملة، فى الفترة من مارس 2002 إلى فبراير 2003 ، و قد تم تحديد نسبة الإصابة بطفيليات الحلم مع التعرف على أنواع الحلم و دراستها. و بالتالى فإن حلم النحل ينحصر فى نوعيين هما:

- حلم الفاروا أو إيدز النحل:
  و هو يتطفل خارجيا على شغالة النحل و على الحضنة و تصل نسبة الإصابة 21% لكل الخلايا.
- حلم القصبة الهوائية:
  و يعيش داخل الجهاز التنفسى للنحل البالغ و الصغير، و لابد من تشريح حشرة النحل لرؤيته و التعرف علية . و كانت نسة الإصابة به 5% لكل خلية.

عندما تعرضت عشر خلايا إلى المجال المغناطيسى الناتج عن الدائرة الكهربائية التى تم لصقها أسفل الخلية النحلية، بمقدار 4540 جاوس الناتج من تيار كهربائى شدده 5 فولت، لمدة 20 دقيقة صباحا و 20 دقيقة آخرى فى نهاية اليوم، قبل غروب الشمس، لمدة شهر، وجد أن الإصابة بطفيل الحلم إنعدمت تماما.

EFFECT OF PHYCOCYANIN PIGMENT ON ACUTE RENAL FAILURE INDUCED BY MERCURIC CHLORIDE

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ABSTRACT

C-phycocyanin is a biliprotein pigment found in blue green algae. It has nutritional, medicinal value as well as nephroprotectant agent. For evaluating its efficiency in curing nephrites against mercuric; five groups of mice (10 mice for each) were intraperitoneally injected as follow: **(I)** Control mice were injected with 1% phosphate saline buffer, pH 7.2; **(ii)** The animals were injected with C-PC three times at dose 4 mg/Kg body weight for each injection; **(iii)** The animals were injected one time with $HgCl_2$ (5 mg/kg body weight); **(iv)** The animals were injected with C-PC three times at dose 4 mg/kg body weight and followed with injection $HgCl_2$ at the dose 5 mg/kg body weight once. **(v)** The animals were injected firstly with $HgCl_2$ at the dose 5 mg/kg body weight and injection with C-PC three times at dose 4 mg/kg body weight. The animals were sacrificed following 15 and 30 days from the beginning of experiment. Results of blood alteration following $HgCl_2$ injection evoked a significant elevation ($P<0.001$) in serum urea and creatinine accompanied with highly significant decrease in hemoglobin (Hb) content, oxygen capacity and R.B.Cs count. Histopathological examination revealed hydropic degeneration, swollen nuclei and sloughing of some epithelial cells of tubular epithelium and irregular contours of Bowman's capsule as well as detached of some cells of glomeruli with obvious destruction in renal corpuscle in the kidney of mouse. The previous results were counteracted especially in C-PC treated mice before their treated with $HgCl_2$ (C-PC pre and post with $HgCl_2$). Hence it could be concluded that C-PC suppresses the lipidperoxidation and antagonized $HgCl_2$ intoxification nephrites (nephroprotective agents).

Key words: Phycocyanin; Blue green algae; Mercury; Renal index, Histopathological alteration.

INTRODUCTION

Inorganic mercury present in the environmental as a well established toxicant to human health. It is being widely used in the industrial, medical, agriculture and other fields **(ATSDR, 1989).** It is well-known human and animal nephrotoxicant. (**WHO, 1991 and Jarup, 2003).** Slight exposure to $HgCl_2$ leads to acute renal failure (ARF) where as acute oral or parenteral exposure induces extensive kidney damage (**Hostetter *et al.*, 1983 and Woods, 1989).** Paralliy, it induced biochemical alteration in the blood of albino mice **(Sharma *et al.* 2005).** Studies in vivo and in vitro have demonstrated that mercury induces lipidperoxidation and enhances renal mitochondrial hydrogen peroxide formation in vivo and in vitro **(Lund *et al.*, 1993)**. Moreover, it promotes the formation of reactive oxygen species (ROS) such as hydrogen peroxide which enhances the lipidperoxidation and reactive hydroxyl radicals **(Kim and Sharma, 2003 and Sener *et al.*, 2003).** The previous radical plays a cortical role in renal cell injury **(Lund *et al.*, 1991).** Whereas other investigators showed that lipid peroxidation is not directly responsible for mercury-induced cell injury in hepatocytes and renal cells **(Strubelt *et al.*, 1996).** The mechanisms underlying the onset of $HgCl_2$-induced ARF have been considerably elucidated for the last two decades. Mercuric ions have a high affinity for sulfhydryl-containing molecules such as albumin, glutathione, and cysteine **(Bohets *et al.*,**

1995 and Zalups, 2000). New a days it is considered that mercuric ions present in the proximal tubular lumen following $HgCl_2$ exposure may exist at least in part in the form of mercuric conjugates of glutathione (GSH—Hg–GSH) and cysteine (CYS—Hg–CYS) **(Zalups, 2000).** Also, there is available evidence indicating that mercuric conjugates of glutathione, cysteine, and other small molecules, such as *N*-acetylcysteine, that are formed in plasma may be taken up at the basolateral membrane of proximal tubules **(Zalups, 2000).** Moreover it has been reported that phycocyanin is a potent free radical scavenger (hydroxyl and peroxyl radicals) inhibiting microsomal lipidperoxidation (LPO) **(Pinero *et al.*, 2000)** and other effects. Moreover **Rimbau *et al.*, (1999)** indicating that C-PC has antioxidant, anti-inflammatory and neuroprotective effects. This work aims to evaluate the counteraction effect of $HgCl_2$ toxicity for renal cells by C-PC.

MATERIAL AND METHODS

Animals

Random–bred, mal Swiss albino mice (6-8 weeks old), were taken from animal house of faculty of Veterinary medicine, Zagazig University. They were kept under controlled nutritional and environmental conditions with provision of a 12h light: 12h dark regimen.

Extraction and determination of the C-PC

C-phycocyanin was extracted from blue green alga *Spirulina platensis* according to the method of **Boussiba and Richmond (1979);** approximately 2 g of experimental alga was suspended in 200 ml of 0.1M Sodium phosphate buffer pH 7.2 containing 100µg/ml lyzozyme and 10 mM EDTA. The crude extracted was precipitated with ammonium sulfate at 70% saturation. The precipitated fraction was collected and dialyzed against distilled water and the non dialyzed fraction was used for intraperitoneal injection.

Preparation of C-PC for injection

Stock solution of C-PC in saline buffer pH 7.2 was prepared and diluted with the same buffer to obtain the intraperitoneal injected dose. It was 12 mg/kg body weight.

Preparation of $HgCl_2$ for injection

An equivalent weight of mercuric to (5.0 mg/kg body weight) was estimated and dissolved in saline buffer pH 7.2 according to **Sharma *et al.* (2005).**

Experimental protocol

The albino mice were divided into 5 groups each having 10 animals.

Group (1): Control mice were injected intraperitoneally with saline buffer pH 7.2.

Group (2): The animals were injected intraperitoneally with C-PC three times at dose 4 mg/kg body weight, two days apart.

Group (3): The animals were injected once with $HgCl_2$ at the dose 5 mg/kg body weight.

Group (4): The animals were injected with C-PC three times at dose 4 mg/kg body weight; two days apart and left 7 days then injected with $HgCl_2$ at the dose 5 mg/kg body weight once.

Group (5): The animals were injected firstly with $HgCl_2$ at the dose 5 mg/kg body weight then left 6 days then injected with C-PC three times at dose 4 mg/kg body weight; two days apart.

All half the animals scarified at 15 days and the others at 30 days from the beginning of experimental.

Biochemical analysis

Serum **urea** was enzymatically determined by colorimetric method according to **Patton and Crouch (1977)** and Serum **creatinine** was quantitatively determined kinetically according to **(Henry, 1974).**

Hemoglobin content (Hb %) was calorimetrically determined according to **(Van and Zijlstra, 1961 and Brit, 1967).**

N.B: 1gm of Hemoglobin is capable to combine with 1.36 ml of O_2 under volume optimal condition. So **oxygen capacity** = hemoglobin concentration x 1.36 according to **Bernard, (1976).**

Enumeration of Erythrocytes (R.B.Cs count)

The blood was diluted with saline buffer (0.9 % NaCl) and red blood cells were counted using Bright-line Improved Neubauer Haemocytometer counting chamber (**ASO Scientific Instruments**).

Histopathological investigation

Histopathological investigations of different samples were carried out using Hematoxylin and Eosin (H & E) according to **Drury and Wallington (1980).**

Statistical analysis

Statistical analysis of variance (ANOVA) and Student–Newman–Keul multiple comparison test were applied to determine the significant differences among the groups. $P > 0.05$ was considered significant.

RESULTS

Serum urea and creatinine

The results listed in table (1) showed that injection with $HgCl_2$ elevates the value of both serum urea and creatinine to the maximum level as they compared with their corresponding controls (50 mg/dl) and (1.66 mg/dl) especially after 30 days old mice. Whereas C-PC did not induce any shifting in the values of serum urea and creatinine as compared with their controls. On the other hand, preinjection with C-PC three times followed with injection $HgCl_2$ or preinjection with $HgCl_2$ followed with three injections with C-PC gave more or less similar results with an obvious elevation in both parameters as the mice treated once with $HgCl_2$ followed with C-PC as compared to the irrespective controls. This indicates that C-PC has a counteractive effect to the intoxification of $HgCl_2$ either in the mice scarified following 15 and 30 days old mice. In this regard, the renal index in 15 and 30 days old mice were parallel with their corresponding controls.

Changes in Hemoglobin (Hb) content and R.B.Cs count

Here again results in table (2) revealed that $HgCl_2$ intoxication gave very highly significant depletion in hemoglobin (Hb) content and R.B.Cs count in mice under investigation the percentage of depletion was obviously appeared in 30 days old mice (30% and 37%) as compared with their respective controls. Contrarily C-PC injection alone did not induce any effect on both parameters (similar to control). On the hand, preinjection with C-PC three times followed by injection with $HgCl_2$ one time gave an improve in the toxicity of $HgCl_2$ since hemoglobin (Hb) content and R.B.Cs count show slight decline in their values as compared with their controls especially in the group of 30 days old mice. The percentages of decreases were 4% and 3.8% respectively. Meanwhile as the mice treated with $HgCl_2$ followed by the injection with C-PC gave an obvious reduction in 15 days old mice which

were improved as the animals left to 30 days. The previous results indicates C-PC contract the mercuric toxicity.

In parallel pattern, the oxygen capacity was found to be dependent on the hemoglobin value. Whereas injection with $HgCl_2$ lead to a very highly significant ($P < 0.001$) decrease especially after 30 days following injection. Such depletion was antagonistic following preinjection with C-PC three times followed with injection $HgCl_2$ as compared with the corresponding control.

Table (1): Effect of separate doses of $HgCl_2$ and C-PC, as well as a combination of both on some renal failure index following 15 and 30 days post injection:

Treatment	Urea	Creatinine	Urea	Creatinine
	15 days		30 days	
Control	25.000 ± 0.577	0.83 ± 0.017	25.00 ± 0.231	0.83 ± 0.047
C-PC alone	25.000^{NS} ± 1.155	0.82^{NS} ± 0.048	25.00^{NS} ± 1.527	0.81^{NS} ± 0.032
$HgCl_2$ alone	45.000^{+3} ± 1.155	1.49^{+3} ± 0.037	50.00^{+3} ± 1.527	1.66^{+3} ± 0.049
C-PC Then $HgCl_2$	29.00^{+2} 0.577	0.97^{NS} ± 0.067	26.00^{NS} ± 0.577	0.87^{NS} ± 0.015
$HgCl_2$ Then C-PC	34.00^{+2} ± 0.55	1.30^{+2} ±0.03	29.00^{+2} ± 0.565	1.08^{+1} ± 0.077

- (NS) Not significant, $P > 0.05$.
- ($^{+1}$) Significant, $P < 0.05$
- ($^{+2}$) Highly significant, $P < 0.01$.
- ($^{+3}$) Very highly significant, $P < 0.001$.

Table (2): Effect of separate doses of $HgCl_2$ and C-PC, as well as a combination of both on Hb, oxygen, R.B.Cs following 15 and 30 days post injection:

Treatment	Hb	O_2	R.B.Cs	Hb	O_2	R.B.Cs
	15 days			30 days		
Control	15.00 ± 0.252	20.39 ± 0.34	5.00 ± 0.08	15.00 ± 0.252	20.37 ± 0.32	5.00 ± 0.085
C-PC alone	15.00^{NS} ± 0.681	20.40^{NS} ± 0.92	5.00^{NS} ± 0.265	15.00^{NS} ± 0.404	20.40^{NS} ± 0.55	5.00^{NS} ± 0.137
$HgCl_2$ alone	10.50^{+3} ± 0.378	14.23^{+3} ± 0.49	3.49^{+3} ± 0.128	9.60^{+3} ± 0.265	13.00^{+3} ± 0.38	3.17^{+3} ± 0.088
C-PC Then $HgCl_2$	12.30^{+3} ± 0.115	16.72^{+3} ± 0.16	4.12^{+3} ± 0.061	14.50^{+1} ± 0.288	19.72^{NS} ± 0.39	4.81^{NS} ± 0.116
$HgCl_2$ Then C-PC	11.20^{+3} ± 0.17	15.50^{+3} ± 0.23	3.7^{+3} ± 0.06	14.00^{+3} ± 0.051	19.04^{+3} ± 0.26	4.66^{+2} ± 0.08

- (NS) Not significant, $P > 0.05$.
- ($^{+1}$) Significant, $P < 0.05$.
- ($^{+2}$) Highly significant, $P < 0.01$.
- ($^{+3}$) Very highly significant, $P < 0.001$.

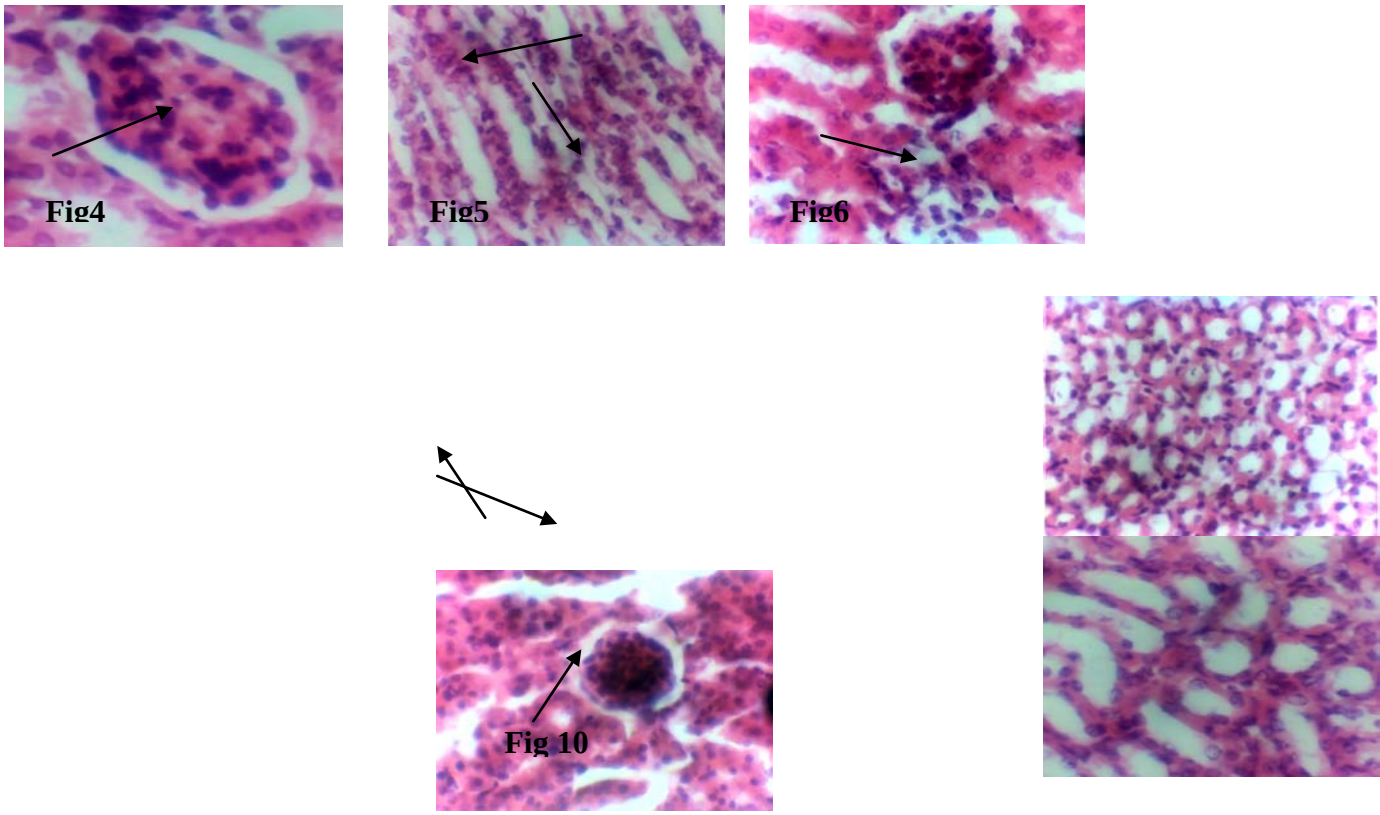

Fig.1. Histopathological appearance of kidney sections stained with H&E **(1)** Well organized medullary tissue of mouse kidney exhibiting normal collecting tubules with a good viewed nuclei, normal blood capillaries between the tubules after 15 days of intraperitoneal injection with saline buffer (pH 7.2), (X300). **(2)** Revealing normal renal corpuscles, normal tubules and good nuclei in cortical tissue of mouse kidney after 30 days of intraperitoneal injection with saline buffer (pH 7.2), (X300). **(3)** Showing a good epithelium of tubules and marked increase in the number of nuclei of medullary tissue of mouse kidney appeared after 15 days of injected with C-PC, (X600). **(4)** The same as control with a good picture of renal corpuscle and increased a space of renal corpuscle after 30 days of C-PC injection in mouse kidney, (X600). **(5)** The a ascending and descending tubules of mouse kidney showing degeneration in the epithelium of tubules and swollen nuclei after 15 days of $HgCl_2$ injection appeared in the medullary tissue (X300). **(6)** Showing hydropic degeneration and sloughing of some epithelial cells of tubular epithelium, irregular contours of Bowman's capsule as well as detached of some cells of glomeruli with destructed renal corpuscle below the photomicrography (arrow). In the cortical tissue of mouse kidney after 30 days of $HgCl_2$ injection, (X300). **(7)** Showing regenerated epithelium of tubules, no swollen nuclei and no hemorrhage between the tubules inside medullary tissue of mouse kidney due to application of injected with C-PC then $HgCl_2$ after 15 days. (X300). **(8)** Showing well organized renal corpuscle, with absence of all abnormalities shown fig **(6)** in the cortical tissue of mouse kidney after injected with C-PC then $HgCl_2$ after 30 days. (x600). **(9)** Showing recovery of epithelium of tubules with few swollen nuclei, without hemorrhage in the interstitial tissue of mouse kidney after injected with $HgCl_2$ then C-PC after 15 days. (X300). **(10)** Showing a good picture with no signs of hemorrhage and with few degenerated tubular epithelium in cortical tissue of mouse kidney due to application of injected with $HgCl_2$ then C-PC after 30 days, (X300).

DISCUSSION

The present work revealed that mercuric intoxication causes a signification increase in urea and creatinine levels moreover, an obvious significant decrease in hemoglobin (Hb) content, Total $_{O2}$ volume and R.B.Cs count (tab). Results are in parallel with **Yanagisawa *et al*. (2002)** who found that an increase in serum creatinine and blood urea nitrogen (BUN) in rats preinjected with mercuric chloride. Moreover **Fowler and Wood (1977)** and **Woods (1989**) have been demonstrated that mercuric chloride is a well-know human and animal nephrotoxicity that induces extensive kidney damage. **Ahn, *et al* (2002**).reported that Mercuric chloride injection in rabbits was accompanied with an obvious increase in serum creatinine level after 24h post administration. Moreover, **Lund *et al* (1991)** demonstrated that mercuric chloride induces lipid peroxidation which accompanied with oxidative stress that plays a critical role in mercuric chloride induced renal failure. In a similar way **Lund *et al* (1993)** explained the toxicity of mercuric ion on the basis that mercuric induced cell injury as well as depletion of mitochondrial reduced glutathione content and increased the formation of $H_2O_2$by mitochondrial electron transport chain in vivo. The accumulation of H_2O_2 leads to nephrotoxicity and increase renal failure risk (**Ohsugi *et al*., 1999**). The significant reduction in red blood corpuscles (R.B.Cs) count of mice treated with mercuric ion related to the retardation of Erythropoiesisis process which controlled by kidney producing hormone erythropoietin under severe renal damage (**Sharma, 2002)**.In **a** parallel process the formation of red blood corpuscles (RBC) is disturbed as a result to the sacristy of iron absorption (**Sharma, 2005).** Results in table **(1)** indicated that mice treated with a combination between C-PC and mercuric chloride are able to decrease blood urea nitrogen (BUN) and serum creatinine levels than the corresponding values in mice treated with mercuric chloride alone where as ,hemoglobin (Hb) content , total $\mathbf{o_2}$volume and RBC s were significantly increase and return to their normal levels with respect to their corresponding control Tab **(2)** .The previous results lead to emphasis that C-PC counteracts the renal toxicity induced by mercuric chlorides (**Yamane, 1988 and Fukino, 1990)** where C-PC provide the mice a sort of protection against antioxidant injuries **Farooq *et al.* (2004**). In the same line (**Lissi *et al*., 2000**) indicate that C-PC is a potent free radicals inhibiting microsomal lipid peroxidation and other effects scavenger in renal cells. **Farooq *et al.* (2004**) evaluates that C-PC has nephroprotective efficiencies through its ability to combat reactive oxygen species (ROS). Moreover **Pinero *et al*. (2000)** proved that C-PC has the ability to scavenger the free hydroxyl; alkoxyl and peroxyl radicals. Regarding to hemoglobin content it could be indicated that the significant increases in this parameter in presence of C-PC may be related to that C-PC enhances erythropoietin activity and bone marrow hematopoietic progenitor cell in normal (**Sharma, 2005**). The mercury chloride treatment significantly decreases the red blood corpuscles (R.B.Cs) count (**Patni *et al*., 2001**). Erythropoiesisis controlled by kidney producing hormone erythropoietin and Mercury causes severe renal damage (**Sharma, 2002).** So due to renal damage, Erythropoiesisis is cannot be synthesized and process of red blood corpuscles (R.B.Cs) formation is disturbed. So, decrease in (RBC) count may be correlated with the decrease in serum iron level (**Sharma, 2005**). The previous studies investigated that Histopathological analysis in addition to necrosis of proximal tubule epithelial cells, necrosis of macula densa cells and swelling of glomerular epithelial cells were observed in the renal cortex of rats with $HgCl_2$-induced ARF (**Yanagisawa *et al*., 2002**). The inorganic mercury taken up may be accumulated into proximal tubules epithelial cells and may finally causes acute tubular necrosis (**Zalubs, 2000)**. Results in figures indicated that mice treated with a combination between C-PC then $HgCl_2$ or $HgCl_2$ then C-PC are able to regenerate epithelium of tubules, no hemorrhage in the interstitial tissue and well organized renal corpuscle, with absence of all abnormalities inside tissue of mouse kidney. The previous studies demonstrated

that the antioxidant and **a**ntiurolithic potential of phycocyanin thereby projection it as a promising it as a promising therapeutic agent against renal cell injury associated kidney stone and renal failure **(Farooq *et al*.,2004 and Sharma *et* al., 2005).** Hence it could be concluded that C-PC suppresses the lipidperoxidation and antagonized $HgCl_2$ intoxification nephrites (nephroprotective agents).

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تأثير صبغة فيكوسيانين على الفشل الكلوي الحادّ المستحث بالكلوريد الزئبقي.

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****قسم علم الأنسجة- كليّة الطبّ البيطري- جامعة الزقازيق.**

صبغة فيكوسيانين موجوده في الطحالب الخضراء الزرقاء. له قيمة طبية وغذايه عاليه بالاضافه الى انه عامل مضاد للفشل الكلوى الحاد. ولتقييم كفاءته في معالجة الفشل الكلوى الحاد ضدّ الكلوريد الزئبقي قد قمنا باجراء تجارب تضمنت خمسة مجموعات من الفئران (10 فئران لكلّ مجموعه) وحقنت الفئران (داخل البريتون) وهى كالتّالي:

(1) فئران طبيعيه غير معالجه حقنت (1% محلول فوسفات الملحى, قلويه7.2%).
(2) فئران حقنت بصبغة (فيكوسيانين) ثلاث مرات في كل جرعة (4 ملجم / كيلوجرام) لكلّ جرعه.
(3) فئران حقنت بالكلوريد الزئبقى مره واحده (5 ملجم/كيلوجرام).
(4) فئران حقنت بصبغة (فيكوسيانين) ثلاث مرات في كل جرعة (4 ملجم / كيلوجرام) ثم اتبعت بالحقن بالكلوريد الزئبقى مره واحده (5 ملجم/كيلوجرام).
(5) فئران حقنت أولا بالكلوريد الزئبقى مره واحده (5 ملجم/كيلوجرام) ثم اتبعت بالحقن بصبغة (فيكوسيانين) ثلاث مرات في كل جرعة (4 ملجم / كيلوجرام) لكلّ جرعه.

ثم قمنا بذبح الفئران يومى 15و 30 من بداية التجربه. وقد أشارت نتائج التحاليل **البيوكيميائيه** انه عند حقن الفئران بالكلوريد الزئبقى يؤدى الى ارتفاع نسبة اليوريا والكرياتينين مسحوبا بالنقصان الحاد في نسبة الهيموجلوبين , الأوكسجين و كرات الدم الحمراء فى الدم. اما الفحص **الهيستوباثولوجيكال** يشير الى زيادة حجم الانويه داخل الخلايا ونزع بعض الخلايا الطلائية للانابيب الكلويه وتشوه كبسولة بومان وذلك بألتصاق بعض خلايا الكبيبات بها مع الدمار الواضح في كرية الدم الكلوية في كلية الفأر. اما عند حقن الفئران بصبغة الفيكوسيانين ثم الكلوريد الزئبقى والعكس نلاحظ انخفاضا ملحوظا فى نسبة اليوريا والكرياتينين مسحوبا بالزياده في نسبة الهيموجلوبين , الأوكسجين و كرات الدم الحمراء فى الدم. اما الفحص **الهيستوباثولوجيكال** يشير الى تحسن الأنابيب الكلويه مع غياب كلّ حالات الشذوذ فى كرية الدم الكلوية وعدم وجود نزيف في النسيج الفراغي لكلية الفأرالتى حقنت بالكلوريد الزئبقى.

ونستنتج من هذه التجارب السابقه ان صبغة الفيكوسيانين عامل مؤكسد قوى ضد اكسدة الليبيدات داخل النسيج الكلوى **وعامل وقائى و مضاد للفشل الكلوى الحاد المستحث بالكلوريد الزئبقى.**

PROCEEDING OF THE SECOND SCIENTFIC ENVIRONMENTAL CONFFER, 2007, ZAGAZIG UNI., 97-113

CHITOSANASE PRODUCTION USING SOME FUNGI OPTIMIZATION OF FERMENTATION CONDITIONS OF CHITOSANASE PRODUCED BY *ASPERGILLUS ORNATUS*

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ABSTRACT

The potentiality of 26 fungal species belonging to 10 genera and unidentified two species isolated and identified from Egyptian soils to produce chitosanase in their culture filtrates under submerged culture conditions using colloidal chitosan as the sole carbon source were tested. Aspergilli, particularly *Aspergillus ornatus* were distinguished by its capacity to release exo-chitosanase when grown on a selected medium. Best results were achieved after 7 days of incubation at 30°C and pH 5.0. The impact of aeration, mechanical agitation as well as the volume and age of inoculum upon chitosanase and biomass production were also discussed briefly.

The present paper gives an account of observations made on the production of chitosanase and biomass in relation to the chemical constituents of fermentation medium. 1% colloidal chitosan followed by crystal chitosan were more initiative for chitosanase production than any other carbon compounds. Peptone (0.45%) followed by yeast extract were the best nitrogen source for both biomass and chitosanase production. The optimum chitosanase and biomass production were achieved on medium containing 0.1% KH_2PO_4, 0.5% KCl and 0.5% $MgSO_4.7H_2O$. The impacts of the levels of glucose and NaCl on both experimental parameters were also examined. In addition, the supplementation of various B-group vitamin and some trace elements individually to the bioprocess caused no significant effects on chitosanase production. However slight inhibition was obtained with a mixture of tested metal ions. The productivity of chitosanase supported by addition of tween 80 as surfactant agent and enhanced on sand and soil extracts than other natural sources investigated.

INTRODUCTION

Chitosan, a linear copolymer composed of β 1, 4-linked glucosamine (GlcN) residues with various degrees of *N*-acetylated residues, is a deacetylated derivatives of chitin, an insoluble linear β 1, 4 linked polymer of *N*-acetylglucosamine (Glc.NAc) and is the most abundant polymer, next to cellulose in nature **(Zhu *et al,* 2003)**. Chitosan is present in the mycelial and sporangiophore walls of many fungi and the exoskeletons of insects and crustacean **(Kim *et al,* 2004)**. Actually, chitosan is applied widely to health food, such as for the treatment of hyperuricemia and as an antimicrobial agent, preservative agent and edible film **(Chen *et al,* 2005)**. Recently, much attention has been paid to converting chitosan to save and functional chitooligosaccharides. These chitooligosaccharides produced by hydrolyzing chitosan with chitosanase and have various physiological activities such as antitumer and antimicrobial activities as well as immuno-enhancing effects **(Qin *et al,* 2004 and Chen *et al,* 2005).** The productions of microbial chitosanases have received attention as a step in a proposed

bioconversion process to produce low molecular weight chitosans and chitooligomers **(Liu and Xia, 2006)**.

Chitosanases (EC.3-2.1.132) are glycosyl hydrolases that catalyses the hydrolysis of β 1, 4 glycosidic bonds of chitosan and have been found in a variety of microorganisms, including bacteria and fungi **(Somashekar and Joseph, 1996, Yun *et al*, 2005 and Chen *et al*, 2006)**. Fungal chitosanolytic enzymes have been produced and characterized from different species of the following genera; *Aspergillus* **(Kim *et al*, 1998, Cheng and Li, 2000, Zhang *et al*, 2000, Eom and Lee, 2003 and Chen *et al*, 2005)**, *Fusarium* **(Shimosaka *et al*, 1993)**, *Mucor* **(Alfonso *et al*, 1992)**, *Paecilomyces* **(Chen *et al*, 2005)**, *Penicillium* **(Fenton and Eveleigh, 1981)** and *Trichoderma* **(Nogawa *et al*, 1998)**. Also fermentation conditions of microbial chitosanase production have been reported **(Somashekar and Joseph, 1996, Zhang *et al*, 2000 and Zhu *et al*, 2003)**. It appears from the literature that chitosanase production by moulds received less attention than bacteria. Thus, the present study aimed to investigate the chitosanolytic activity of some Egyptian soil fungi as well as the optimal fermentation conditions leading to maximum yield of exo-chitosanase by the most active fungal isolate.

MATERIALS AND METHODS

Materials: Chitosan and glucosamine were purchased from Sigma (St-Louis, MO, USA). Commercial chitosan was prepared from shrimp chitin in our laboratory as described by **Hejazi and Amiji (2003).** Colloidal chitosan was prepared by the method of **Fen *et al* (2006).** Folin reagent was purchased from LOBA chemie (Mumbai, India). All other chemicals used were of analytical grade.

Isolation and identification of chitosan degrading fungi: Different fungal isolates were isolated from soil as well as agriculture waste samples collected from Sharkia Governorate, Egypt. The dilution plate method essentially as described by **Johnson *et al*, (1959)** with some modification was employed. Chitosan (1%) Czapek's Dox agar medium was used for chitosanase producing fungi **(Zhang *et al* ,2000)** to which were added both rose bengal (65 ppm) and dihydrostreptomycin (20 ug/ml) as bacteriostatic agents, **(Smith and Dawson, 1944)**, was used as an isolation medium . The plates were incubated at 30ºC for up to 10 days, during which the developing fungi were isolated. The pure fungal isolate were identified by consulting, **Barron (1968), Ellis (1971), Raper and Fennell (1977), Pitt (1979), Carmichael *et al* (1980), Domsch *et al* (1980), Nelson *et al* (1983)** and other. The fungal cultures were maintained on potato dextrose (Difco) agar plates and incubated at 30ºC for 7 days **(Zhang *et al*, 2000).** The conidial suspension was prepared by suspending the conidia from the slants in 10 ml of sterilized distilled water.

Fermentation medium and culture conditions : The fermentation medium used for chitosanase production was modified Czapek's - Dox medium containing chitosan as carbon source **(Zhang *et al*, 2000)** ,with some modifications , composed of (g/L): 10.0 colloidal chitosan , 2.0 $NaNO_3$, 5.0 yeast extract, 1.0 KH_2PO_4 , 0.5 $MgSO_4.7\ H_2O$, 0.5 KCl, 0.01 $FeSO_4.7H_2O$ and 1000 ml distilled water . The pH adjusted to 5.0. The fermentation medium was dispensed in 250 ml conical flasks, each containing 50 ml. The flasks were inoculated

with 1ml spore suspension of each tested fungal species. The submerged flasks were shaken at 3.7 H_Z and 30ºC for 7 days (using shaker incubator, ***New Brunswick Scientific, Edison N.J. USA***).

Estimation of biomass production : Each fungal culture was filtered on Whatman No 1 filter paper and the pellets were washed several times , then dried at 90ºC till constant weight and the dry biomass was estimated in g/L of productive medium **(Shindia,1997)**.

Chitosanase assay: Assay of chitosanase activity was carried out as described by **Fenton and Elveleigh (1981)** as well as **Uchida and Ohtakara (1988)** with some modification. The culture filtrate was centrifuged at 5000 r.p.m for 10 min at 40ºC and the supernatants were used as crude enzyme. The standard assay mixture containing 1 ml of crude enzyme preparation and 1ml of 1% colloidal chitosan in citrate phosphate buffer (pH 5.0) was incubated at 40 ºC for 1h. The amount of glucosamine of the enzymatic reaction mixture was then determined using the Nelson-Somogi method **(Nelson, 1944)**. The intensity of the colored solution was quantified in spectrophotometric (Spekol- spectrocolorimeter) at 700 nm. The reducing sugar concentration produced in the reaction mixture was measured based on standard curve obtained with glucosamine as standard. One chitosanase unit (U) is defined as the amount of enzyme that liberates 1µ mol of reducing sugar per minute at 40ºC **(Zhang *et al*, 2000).**

Determination of protein: The protein content of the crude enzyme preparation was estimated colorimetrically according to the method adapted by **Lowery *et al* (1951)**.

RESULTS AND DISCUSSION

Potentiality of production of chitosanase among tested fungi: Twenty six fungal species belonging to 10 genera in addition to two unidentified species were examined to investigate their capacity to produce chitosanase in their culture filtrates (Table, 1). The results show that the chitosanolytic activity among the fungal species of the same genus is quite different. Moreover, not all the fungal species possess the same chitosanolytic activity. *A. ornatus* was obviously the best producer of highest chitosanase activity followed by *A. ochraceous, P. citrinum, T. viride, A. fumigatus, P. chrysogenum, Paecilomyces varioti* and *T.* sp. The fungal isolates with chitosanase activity below 25% of that *A. ornatus* were considered low producers of enzyme and will not be considered for further discussion. Four fungal isolates showed no chitosanase activity (Table, 1). In connection with our screening study, several species particularly Aspergilli were listed by other investigators as chitosanase producers during their course of screening of fungal strain with chitosanase activity such as *A. oryzae, A.* sp J22-326, *A. fumigatus, A.* sp Y2K and *A. flavus* and others (**Zhang *et al*, 2000 and 2001, Cheng and Li, 2000, Eom *et al*, 2003, Liang *et al*, 2005, Chen *et al*, 2005).** The studies of other investigators **(Fenton and Eveleigh, 1981, Alfonso *et al*, 1992, Shimosaka *et al*, 1993, Nogawa *et al*, 1998 and Chen *et al*, 2005)** support our conclusions about chitosanolytic activities of the reported fungal strains. These activities that showed a wide range of variation and these natural differences proved the complementary action of these organisms towards each other in their living ecosystem. This may be attributed to the inherited differences in the biological activities of different fungal strains surviving single environmental niche. On the other hand, it is revealed that the most isolated fungal species in

our screening have been recorded as chitosanolytic fungi by the aid of compendium of soil fungi **(Domsch *et al*, 1980).**

Aspergillus ornatus gave the highest chitosanase activity under bioprocess conditions. These findings justified the selection of *A. ornatus* for further experimentation in order to enhance its productive capability.

Time course of growth and chitosanase productivity of *Aspergillus ornatus*: The growth of *Aspergillus ornatus* and its production of chitosanases in culture filtrates were determined during the incubation period which prolonged for 14 days. The result in Fig (1) reveals a correlation between level of chitosanases production and extent of fermentation period. Negligible production of chitosanases was observed in the initial 48 h of fermentation process, though adequate growth was maintained under these conditions. Optimum chitosanase production was achieved after 7 days of fermentation. Beyond this period the chitosanase was found to drop gradually with further extension of the fermentation period to 14 days. The data also appeared that, no correlation was observed between the released chitosanase in culture filtrates and the biomass of *Aspergillus ornatus*. Optimum biomass was obtained after 8 days of fermentation periods, above which the fungal biomass production dropped. The late appearance of chitosanases could be the result of induction as colloidal chitosan eventually becomes available after consumption of proteins. Previous investigators found that an incubation period from 4 to 5 days was optimal for chitosanase production by some *Aspergillus* sp (**Cheng and Li, 2000 and Chen *et al* 2005**) and *Bacillus* sp **(Choi *et al*, 2004)**. On the other hand, cultures of *Aspergillus oryzae* showed the highest chitosanase and growth rate after 60 hours of incubation **(Zhang *et al*, 2000)**.

These differences in optimum production of chitosanases by different fungal and bacterial species may ascribe to either the condition of cultivation or special differences.

Effect of initial pH-value: Chitosanase and dry biomass profile with respect of initial pH of the fermentation medium is shown in Fig (2). It is evident that, the growth of *Aspergillus ornatus* and its ability to produce chitosanase respond differently to the reaction of the basal medium. The results revealed that an initial pH of 5.0 was found to be optimal for both growth of *Aspergillus ornatus* and chitosanase production. Above and below this pH value the yield of biomass and chitosanase was substantially lower. It is also clear from the data that, the experimental fungus has the ability to survive and release extracellular chitosanase in its culture filtrate at wide range of pH's value. These observations agree with the findings of **Alfonso *et al* (1992)** who found that the pH 5.0 was the best for growth and chitosanases production by *Mucor rouxii*. The most of fungal chitosanases showed optimum productivity close to 5.0 **(Zhang *et al*, 2000 and Chen *et al*, 2005). Effect of incubation temperature:** The incubation temperature has a significant influence on both chitosanase production and growth of *A. ornatus* (Fig, 3). The optimum temperature for efficient fermentation was found to be 30º C. Above and below this temperature the biomass and chitosanase production dropped. It is also found that the tested organism failed completely to develop mycelia and hence no chitosanase activity at 50ºC. Therefore, all further optimizing efforts using *A. ornatus* were tried at 30ºC which seems to be the optimum for chitosanase production and dry biomass of tested fungus. In accordance with these findings are those obtained by **Somashekar and Joseph (1992), Tanabe *et al* (2003), Choi *et al* (2004) and Chen *et al* (2005)** who reported that 30ºC was optimal for the growth and chitosanase production by different microorganisms.

Table (1): Chitosanolytic activity and protein production of different fungal isolates grown on chitosan as carbon source at 30°C.

	Fungal isolate	Chitosanase activity (U/ml)	Protein (mg/ml)
1	*Aspergillus awamori*	1.11	0.145
2	*A. carbonarius*	1.28	0.170
3	*A. carneus*	1.95	0.203
4	*A. flavus*	3.21	0.311
5	*A. fumigatus*	2.36	0.255
6	*A. niger*	1.17	0.150
7	*A. ochraceous*	2.93	0.218
8	*A. ornatus*	4.46	0.401
9	*A. restrictus*	1.26	0.165
10	*A. tamari*	3.11	0.305
11	*A. terreus*	2.06	0.211
12	*Chaetomium globosum*	ND	0.05
13	*Chaetomium sp*	ND	0.10
14	*Penicillium chrusogenum*	2.34	0.228
15	*P. citrinum*	2.68	0.256
16	*P. elegans*	1.70	0.188
17	*P. funiculosum*	2.0	0.205
18	*Cladosporium cladosporioids*	ND	0.08
19	*Fusarium moniliforme*	1.66	0.177
20	*F. oxysporum*	1.95	0.201
21	*Paecilomyces varioti*	2.31	0.223
22	*Trichoderma konigii*	2.10	0.210
23	*T. viridie*	2.50	0.241
24	*T.* sp	2.23	0.215
25	*Cunninghamella echinulata*	ND	0.06
26	*Mucor circinoloids*	0.5	0.101
27	*M. racemosus*	0.7	0.110
28	*Rhizopus oryzae*	0.1	0.09

Effect of aeration rate: Data showing the influence of aeration rate (volume of fermentation medium/volume of fermentation flask) on biomass yield and chitosanase production by *A. ornatus* are presented in Fig (4). Under our experimental conditions, 50 ml of fermentation medium in 250 ml flask (1/5 v/v); allow optimal dry biomass output and extracellular chitosanase biosynthesis. This may be due the compromises between mass transfer and shearing stress at this volume. Further increase in the volume of fermentation medium (decreased O_2 levels) causes gradual decrease in biomass production as well as extracellular productivity. As the volume of fermentation medium increased, the shearing stress may be increased and causal hazardous effect on fungal pellets **(Liu *et al*, 2003).** These observations are in line with that previously reported **(Tanabe *et al*, 2003 and Kim *et al*, 2004).**

Effect of agitation rate: From the result in the Fig (5), it can be noticed that the growth of *A. ornatus* was induced with agitation speed compared to static culture and gave the maximum biomass at 150 r.p.m (5.0 g/L) after which gradually decreased were obtained up to 300 r.p.m . At the same agitation rate (150 r.p.m) the optimum chitosanase activity was also recorded by tested organism and dropped thereafter. Generally, the major roles of providing agitation rate were in improving the mixing, mass and heat transfer in submerged bioprocess. This is compatible with the findings previously concluded by **Yoon *et al*, (2001)** and **Chen *et al* (2005)** for optimal chitosanases production by *B.* sp CK4 and *A.* sp CJ22-326 respectively. However, at higher agitation rates the biomass and chitosanase production by tested fungus decreased. This may be due to over increasing in mass and heat transfer or/and may have negative effects on morphological states such as rupture cells, vaculation and outlysis as well as changes in fungal pellets **(Cui *et al* , 1997) .**

Effect of the amount and the age of the inoculum: The strength of the inoculum and age distribution of mycelium is known to impact the growth rate as well as the enzymatic activities of fungi grown under special conditions.

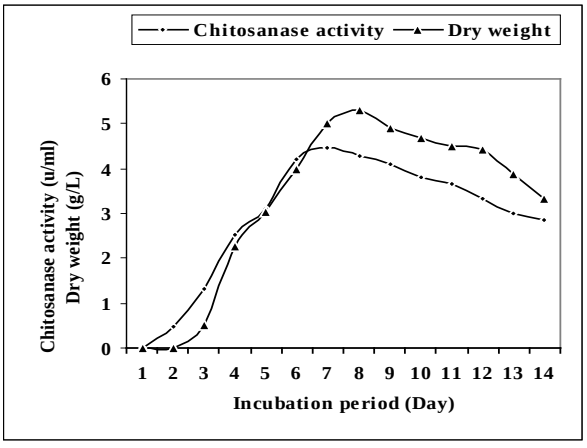

Fig.1: Time course of growth and chitosanase productivity of *A. ornatus*:

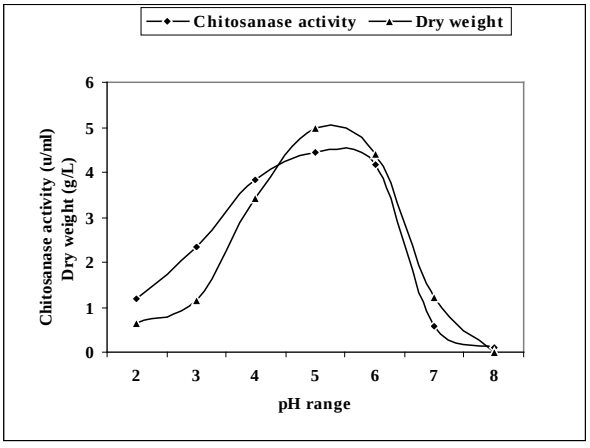

Fig.2: Effect of initial pH-value on growth and chitosanase production by *A. ornatus.*

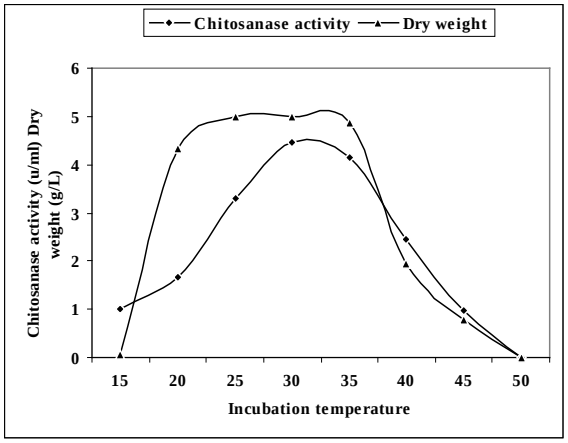

Fig.3: Effect of incubation temperature on growth and chitosanase production by *A. ornatus*.

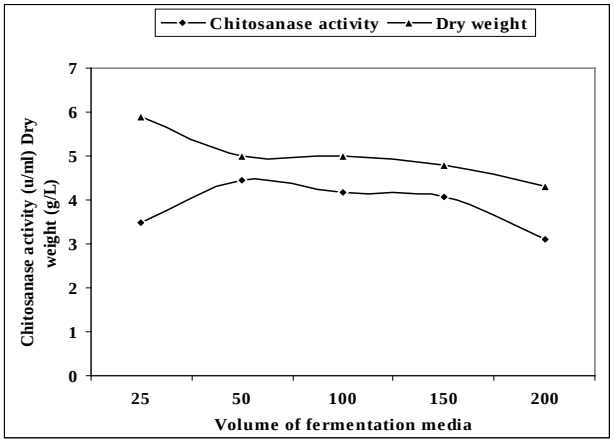

Fig.4: Effect of aeration rate on growth and chitosanase production by *A. ornatus*

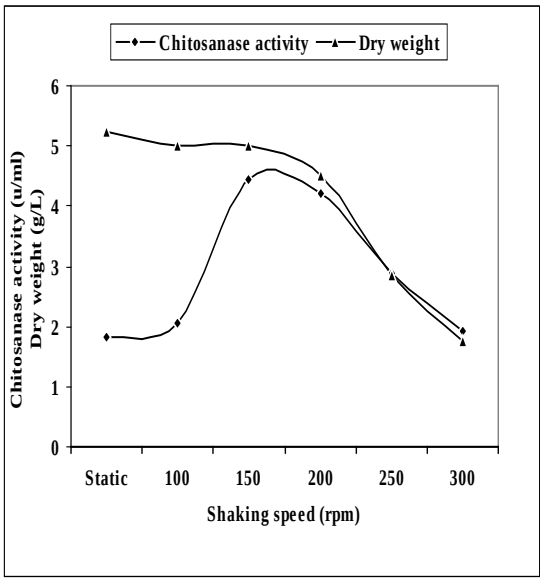

Fig.5: Effect of agitation rate on growth and chitosanase production by *A. ornatus.*

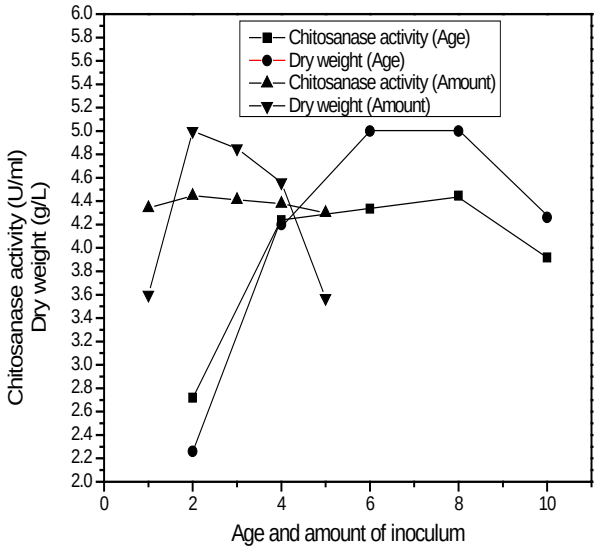

Fig.6: Effect of the age and amount of inoculums on growth and chitosanase production by *A. ornatus*

(**Gottleib and van Etten, 1965)**. The aging of inoculum used to inoculate fermentation medium, (Fig, 6) exerted changes in biomass and chitosanase production. The optimum yields of both parameters were obtained by using 8 day-old inoculum of *A. ornatus*. On the other hand, chitosanase activity of this fungus was not influenced greatly by the size of inoculum in the range from 2 to 10% (Fig, 6). Also, the yield of biomass was not influenced appreciably within the range of 4 to 8% of inoculum but above and below this range a marked decline in production of biomass occurred. It seemed probable that certain substances present in large amounts of inocula may tend to inhibit the growth of microorganism. These

result coincide with these previously reported for different fungal species and their chitosanolytic activity **(Zhang *et al*, 2000 and 2001, Zheng and Xiao, 2004)**.

Effect of different carbon sources: As shown in Table (2), the relation between the carbon substrates and extracellular chitosanase production as well as growth rate of the experimental organism were investigated. The data indicated that the optimum exochitosanase levels were detected as *A. ornatus* was grown on media containing chitosan, but not with any other tested carbon compounds. Colloidal chitosan induced the best chitosanase productivity of tested organism followed by crystalline chitosan and fungal chitosan. While, cell free filtrate of culture growth with non chitosan substrates were devoid of this activity. On the other hand, the monomer glucose and glucoseamine supported fungal growth, but chitosanase production was not detectable in culture filtrates of tested fungus.

Table (2): Effect of different carbon sources on growth and chitosanase production by *Aspergillus ornatus*:

Carbon source	Chitosanase activity (U/ml)	Dry wt. (g/L)
Glucose	-	12.4±0.0336
Fructose	-	7.8±1.025
Mannose	-	7.62±1.094
Xylose	-	8.64±0.684
Glucoseamine	-	10.96±1.087
Sucrose	-	8.52±2.002
Lactose	-	6±1.064
Maltose	-	9.8±1.035
CMC	-	2.05±1.023
Cellulose	-	0.94±0.756
Pectin	-	6.14±0.054
Starch	-	6.93±0.08
Crystalline chitin	-	1.26±0.006
Colloidal chitin	-	3.92±0.21
Crystalline chitosan	2.2±0.0963	2.19±0.074
Colloidal chitosan	4.46±1.82968	5.0± 0.13
Fungal chitosan	1.6±0.9824	3.23±0.495

The selectivity in action of carbon sources led us and others **(Kim *et al*, 1998 and Chen *et al*, 2005)** to conclude that the apparent increased represence of the enzymes to the carbon sources was an inducible one. The preference in the usage of one carbon source by different chitosanolytic microorganisms was reported by several investigators **(Mitsutomi *et al*, 1998,**

Zhu *et al*, 2003, Kim *et al*, 2004 and Chen *et al*, 2006). For example, **Cheng and Li (2000)** indicated that high chitosanase production was found only in culture of *A.* sp Y2K supplied with soluble chitosan. The highest production of enzyme with colloidal chitosan may be related to greater accessibility to enzymatic attack probably resulted from the largest surface area of solubilized chitosan. Like other inducible enzyme systems chitosanase formation can be repressed by excess soluble metabolites in the presence of inducer **(Davis and Eveleigh, 1984 and Tanabe *et al*, 2003)**. The induction of *A. ornatus* chitosanase, in the present study is repressed by addition of different concentrations of glucose (at zero time) to the chitosan basal medium as shown in Fig (7). These results suggest that *A. ornatus* chitosanase is controlled by an inducer repressor system. These finding were correlate well with the previous observations of **Kim *et al*, (1998) and Chen *et al*, (2005)**. In the presence of adequate concentration of easily metabolites monosaccharides, a number of catabolite pathways involved in hydrolysis of polysaccharides are repressed **(Atlas, 1984 and Angell *et al*, 1992)**. In contrast to these inducible enzymes, chitosanase which are produced constitutively are also reported for different microorganism **(Alfonso *et al*, 1992 and Somashekar and Joseph, 1992)**.

The results in Fig (8) show that the excretion of chitosanase and growth of *A. ornatus* were not only affected by the kind of carbon source supplied but also were sensitive to the concentration of the specific carbon used (colloidal chitosan). It was found that 1.0% of colloidal chitosan gave the maximum production of enzyme while the lowest productivity was recorded at 3.0% chitosan. It was reported that the yield of chitosanase depend on microorganism as well as nature of chitosan and its levels **(Somashekar and Joseph, 1996)**. For maximum chitosanase productivity, the optimal concentration of colloidal chitosan was 1.0% for different microorganisms **(Zhu *et al*, 2003 and Chen *et al*, 2005)**.

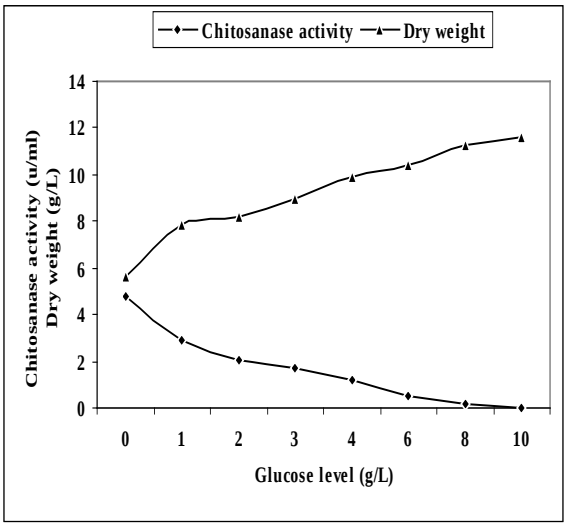

Fig.7: Effect of glucose levels on growth and chitosanase production. by *A. ornatus.*

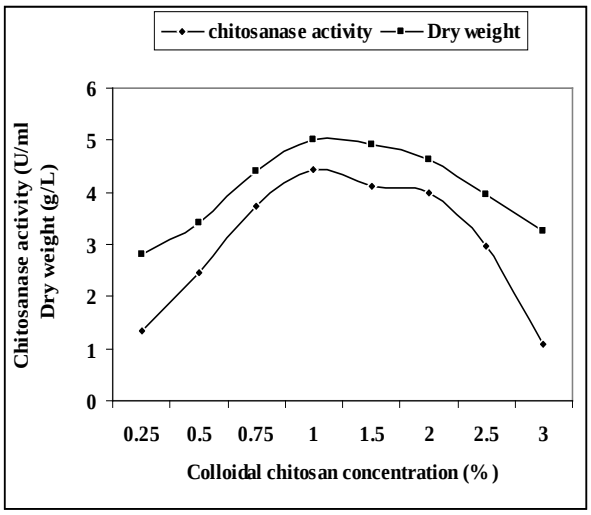

Fig.8: effect of colloidal chitosan concentration on growth and enzyme production by *A. ornatus*

Effect of different nitrogen sources:

The impact of a range of nitrogen sources on chitosanase productivity and growth rate of *A. ornatus* was shown in Table (3). All 8 nitrogenous tested compounds were able to support growth; the range in variation in final biomass yield was only 3.63 g/L except for urea nitrogen containing media, in which it is reduced about 7 fold from 5.8 to 0.78 g/L of biomass. This may be due to its toxic effect in high doses used or/and the pH variations occurring after the addition of it **(Reid, 1983)**. The final extracellular chitosanase production varied considerably; however, maximum production of enzyme was evident in presence of peptone followed by yeast extract.

Table (3): Effect of different nitrogen sources on growth and chitosanase production by *Aspergillus ornatus:*

Nitrogen source	Chitosanase activity (U/ml)	Dry wt. (g/L)
Without	1.854± 3.3867	0.75± 0.0586
Control*	4.46±1.82968	5.0± 0.13
$NaNO_3$	2.066± 1.54	2.17± 0.089
NH_4Cl	2.324± 0.20785	2.3± 0.0058
KNO_3	2.756± 2.81233	2.96± 0.0186
$(NH_4)NO_3$	3.173± 2.09249	4.4± 0.123
$(NH_4)_2SO_4$	2.18 ±0.0	2.26± 0.141
Urea	1.86 ± 0.0	0.78±0.080
Peptone	4.474± 1.97099	5.8± 0.235
Yeast extract	4.13± 0.43466	5.65± 0.1389

*Mixture of yeast extract (0.5%) and $NaNO_3$ (0.3%).

Minimum chitosanase production was obtained only in the presence of urea. Other tested inorganic and organic nitrogen compounds were favorable for chitosanase production by tested organism but, comparatively less inducible than control. It is interest to note that, in absence of exogenous supply of nitrogen compound, the biomass and chitosanase productivity of *A. ornatus* posed low yield, indicating that, it is able to utilize the colloidal chitosan as carbon and nitrogen sources. These observations confirmed the early reports that certain microorganisms produced large amounts of extracellular chitosanases in the presence of complex organic nitrogen such as peptone and yeast extract **(Mitsutomi *et al*, 1998 and Yoon *et al*, 2001)**. The prominent effect of peptone may be attributed to the fact that such complex organic nitrogen gives on hydrolysis a number of some intermediate compounds structurally available as precursors of enzymes biosynthesis as well as major, minor element, and growth factors that may be used for inducing growth and biosynthesis of enzymes **(Anonymous, 1958)**. Also, the preferability of one nitrogen source by chitosan degrading microorganisms has been reported **(Fenton and Eveleigh, 1981. Cheng and Li, 2000, Zhang *et al*, 2000 and Kim *et al*, 2004)** and their findings were in connection with our results.

Different levels of peptone (0.25-0.5%) as the best nitrogen source were tested (Fig, 9). The best levels of peptone for maximum extracellular chitosanase release and biomass production by tested organism were found to be 0.45%. Above and below this optimal

concentration of nitrogen source, the biomass and chitosanase productivity almost reduced. Others indicated that both the nature of nitrogen source and its level in bioprocess are important in controlling fungal development and biosynthesis of enzymes **(Shindia *et al*, 2001)**. Also, similar results have been reported by several workers connecting the nitrogen concentration dependence of both chitosanase and biomass of microorganisms **(Zhang *et al*, 2000 and Chen *et al*, 2005).**

The effects of different levels of essential salts in culture medium (MgSO4.7H2O, KH2PO4 and KCl), one at a time, were also investigated.

It well known that phosphorous especially KH_2PO_4 plays an important roles in fungal cell metabolism particularly utilization and metabolism of carbohydrates **(Jennings, 1995)**. The highest chitosanase activity (4.74 u/ml) was obtained with 0.1 g/L of KH_2PO_4 (Fig, 10) as source of phosphate in bioprocess with more than two-fold increase than the phosphate depleted medium. Further increase in phosphate levels supply led to a decrease in both chitosanase and biomass production by experimental organism. Other investigators reported different optimal levels according to the kind of phosphorus source as well as their tested microorganisms **(Fenton and Eveleigh, 1981, Cheng and Li, 2000, Zhu *et al*, 2003 and Chen *et al*, 2005)**. Also, **Zhang *et al*, (2000) and Tanabe *et al*, (2003)** found that, the maximum chitosanase production by *A. oryzae* IAM 2660 and *Streptomyces griseus* HUT 6037 were obtained in the presence of 0.1% KH_2PO_4 in fermentation media.

The vital importance of Mg^{+2} ions and other bivalent ions as growth factors had been discussed by **Jennings (1995)**. The optimal levels of $MgSO_4.7H_2O$ supporting the highest chitosanase activity and biomass yield dropped markedly with higher salt levels. Others reported that, the optimal Mg^{2+} ions concentration employed varied with the different organism being 0.5g/L for *A. oryzae* IAM 2660 and *A.* sp CJ22-326 **(Zhang *et al*, 2000 and Chen *et al*, 2005)** and 0.7 g/L for *P. islandicum* **(Fenton and Eveleigh, 1981)**.

Similarly the presence of KCl in bioprocess exerted a significant impact on chitosanase activity and biomass production (Fig, 10). The presence of 0.5g/L KCl maximum chitosanase and mycelial dry weight production by *A. ornatus* were recorded with an increase of abut 3.7-fold compared to the medium omission of the salt. Similar results have been previously recorded by **Zhang *et al*, (2000)** who found that, the suitable amount of *A. oryzae* IAM 2660 chitosanase was achieved in the presence of 0.5 g/L KCl in bioprocess.

Trace elements have been shown to exert a low impact on chitosanase production as well as on growth of tested organism, in general, as individually added (Fig, 11). No significant increase in chitosanase and biomass production was observed by the addition of $CoCl_2$ and $FeSO_4.7H_2O$ to the fermentation medium. However, a remarkable inhibition in both fermentation parameters were obtained by the addition of mixture of tested heavy metals and $ZnSO_4.7H_2O$ and ZnCl salts. These results partially were in agreement with those obtained by **Zhang *et al*, (2000) and Kim *et al*, (2004).** The support of some heavy metals ions in the enzyme yield and growth of microorganisms is either related to the actual increase in the enzyme yield or simply to enhancement of enzyme activity **Harper *et al*, (1977).**

Generally, fungi need only water soluble vitamins of B-complex series **(Bilgrami and Verma, 1981)**. Thus, the impact of some vitamins on the production of chitosanase and growth of *A. ornatus* were investigated as shown in Fig (12). No significant increase in enzyme production was observed by the addition of each vitamin individually to the fermentation medium. Both chitosanases and biomass production are ineffective by B_2 but retarded by B_{12}, thiamin and B_6 in fermentation media. These findings are in connection with those previously reported for different enzymes **(Shindia *et al*, 2001)**.

The results (Fig, 13) revealed that, the chitosanase and biomasss production by *A. ornatus* gradually decrease with an increasing the levels of NaCl up to 0.5%. This is not compatible

with the results reported by **Cheng and Li, (2000)** who found that 0.05% NaCl enhanced the productivity of *A.* sp Y2K chitosanases.

Supplementing the bioprocess with individual surface agents exerted different effects on both chitosanase and biomass production by tested organism (Fig, 14). Addition of 2.0% tween 80 supported fair amount of chitosanase and biomass output, however, Tween 40 retarded the two fermentation parameters. Tween 80 stimulates enzyme production, release of enzyme and enhancement of the O_2 supply to the fungal cells as discussed by **Perdih and Lestan, (1993)**. Similarly, **Kim *et al*, (2004)** found that the addition of tween 80 enhanced the release of chitosanase in fermentation media.

The experiments were extended to investigate the impact of natural products on the chitosanase and biomass production by tested fungus (Fig, 15). The results of this study showed that sand and soil extracts incorporated separately, exhibited comparable values of both chitosanase and biomass production as control. However, malt extract recorded a general lower productivity of both parameters. The superiority of sand and soil extracts might attribute to the availability of appreciable amounts of soluble salts as well as oligomers that induce enzymes and growth of microorganisms **(Sabry *et al*, 1992)**.

Finally, from the above results, it could be concluded that, *A. ornatus* proved to be the most active fungus for chitosanase production and its productivity affected by environmental and nutritional conditions of the culture media.

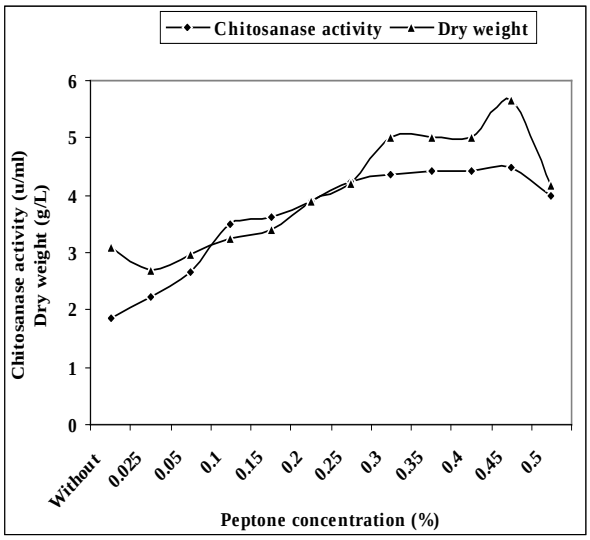

Fig.9: Effect of different concentrations of peptone on growth and enzyme production by *A. ornatus*.

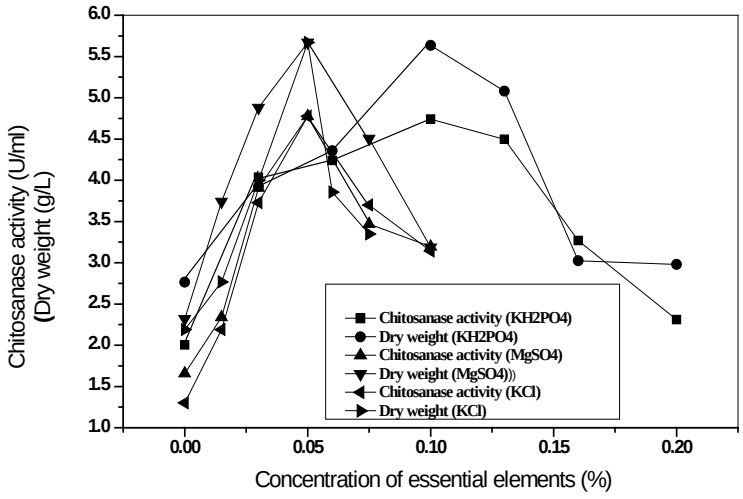

Fig.10: Effect of essential elements on growth and chitosanase production by *A. ornatus.*

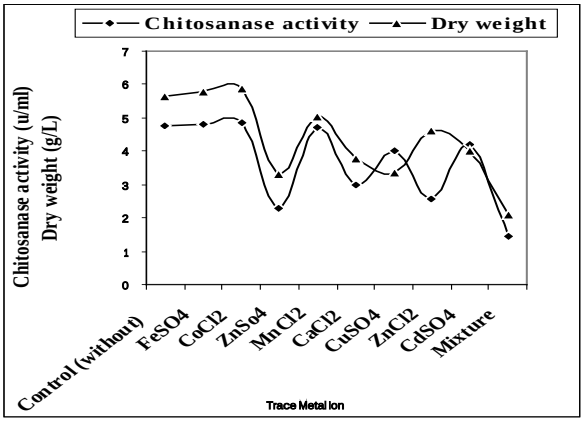

Fig.11: Effect of heavy metal ions on growth and chitosanase production by *A. ornatus*.

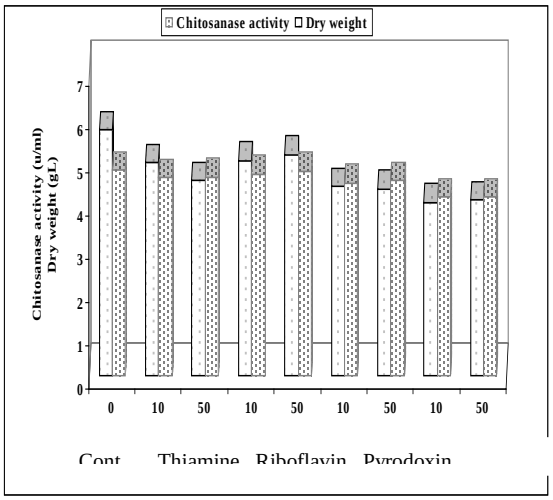

Fig.12: Effect of different vitamins on growth and chitosanase production by

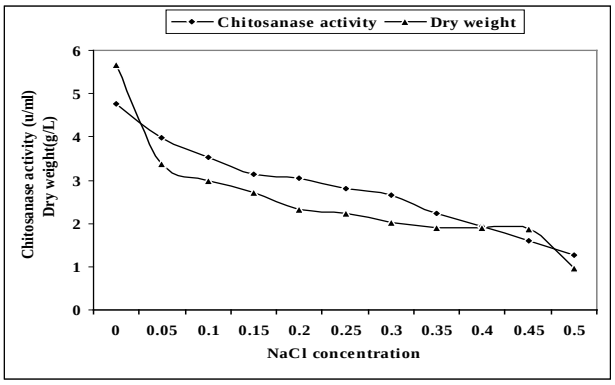

Fig.13: Effect of different concentrations of NaCl on growth and chitosanase production by *A. ornatus*.

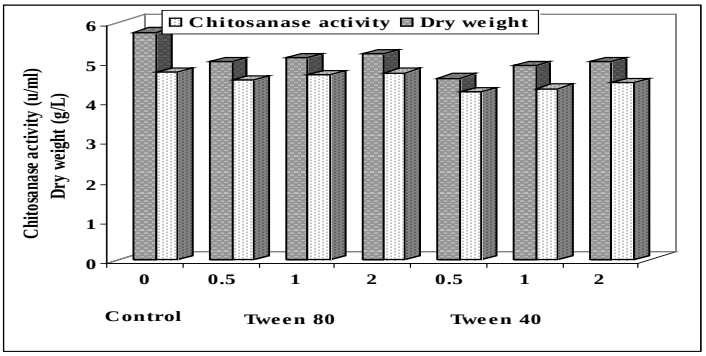

Fig.14: Effect of some surfactants on growth and chitosanases production by *A. ornatus*.

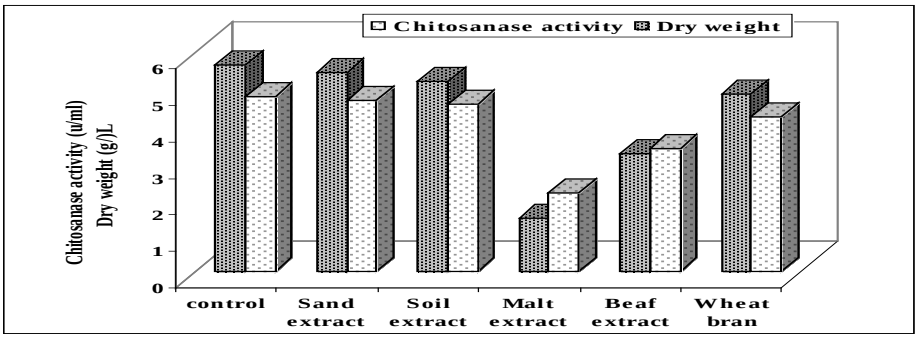

Fig.15: Effect of different natural additives on growth and chitosanase production by *A. ornatus*.

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CHITOSANASE PRODUCTION USING SOME FUNGI OPTIMIZATION

إنتاج إنزيم الكيتوزينيز من بعض الفطريات

دراسة ظروف التخمر المثلي لإنتاج إنزيم الكيتوزينيز من اسبرجيلس اورناتس

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تم في هذا البحث عزل وتعريف 26 نوعا من الفطريات و نوعين غير معرفين تنتمي إلي 10 أجناس من التربة المصرية واختبار قدرتها علي إنتاج إنزيم الكيتوزينيز خارج الخلايا في مرشحاتها المزرعية المغمورة باستخدام الكيتوزان كمصدر وحيد للكربون. تميزت انواع الاسبرجيلس بقدرتها العالية علي إنتاج الإنزيم وتم اختيار الاسبرجيلس اورناتس كأحسن فطر في إنتاج إنزيم الكيتوزينيز. وجد أن أحسن نتائج تم الحصول عليها بعد فترة تحضين 7 أيام عند درجة حرارة 30 م° و اس هيدروجيني 5. وتم ايضا اختصار مناقشة تأثير التهوية و الهز الكهربائى وكذلك كمية و عمر اللاقحة المستخدمة علي تكوين إنزيم الكيتوزينيز و الكتلة الحيوية للفطر المستخدم.

أشار البحث لبعض النتائج عن علاقة إنتاج إنزيم الكيتوزينيز و الكتلة الحيوية للفطر و المكونات الكيميائية المستخدمة في وسط التخمر ووجد أن 1% كيتوزان غروي يتبعه الكيتوزان الخام كانت أحسن المصادر الكربونية المختبرة تنشيطا لإنتاج الإنزيم. و أن 0.45% ببتون يليه مستخلص الخميرة هما أحسن المصادر النيتروجينية المستخدمة لإنتاج الإنزيم و الكتلة الحيوية. تبين أيضا أن أعلي إنتاج لإنزيم الكيتوزينيز يمكن الحصول عليه بتنمية العزلة المختارة علي بيئة الكيتوزان السائلة التي تحتوي علي 0.1% فوسفات البوتاسيوم ثنائي الهيدروجين , و 0.05% كبريتات ماغنسيوم, و 0.05% كلوريد بوتاسيوم. كما تم أيضا دراسة تأثير التركيزات المختلفة لكلوريد الصوديوم وكذلك الجلوكوز علي الإنزيم والكتلة الحيوية. وبدراسة تأثير بعض العناصر الصغرى وفيتامينات المجموعة (ب) عند إضافتها للوسط الغذائي السابق وجد أنها لا تؤثر تأثيرا معنويا علي زيادة إنتاج الإنزيم ولكن عند إضافة خليط من هذه العناصر لوحظ تثبيطا طفيفا في إنتاج الكيتوزينيز. ولوحظ زيادة إنتاجية الإنزيم في وجود مادة التوين 80 ذات النشاط السطحي في وسط التخمر. كما أدى إضافة كلا من مستخلص التربة الرملية و الطينية إلى زيادة إنتاج الإنزيم مقارنة بالمصادر الطبيعية الأخرى.

DEVELOPMENTAL PERIODS AND FECUNDITY OF *LEPIDOGLYPHUS DESTRUCTOR* (SCHRANK) ACARI: GLYCYPHAGIDAE WHEN FED ON DIFFERENT FUNGI AT DIFFERENT TEMPERATURES

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ABSTRACT

Lepidoglyphus destructor (Schrank) was reared individually on fungal colonies of *Aspergillus niger*, *Alternaria alternata* and *Fusarium oxysporum*. For the developmental test, tritonymphs were reared on the colony of each fungus individually at four different temperatures (20, 25, 30 and 35 °C and 75 % R.H.) and corresponding observations were conducted. The experiment showed that *Lepidoglyphus destructor* passed through (egg – larva – protonymph – tritonymph) before reaching adulthood at 20, 25 and 30 °C. The hypopus (deutonymph appeared at 35 °C only which is produced as a response to environmental stress. All motile developmental stages are similar in their appearance. As soon as females emerged on the colonies, males were introduced for mating. When three fungi were compared, the life cycle of female was long when reared on the fungus *F. oxysporum* 17.21 days but for male it reached 10.65 days when fed on *A. alternata* at 20. The adult mites lived longer on the colony of *Aspergillus niger* (35.8 days for female and 20.25 days for males at 20 ºC. than on colonies of *Alternaria alternata* and *Fusarium oxysporum*. The fungus *F. oxysporum* showed the best host for mite egg laying (351 eggs) at 35 ºC, while the fungus *A. niger* was the unfavorable one for egg laying (172 eggs) at 35 °C.

INTRODUCTION

Mites are found everywhere; such as in food kept in store for any length of time, and increased to numerous numbers under optimum conditions where contaminate it with their bodies and extra. Mites belonging to suborder Astigmata was first recorded by different authors around the world i.e. **Rivard** (1961), **Sinha** and **Wallace** (1966), **Zdarkova** (1967), **Shen** (1975), **Hughes** (1976), **Jefrrey** (1976), El-Naggar (1977); **Maurry** and **Jamil** (1982), **Mostafa** and **Shokeir** (1993), **Fawzy** (1996), **El-Sanady** (1999) , **Mohamed** (2003) . Many species of relatively desiccation-tolerant astigmaid mites which are pests in stored products have been successfully reared in the laboratory through their full ontogeny, **Grifth**, 1964; **Sinha**., 1964, Hughes, 1976 and **Okabe** and **Oconor**, 2001.The glycyphagid mites are a widely distributed species, often occurring in large numbers on dried plant and animal remains in houses and stables but not in grass land, **Griffith**, 1960. It has been found in flour, wheat, hay, rice, dried milk, linseed, tobacco, cheese, **Chmielewski**, 1969 and poultry feed mixture of birds & chicken, **Gigia**, 1964. and **Parkinson** *et al*. 1991.

Lepidoglyphus destructor found in large number on most of the stored products; this mite species was abundant where other infestations and secondary fungus infestations, and insect excretions exist (**Hughes**, 1976). *Lepidoglyphus* was able to survive when kept with cultures of stored product fungi (**Sinha**, 1968) That has been used in this experiments. Several species of common fungi were assessed as food for fungivorous astigmatid mites (**Okabe** and **Oconor**, 2001). They noticed that *Hypocerea nigericans*, *Botrytis cinerea* and *Flammulina relutipes* were generally good food sources for most mites examined.

A few contributions have been made to evaluate the effect of temperature and relative humidity on the acarid mites under controlled conditions in the laboratory. In this study the trials were conducted in the laboratory conditions 20, 25, 30 and 35 ºC and relative humidity 75 $\pm$ 5 % R.H. to

study the different biological aspects of the astigmatid glycyphagid mite, *Lepidoglyphus* sp. n when fed on different fungi (*Aspergillus niger*, *Alternaria alternata* and *Fusarium oxysporum*).

MATERIALS AND METHODS

Lepidoglyphus destructor (Glycyphagidae: Astigmata) was maintained at 25 ºC on colonies of (*Aspergillus niger, Alternaria alternate* and *Fusarium oxysporum),* which had been cultured on potato dextrose agar (PDA) medium (17 – 20 gm agar + 20 gm dextrose sugar + 200 gm crushed potato. A colony of fungus was put in a glass ring (1.5 cm in diameter and 1 cm high) and a single mite was introduced to each colony. High vacum silicon grease was applied and a cover glass was put onto each glass ring to keep the mites from escaping. Petri dishes (9 cm dia.) containing 10 glass rings were placed in incubators, which were set up at various temperatures and a photoperiod of 16 L - 8 D. For the developmental test, tritonymphs were reared on the colony of *Aspergillus niger* or *Fusarium oxysporum* individually at each temperature and corresponding observations were conducted. As soon as females emerged on the colony, males were introduced for mating. Eggs that have been oviposited within 12 hours were used for experiments at 20 and 25° C, where the eggs oviposited within 6h., were selectively used for experiments at 30 and 35°C., to minimize the relative effects of variance to shorten egg periods at high temperatures. The eggs were individually placed into a glass ring using a painting brush. Fifteen eggs were initially used for each temperature condition, and their development was observed every 12 h. until the mites reached maturity or died. Females which reared individually from tritonymphs and become adults within
12 h. were used for the experiments on fertility and longevity. The colony of fungi were never exhausted and were kept clean throughout the experiment. Observation was terminated when all females had died. All presented data were subjected to one way of variance (ANOVA) and means were separated by Duncan´ s multiple range test, **Duncan** (1955).

RESULTS

In this study, the trials were conducted under the laboratory conditions 20, 25, 30 and 35 ° C and relative humidity 75 $\pm$ 5 % and three different fungi to study the different biological aspects of the glycyphagid mite, *Lepidoglyphus destructor* which found in large numbers on most of stored products at different areas of Egypt.

Habitat and Feeding: Field observations showed that this mite was found inhabiting stored products in Egypt. Observation showed that, they were generally found in Qaha, (Qaluobia Governorate); Giza; Ismaeilia, and Sharkia Governorates. This mite passed through four different stages before reaching adult (egg, larva, protonymph, and tritonymph). The deutonymph (hypopus) appeared only at 35 °C only for both sexes.

Hatching: Eggs were laid randomly in cracks and substrate of rearing cells, under the granules of food. Egg was elongated and translucent when developed, and then changes to dark whitish colour before hatching. During hatching, the egg ruptured longitudinally and larvae crawled out from the medium slit with its hind legs at first, then larva librated outside and immediately moved to search for food. The hatching process lasted about 25 minutes.

Moulting: When full-grown, every moving immature stage of *L. destructor* enters to quiescent stage in which it seeks a dry hole or crack in the substrate of rearing chamber and completely ceases feeding and movement. The body swelled and enlarged which made the cuticle highly stretched. The legs become shrieked and contracted under the body surface. Body colour becomes pale-white. Quiescent individual never responses to any stimulation before moulting, the anterior part of the body becomes translucent, this area increases gradually covering the whole body, the old skin ruptured along transversal line behind the hysterosomal region, the hind legs appears from

the old skin at first. Then, the new stage crawls backward coming out of the rest of the exuvia. Newly emerged individuals kept quite beside its old skin for a short period, then started to move actively searching its food after 10 minutes. The deutonymph (hypopus stage) appeared only at 35 C for both sexes (male ad female).

This period spin for (0.9,0.7, 0.65) days for female individuals , changed to (029 ,0.37, 0.43) days for males when reared on different fungi at different temperatures..

Mating: During copulation the male assumed a dorsoposterior position with his aedegus; this is mid ventral, inserted into the female's posterior bursa copulatrix. The male tightly clasped the mid dorsum of the female with legs I and II. Legs III clasped her ventral posterior part, while leg IV hung leg limb and clasped female. The female was active and crawled, while male was immovable. The process lasted about one hour. Both sexes accepted copulation immediately after emergence. No parthenogenesis occurred in this species. In both sexes, female and male copulation occurred more than once.

Duration of stages

Incubation period: The incubation period of *Lepidoglyphus destructor* lasted for average 6.63 days (the longest period) when the mite female fed on *Aspergillus niger* at 20 °C , decreased to reach 2.1 days (the lowest period) when the mite fed on *Fusarium oxysporum* at 35 °C, Table (1) and Fig. (1). The statistical analysis of data showed that there was very highly significant difference between the mites fed on different fungi at different temperature. L.S.D. at 0.05 level = 0.25 and 0.29 for diets and temp., respectively in case of females.

On the other hand, the eggs which give rise to male took the longest period when the individuals fed on *Alternarium alternata* 5.2 days at 20 °C decreased to 1.45 days when the mite fed on *Fusarium oxysporum* at 35 °C. L.S. D. at 0.05 level = 0.22 for diet and 0.26 for temp, Table (2) and Fig. (2).

Life cycle

As shown in Table (1) the female durated 17.21 days for reaching adulthood (the longest period) when fed on *Fusarium oxysporum* at 20 °C changed to 5.4 days for the same individuals when fed on the same diet at 35 ° C. On the other hand the male individuals took 10.65 days when fed on *A. alternata* at 20 °C (the longest period) changed to 3.25 days at 35 °C on the fungus *F. oxysporum*. The statistical analysis of data showed that there was significant difference when the mites fed on different temperature on different diets. L.S.D. at 0.05 level =0.51 and 0.59 for diets and temp. in case of females and 0.50 and 0.57 for males, respectively.

Longevity: Concerning the adult longevity of *Lepidoglyphus destructor*, Tables (1 & 2), the statistical analysis using L.S.D. at 0.05 level value pointed out that the longevity of the resulted females and males was highly affected by temperatures and diets on this period. The data showed obvious differences in this period. However, feeding on *Aspergillus niger* during the longevity period lasted 35.8 and 20.25 days at 20 °C for female and male, respectively decreased to 7.7 days and 7.1 days on *F. oxysporm* at 35 ° C for female and male, respectively. L.S.D. at 0.05 level = 0.38 and 0.44 & 0.31 and 0.36 for diets and temperature for females and males, respectively..

Fecundity: The diets and temperatures suitability were obviously affected on the number of deposited eggs laid by adult females of *Lepidoglyphus destructor* .It was noticed in Table (1) that the number of eggs was 351 eggs at 35°C (the largest number) when the individuals fed on *F. oxysporum* while the lowest one recorded when the female fed on the *A. niger* at 20 °C (172 eggs). Generally, the biological aspects of *Lepidoglyphus destructor* when fed on different diets (three fungi) were affected by changing temperature from 20 to 35 °C. The statistical analysis of obtained data showed that there was a very highly significant difference between the numbers of laid eggs on different fungi. L.S.D. at 0.05 level = 1.57 and 1.81 for diets and temperature, respectively.

DISCUSSION

From the present study, it was observed that the incubation periods of astigmatid mite, *Lepidoglyphus destructor* were longer at 20 º C than at 35 ºC when fed on the different tested fungi. On the other hand life cycle, adult longevity and life span of females and males lasted longer periods at 20 C than at 35 º C and these periods were longer when mites reared on *Aspergillus niger* than other fungi. Female fecundity was higher at 35º C and when fed on *Fusarium oxysporum* than other temperatures and other diets. Similar results were obtained by **Boczek** and **Davis** (1993) who mentioned that the transferring to a higher temperature caused a significant decrease in fecundity and longevity and the mite was unable to adapt to this treatment. These results also are in agreement with that obtained by **El-Naggar** (1977) studied the effect of different food types on the biology of *T. putrescentiae* at 26°C. The female incubation period lasted for 2.53 days on *Penicillium* sp. and 2.6 days on sound potatoes. **Abdel-Azeim**, (1999) reared *Lepidoglyphus* sp. on hazel nuts. The female life cycles were 16.75, 13.10, 10.75 days, while male life cycles were 15.00, 11.90, 9.80 days at 20, 25, 28°C, respectively. The total female life-span durated for 60.6, 56.3, 47.0 days, but male life-span was 53.3, 46.4, 43.0 days at the same temperatures, respectively. **Franzolin** *et al*. (1999) studied the ability of *T. putrescentiae* to spread the toxigenic fungus *Aapergillus flavus* from contaminated maize to sterile grains under controlled conditions. They added that the current data confirms *Lepidoglyphus* sp. is a means of dispersal for toxigenic fungi in stored grain kept under warm and moist conditions.

Franz *et al*. (2000) noticed that *Glycyphagus domesticus* passed through (egg- larva- prelarva, protonymph- tritonymph) before reaching adulthood. The life cycly of this mite lasted 17 days at 25 C and 75 % R.H.

In this trend, **Chmielewski** (2002) collected and reared *Lepidoglyphus destructor* on bruised buck wheat grain at 20 C and 85 % R.H. From the experiment the life cycle of mite was completed in 17.7 days. The number of laid eggs by female during its life span was 78.1days.

Also, similar results were obtained by **Corente** and **Knulle** (2003) who noticed a unique (synapomrphic) characteristic of astigmatic mites is heteromorphic deutonymph (hypopus). It is a non-feeding and facultative instar between protonymph and tritonymph adopted for dispersal and also for dormancy, as *Lepidoglyphus destructor* and suggested that as food quality decreases, development lasts longer and hypopodes are produced in a great number.

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Fig. (1) : Developmental periods of *Lepidoglyphus destructor (Shrank)* female when fed on different diets at different temperatures

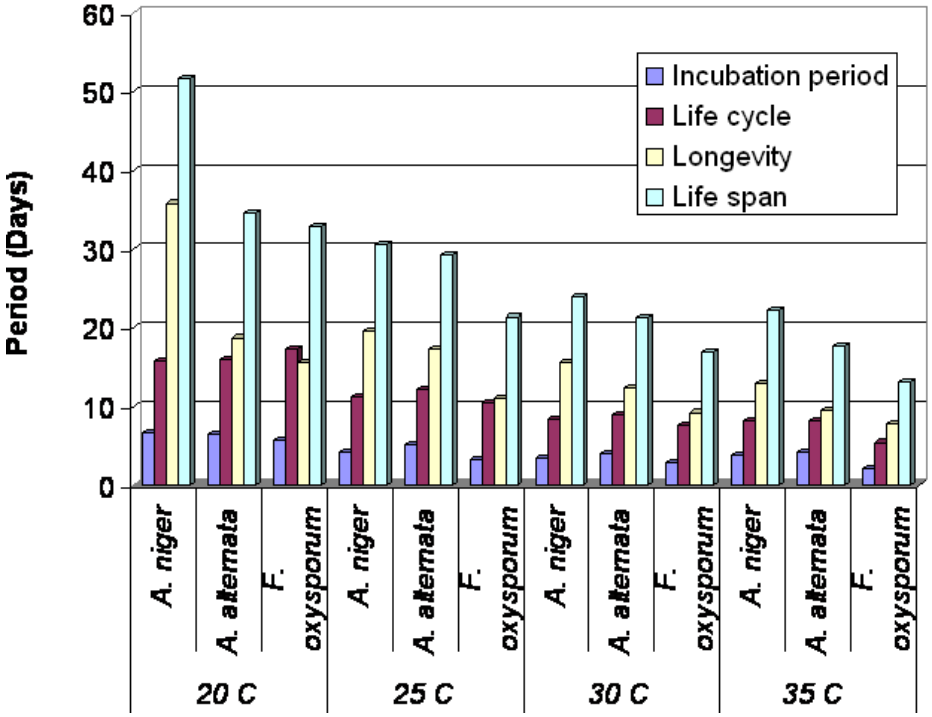

Fig. (2) : Developmental periods of *Lepidoglyphus destructor (Shrank)* male in different fungi at different temperatures

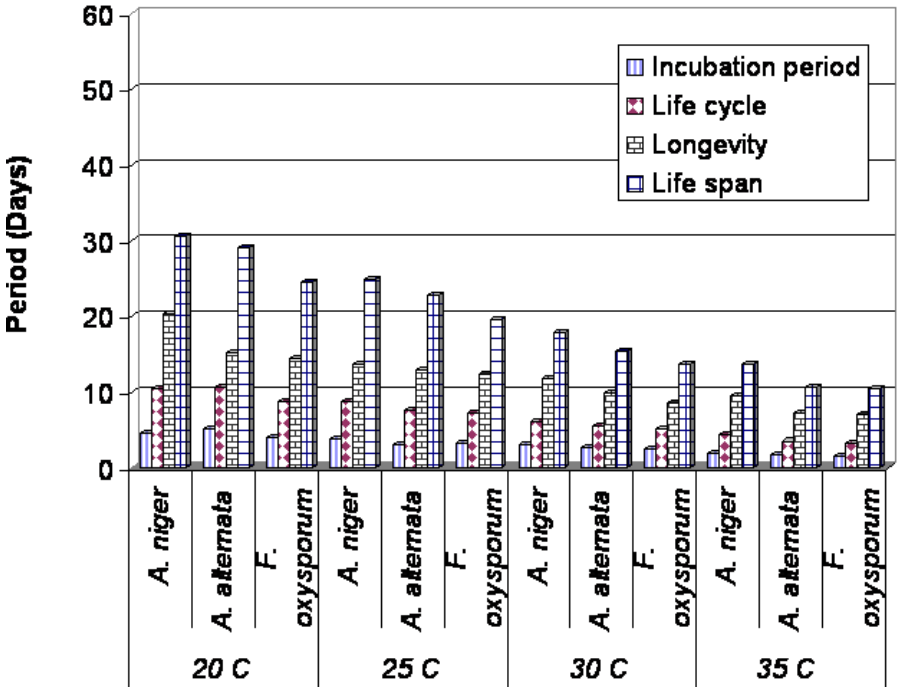

Table (1): Developmental periods and fecundity of *Lepidoglyphus destructor* female when fed on different diets at different temperatures

Biol. Aspects.	20 ºC			25 ºC			30 ºC			35 ºC			L.S.D. at 0.05	
													Diets	Temp.
	A	B	C	A	B	C	A	B	C	A	B	C		
Incubation period	6.63 ± 0.71	6.38 ± 0.77	5.73 ± 0.66	4.13 ± 0.99	5.2 ± 1.27	3.13 ± 0.77	3.43 ± 0.40	3.9 ± 0.88	2.8 ± 0.65	3.78 ± 0.78	4.2 ± 0.97	2.1 ± 0.41	0.250	0.2887
Life cycle	15.8 ± 1.77	15.96 + 1.25	17.21 ± .25	11.13 ±1.03	12.06 ± 1.53	10.36 ±1.42	8.36 ± 0.43	8.91 ±0.97	7.65 ± 0.88	9.14 ± 0.72	8.91 ± 0.76	6.05 ± 0.53	0.5080	0.5866
Longevity	35.8 ±5.1	18.7 ±3.4	15.6 ±3.7	19.5 ±3.0	17.2 ±2.2	11.6 ±3.2	15.6 ± 2.30	12.3 ± 1.9	9.2 ±1.9	12.9 ± 1.9	9.5 ± 1.3	7.7 ± 1.1	0.3781	0.4366
Preoviposition	5.75 ± 0.32	6.7 ± 0.49	3.9 ± 0.11	4 ± 0.46	4.2 ± 0.40	2.9 ± 0.3	3 ± 0.06	3 ± 0.63	1.4 ± 0.65	4 ± 0.69	3 ± 0.61	1.6 ± 0.1	-	-
Oviposition	20 ± 1.7	9 ± 0.43	11 ± 0.79	10 ± 1.0	11 ± 1.50	8.1 ±3.2	8.6 ± 1.14	8 ±0.95	7.3 ±0.95	6.9 ± 0.43	5 ±0.84	5.7± 0.88	-	-
Postoviposition	10 ± 1.1	3 ± 0.1	0.7 ± 0.08	5.5 ± 0.15	2 ± 0.09	0.6 ± 0.0.3	4 ± 0.11	1.3 + 0.1	0.5± 0.09	2 ± 0.1	1.5± 0.06	0.4 +0.01	-	-
Fecundity	172 ±16.6	190 ± 29.75	183 ± 34.2	243± 19.64	259 ± 26.3	220 ± 24.56	261 ± 16.97	270 ± 16.89	286 ± 18.3	287 ± 22.5	320 ± 20.1	351 ± 19.68	1.570	1.8132
Life span	51.60 ±4.56	34.48 ± 3.21	32.81 ± 2.14	30.63 ± 1.5	29.25 ± 2.54	21.36 ± 1.89	23.96 ± 3.21	21.21 ± 1.69	16.85 ± 1.11	22.14 ± 2.21	17.71 ±2.01	13.1 ± 0.99	0.7343	0.8478

A = *Aspergillus niger* B = *Alternaria alternata* C = *Fusarium oxysporum*

Table (2):Developmental periods of *Lepidoglyphus destructor* (Shrank) male when fed on different diets at different temperatures

Biol. Aspects.	20 ºC			25 ºC			30 ºC			35 ºC			L.S.D. at 0.05	
													Diets	Temp.
	A	**B**	C	**A**	**B**	**C**	A	**B**	C	A	B	C		
Incubation period	4.5 ± 0.32	5.2 ± 0.11	4.05± 0.26	3.8 ± 0.32	3.1± 0.16	3.2± 0.11	2.95 ±0.11	2.70 ±0.21	2.5± 0.11	1.85 ±0.08	1.66 ±0.11	1.45±0. 21	0.222	0.257
Life cycle	10.35 ±1.5	10.65 ±1.98	8.8± 1.6	8.65 ±1.65	7.5 ±2.2	7.25± 1.65	6.15 ± 0.82	5.45 ± 1.1	5.15± 0.11	4.72 ±0.32	3.79± 0.3	3.68± 1.1	0.495	0.572
Longevity	20.25 ±3.4	15.2 ±2.6	14.5± 3.0	13.7 ± 3.1	12.9 ± 2.2	12.3± 2.5	11.7 ± 2.1	9.9 ± 1.9	8.5± 1.3	9.4 ± 3.2	7.25 ±1.0	7.1± 2.2	0.310	0.358
Life span	30.6 ±4.6	29.05 ± 2.8	24.45 ± 4.1	24.8 ± 2.5	2.2 ± 3.54	19.55 ±3.4	17.85 ± 1.65	15.3± 1.41	13.65 ± 2.4	13.75 ± 1.4	10.7 ± .65	10.35 ± 1.87	0.530	0.612

A = *Aspergillus niger* B = *Alternaria alternata* C = *Fusarium oxysporum*

فترات النمو وخصوبة الاكاروس *Lepidoglyphus destructor* (Shrank) (اكارى : جليسفاجيدى) عند التغذية على عدة فطريات ودرجات حرارة مختلفة

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الملخص العربى

تمت تربية الاكاروس *Lepidoglyphus destructor* والمنتمى الى تحت رتبة عديمة الثغر التنفسى على عدة مستعمرات من الفطريات عند درجات حرارة20و 25 و 30 و35 درجة مؤية و 75 % رطوبة نسبية وذلك فى المعمل.

أظهرت النتائج المتحصل عليها ان الافراد الذكور والاناث مرت بالاطوار الاتية بيضة – يرقة – حورية اولى – حورية ثالثة قبل الوصول الى الفرد البالغ عند درجات الحرارة 20و 25 و 30 درجة مؤية. اما عند درجة الحرارة 35 درجة مؤية ظهرت الحورية الثانية (Hypopus) وذلك نتيجة الظروف الغير مناسبة حيث استغرقت الحورية الثانية(0.65،0.7،0.9) يوم للأنثي بينما للذكر فأستغرقت(0.43،0.29،0.37) يوم عند التغذية علي الفطريات (*Fusarium oxysporum* و *Alternaria alternata* و *Aspergillus niger*) علي التوالي .

واتضح من النتائج المتحصل عليها ان دورة حياة الانثى كانت عالية عند التغذية على الفطر *Fusarium oxysporum* (17.21 يوما) اما الذكور فاستغرقت (10.65 يوما) عند التغذية على الفطر *Alternaria alternata* عند درجة الحرارة 20 م . واتضح ايضا من الدراسة ان الافراد الذكور والاناث البالغة عاشت فترة اطول عند التغذية على الفطر *Aspergillus niger* عند درجة الحرارة 20 درجة مؤية (35.8 و 20.25 يوما على التوالى) اكثر من الفطريات *Alternaria alternata* و *Fusarium oxysporum* . وكان الفطر *F. oxysporum* أفضل العوائل من حيث عدد البيض الموضوع للانثى عند 35 درجة مؤية (351 بيضة) بينما اعطت الانثى عند التغذية على الفطر *A. niger* (172 بيضة) عند 20 م .