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Editorial Preface

It is my great pleasure to publish this issue of volume four of the Jordan Journal of Biological Sciences (JJBS), published by the Hashemite University in cooperation with the Jordanian Scientific Research Support Fund (SRF)- Ministry of Higher Education and Scientific Research- Hashemite Kingdom of Jordan. JJBS is a peer reviewed quarterly international journal aims to provide readers worldwide with high quality peer-reviewed scholarly articles on a wide variety of issues related to cell biology, genomics, microbiology, immunology, molecular biology, biochemistry, embryology, immunogenetics, cell and tissue culture, molecular ecology, genetic engineering and biological engineering, bioremediation and biodegradation, bioinformatics, biotechnology regulations, gene therapy, organismal biology, microbial and environmental biotechnology, and marine sciences.

I am honored to have six editorial board member: Ahmad Khalil (Yarmouk University), Abdul- Karim Sallal (Jordan University of Science and Technology), Khaled Tarawneh (Mutah University), Sawsan Oran (University of Jordan), Shtaywy Abdallah (University of Jordan) and Suliman Saleh (Hashemite University). I'm also delighted with our group of International Advisory Board members consisting of scholars from 12 countries worldwide that are actively serving on scientific fields. With our editorial board's cumulative experience on research and education, this journal brings a substantial representation of the field of biological studies in the disciplines noted. Without the service and dedication of our editorial board, JJBS would have never continued. The Editorial Board is very committed to build the Journal as one of the leading international journals in biological sciences in the next few years.

I am glad to witness the recent expansion of JJBS and the attention it has generated among researchers. This issue contains a very interesting perspective articles from Jordan, Malaysia, Algeria, Nigeria, India and Iraq. JJBS has introduced a new type of papers under category of **Short Communication**. This category of papers has the objective of disseminating and sharing our researchers' most current results and developments in the shortest possible time, and may include for example pilot studies results that will lead to more detailed future research.

I am also delighted to announce that Jordan Journal of Biological Sciences articles are now abstracted/indexed in CAS (Chemical Abstracts), CAB International Abstracts, Zoological Records. I am also glad to announce that the EBSCO Publishing has recognized JJBS and agreed to consider it as a prestigious scientific journals and this will be accomplished upon signing agreement, which will entitle JJBS to appear in Scopus and later in ISI, enhancing the journal's impact.

On this occasion, I would like to thank Professor Naim S. Ismail on his excellent work as Editor- in- Chief of this Journal over the past 4 years. I would also like to express my thanks and appreciation to the authors, referees, and the members of the editorial board for their support and follow-up. My thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous support to Jordan Journal of Biological Sciences.

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Glutathione as Potential Target for Cancer Therapy; More or Less is Good? (Mini-Review)

Maher Y. Abdalla

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Abstract

Glutathione (GSH) plays important roles in antioxidant defense, nutrient metabolism, and regulation of cellular processes, including cell differentiation, proliferation and apoptosis. Glutathione deficiency contributes to oxidative stress, which plays a key role in aging and pathogenesis of many diseases, one of which is cancer. The GSH content of cancer cells is associated with multidrug and radiation resistance. Just as low intracellular GSH levels decrease cellular antioxidant capacity, elevated GSH levels generally increase antioxidant capacity and resistance to oxidative stress, and this phenomenon is observed in may cancer cells as compared to normal ones. The present review will address the following questions: what is cancer-glutathione relation? Can glutathione play a role in treating or preventing cancer?

Keywords: Glutathione, antioxidant, cancer, prevention.

1. Introduction

It is amazing how a tripeptide composed of cysteine, glutamic acid and glycine can be of this importance for cellular function. Glutathione (L-g-glutamyl-L-cysteinylglycine) is the principal tripeptide thiol involved in the antioxidant cellular defense (Clark et al., 1984; Vojislav et al., 2001; Ganesaratnam et al., 2004). The most two important structural features of GSH are: γ-glutamyl linkage and sulphydryl group (–SH). It is the thiol of cysteine residue that composes the active group (Figure 1) (Kaplowitz et al., 1985).

GSH is a tripeptide produced by the liver and is able to detoxify the lungs, RBCs, liver and the intestinal tract; it also removes a wide range of toxins, including those produced by heavy metals, cigarette smoke, alcohol, radiation and cancer chemotherapy. Glutathione neutralizes oxygen molecules before they cause damage to cells. It is found in two forms: free or bound to proteins. Free form is present mainly in its reduced form (GSH), which can be converted to the oxidized form (GSSG) during oxidative stress, and can be reverted to the reduced form by the action of the enzyme glutathione reductase (Ames 1989; Ames et al., 1993) (Figure 2).

In normal conditions, the GSH concentrations in mammalian cells can range between 1 and 10 mM, with the reduced GSH predominating over the oxidized form (Hassan and Fridovich, 1980). Maintaining optimal GSH:GSSG ratios in the cell are critical for survival, and a deficiency of GSH can result in oxidative damage. This ratio exceeds 100 in a normal resting cell, whereas in various models of oxidative stress, this ratio was reported to decrease to values between 10 and 1 (Hassan and Fridovich, 1980).

Glutathione is synthesized in the cell by the sequential actions of γ-glutamylcysteine synthetase (GCS) and glutathione synthetase (GS) in a series of six-enzyme-catalysed reactions (Meister and Anderson, 1983). This review will highlight the importance of GSH homeostasis in cancer therapy.

2. Reactive Oxygen Species and Human Diseases

Due to different roles of reactive oxygen species (ROS) in cell signaling and many human pathological processes, imbalance of GSH is observed in a wide range of pathologies, including cancer, neurodegenerative disorders, cystic fibrosis (CF), HIV, and aging (Townsend and Tew, 2003; Ganesaratnam *et al.*, 2004; Ken *et al.*, 2004; Hayes *et al*., 2005). Maintaining proper GSH levels and oxidation state are important for cell function and their disruptions are observed in many human diseases. GSH deficiency leads to an increased susceptibility to oxidative stress and, thus, progression of many disease states (Townsend and Tew, 2003; Ganesaratnam *et al.*, 2004; Ken *et al*., 2004; Hayes *et al*., 2005). On the other hand, elevated GSH levels increase antioxidant capacity and resistance to oxidative stress and this is observed in many types of cancer (Townsend and Tew, 2003; Ganesaratnam *et al*., 2004; Ken *et al*., 2004; Hayes *et al*., 2005).

Free radicals produced by normal cellular metabolism can lead to extensive damage to DNA, protein, and lipid (Olinski *et al*., 1992; Okamoto *et al*., 1994; Devi *et al*., 2000; Wu *et al*., 2004).

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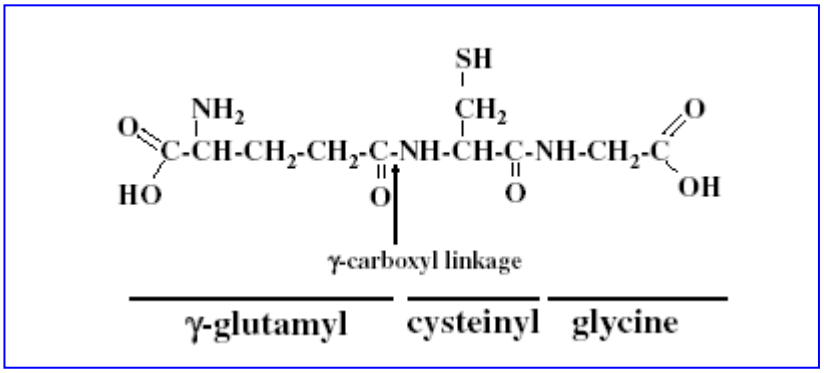

Figure 1. Structure of GSH or γ-glutamylcysteinyl glycine. The N-terminal glutamate and cysteine are linked by the γ-carboxyl group of glutamate (Kaplowitz, *et al.*, 1985).

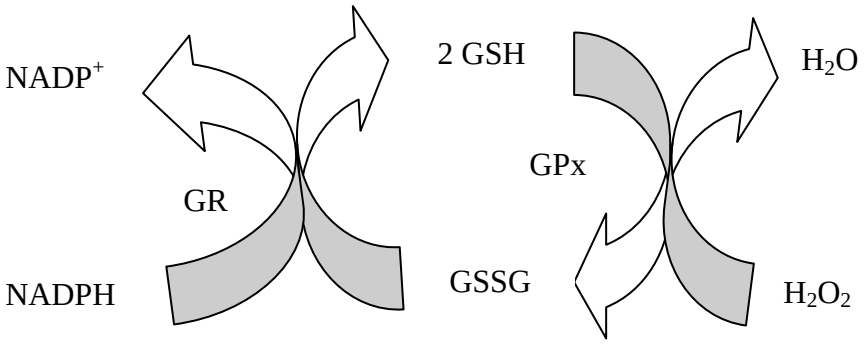

Figure 2. Pathway of ROS clearance (Adopted from Droge, 2002). Oxidants such as H_2O_2 is converted to H_2O by the action of GPX (or Catalase) using GSH. Regeneration of GSH requires NADPH and GR enzyme.

GR=glutathione reductase

GPx=glutathione peroxidase

DNA accumulates oxidative damage induced by ROS generated by endogenous and exogenous sources. This damage is a major contributor to diseases such as cancer, heart disease, cataracts, brain dysfunction, and aging (Ames, 1989; Ames, Shigenaga *et al*., 1993). It is estimated that the number of oxidative hits to DNA per cell per day is around 100,000 in the rat and 10,000 in the human (Ames, Shigenaga *et al*., 1993). It is possible that oxidative lesions in endogenous mammalian DNA exceeds 100 different types, of which 8-hydroxyguanine (8-oxoG) is one of the most abundant (Ames, 1989; Ames, Shigenaga *et al.*, 1993). In normal functional cells, DNA repair enzymes efficiently remove most of the lesions formed by ROS. Several different methods are used to remove any mutation or mismatch (Demple and Harrison, 1994). In 2008, Petta *et al*., have shown the role of human DNA polymerase iota in protecting cells against oxidative stress (Petta *et al.*, 2008). However, increased ROS generation in cancer cells leads to the accumulation of oxidative products of DNA, proteins, and lipids in tissues, and their release into the blood and urine. DNA oxidative products (8-oxoG), and lipid peroxidation have been detected in many cancer tissues, such as colorectal adenocarcinomas, mammary ductal carcinomas, renal cell carcinoma, and blood samples from leukemia patients (Olinski *et al*., 1992; Okamoto *et al*., 1994; Devi *et al*., 2000; Wu *et al*., 2004).

3. GSH and Cancer

Many types of cancer cell have increased levels of free radicals and ROS compared with their normal counterparts (Toyokuni, Okamoto *et al.*, 1995; Kawanishi, Hiraku *et al.*, 2006). However, several studies using primary cancer tissues have revealed increased levels of ROS-scavenging enzymes and antioxidant compounds (Goodwin and Baylin, 1982; Oltra *et al*., 2001). This increase could be a result of an adaptive response to intrinsic ROS stress.

While GSH is important in the detoxification of carcinogens, its elevated state in many types of tumors may also increase resistance or alters the cytotoxicity of many chemotherapy drugs or radiation (Clark *et al*., 1984; Vojislav *et al*., 2001; Ganesaratnam *et al*., 2004). One example is human fibroblast tumor cell lines which has higher levels of cellular GSH than did normal human fibroblasts (Goodwin and Baylin, 1982; Carney *et al*., 1983). This increased GSH may be an important factor in chemo- or radiotherapy resistance seen in these cells (Yu and Brown, 1984; Guichard *et al.*, 1986).

Manipulation of intracellular GSH using drugs such as 2-oxothiazolidine-4-carboxylate (OTZ) (a compound that stimulates GSH synthesis (Williamson *et al.*, 1982) or L-buthionine-(S,R)-sulfoximine (BSO) (a compound that inhibits GSH synthesis (Griffith *et al*., 1979)) has been used to increase the sensitivity of different tumor cell lines to therapy and showed that selective differential

chemotherapy responses of normal versus tumor cells is possible (Griffith *et al.*, 1979; Williamson *et al.*, 1982).

It has been shown that manipulating intracellular oxidant status of tumor cells can be of clinical value. Increasing ROS or decreasing free radical scavengers such as GSH was shown to be toxic to tumor cells. Weydert *et. al*.(2008) have shown that combining GSH depletion using 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) chemo-therapy with superoxide dismutase (SOD) gene therapy could be extremely successful in the treatment of breast cancer. Simon *et al*. (2007) have also shown that BSO sensitizes cancer cells to chemotherapy agents. Combining agents that induce mitochondrial dysfunction, such as AZT, and GSH depletion with BSO causes significant toxicity in head and neck cancer. However, it's important to note that different cells respond differently to oxidative stress inducing therapies (Mattson *et al.*, 2009).

Other factors might play an important role in the GSH therapy mechanism and should be considered when using GSH manipulation drugs. One important factor is a group of transferases enzymes called glutathione-S-transferases (GSTs). Elevated levels of GST in many tumor cell types have been demonstrated to limit the effectiveness of chemotherapy (Blair *et al*., 1997; Cullen *et al*., 2003). Moreover, GSTs have been associated with multidrug resistance of tumor cells, and over expression of GSTs can increase susceptibility to carcinogenesis and inflammatory disease (Townsend and Tew, 2003; Ganesaratnam *et al.*, 2004; Ken *et al*., 2004; Hayes *et al*., 2005). One mechanism by which chemotherapy resistance may occur is by gene amplification of GST(s). It has been shown that over expression of the gene products of GST-π, can provide a tumor cells with survival advantage relative to normal cells. High GST-π, expression was associated with poor overall survival and may be associated with a more aggressive phenotype in head and neck squamous cell carcinoma (Shiga *et al.*, 1999; Ulrike *et al.*, 2002; Cullen *et al.*, 2003).

4. GSH level, more or less is better?

GSH is involved in a variety of cellular functions such as DNA repair, cell cycle, regulation of cell signaling and transcription factors (Arrigo, 1999). It has been reported that GSH can modulate the activity of multiple stress genes which act to regulate the genes of cell proliferation, differentiation and apoptosis (Wiseman and Halliwell, 1996).

The fact that the changes in the intracellular GSH/GSSG ratio are critical for activation of cell proliferation and cell death makes it a very important to consider when using any treatment that has an effect on intracellular GSH levels. As shown in Figure 3, a higher level of GSH (left side of Figure 3) is important for normal cellular functions, signal transduction and protection against certain carcinogens. However, this high level (whether induced by certain drugs or as normal response to stimulants) can slow down any effective cancer treatment that works by increasing intracellular ROS (Figure 3).

On the other hand, when intracellular GSH levels are low (using certain drugs such as BSO), the cells are more vulnerable to ROS attacks. Increased ROS might activate different intracellular oncogenic pathways or mutate a tumor suppressor gene pathway, which will activate a tumorigenesis process (Irani *et al.*, 1997; Komatsu *et al*., 2008). Because the increase of ROS in cancer cells maybe part of the initiation and progression of cancer, such intrinsic oxidative stress is often viewed as an adverse event. However, as excessive levels of ROS stress can also be toxic to the cancer cells and cells are likely to be more vulnerable to damage by further ROS induced by exogenous drugs and make them more responsive to ROS producing cancer treatments (Figure 3). Therefore, changing ROS levels by GSH modulation is a way to selectively kill cancer cells without causing significant toxicity to normal cells (Trachootham *et al.*, 2009). It is important to take into consideration that under increased levels of ROS, certain cancer cells may acquire some cancerous measures such as: proliferation, immortalization, and metastasis (Behrend *et al.*, 2003; Hu *et al.*, 2005; Makiya 2008).

5. Recommendations

It is clear that different cancer cells respond differently to certain cancer therapies. This difference could be due to inherent features of these cells or could be due to the nature of the action of drugs. In general, future drugs should be able to increase ROS production and used in combination with other drugs that interfere with ROS scavenging at the same time. It is important to find out whether these cells have a drug resisting mechanisms. These mechanisms can reverse the drug effect and implicate the need of higher doses.

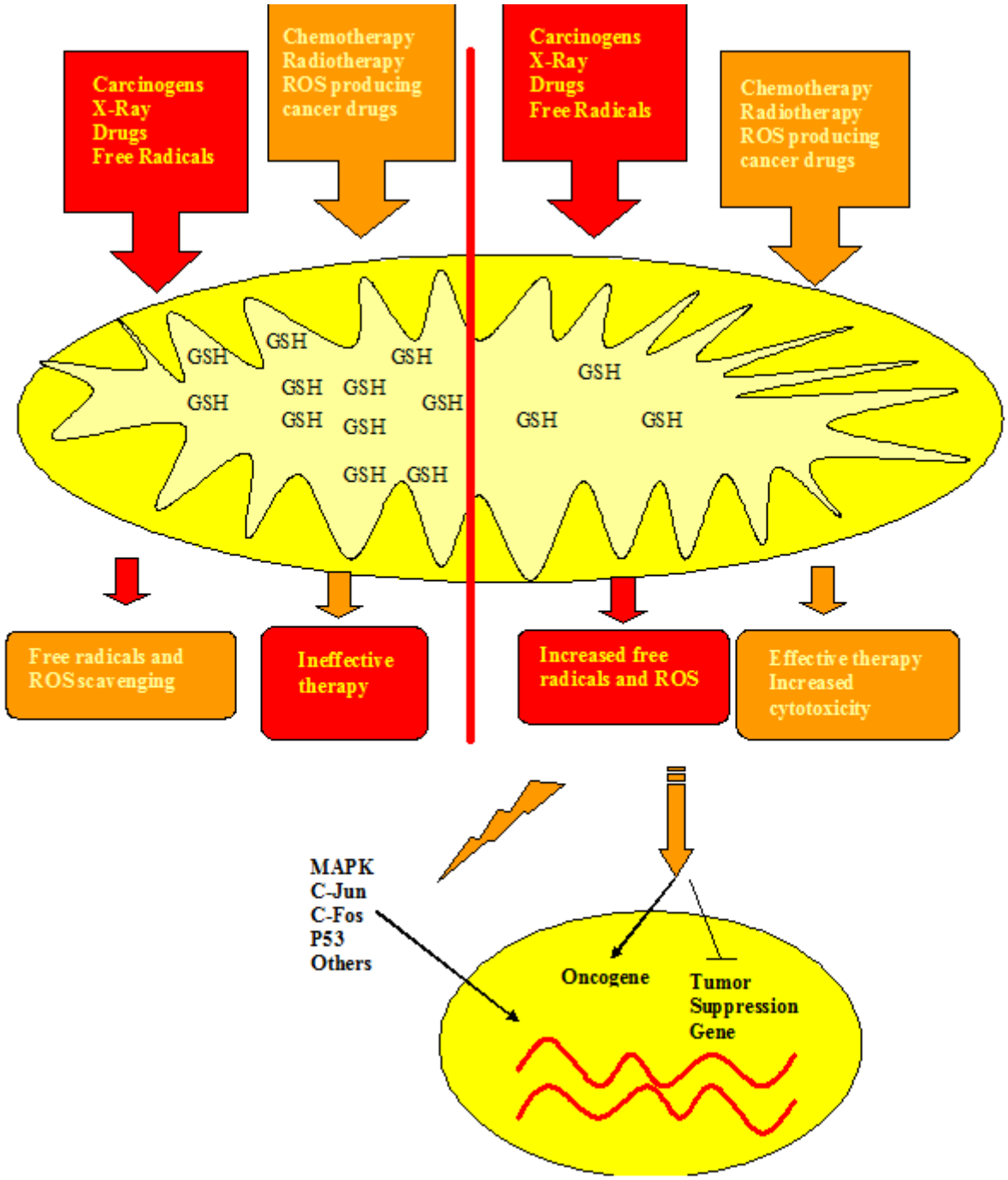

Figure 3. Glutathione level can affect expected outcome. High level of GSH (left side) is needed for cellular functions, signal transduction and protection against certain carcinogens. However, it can slow down any effective cancer treatment that works by increasing intracellular ROS. Low GSH levels (right side) renders cells more vulnerable to ROS attacks. Increased ROS activates intracellular oncogenic pathways or mutate a tumor suppressor gene pathway, which will activate a tumorigenesis process.

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Antibiogram and Heavy Metal Resistance Pattern of *Salmonella* spp. Isolated from Wild Asian Sea Bass (*Lates calcarifer*) from Tok Bali, Kelantan, Malaysia

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Abstract

The aim of this study is to characterize antibiogram and heavy metal resistance pattern of *Salmonella* spp. isolated from wild Asian sea bass (*Lates calcarifer*). *Salmonella* spp. is recognized as a food borne pathogen to humans. Therefore, this study was carried out to determine the suitable antibiotic in controlling *Salmonella* spp. isolated from Asian sea bass, Malaysian favorite seafood. In the present study, *Salmonella* spp. was isolated using Xylose Lysine Desoxycholate (XLD) medium. The bacterial isolates were then identified using conventional biochemical tests and conformed to commercial identification kit. A total of 150 isolates of *Salmonella* spp. were randomly selected for antibiotic and heavy metal tolerance tests. Fourteen antibiotics, namely oxolinic acid (2 µg), ampicillin (10 µg), erythromycin (15 µg), furazolidone (15 µg), lincomycin (15 µg), colistin sulfate (25 µg), oleandomycin (15 µg), doxycycline (30 µg), nitrofurantoin (50 µg), fosfomycin (50 µg), florfenicol (30 µg), flumequine (30 µg), tetracycline (30 µg), and spiramycin (100 µg) and four heavy metals; mercury (Hg^{2+}), cadmium (Cd^{2+}), chromium (Cr^{6+}) and copper (Cu^{2+}) were tested in the present study. The results of the present study indicating that oxolinic acid were found the most effective in controlling present bacterial isolates in which 85.3 % of the present bacterial were sensitive to them. This was followed by tetracycline and nitrofurantoin in which 85.2 % of the bacterial isolates were sensitive to it. On the other hand, all the bacterial isolates were resistant to lincomycin and oleandomycin. The findings of the present study indicate that the samples may be highly exposed to the tested antibiotics and heavy metals.

Keywords: Dark-light cycle, antibiogram, heavy metal, *Salmonella* spp., Asian sea bass, *Lates calcarifer*

1. Introduction

Food poisoning is a case that occurs when a patient is exposed to or consumes food or beverage contaminated with bacteria, parasites, viruses, or a toxin produced by microorganism. *Salmonella* spp. is one of the pathogens that are recognized as a causative agent of food poisoning. Recently, a local newspaper in Malaysia has reported that food poisoning cases of Kota Bharu, Kelantan, Malaysia increased 100% and the sampling sites of the present study were only about 100 km away from Kota Bharu. Salmonellosis due to *Salmonella* spp. was recognized as a public health problem associated with a significant morbidity and mortality in those infected with the pathogens (Lunestad *et al*., 2007). These bacteria are ubiquitous was and are reported to be commonly found in food products and water samples (Baudart *et al*., 2000). Products such as fish meal, meat, bone meal, maize and soy products may be contaminated with this bacterium at high prevalence (Jones and Richardson, 2004). Thus, fish may be one of the sources of *Salmonella* spp. harboring in the cultured fish from aquaculture sites. Although *Salmonella* spp. was not recognized as fish pathogen, this bacterium was reported as persistently found in the gastro intestinal, internal organs and muscle of fish after the fish was exposed orally to high dose to this bacterium (Hagen, 1966; Buras *et al*., 1985). Therefore, this paper discusses the presence of *Salmonella* spp. in the wild of *Lates calcarifer* as well as their antibiogram and heavy metal resistance pattern. Furthermore, the implications of such *Salmonella* contamination on fish and human health are assessed.

2. Materials and Methods

A total of 50 of wild caught Asian sea bass, *Lates calcarifer* with the size 20 to 25 cm were sampled at Tok Bali seaside, Kelantan, Malaysia. The water parameters of the sampling sites were measured using pH meter (YSI, USA). The temperature, dissolved oxygen, pH, and salinity of the sampling sites were 29.52 ^{o}C, 6.87 mg/l, 8.81, and 28.31 ppt, respectively.

A total of 10 g of the minced flesh of *L. calcarifer* was diluted in 100 ml sterile physiological saline followed by a

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serial dilution in sterile physiological saline and plated on two types of medium; Tryptic Soy Agar (TSA) and Xylose Lysine Desoxycholate (XLD) (Merck, Germany). All the inoculated media were incubated at room temperature for 24 to 48 h. The bacterial colonies that grew on the selective media were further selected for the identification test. The bacterial isolates were identified using conventional biochemical tests (Holt *et al.*, 1994) and conformed to commercial identification kit (BBL, USA). All the bacterial isolates were identified as *Salmonella* spp.

The isolates (n = 150) were cultured in Tryptic Soy Broth (TSB) (Oxoid, England) for 24 h at room temperature. The bacterial cells were then centrifuged at 14,500 rpm for 5 min by using minispin (Eppendorf, Germany). The concentration of the bacterial cells were adjusted to 10^6 colony forming unit (CFU) using saline and monitored with Biophotometer (Eppendorf, Germany) before being swabbed onto the prepared Mueller Hinton Agar (MHA) (Oxoid, England). Antibiotic susceptibility test was conducted according to Kirby–Bauer disk diffusion method using MHA (Bauer *et al.*, 1966). Antibiotics tested were:OA2; oxolinic acid (2 µg/disk), F50; nitrofurantoin (50 µg/disk), AMP10; ampicillin (10 µg µg/disk), E15; erythromycin (15 µg/disk), FR15; furazolidone (15 µg/disk), MY10; lincomycin (10 µg/disk), CT25; colistin sulfate (25 µg/disk), OL30; oleandomycin (30 µg/disk), FOS50; fosfomycin (50 µg/disk), DO30; doxycycline (30 µg/disk), FFC30; florfenicol (30 µg/disk), UB30; flumequine (30 µg/disk), TE30; tetracycline (30 µg/disk) and SP100; spiramycin (100 µg/disk) (Oxoid, England). Interpretation of the results, namely sensitive (S), intermediary sensitive (I) and resistance (R), was made in accordance to the standard measurement of inhibitory zones in millimeter (mm) (CLSI, 2006).

Multiple antibiotic resistance (MAR) index of the isolates against the tested antibiotics was calculated based on the following formula (Sarter *et al.*, 2007; Lee *et al.*, 2009; Lee *et al.*, 2010):

MAR index (multiple antibiotic resistance) = X / (Y x Z)

X = total number of antibiotic resistance cases

Y = total number of antibiotics used in the study

Z = total number of bacterial isolates

A MAR index value of equal or less than 0.2 was defined as those antibiotics that were rarely or never used for the animal in terms of treatment, but if the MAR index value was higher than 0.2 it is considered as that when the animals have received high risk of exposure to those antibiotics.

Heavy metal resistance test was carried out as described by Miranda and Castillo (1998). Bacterial tolerance to four elements of heavy metal: mercury (Hg^{2+}), cadmium (Cd^{2+}), chromium (Cr^{6+}) and copper (Cu^{2+}) was determined by agar dilution method. Overnight bacterial suspension was spread onto plates of TSA medium incorporated with different concentrations of $HgCl_2$, CdCl, $K_2Cr_2O_7$, and $CuSO_4$ (Fluka, Spain). By two-fold dilutions, concentration of both Cd^{2+} and Cr^{6+} ranged from 25 to 400 µg/mL while concentration of Hg^{2+} and Cu^{2+} ranged from 2.5 to 40 µg/mL and 150 to 2400 µg/mL, respectively. For the purpose of defining metal resistance, the isolates were considered resistant if growth was obtained at concentrations of 10 µg/ mL (Hg^{2+}), 100 µg/ mL (Cd^{2+} and Cr^{6+}), and 600 µg/mL (Cu^{2+}), respectively (Allen *et al.*, 1977). The operational definition of tolerance used in this study was based on the positive bacterial growth when the concentration of each heavy metal was above the stated concentration for resistance.

3. Results

The total plate count of *Salmonella* spp. from the water sample in the Asian sea bass hatchery was 1.0 X 10^3 colony forming unit (CFU)/ml. In the present study, all bacterial isolates were found resistant to lincomycin and oleandomycin. However, more than 46 % of the bacterial isolates were found to be resistant to fosfomycin, furazolidone, ampicillin, doxycycline and spiramycin (Fig. 1). On the other hand, more than 70% of the bacterial isolates were sensitive to nitrofurantoin, tetracycline, and oxolinic acid. More than 50% of the present bacterial isolates were sensitive to colistin sulfate, erythromycin, and florfenicol. Overall, in the antibiotic susceptibility test, 49.5 % was recorded as antibiotic resistance case and 4.8% and 45.7 % was recorded as intermediary sensitive and sensitive case. The MAR value of the present study was 0.50. With regards to the heavy metal tolerance test, 66.7% and 73.3% of present bacterial isolates were sensitive to Cd^{2+} and Cu^{2+} respectively. 33.3 % of the bacterial isolates were sensitive to Hg^{2+}. All the bacterial isolates were found to be resistant to Cr^{6+}.

4. Discussion

The results of the present study reveal the existence of *Salmonella* spp. in the flesh of the Asian sea bass. However, the population of the bacteria in the sample is still within the safety level for human consumption. As long as the hygiene procedure was taken during the food preparation, the risk of transmission of *Salmonella* spp. to humans via fish products from that sampled area is minimal. In the present study, 46.7% of *Salmonella* spp. was found to be resistant to florfenicol. This may due to the bacterial strains that developed a florfenicol resistance gene in their genomic property. The existence of this florfenicol resistance gene was detected as early as 1969; this gene was found in a plasmid of *Klebsiella pneumoniae* in France (Smith, 2008). Furthermore, it was found in *S. enterica* serovar *typhimurium* DT 104 in the United States in 1985 (Smith, 2008), and in a plasmid of *Vibrio damsela* that infected fish farms in Japan in 1990s (Kim *et al.*, 1993). Hence, we may conclude that this antibiotic resistance gene can be found in various species of bacteria. Subsequently, the incidence of florfenicol resistance was widely spread among bacteria species including the bacterial strains in the present study. All the present bacterial isolates were found resistant to oleandomycin and lincomycin. Therefore, we suggested that these two types of antibiotics may be used as supplement for *Salmonella* spp. isolation medium to inhibit the growth of other microorganisms such as fungal.

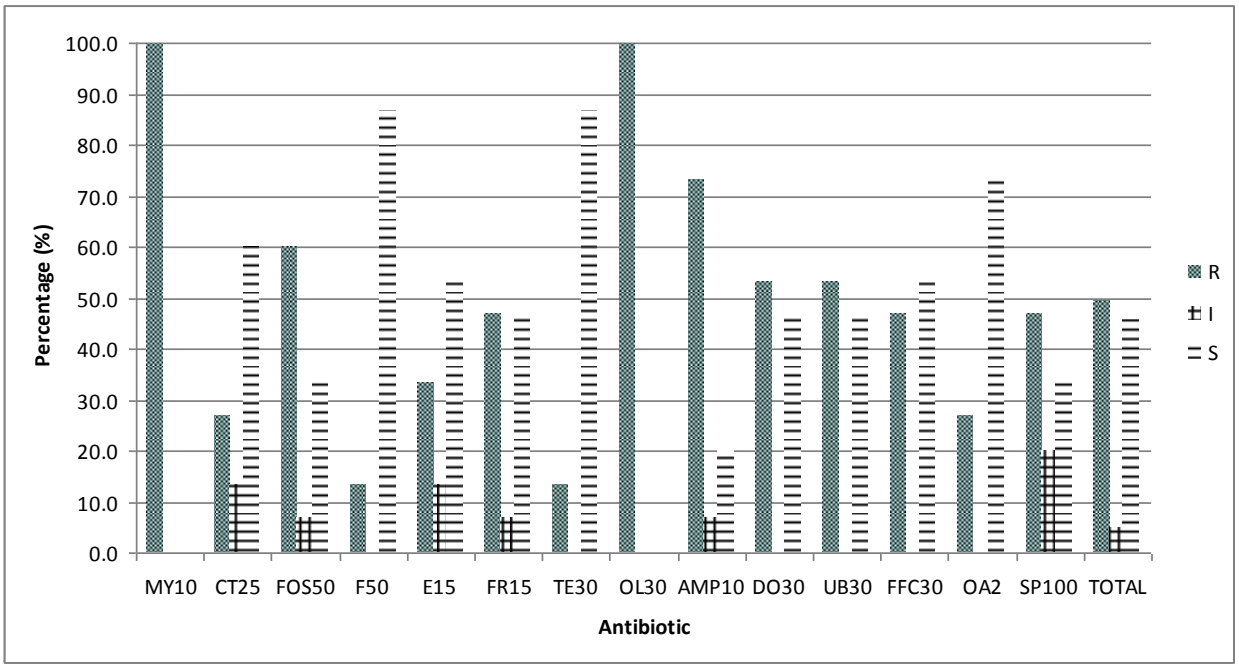

Figure 1. Antibiotic sensitivity of *Salmonella* spp. isolated from wild Asian sea bass.

OA2; oxolinic acid (2 µg/disk), F50; nitrofurantoin (50 µg/disk), AMP10; ampicillin (10 µg µg/disk), E15; erythromycin (15 µg/disk), FR15; furazolidone (15 µg/disk), MY10; lincomycin (10 µg/disk), CT25; colistin sulfate (25 µg/disk), OL30; oleandomycin (30 µg/disk), FOS50; fosfomycin (50 µg/disk), DO30; doxycycline (30 µg/disk), FFC30; florfenicol (30 µg/disk), UB30; flumequine (30 µg/disk), TE30; tetracycline (30 µg/disk) and SP100; spiramycin (100 µg/disk)

Furthermore, oleandomycin was recognized as fungicidal antibiotic as early as 1958 (Holzer, 1958). Several studies reported that most of the bacterial isolated obtained from aquatic animals were found highly resistant to oleandomycin and lincomycin. For instance, Lee *et al* (2009a) claimed that *Vibrio* spp. isolated from *Litopenaues vannamei* were highly resistant to these two types of antibiotics. Similar finding was also observed in the study of Lee *et al.* (2009b) (bacteria isolated from giant freshwater prawn), Lee *et al.* (2009c) (bacteria isolated from Asian seabass) and Lee *et al*. (2009d) (bacteria isolated from American bullfrog). A similar resistance pattern was also documented of bacteria isolates from freshwater Asia seabass fingerling Lee *et al*. (2010a) and silver catfish and red hybrid tilapia (Lee *et al*., 2010b). Hence, we may conclude that most of the bacterial isolates from aquatic animals were found resistant to oleandomycin and lincomycin. The high value of MAR that was observed in the present study indicates that the fish of the present study was under high risk of being exposed to the tested antibiotics in which the result can give us information on the existence or contamination of the tested antibiotics residues in the sampling areas.

In addition to antibiotic test, however, a low percentage of the present bacterial isolates was resistant to Cd^{2+} and Cu^{2+}, but a high percentage of the present bacterial isolates was resistant to Hg^{2+} and Cr^{6+}. This indicates that the sampling areas in our study may have been exposed to these heavy metal residues and this may be due to agricultural activities that surrounded the sampled area in which the discharged agricultural wastes such as fertilizer may contain heavy metal residues. Many studies on the heavy metal resistance pattern of bacteria isolated from various aquatic animals namely Giant freshwater prawn, *Macrobrachium rosenbergii* (Lee *et al*., 2009a), Asian seabass, *Lates calcarifer* (Lee *et al.*, 2009b), American bullfrog, *Rana catesbeiana* (Lee *et al*., 2009c), Silver catfish, *Pangasius sutchi* (Lee *et al.*, 2010a) and red hybrid tilapia, *Tilapia* sp (Lee *et al*., 2010b) were reported in the literature.

In conclusion, the results of the present study indicate that the sampling areas may be contaminated with the tested antibiotic and heavy metal residues. Therefore, we proposed that further study should be carried out in the near future to reveal the actual status of contamination of the sampling sites before we can come to a final decisive conclusion.

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Micromycetes in Sand and Water along the Algerian Western Coastal Areas

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Abstract

The littoral represents the final compartment of the entropic rejections generating great quantities of organic matters. These areas represent an ideal place for the development of saprotrophic fungal communities. The distribution and diversity of microfungi in the littoral region of the Algerian Western Coast was investigated for the first time. Sixty four samplings of sand (surface and 5 cm deep) and water (surface and 1 m deep) were carried out during the dry and rainy seasons. From the study, more than 250 strains belonging to 15 different genera had been isolated from water and sandy beaches. Data indicated a clear abundance (85%) of *Penicillium*, *Aspergillus*, Muccorales, *Cladosporium*, *Trichoderma* and *Fusarium* in the littoral region of the Algerian Western Coast.

Keywords: Filamentous fungi, sea water, sand beaches, Algerian western coast, littoral.

1. Introduction

In the seas and oceans, the fungus occupied all the habitats of surface water, and some fungal biotypes are adapted to living deeply in the ocean. There are many environmental problems, like marine erosion, pluvial water and domestic waste disposal on the beach, causing the water to be inappropriate for bathing and contact sports in some areas.

Fungi are important components of ecosystems as they are cosmopolitan and usually isolated from tropical, subtropical and temperate countries (Smith and Berry, 1975). They are considered the most active microorganisms in the decomposition of organic compounds both in sand and water (Moore-Landecker, 1996). When compared to thousands of fungal species known from terrestrial environments, only 500 have been described for oceans and estuaries although they comprehend the largest part of the Earth's surface (Kohlmeyer and Kohlmeyer, 1979; Kohlmeyer and Volkmann-Kohlmeyer, 1991). The described species are mainly the anamorphous of Ascomycota and Basidiomycota, including some marine yeast. Some of these marine filamentous fungi are parasites of marine algae or marine angiosperms, or they grow symbiotically with brown algae (Kingham and Evans, 1986). Most of the publications referring to filamentous fungi in marine environments are centered on Europe and North America (Dabrowa *et al.*, 1964, Kishimoto and Baker, 1969; Kirk, 1983; Tan, 1985). However, in Brazil, studies such as Faraco and Faraco (1974), Mattede *et al.* (1986), Purchio *et al.* (1988), Pinto *et al.* (1992), and Sarquis and Oliveira (1996) may be highlighted. Very recently, a special interest, by certain scientists in the world, was established to investigate marine fungi because of their important utility in agroalimentary and health and their great harmful effects through producing toxic substances that cause digestive dysfunctions to humans.

By following up the causes behind many food poisoning cases recorded in Canada and France, researchers identified marine fungi as the only reason behind these pathological signs that usually appear in individuals who consume seafood containing shells (Brewer *et al.*, 1993). Many authors emphasized that fungal species in marine environment are known to produce mycotoxins. Among those authors, Landreau (2001) studied the metabolites of *Trichoderma koningii Oudemans*; Grovel (2002) and kerzaon *et al.* (2008) characterized the gliotoxin secreted by *Aspergillus fumigatus*; Petit *et al.* (2004) investigated the griseofulvin of *Penicillium waksmanii*, and Mohamed-Benkada (2006) evaluated the fungal risk in conchylaceous zone of *Trichoderma* genus.

Oran city has approximately two million inhabitants and about 70% of the population is served by a sewage collection system. The sewage is discharged in the sea without treatment. Ain Eturk, Eden, Andalouses, and Madagh represent the main beaches in Oran. However,

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they present serious environmental problems as they are intensively visited by tourists and local visitors (Boutiba *et al*., 2003). The mycota of these beaches is practically unknown, especially with respect to human pathogens. Therefore, considering the lack of studies on the diversity of filamentous fungi in Oran beaches, the aim of this work was to isolate and identify filamentous fungi from four sandy beaches during the dry and rainy seasons.

2. Material and Methods

2.1. Study area

Oran city is approximately 2121 km² located at 35°43'N 0°37'W. The beaches of Ain Eturk (S1), Eden (S2), Andalouses (S3), and Madagh (S4) are the main beaches in Oran (Fig. 1). As urban beaches, they are intensively visited by tourists and locals, and present serious environmental conflicts (Boutiba *et al*., 2003).

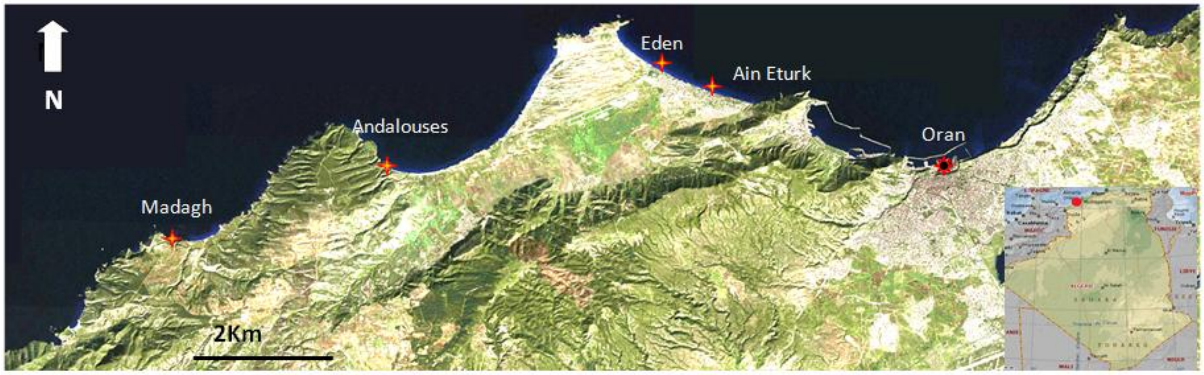

Figure 1. A map showing the study area, Oran city and its main beaches.

The sampling points were chosen in the regions that have the greatest access of bathers and the highest release of domestic sewage.

2.2. Sand and water samplings

Sand and water samplings were concomitantly collected in Ain Eturk, Eden, Andalouses and Madagh beaches, during rainy season (December/2008 and January/2009**)** and during dry season (June and July/2009). Sixty four sand and water samples were concomitantly collected. The sand sampling in each site was carried out using a little garden shovel, in mid-seashore at one meter from the tideline. Samples were taken from surface soil and below at 5 *cm* deep. The samples were placed in labeled plastic bags.

In each sampling site, water samples were collected from surface water and 1 *m* deep using sterile labeled glass tubes. The sand and water samples were kept at room temperature and transferred to the laboratory for later experimental work.

2.3. Isolation, purification and identification of filamentous fungi

Each sand sample (50 g) was diluted in 90 ml of sterilized distilled water, with addition of some drops of Tween 80 making it possible to suspend the spores which could adhere to the grains of sand. The solution was centrifuged at 2500 r/min for 15 min, and then 0.5 ml of the supernatant was spread, in triplicate, onto Petri dishes containing Sabouraud's dextrose Agar (SDA) with chloramphenicol (500 mg/l). Each water sample (0.5 ml, undiluted) was also spread onto Petri dishes as above. The cultured plates were incubated at 27 °C (±2 °C). As soon as the first colonies were developed, they were transferred to test tubes containing SDA. After the purity of the colonies was confirmed, they were subcultured onto Potato Dextrose Agar, or Czapeck's Agar, in glass tubes. Fungal identification was carried out by macroscopic and microscopic observations of colonies and when needed, mainly for identification of species level, by microculture on a microscopic glass slides (Riddell, 1950; and Pitt, 1985)

2.4. Sand and water abiotic analysis

Sand and water pH and temperature were measured in the morning with a digital pH-Meter and digital thermometer (Hanna), respectively. Salinity was obtained using a handheld salinity refractometer.

3. Results and Discussion

3.1. Sand and water abiotic data for Ain Eturk, Eden, Andalouses and Madagh beaches

Factors such as water salinity, temperature and pH may influence the activity, abundance and distribution of marine fungi (Dix and Webster, 1995; Sridhar, 2009). Moulds can grow between 0 and 35°C. Certain species are able to grow with extreme temperatures; *Cladosporium herbarum* can develop with temperatures lower than 0°C, and *Aspergillus flavus* or *Aspergillus fumigatus* can tolerate temperature until 60°C (Bourgeois et al., 1996).

The water temperature of Ain Eturk, Eden, Andalouses and Madagh beaches ranges from 20.9 °C to 23.6 °C in the dry season (June and July). In the rainy season (December and January), the minimum water temperature was 13.3 °C while the maximum temperature was 18.2°C. The sand temperature in the dry season reached its minimum at 21.1 °C and maximum at 23.6 °C, and in the rainy season, it was between 13.1 and 19.4 °C. Salinity and the temperature are the most important factors affecting the marine fungi diversity (Booth and Kenkel, 1986).

In Ain Eturk, the water and sand salinity was of 24 and 38.1‰ in the rainy and dry seasons, respectively. Water and sand salinity in Eden was of 20 and 37.8% in the dry and rainy seasons, respectively. Water and sand salinity in Andalouses was of 24 and 37% for Madagh, water and sand salinity was between 15 to 37.4% (Table 1). All water

and sand samples had a slightly alkaline pH, varying from 7.78 to 9.21.

In general, marine fungi need high temperatures (usually between 25 and 30 °C) to reproduce (Griffin, 1981). According to Gambale *et al*. (1977), salinity has a great influence in the microbiota of the estuaries. Different studies showed that salinity seemed to affect the metabolism of fungi, as unusual physiological or morphological effects were observed on terrestrial fungal strains when grown on media containing high sodium chloride concentrations (Tresner and Hayes, 1971). Sodium chloride also induced inhibition of cellulolytic ability of fungi belonging to the genus *Aspergillus* (Malik et al., 1980), and Frisvad and Samson (2004) proposed the use of a culture medium with 5% of sodium chloride for the study of fungal secondary metabolites to enhance their production. The tolerance of fungal growth toward salt in the medium seemed to change from species to species and it appeared to be dependent on the culture temperature (Rai and Agarwal, 1973; Mert and Dizbay, 1977; Mert and Ekmekci, 1987).

Table 1. Temperature, pH and salinity of the water and sand from Ain Turk, Eden Andalouses and Madagh beaches.

	Sampling date	Ain Turk						Eden					
		water			Sand			water			Sand		
		pH	T (°C)	S (%)	pH	T (°C)	S (%)	pH	T (°C)	S (%)	pH	T (°C)	S (%)
Rainy saison	12/04/08	7.84	13.3	30	8,15	15,2	25.0	7.78	14.3	29	8,36	13,1	20
	12/20/08	7.87	13.3	31	8.60	16.5	26.0	7.88	14.5	28	8.70	15.2	20.5
	01/03/09	7.88	13.4	29.5	8.61	18.9	24.0	7.94	13.6	27.5	8.85	13.9	30
	01/29/09	7.94	17.0	32	8.70	19.4	28.0	7.94	18.2	30	8.80	14.0	36
Dry saison	06/04/09	8.12	21.0	37	8.82	21.1	26.0	7.98	20.9	36	8.85	22.0	37
	06/23/09	8.17	21.3	37	8.80	21.9	32.0	7.97	22.0	36.2	8.83	22.3	37
	07/01/09	8.25	21.5	37.5	8.85	22.5	36.5	8.12	22.2	36.5	8.85	23.4	37.5
	07/18/09	8.56	22.2	38	9,18	22,9	38.1	8.22	22.3	37.4	9,21	23,6	37.8

	Sampling date	Andalouses						Madagh					
		Water			Sand			Water			Sand		
		pH	T (°C)	S (%)	pH	T (°C)	S (%)	pH	T (°C)	S (%)	pH	T (°C)	S (%)
Rainy saison	12/04/08	7.87	14.1	32	8.34	16.5	24.0	7.88	14.3	34	8.72	16.5	15
	12/20/08	7.84	14.3	31.5	8.60	16.4	26.0	7.98	14.0	33	8.75	16.7	20
	01/03/09	7.94	17.4	33	8.60	18.0	29.5	8.12	18.0	34	8.80	17.3	30
	01/29/09	8.07	16.0	34	8.65	18.4	32.0	8.22	18.2	35	8.82	17.0	33
Dry saison	06/04/09	8.12	21.5	35	8.72	23.0	35.0	8.94	21.9	36	8.88	22.3	36
	06/23/09	8.15	22.0	35	8.83	25.9	36.0	8.94	22.0	36.5	8.73	22.6	36.4
	07/01/09	8.46	22.3	36	8.95	23.4	37.6	8.97	22.3	37	8.95	23.4	37
	07/18/09	8.88	22.3	36.5	9.17	23.5	37.0	9.18	22.4	37.4	9.16	23.5	37.1

Furthermore, those strains from saline environment also showed a reduction in growth rate related to the increase of salt concentration in solid medium (Tresner and Hayes, 1971; Atapattu and Samarajeewa, 1990; Rojas *et al.*, 1991; Tepsic *et al.*, 1997).

Cantrell *et al.* (2006) found 86 isolates from hypersaline environments: *Cladosporium*, *Aspergillus* and *Penicillium.* In Portugal, Barata (2006) reported that the water temperature was (12 to 24°C), the pH (6.6 to 8), and the salinity (2.57 to 31.81). High salt concentrations also affected intracellular growth activities of a strain of *A. foetidus* grown successively on culture media with increasing salt concentrations. It was assumed that this phenomenon would result from the requirement of the organism to synthesize osmo-protective compounds for cell-wall integrity and vacuolar morphology (Thangavelu *et al.*, 2006).

According to Kerazon *et al.* (2008), while fungal development is limited to *A. fumigatus* strains occurring in marine environment, their excretion of gliotoxin is stimulated by a salinity of 33 g/L which would lead to an amplification of toxin release in marine surroundings. In Brazil, Gomes *et al.* (2008) found that the temperature range of water and sand in both rainy and dry seasons was (24.3 to 28.8°C); however the range of pH was 7.70 to 8.22 and the range of salinity was 15 to 40%.

Table 2. Number of strains isolated from each sample collected from the Algerian western coastal areas. S1: Ain Eurk, S2: Eden, S3: Andalouses , S4: Madagh.

			Water								Sand							
			Rainy season				Dry season				Rainy season				Dry season			
	Total	%	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4
Penicillium	105	41.66	3	1	2	1	3	2	2	1	19	10	13	6	15	11	7	9
Aspergillus	49	19.4	2	1	1	−	1	1	1	2	9	7	6	4	5	4	2	3
Muccorales	29	11.50	2	−	−	−	1	−	−	1	4	3	2	3	5	3	1	4
Cladosporium	16	6.34	2	1	−	−	2	−	2	1	2	2	1	0	2	0	1	0
Trichoderma	8	3.17	−	−	−	−	−	−	1	−	3	2	1	0	0	0	0	1
Fusarium	7	2.77	−	−	−	−	−	−	−	−	2	3	0	0	1	1	0	0
Alternaria	6	2.38	−	−	−	−	−	−	−	−	2	1	1	0	1	1	0	0
Pullularia	4	1.58	1	−	−	−	−	−	−	−	2	0	0	0	0	0	0	1
Verticillium	3	1.19	−	−	−	−	−	−	−	−	1	1	0	0	0	0	0	1
Geotrichum	3	1.19	−	−	−	−	−	−	−	−	2	1	0	0	0	0	0	0
Scopulariopsis	3	1.19	−	−	−	−	−	−	−	−	1	2	0	0	0	0	0	0
Gliocladium	2	0.79	−	−	−	−	−	−	−	−	1	0	0	0	1	0	0	0
Phialophora	2	0.79	−	−	−	−	−	−	−	−	1	0	0	0	1	0	0	0
Acremonium	1	0.39	−	−	−	−	−	−	−	−	1	0	0	0	0	0	0	0
Chrysosporium	1	0.39	−	−	−	−	−	−	−	−	1	0	0	0	0	0	0	0
Unknowns	13	5.15	2	1	1	−	2	−	1	3	0	0	0	0	1	0	1	1
Total	252		12	4	4	1	9	3	7	8	51	32	24	13	32	20	12	20

About 252 fungal strains were isolated (Table 2, Figure 2) and identified from Algerian Western coastal areas for all types of samples combined, belonging to 15 genera of Ascomycetes subphylum. Fungal communities were represented predominantly by strains of *Penicillium* (41.66%), *Aspergillus* (19.44%), Muccorales (11.50%), *Cladosporium* (6.34%), *Trichoderma* (3.17%), *Fusarium* (2.77%), *Alternaria* (2.38%), *Pullularia* (1.58%), *Verticillium* (1.19%), *Geotrichum* (1.19%), *Scopulariopsis* (1.19%) *Gliocladium* (0.79%), *Phialophora* (0.79%), *Acremonium* (0.39%), *Chrysosporium* (0.39%), and "unknowns" (5.15%). These results are in agreement with Oliveira (1996) who particularly studied the diversity of filamentous fungi on Ipanema beach, Sarquis and isolated 34 genera and 170 species. The genera with the most frequent species were: *Aspergillus* (30.4%) and *Penicillium* (16.2%). In Brazil, fifty seven species were isolated with *Aspergillus*, and *Penicillium* were the most frequent genera in both sand and water, with a total of 11 and 19 species, respectively (Gomes *et al.*, 2008). Similar to the present study, Tauk-Tornisielo (2005) isolated soil filamentous fungi from the Ecological Park of Juréia-Itatins, finding that *Aspergillus* and *Penicillium* were the genera with the highest diversity of species. Studying the incidence of anemophilous fungi isolated from Praia do Laranjal, Pelotas, RS, Bernardi and Nascimento (2005) identified 18 genera. The authors found that *Cladosporium* (18.22%), *Alternaria* (13.84%), *Penicillium* (10.20%), *Curvularia* (7.47%) and *Aspergillus* (3.28%) were the genera with the species most frequently found. Moreover, Migahed (2003) studied the fungal genera of highest incidence and their respective numbers of species, concluding that *Penicillium* and *Aspergillus* were the most. Thirty-two manglicolous marine fungi belonging to 23 ascomycetes, 1 basidiomycete and 8 mitosporic fungi were observed from South India coast (Gayatri *et al.*, 2008). It should be noted that a large number of strains isolated from seawater samples could not be identified because their mycelium remained sterile (Gomes *et al.*, 2008).

During the dry and rainy seasons, from sea water and sand, 104 isolate of filamentous fungi, mostly anamorphous, were isolated and identified from S1, 59 from S2, 47 from S3 and 42 from S4 (Figure 2). Some species were common for all the beaches, like *Penicillium, Aspergillus*, Muccorales *and Cladosporium.*

The number of genera in S1 was higher than that in other beaches; this may be due to the fact that this beach is more frequently visited by tourists and local bathers than the other beaches. It may be also due to the proximity of this site to sources of waste water rejections, which certainly contributes to increasing pollution. Similar facts were observed in a study carried out by Purchio *et al.* (1988) and Gomes *et al.* (2008). Conversely, the opposite was found by Mattede *et al.* (1986). The authors evaluated dry and wet sand samples from polluted and non-polluted beaches of the city of Vitória, Espírito Santo and found that the incidence of fungal genera was greater in non-polluted beaches (55%) than in polluted ones (45%).

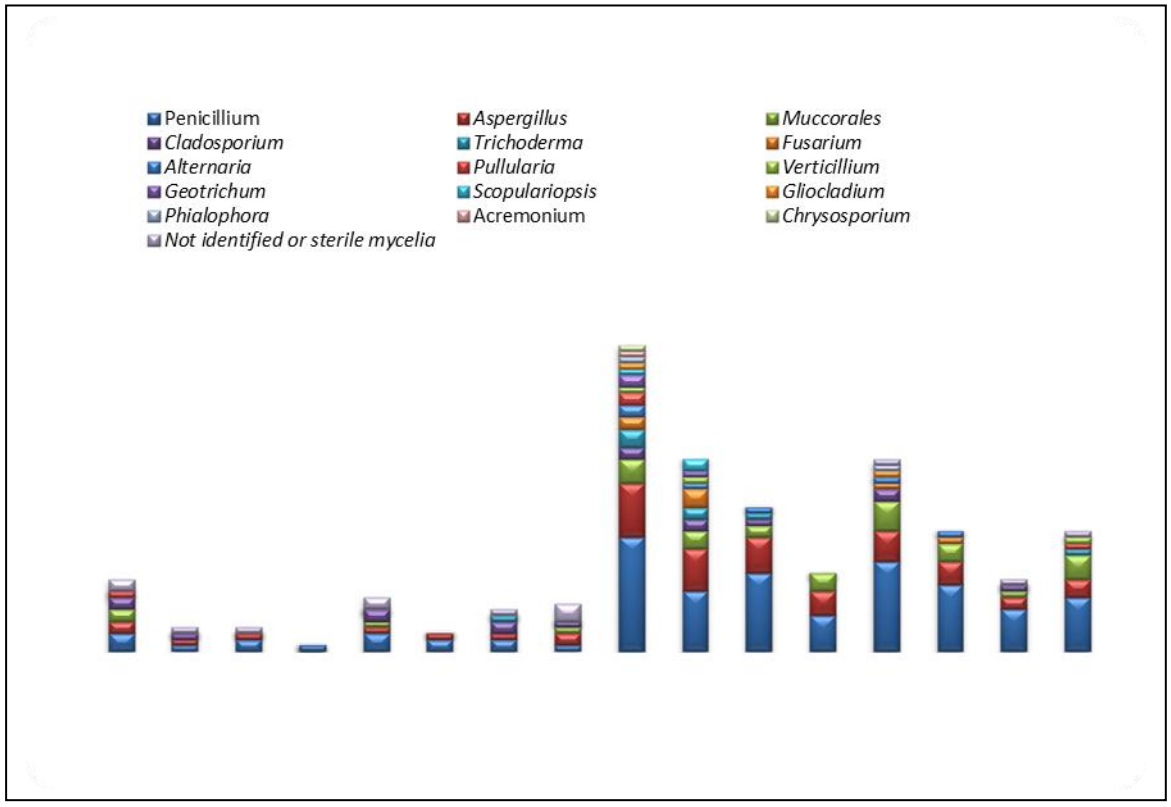

Figure 2. Micromycetes isolated from the four studied beaches of Algerian Western coast.

S1: Ain Eturk, S2: Eden, S3: Andalouses and S4: Madagh

The comparison of the total number of strains isolated by sample type (Table 2) shows that the strain diversity of individual samples was less in seawater (20%) than that of sand (80%). A comparison of the strains found in sand with those isolated from water sand samples had a greater number of strains in which *Penicillium* spp, *Aspergillus* spp, *Cladosporium* spp, Mucorales and *Trichoderma* spp were presented in all types of samples including both sand and water. The similarity in species composition and abundance suggests that these genera are adapted to the marine environment. Genera like *Fusarium*, *Alternaria*, *Pullularia*, *Verticillium*, *Geotrichum*, *Scopulariopsis*, *Gliocladium*, *Phialophora*, *Acremonium* and *Chrysosporium* were isolated only from sand samples (Table 2). Thus, the strains found in sand would appear to be more representative of those really present and well-established in the marine environment (Sallenave, 2000). The number of isolates from samples collected during the rainy season (56%) is greater than that collected during the dry season (44%). The results obtained in the present study agreed with similar studies (Gomes *et al.*, 2008). Harrison and Jones (1975) clearly showed that many mould saprolegniales from fresh water cannot reproduce with salinity higher than 30%. During the dry seasons, when the salinity is raised, marine fungi prevail, contrary to that is the case of the rainy season when the salinity is low and the terrestrial fungi are dominant (Sadaba, 1996).

The investigation carried out by Jessica (2004) on three beaches in Bay Mayagüez to Puerto Rico revealed elevated levels of fungi species during the rainy season. The author correlated this distribution with salinity. Salinity variations had a significant effect upon abundance of fungi, especially during the months of May through August, when the temperatures were higher, causing an elevation of salinity levels in sand due to seawater evaporation.

The main pathogenic fungi to humans and animals can be found within the anamorphic fungi. These fungi are saprophytic and occasionally pathogenic, and can be isolated from water, soil, animals and humans. Species of *Aspergillus*, *Cladosporium* and *Penicillium*, are found in these beaches, and can be a source of infection for superficial and deep mycosis (Sidrim and Moreira, 1999).

In conclusion, this study shows that Algerian Western coastal areas could represent a wide source of fungal communities. Thus, it would appear that littoral coast represents a large fungal reservoir whose role is little understood but is possibly important for animals, plants and the marine and terrestrial ecosystems. Ain Eturk had the highest number of filamenous fungi contamination (41.2%), followed by Eden (20.6%), Andalouses (17.5%), and Madagh (16.6%). The presence of some toxinogenic moulds in marine environment, such as *Penicillium* and *Trichoderma* strains, could represent a potential risk of toxicities for shellfish, involving contaminations and possibly intoxications of shellfish consumers (Sallenave *et al*., 2000). It would be interesting to evaluate the toxicity of some micromycetes isolated from these coastal areas by realising a bioassay screening.

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Production, Purification and Characterization of Protease by *Aspergillus flavus* under Solid State Fermentation

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Abstract

Protease production under solid state fermentation (SSF) was investigated using isolated *Aspergillus flavus*. Different agro-industrial waste products were evaluated to check the possibility of potential utilization of substrates in SSF for protease production by *Aspergillus flavus* using wheat bran as a substrate. The results showed that the optimum conditions for maximum protease production were found to be 7^{th} day of incubation at pH 5.0, temperature 30^{o}C; inoculum size 3%; substrate concentration 3% and 3% KNO_3 as nitrogen source. The purified enzyme produced 5.8 fold with recovery of 3.2% by DEAE-column chromatography and the molecular weight was estimated to be 46kDa by SDS-PAGE. It has a V_{max} value of 60.0 U/mg and K_m value of 0.6 mg/ml at pH of 7. The enzyme activity was found to be stable at 50^0 C and it was stimulated by metal ions like Cu^{2+} and Zn^{2+} and inhibited by Ca^{2+} and Mg $^{2+}$.

Keywords: protease, *Aspergillus flavus*, purification, SDS-PAGE, wheat bran, metal ions, precipitation.

1. Introduction

Protease constitutes a large and complex group of enzymes that plays an important nutritional and regulatory role in nature. Proteases are (physiologically) necessary for living organisms; they are ubiquitous and found in a wide diversity of sources. Protease is the most important industrial enzyme of interest accounting for about 60% of the total enzyme market in the world and account for approximately 40% of the total worldwide enzyme sale (Godfrey and West, 1996; Chouyyok *et al.*, 2005). They are generally used in detergents (Barindra *et al.*, 2006), food industries, leather, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Rao *et al.*, 1998; Paranthaman *et al.*, 2009). They also have medical and pharmaceutical applications.

Microbial proteases are degradative enzymes, which catalyze the total hydrolysis of proteins (Raju *et al.*, 1994; Haq *et al.*, 2006). The molecular weight of proteases ranges from 18 – 90 kDa (Sidney and Lester, 1972). These enzymes are found in a wide diversity of sources such as plants, animals and microorganisms but they are mainly produced by bacteria and fungi. Microbial proteases are predominantly extracellular and can be secreted in the fermentation medium.

Solid state fermentation (SSF) was chosen for the present research because it has been reported to be of more grated productivity than that of submerged fermentation (Ghildyal *et al.*, 1985; Hesseltine, 1972). Economically, SSF offers many advantages including superior volumetric productivity, use of simpler machinery, use of inexpensive substrates, simpler downstream processing, and lower energy requirements when compared with submerged fermentation (Paranthaman *et al.*, 2009). Fungi elaborate a wide variety of proteolytic enzymes than bacteria. The filamentous fungi have a potential to grow under varying environmental conditions such as time course, pH and temperature, utilizing a wide variety of substrates as nutrients (Haq *et al.*, 2006). Several species of strains including fungi (*Aspergillus flavus, Aspergillus melleu, Aspergillus niger, Chrysosporium keratinophilum, Fusarium graminarum, Penicillium griseofulvin, Scedosporium apiosermum*) and bacteria (*Bacillus licheniformis, Bacillus firmus, Bacillus alcalophilus, Bacillus amyloliquefaciens, Bacillus proteolyticus, Bacillus subtilis, Bacillus thuringiensis*) are reported to produce proteases (Ellaiah *et al.*, 2002).

The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of a microbial population. The most important among these are the medium, incubation temperature and pH. The pH of the fermentation medium is reported to have substantial effect on the production of proteases (Al-Shehri, 2004). It can be affect growth of the microorganisms either indirectly by affecting the availability of nutrients or directly by action on the cell surfaces. Another important environmental factor is the

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incubation temperature, which is important to the production of proteases by microorganisms. Higher temperature is found to have some adverse effects on metabolic activities of microorganisms producing proteolytic enzymes (Tunga, 1995). However, some microorganisms produce heat stable proteases which are active at higher temperatures. The thermal stability of the enzymes may be due to the presence of some metal ions or adaptability to carry out their biological activity at higher temperature (Al-Shehri, 2004; Haq *et al.*, 2006).

In the production of protease, it has been shown to be inducible and was affected by the nature of the substrate used in fermentation. Therefore, the choice of an appropriate inducing substrate is of great importance. Different carbon sources such as wheat bran, rice straw, rice bran, cotton and bagasse have been studied for the induction and biosynthesis of protease. However, wheat bran is a superior carbon source for the production of protease by *Aspergillus flavus*. So the further studies were carried out by using wheat bran as carbon source.

The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling (Singh *et al.*, 2009). Major impediments to the exploitation of commercial enzymes are their yield, stability, specificity and the cost of production. New enzymes for use in commercial applications with desirable biochemical and physiochemical characteristics and low production cost have been focus of much research (Kabli, 2007).

The purpose of this study was to isolate, produce and purify protease from *Aspergillus flavus*, and to characterize some properties of the isolated enzymes using wheat bran as a substrate under SSF.

2. Materials and Method

2.1. Cellulosic material

In our preliminary studies, various agro wastes were used as a carbon source, and, hence, it could reduce the cost of enzyme production, which is collected in dried form from cattle shop, Coimbatore. Substrates, like wheat bran, cotton seed, rice bran, rice straw and sugarcane bagasse, were screened for enzyme production, in which wheat bran showed higher protease production, so it was used for the further studies.

2.2. Organism and inoculum preparation

Fungal strains were isolated from soil of sugarcane field Coimbatore, India by serial dilution plate method (Waksman, 1922). Fungus were isolated from 10^{-3} - 10^{-4} dilutions by plating into Potato Dextrose Agar (PDA) medium. Isolated fungal cultures were screened for protease enzyme production. The organisms were identified using lacto phenol cotton blue mounting method (Konemann *et al.*, 1997). The isolated culture (*Aspergillus flavus*) was purified by routine sub-culturing and stored at 4°C for further use.

2.3. Fermentation condition

SSF was carried out in 250ml conical flask contains 10g of substrate with 10 ml of salt solution (g/l). KNO_3 2.0, $MgSO_4.7H_2O$ 0.5, K_2HPO_4 1.0, $ZnSO_4.7H_2O$ 0.437, $FeSO_4.7H_2O$ 1.116, $MnSO_4.7H_2O$ 0.203, pH 7.0 and it was autoclaved at 121°C for 30 min. After sterilization, the flasks were inoculated with 1.0ml of spore solution (10^6 spores/ ml) and incubated at 30°C for eight days in an incubator shaker at 125rpm. At the end of fermentation, cultures were extracted with 100ml of distilled water by shaking for 2hr. The filtrate obtained was centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant used as crude enzyme extract.

2.4. Protease assay

Protease activity was determined according to the modified Anson's method. 1.0 ml of the culture broth was taken in a 100 ml flask and 1.0 ml of pH 7.0 phosphate buffer added to it. One ml of the substrate (2% Hammersten's casein pH 7.0) was added to the buffer-enzyme solution and incubated at 37°C for 10 minutes in a water bath. At the end of 10 minutes, 10.0 ml of 5N TCA (trichloroacetic acid) was added to stop the reaction. The precipitated casein was then filtered off and 5.0 ml of the filtrate were taken in a test tube. To this 10.0 ml of 0.5N NaOH solution and then 3.0 ml of the folin ciocalteu reagent (one ml diluted with 2 ml of distilled water) were added. Final readings were taken in a spectrophotometer at 750 nm. Blanks of the samples were prepared by adding the TCA before the addition of substrate. The effect of various factors like noculums size, carbon source, nitrogen sources, pH and temperature on the production of protease was studied.

2.5. Purification and characterization of Protease

Crude extract was precipitated by 70% saturation with ammonium sulphate and then dialyzed against 50mM phosphate buffer (pH 7.0) for 24 hours at 40°C. The filtrate was loaded onto a DEAE-Cellulose chromatographic column equilibrated with phosphate buffer, 50mM, pH 7.0.The enzyme was eluted with a linear salt concentration gradient (Na Cl, 0-0.4 M) in the same buffer and 3.0 ml fractions were collected at a flow rate of 20 ml per hour.

SDS-PAGE electrophoresis was carried out and molecular weight was determined. The protein content was estimated by the method of Lowry *et al.* (1951). The kinetic parameter of the purified protease enzyme was determined and the optimum pH 3.0 -8.0 [The pH was adjusted using, the following buffers: 50 mM sodium citrate (pH 3.0-6.0) and 50 mM sodium phosphate (pH 7.0 & 8.0)] and temperature (30-70°C) on the activity of the enzyme was also assayed. All experiments were conducted in triplicates and their mean values represented.

3. Results

Enzyme production by micro organisms is greatly influenced by media components, especially carbon and nitrogen sources, and physical factors such as temperature, pH, incubation time and inoculum density. It is important to produce the enzyme in inexpensive and optimized media on a large scale for the process to be commercially viable; hence the studies on the influence of various physico-chemical parameters such as incubation periods, inoculum size, temperature, pH, carbon, and nitrogen

sources. Agricultural byproducts rich in cellulosic biomass can be exploited as cheap raw material for the industrially important enzymes and chemicals (Bigelow and Wyman, 2004). The fermentation medium was inoculated with the fungal strain and incubated for various time intervals (1-8 days). The enzyme production was gradually increased with the passage of time and highest enzyme activity (49.3 U mL^{-1}) was obtained on 7th day of incubation (Fig.1). It was also observed that prolonged incubation decreased the enzyme activity. However the growth of the microorganism was not significantly affected.

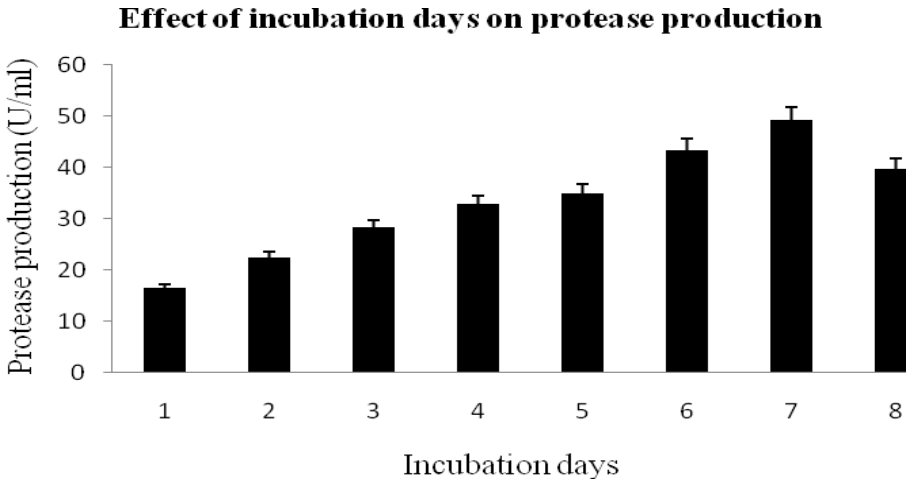


Figure1.Results are mean of three independent determinations. Bars correspond to standard deviation.

Productivity of the enzyme by culture is greatly dependant on pH of the fermentation medium. Therefore, the effect of pH (3.0 - 8.0) was studied for the production of protease by *Aspergillus flavus*. There was a gradual increase in protease synthesis from pH 3.0 to 5.0, and a maximum production of enzyme was observed at pH 4.0 i.e. 48.6 U mL^{-1} (Fig. 2).

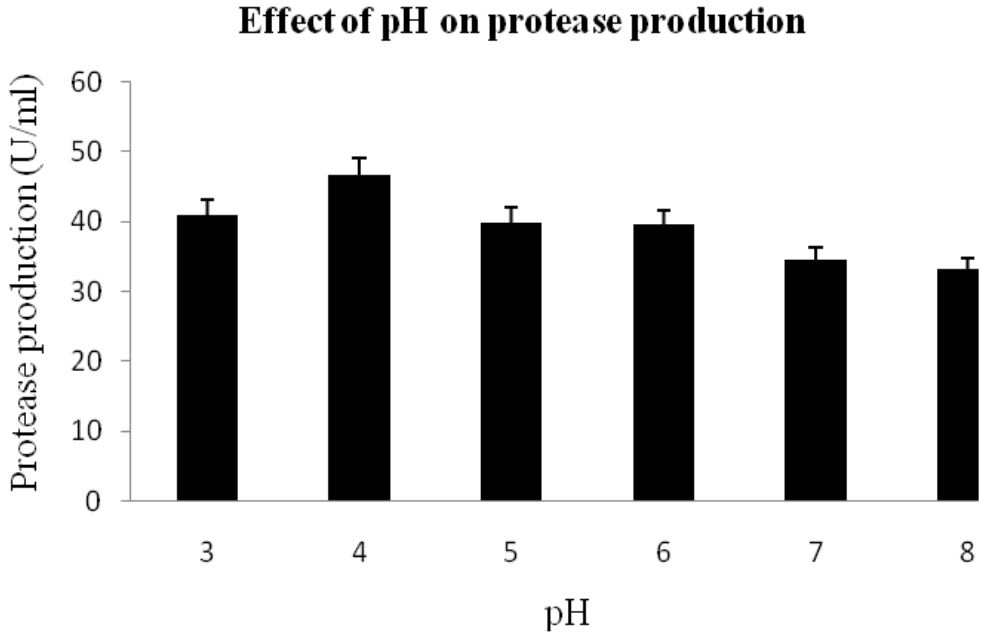


Figure 2. Results are mean of three independent determinations. Bars correspond to standard deviation.

The enzyme production by *Aspergillus flavus* at 20 - 70°C temperature range revealed that there was a sudden increase in protease production when the incubation temperature was increased from 20°C to 30°C. The enzyme production was slightly decreased up to 40°C. So the optimum incubation temperature for the production of protease was found as 30°C (Fig. 3).

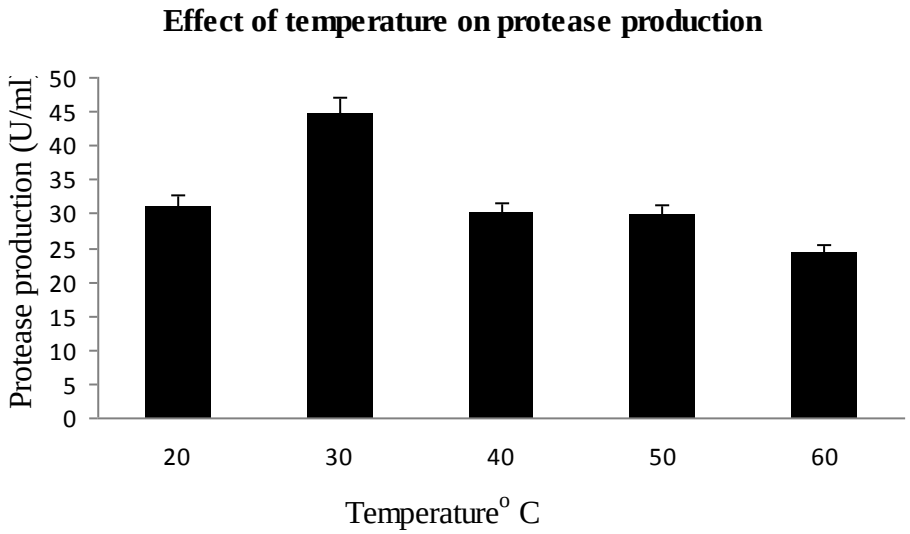


Figure 3. Results are mean of three independent determinations. Bars correspond to standard deviation.

Size of inoculum is an important biological factor in the production of the enzyme. Maximum enzyme production (Fig. 4) was obtained when SSF medium was inoculated with 3.0 ml of inoculum.

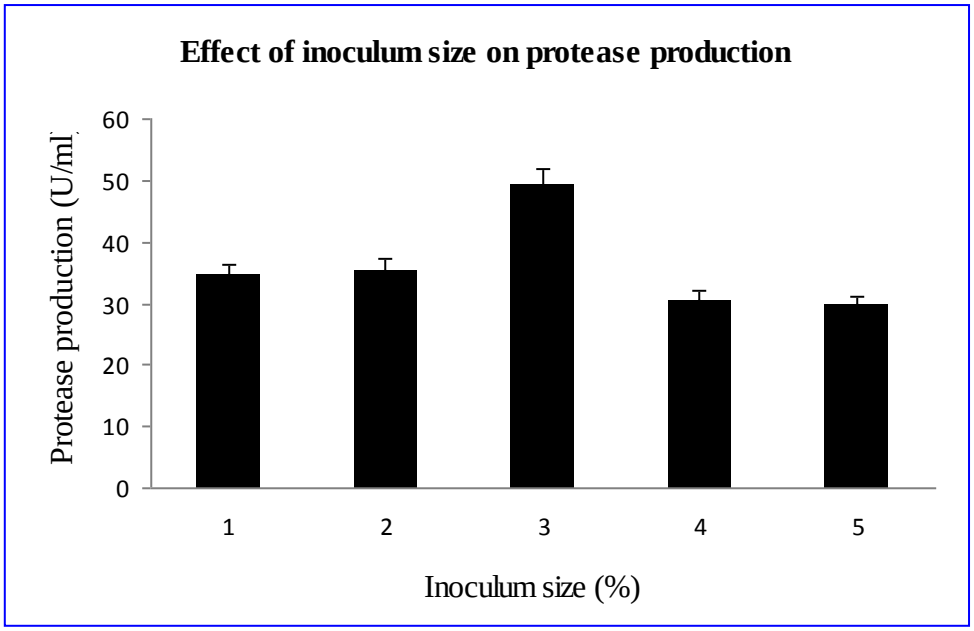


Figure 4. Results are mean of three independent determinations. Bars correspond to standard deviation.

Different agricultural byproducts such as wheat bran, rice bran, rice straw, cotton seed and sugarcane bagasse were tested for the production of enzyme (Fig. 5). Of all the substrates tested, wheat bran was found to be the best substrate for the production of protease. The other substrates gave comparatively less production of protease.

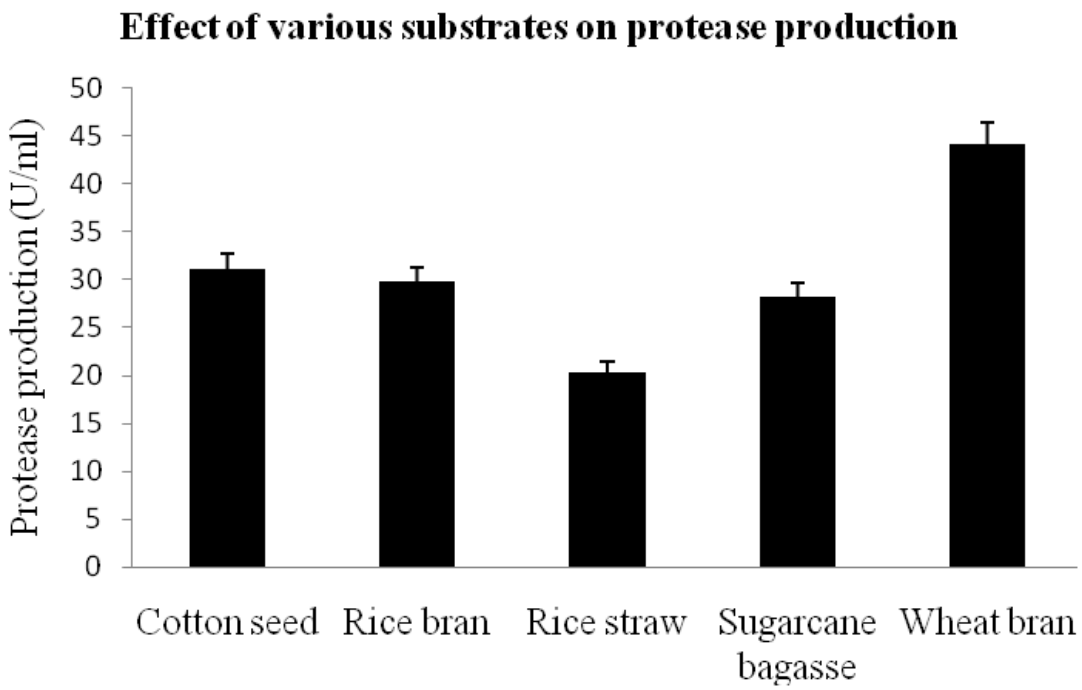


Figure 5. Results are mean of three independent determinations. Bars correspond to standard deviation.

Protease production in fermentation medium was found to be maximal when 3.0% of wheat bran was used (Fig. 6). A further increase in the amount of wheat bran resulted in a decreased production of the enzyme.

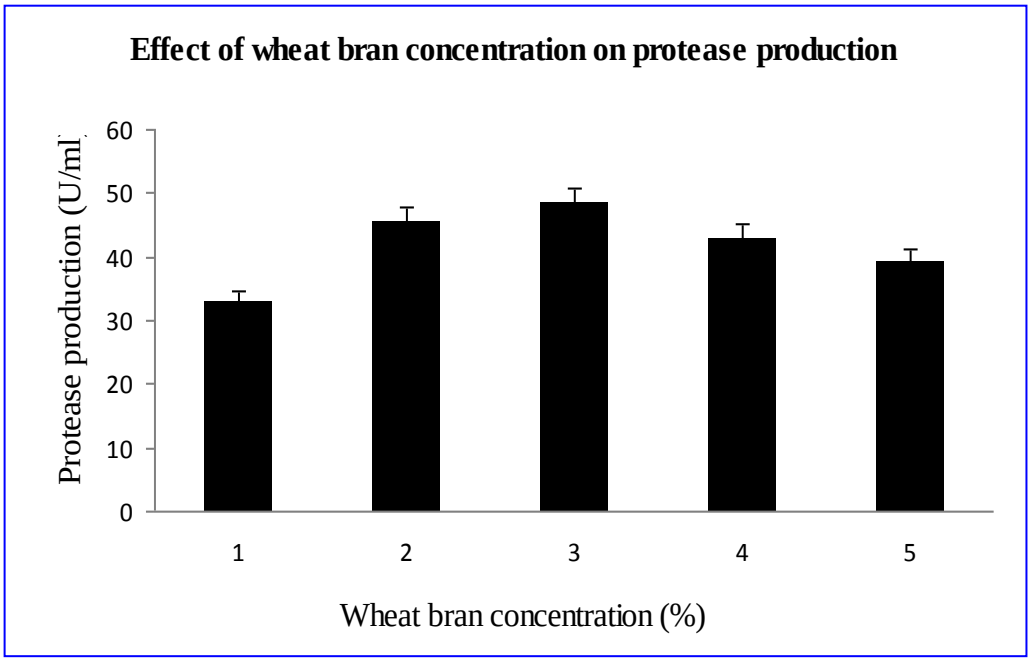


Figure 6. Results are mean of three independent determinations. Bars correspond to standard deviation.

The nitrogen sources beef extract, KNO_3, peptone, yeast extract and urea were screened for synthesis of protease (Fig 7). In that, KNO_3 (at 3% concentration) was found to be the most suitable nitrogen sources for protease production (Fig. 8).

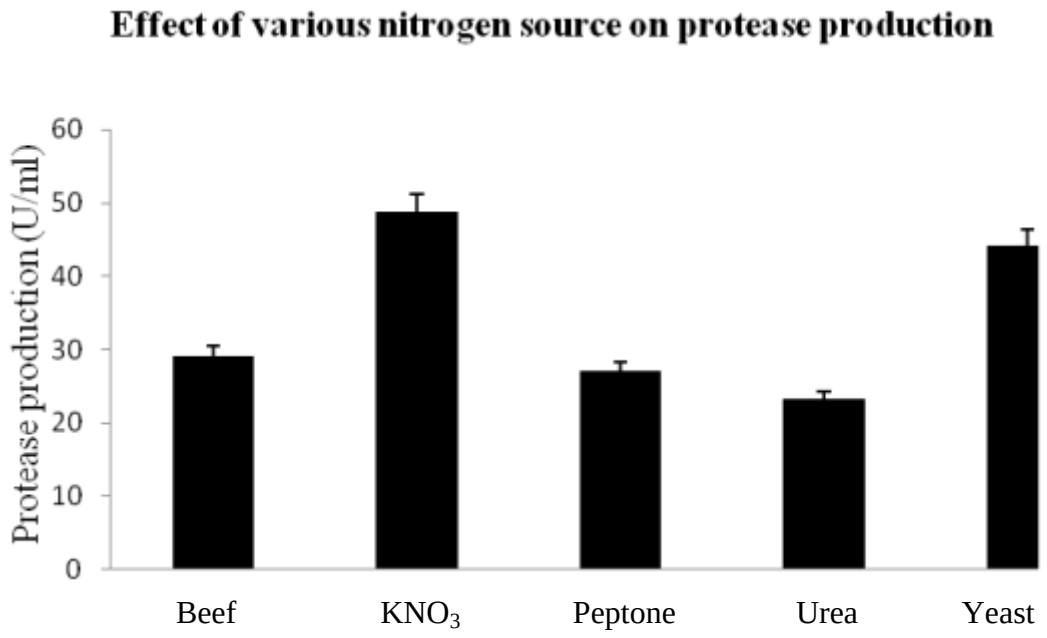


Figure 7. Results are mean of three independent determinations. Bars correspond to standard deviation.

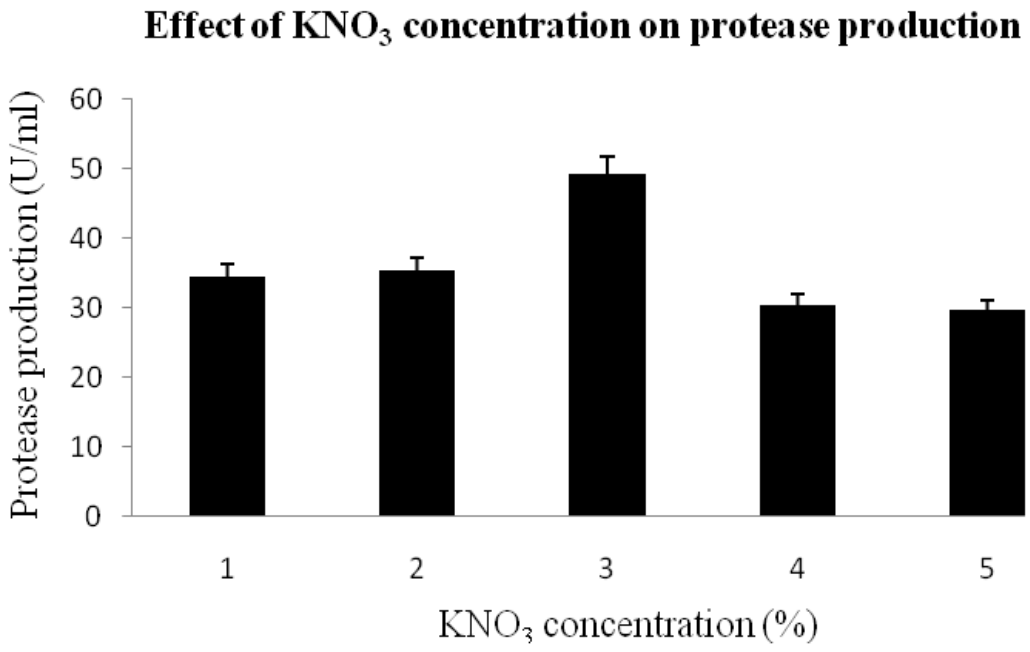


Figure 8. Results are mean of three independent determinations. Bars correspond to standard deviation.

Purification steps for protease production from *A.flavus* are given in Table 1 and Figure 9. The purification of protease resulted in 2 fold purification with 66% of recovery by ammonium sulphate precipitation. The purification of crude enzyme through DEAE cellulose column chromatography gave 5.8 fold increases in purity with 3.2% recovery of protease from *A. flavus*.

Table 1. Purification and recovery of protease from A. *flavus*

Steps	Protease production (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	8670	295.0	29.3	1	100
70% Ammonium sulphate precipitation	5750	124.0	46.3	1.58	66.32
Dialysis	323	1.9	104.17	2.53	3.72
DEAE Cellulose Column Chromatography	284	0.37	170.0	5.8	3.2

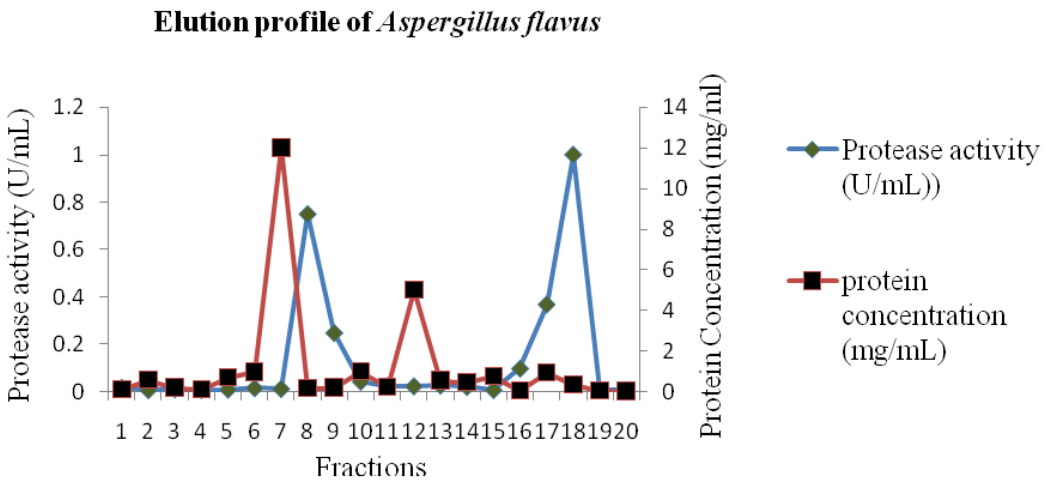


Figure 9. Results are mean of three independent determinations.

Using SDS-PAGE, the partially purified enzyme from *A.flavus* showed a single band (Fig. 10), to confirm it is an enzyme protein band, the protease activity of purified enzyme was also observed and apparent molecular weight of the purified protease was 46KDa.

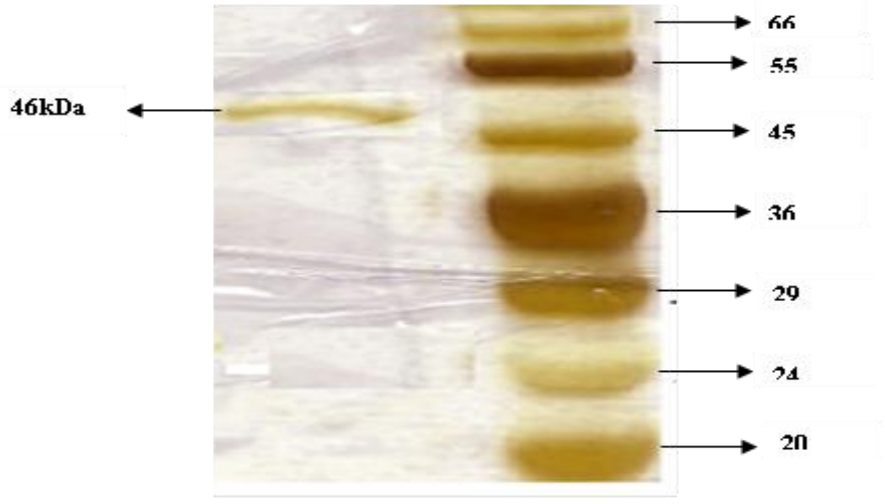


Figure 10. SDS-PAGE analysis of protease purified from *Aspergillus flavus*.

SDS-PAGE analysis of *Aspergillus flavus* strain protease. M-indicates molecular weight markers and P-shows purified protease band of molecular weight approximately 46 kDa.

Thermo stability and stability at wide range of pH are desirable properties of any enzyme for industrial applications. In our case the optimum temperature of enzyme activity was 50°C, while the optimum pH for its activity was recorded as pH 7.0 (Fig. 11 & 12).

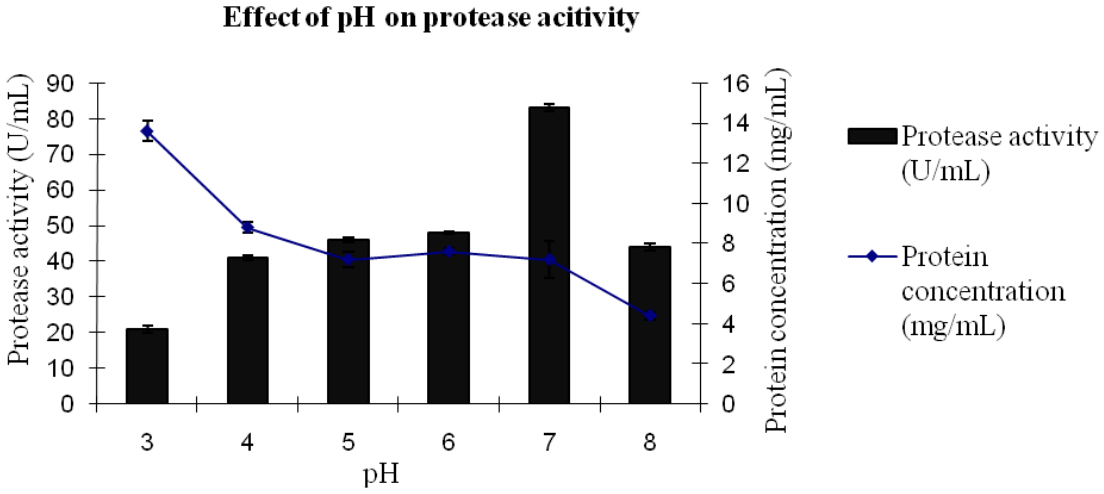


Figure 11. Results are mean of three independent determinations. Bars correspond to standard deviation.

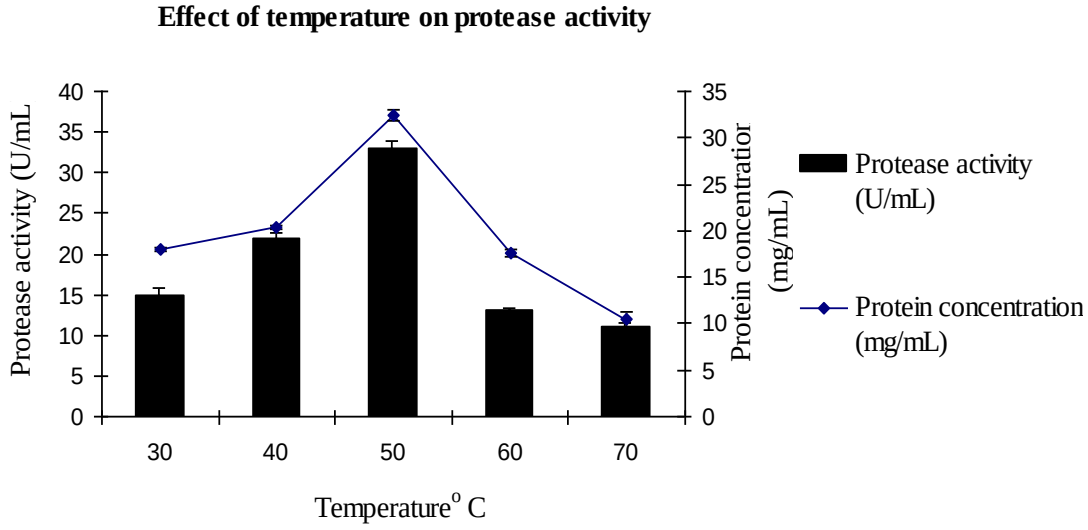
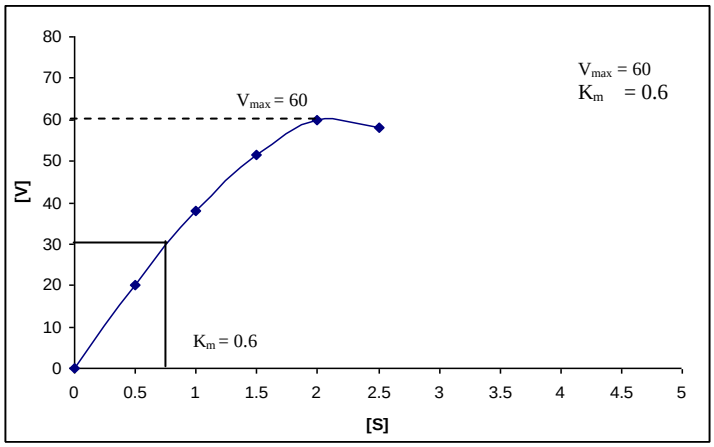


Figure 12. Results are mean of three independent determinations. Bars correspond to standard deviation.

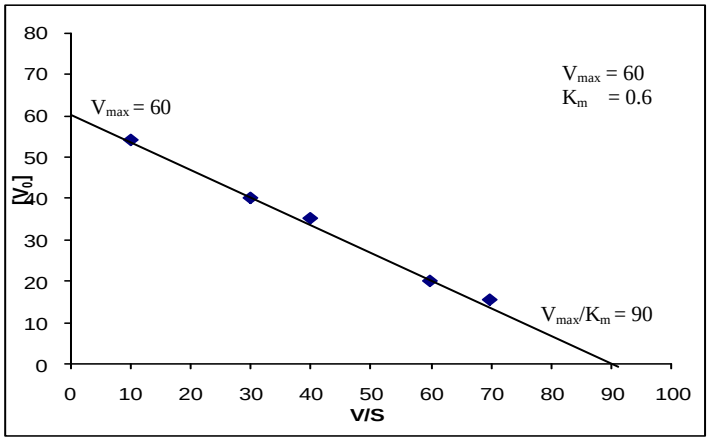
In order to study the effect of substrate concentration various substrate concentrations ranging from 0.5-2.5 % were used. Reaction rate verses substrate concentration curve was plotted to determine whether the enzyme obeys Michaelis-Menten kinetics, and constants were determined from a Line Weaver–Burk plot. The results are presented in Fig. 13.

Figure 13 **Enzyme kinetics of *A. flavus***

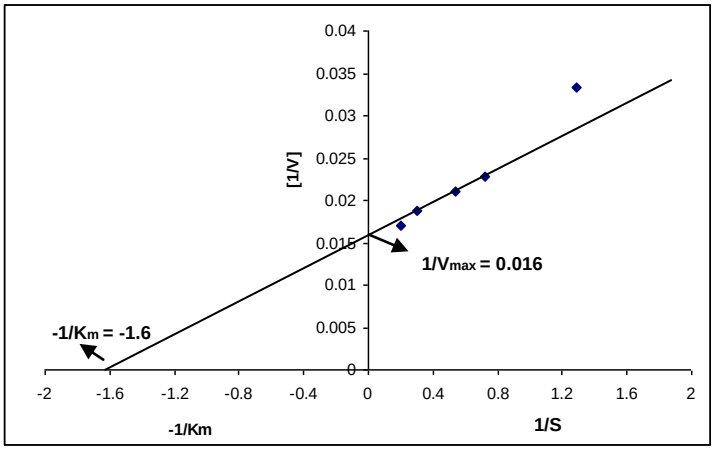
Michaelis-Menten equation



Lineweaver Burk plot



Eadie-Hofstee plot



From Fig.13, it can be seen the V_{max} and K_m values were determined against wheat bran as substrate. *A. flavus* had a higher V_{max} of 60.0 U/mg and K_m of 0.6 mg/ml.

The effects of various metal ions (Zn^{2+}, Mg^{2+}, Ca^{2+}, Na^{+} and Cu^{2+}) on activity of the protease from *Aspergillus flavus* is shown in Fig. 14. It can be shown that the metal ions Zn^{2+} and CU^{2+}supported the maximum enzyme activity whereas Na^{2+} and Ca^{2+} drastically inhibited the protease activity particularly Mg^{2+} was found to be the potent inhibitor of protease.

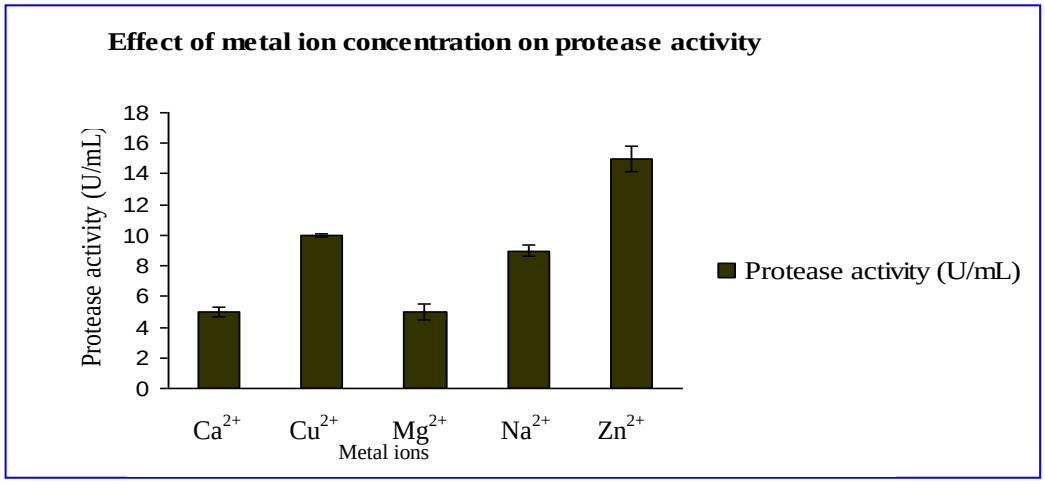


Figure 14. Results are mean of three independent determinations.

4. DISCUSSION

Microbial proteases have a number of commercial applications in industries like food, leather, meat processing and cheese making. A major commercial use is the addition of microbial proteases to domestic detergents for the digestion of pertinacious stains of fabrics (Sharma *et al.*, 1980). It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions. So, it becomes necessary to understand the nature of proteases and their catalytic potentiality under different conditions.

Time course for the production of protease by *A.flavus* was studied at 7th day. These results are supported by Johnvesly *et al.* (2002) and Impoolsup *et al.* (1981) who reported the maximum protease enzyme production, occurred during 7th day of incubation by using *A. flavus.* The incubation period is directly related to production of enzymes and other metabolites to a certain extent. After that, the enzyme production and the growth of the microorganism decreases; this can be attributed to the reduced availability of nutrients and the production of toxic metabolites (Romero *et al.*, 1998).

Protease production by microbial strains strongly depends on the extra-cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production (Elliah *et al.*, 2002). *Aspergillus flavus* showed maximal protease production at pH - 4 (Fig. 2). Identical observations were earlier recorded in *A. flavus, A. oryzae* and *A. candidus* at pH 4.0 (Nasuno and Onara, 1972; Dworschack *et al.*, 1952).

The higher enzyme production was found to be at 30°C. Earlier studies report that different species of *Penicillium* including *P. citrinum, P. perpurogerum* and *P. funculosum* gave highest yield of protease when incubated at 30°C (Sharma *et al.*, 1980). Haq *et al.* (2004) have also reported that maximum production of protease by *P. griseoroseum* was obtained at an incubation temperature of 30°C and the enzyme production was reduced when the incubation temperature was increased above 30°C. Fungal proteases are usually thermolabile and show reduced activities at high temperatures (Sharma *et al.*, 1980). Higher temperature is found to have some adverse effects on metabolic activities of microorganism (Tunga, 1995) and cause inhibition of the growth of the fungus. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure (Conn *et al.*, 1987)

At lower inoculums levels, the yield was very low. The decrease seen with large inoculums size could be due to the shortage of the nutrients available for the large biomass and faster growth of the culture (Hesseltine *et al.*, 1976). In our study, the maximum protease synthesis was noticed with 3% inoculum size whereas at higher concentration there was a decrease, it might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also enzyme release. From the survey of literature it can be seen that the 3% of inoculum size gave maximum production reported by Haq *et al.* (2003) and Kalisz (1988).

There are general reports showing that different carbon sources have different influences on extra cellular enzyme production by different strains. Among the various substrates (cotton waste, sugarcane bagasse, rice bran, rice straw and wheat bran) tested wheat bran was found to be the most effective substrate for the production of protease with the concentration of 3% (Fig. 6). Further increase in this carbon source adversely affected protease production in this *A. flavus* under SSF environment. These results were in accordance with reported protease production in presence of different substrates (Elliah *et al.*, 2002); and different carbon sources have different influences on enzyme production by different strains (Wang & Lee, 1996; Nehra *et al.*, 2002). It might be due to the fact that increase level of substrates decreases the aeration and porosity of the medium, which were very essential for the proper growth of the organism.

In the present study the various nitrogen sources like beef extract, KNO_3, peptone, yeast extract and urea were also studied. In that, KNO_3 showed the maximum protease production with the concentration of 1%. Certain nitrogenous salts tend to decrease the pH of the culture medium and had the adverse effect on enzyme production although they supported the growth of the organism (Wang *et al.*, 1974).

A summary of purification steps for protease from *A. flavus* is given in Table 9. The purification of protease resulted in 2 fold purification with 66% of recovery by ammonium sulphate precipitation. The purification of crude enzyme through DEAE cellulose column chromatography gave 5.8 folds increase in purity with 3.2% recovery of protease from *A. flavus*. The similar observation was reported by Ogundero and Osunlaja (1986) for *A. clavatus.*

Fractions from DEAE-Cellulose column which showed the highest activity were pooled and subjected to SDS-PAGE for determination of molecular weight of the protein. Purified enzyme preparation showed only one band corresponding to molecular weight of approximately 46 kDa (Fig. 10). Our results are more or less similar to that of Akel *et al.* (2009) who reported that the purified protease enzyme revealed a molecular mass of 49 kDa.

The maximum enzyme activity was found to be pH 7.0. Similar results were obtained for the optimum pH for enzymatic activity of other *Bacillus* species: pH 7.5 for *Bacillus subtilis* ITBCCB 148 (Yandri *et al.*, 2008), *Bacillus* sp. HS08 (Huang *et al.*, 2006) and *Bacillus* sp. S17110 (Jung *et al.*, 2007); pH 8.0 for *Bacillus cereus* KCTC 3674 (Kim *et al.*, 2001), Thermophilic *Bacillus* SMIA2 (Nascimento and Martins, 2004) and *B. cereus* BG1 (Ghorbel-Frikha *et al.*, 2005).

The maximum enzyme activity was found to be 50°C. This was supported by Li *et al.* (1997) who reported that alkaline protease isolated from *Thermomyces lanuginose* P134 had a broad temperature optimum of 50°C. Samal *et al.* (1991) also reported an alkaline protease from *Tritirachium albumlimber* to be quite thermostable even up to 50°C. The protease activity was accelerated by Zn^{2+} and it was inhibited by Mg^{2+} and Ca^{2+}. In contrast, Nehra *et al.* (2004) reported that Mg^{2+} was found to be an activator of the alkaline protease enzyme produced by *Aspergillus* sp. suggesting these metal ions had a capability to protect enzyme against denaturation.

V_{max} and K_m values for protease enzyme from *Aspergillus flavus* were determined from Line Weaver and Eadie-Hofstee plots. The results revealed that alkaline protease from *A. flavus* had a V_{max} of 60.0 U/mg of protein and K_m value of 0.6mg/ml. Matta *et al.* (1994) has reported proteases with lower K_m values with casein substrate from *Bacillus alkalophilus* and *Pseudomonas* species, which showed K_m values of 0.4 and 2.5 mg/ml, respectively. A slightly higher K_m value of 3.7 mg/ml has been reported for the enzyme from *B. polymyxa* strain indicating higher affinity of the enzyme towards casein (Kaur *et al.*, 1998).

We have characterized protease from a locally isolated fungus *Aspergillus flavus*. Its desirable characters such as broad substrate specificity, stability at high pH, stability at high temperature are significant characteristics of any enzyme for industrial application. Overall, the study provides that the wheat bran has a good potential to be used as solid state fermentation for protease production using *A. flavus*. The lab-scale study on protease production from wheat bran as major substrate might give the basic information of further development for large scale production.

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Changes in Biochemical Composition of the Gonads of *Donax trunculus* L. (Mollusca, Bivalvia) from the Gulf of Annaba (Algeria) in Relation to Reproductive Events and Pollution

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Abstract

Seasonal changes in biochemical composition of the bivalve *Donax trunculus* L. (Mollusca, Bivalvia) were studied during eight months from January to August 2009 in the gulf of Annaba. The samples were collected monthly at two sites: El Battah, a site far from any source of pollution, and Sidi Salem, a site located near human and industrial wastes. The amounts of carbohydrate, lipid, and protein were determined in gonad-visceral mass. The monthly amounts of the different components in gonads varied from 33.68 to 118.09 for carbohydrates, 6.68 to 44.45 for lipids and 3.56 to 36.72 µg/mg of wet tissue for proteins, respectively. A three-way ANOVA indicated significant effects of time, sex, and treatment. Lowest values of components were observed during the spawning phase. There was a significant difference in amounts of these gonad components between the two sites. Globally, the lowest values of carbohydrate and proteins, and the highest values of lipids were recorded in individuals sampled at Sidi Salem.

Keywords: Gulf of Annaba, *Donax trunculus*, Carbohydrates, Proteins, Lipids, Reproduction, Pollution.

1. Introduction

The continuous anthropogenic pressure exerted on the marine environment constitutes a real environmental problem (Lavado *et al.*, 2006; Sanchez *et al.*, 2007). The gulf of Annaba is the most important touristic and economic coastal zone located in east of Algeria. It is continuously affected by various contaminants from urban, agricultural, harbour and industrial activities (Semadi and Deruelle, 1993; Abdenour *et al.*, 2000). Bivalves play an important role in the ecosystem equilibrium and constitute an important economic endpoint. They are important representatives of the primary consumers in limnic systems and, therefore, an important link in the aquatic food chain. *Donax trunculus* L. (Mollusca, Bivalvia) is widely distributed along the Mediterranean coast and frequently used in marine pollution studies (Gaspar *et al.*, 1999; Moukrim *et al.*, 2004; Usero *et al.*, 2005; Beldi *et al.*, 2006; Sifi *et al.*, 2007). In addition, this species exists in higher densities in the sand beaches of the gulf of Annaba in Algeria (Degiovanni and Moueza, 1972) and is consumed by the local population (Beldi *et al.*, 2006). In Algeria, the growth, population dynamic and reproductive cycle of *D. trunculus* from Algiers bay (Mouëza, 1972; Mouëza and Frenkiel-Renault, 1973) and Annaba bay (Beldi, 2007) were examined. In addition, *D. trunculus* is used for many years ago as a bioindicator species in monitoring programs for the assessment the impact of pollution on marine organisms in the gulf of Annaba (Sifi *et al.*, 2007).

It has been reported that biochemical indicators provide more accurate information about gonad performance and environmental stress (Smolders *et al.*, 2005). Several studies on seasonal variation in biochemical composition of bivalves have been carried out in relation to reproduction (Giese, 1969; Ansell *et al.*, 1980; Zandee *et al.*, 1980; Baber and Blake, 1981; Polak *et al.*, 1987; Pazos *et al.*, 1997**;** Ojea *et al.*, 2004). For the gulf of Annaba, no corresponding data have been found in the literature. Therefore, the present study was undertaken in order to evaluate monthly variations of the main gonad components (carbohydrates, lipids, proteins) in the edible mollusk *D. trunculus* sampled at two sites in the gulf of Annaba. The observed changes are discussed in relation to reproductive events as well as to pollution since contamination status was different in the two studied sites (Beldi *et al.*, 2006; Sifi *et al.*, 2007).

2. Materials and Methods

2.1. Sampling sites

The gulf of Annaba is located in the East of Algeria. It is limited by the Rosa Cap (8° 15 ' E and 36° 38 ' N) in the East, and by the Garde Cap (7° 16 ' E and 36° 68 ' N) in the West. El Battah site (36° 50 ' N - 8° 50 ' E) is located

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about 30 km to the East of Annaba, far from any source of pollution and expected as a relatively clean site. Sidi Salem site (36° 50 ' N - 7° 47 ' E) is situated about 1 km to the East of Annaba city and considered affected by urban, harbor and industrial wastes (Fig. 1).

2.2. Samples collection

D. trunculus of standardized shell size (length: 27 ± 1 mm) were collected monthly from January to August 2009, from the two selected sites in the gulf of Annaba. Sex was analyzed by macroscopic inspection according to the color of the gonad: violet in the females, yellowish-orange in the males (Gaspar *et al.*, 1999). Samples were taken from the same part of the gonad. Thus, the middle part of gonad was dissected and stored at –20°C until analysis.

2.3. Analytical methods

Protein, carbohydrate and lipid were extracted from the same gonad sample following the procedure of Shibko *et al.* (1966). In brief, each sample of gonad (weight: 37-45 mg) was individually homogenized in 1 ml of trichloroacetic acid (20%) and then centrifuged (5,000 g for 10 min). The supernatant was used for the carbohydrates determination as described by Duchateau and Florkin (1959) using anthrone as reagent and glucose as standard, while the pellet added with a mixture of ether and chloroform (1V/1V) was subjected to a second centrifugation (5.000 g for 10 min). The resulted supernatant was used to quantify the lipids based on the vanillin method of Goldsworthy *et al.* (1972), Finally, protein concentration was determined in resulting pellet using the Bradford (1976) assay with blue brilliant of coomassie (G 250, Merck) as reagent and bovine serum albumin (Sigma) as standard.

2.4. Statistical analysis

The normality of data was verified using the Kolmogorov-Smirnov test, and the homogeneity of variances was checked by Levene's test. Data were expressed as Mean ± Standard Deviation (M ± SD). Comparison of mean values was estimated by Student's t-test. The effects of time, sampling sites and sex were tested by a three-way analysis of variance (ANOVA). The relation between the gonad components was also examined. All statistical analyses were performed using MINITAB Software (Version 14, PA State College, USA) and $p< 0.05$ was considered to be a statistically significant difference.

3. Results

3.1. Changes in carbohydrate contents

Monthly variation of the carbohydrate amount is shown in figure 2. Changes in carbohydrate amounts showed a decrease in March followed by two peaks; the first one occurred in April, and the second in July for females and August for males, respectively. The monthly amounts of carbohydrate in gonad ranged from 33 (March) to 119 (August) µg/mg tissue. The amounts of carbohydrates were higher in the female gonad than in the male gonad (Fig. 2). The differences between males and females are significant in January, February, March, April, May, June, July and August for El Battah ($p< 0.01$), and in January, February, March, May, June, July and August for Sidi Salem ($p< 0.001$), respectively. In addition, greater values were also recorded in El Battah compared to Sidi Salem. Significant differences ($p< 0.001$) between the two studied sites were observed in January, March, April, May, June, July and August for males, and January, February, March, April, May, June, July and August for females. This was confirmed by statistical analysis. Indeed, three-way ANOVA indicated significant effects ($p< 0.001$) of both time ($F= 887.95$; $df= 7, 90$), sex ($F= 978.14$; $df= 1, 90$) and site ($F= 22.67$; $df= 1, 90$).

3.2. Changes in lipid contents

Biochemical data related to lipid amounts are summarized in figure 3. Variations in lipid levels presented two decreases: in spring (March for males; April for females) and at the beginning of summer (June for males; July for females). The values varied from 6.68 µg/mg tissue in March to 48.45 µg/mg tissue in August. Data subjected to three-way ANOVA revealed significant effects ($p< 0.001$) of both time ($F= 2184.77$; $df= 7, 116$), sex ($F= 4379.45$; $df= 1, 116$) and site ($F= 2776.85$; $df= 1, 116$). The highest values were observed in females in the two sites (Fig. 3). Significant differences between males and females are recorded for all months in El Battah ($p< 0.01$) and Sidi Salem ($p< 0.001$). Moreover, values from Sidi Salem were significantly ($p< 0.01$) higher as compared to those of El Battah (Males: in January, March, February, April, May, July and August; Females: in January, February, May and July).

3.3. Changes in protein contents

Results from protein analysis are presented in figure 4. Changes in the protein amounts during the reproductive period showed that the lowest values were recorded in spring (April). The highest values were observed in summer (June-August). A three way ANOVA revealed significant effects ($p< 0.001$) of both time ($F= 1078.04$; $df= 7, 115$), sex ($F= 286.76$; $df= 1, 115$) and site ($F= 1971.70$; $df= 1, 115$). Amounts of protein were significantly higher in females than males in January, February, March, June and July for El Battah ($p< 0.01$), and in February, March and July for Sidi Salem ($p< 0.001$) (Fig. 4). The differences between the two sites are significant in both males and females for all months ($p< 0.001$). Statistical analyses on the relation between the gonad components were also analyzed (Table 1). We observed significant ($p< 0.05$) correlations between the different components except at El Battah between carbohydrates-proteins in males ($R= -0.004$; $p= 0.985$) and lipids-proteins in females ($R= -0.139$; 0.528), and at Sidi Salem only between carbohydrates-proteins in females ($R= 0.357$; $p= 0.087$).

4. Discussion

In Algiers Bay, the sexual cycle of *D. trunculus* includes a sexual rest (November - January) and a reproductive period (February-September) (Mouëza and Frenkiel-Renault,1973).

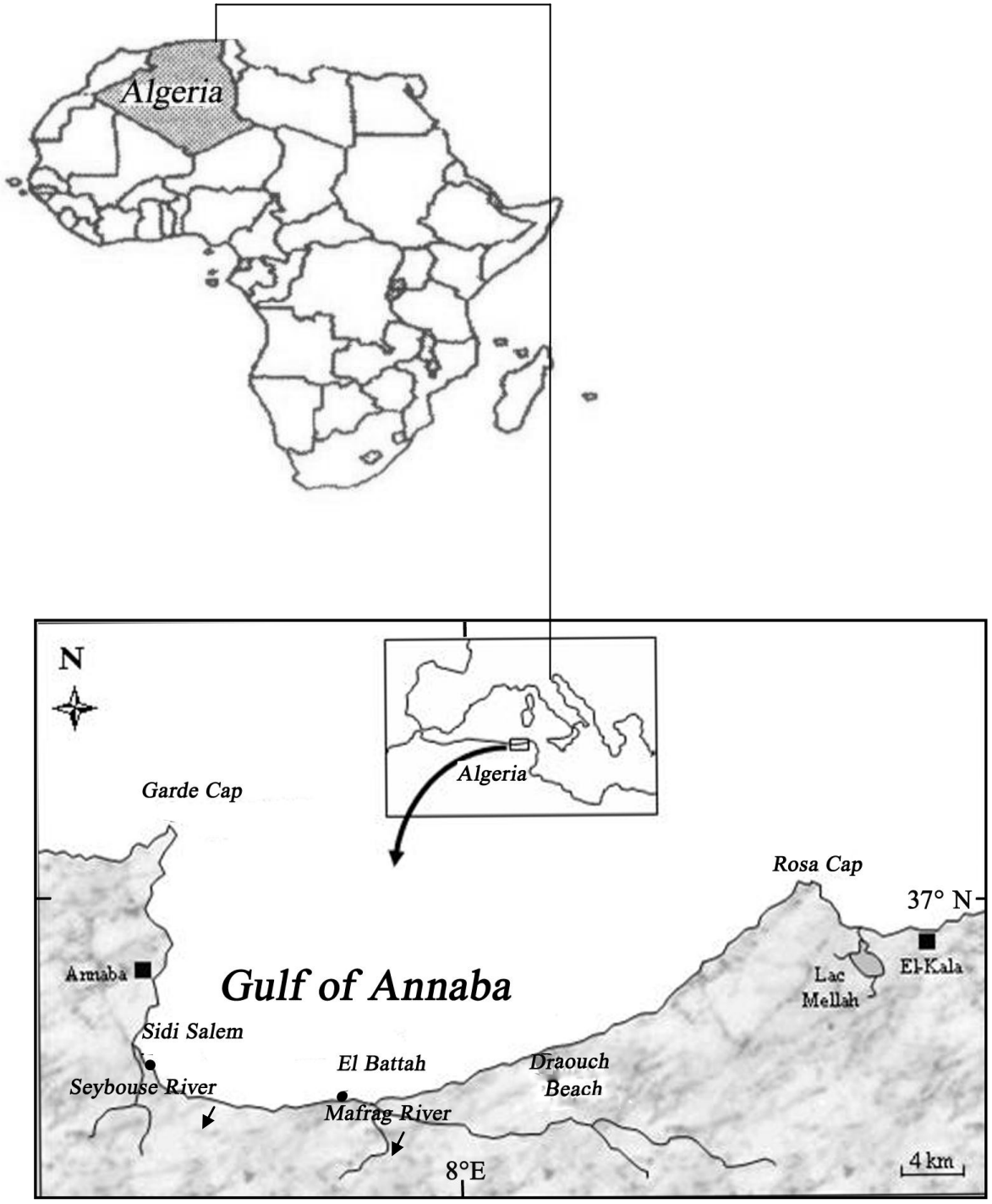

Figure 1. Location of the studied area in Algeria and of the two sampling sites in the gulf of Annaba.

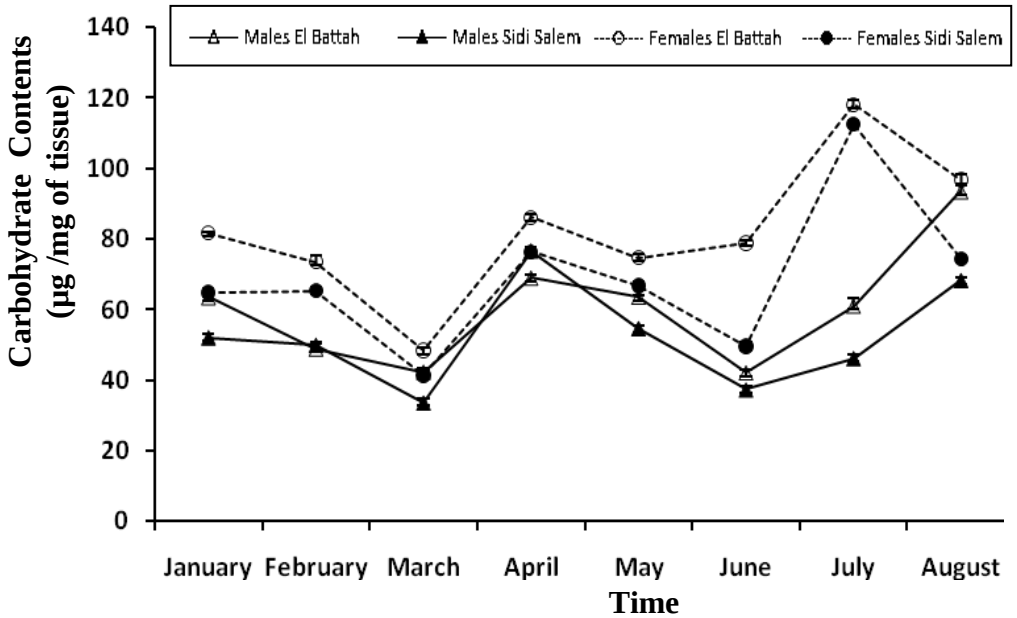

Figure 2. Carbohydrate contents (µg/mg of tissue) in male and female gonads of *Donax trunculus* collected during the reproductive period at two sites (El Battah and Sidi Salem) in the gulf of Annaba in 2009 (mean ± SD; n= 4-6).

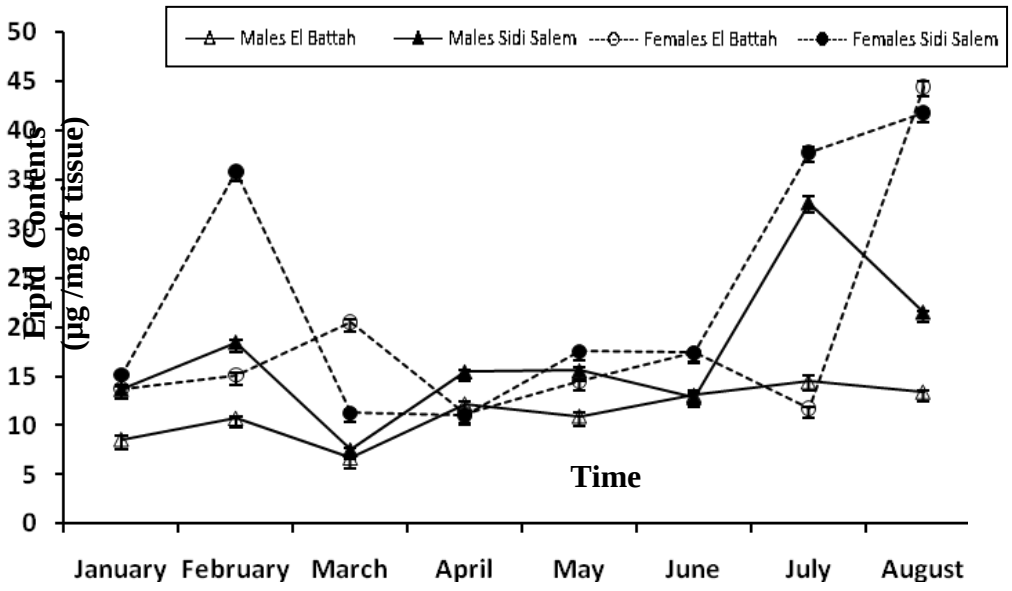

Figure 3. Lipid contents (µg/mg of tissue) in male and female gonads of *Donax trunculus* collected during the reproductive period at two sites (El Battah and Sidi Salem) in the gulf of Annaba in 2009 (mean ± SD; n= 4-6).

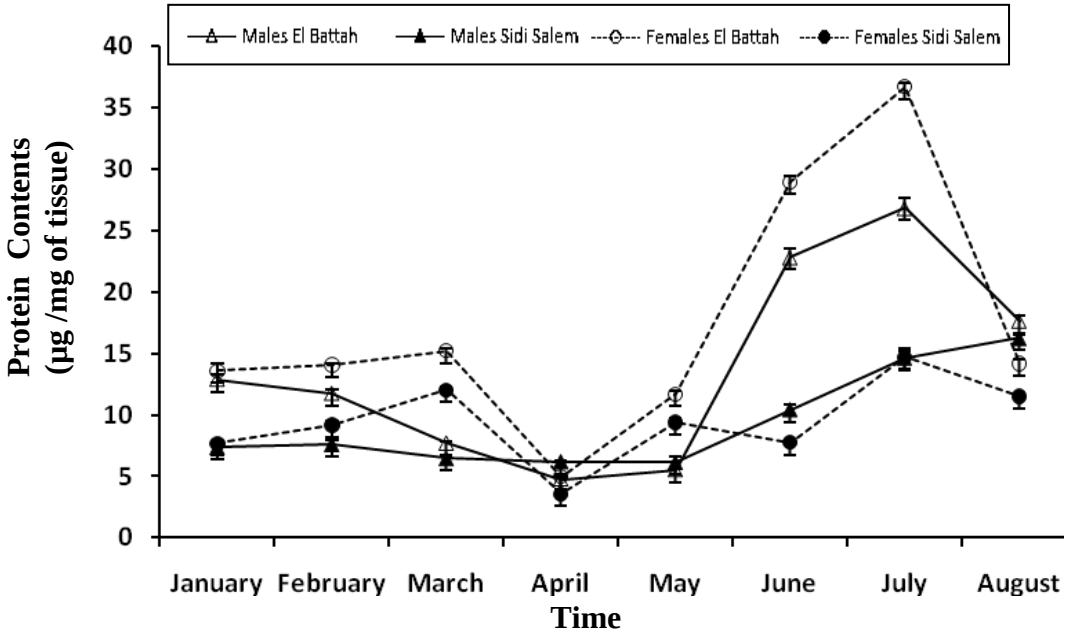

Figure 4. Protein contents (µg/mg of tissue) in male and female gonads of *Donax trunculus* collected during the reproductive period at two sites (El Battah and Sidi Salem) in the gulf of Annaba in 2009 (mean ± SD; n= 4-6).

Table 1. Relation between the different gonad components of *Donax trunculus* collected at El Battah (EB) and Sidi Salem (SS) in the gulf of Annaba (R: coefficient of correlation; *p*: significance level).

Sites	Relation	Male gonads		Female gonads	
		R	*P*	R	*P*
EB	Carbohydrates-Lipids	0.430	0.036	0.427	0.038
	Carbohydrates-Proteins	-0.004	0.985	-0.568	0.004
	Lipids-Proteins	0.651	0.001	-0.139	0.518
SS	Carbohydrates-Lipids	0.697	0.000	0.547	0.006
	Carbohydrates-Proteins	0.701	0.000	0.357	0.087
	Lipids-Proteins	0.798	0.000	0.600	0.002

Gonadal development begins in February and maturity is achieved in spring. -Spawning and gametogenesis occur in summer (Mouëza and Frenkiel-Renault, 1973). Concerning the gulf of Annaba, the reproductive period is observed from January to August, the resting period occurs from September to December and two periods of recruitment are observed: the first in spring and the second in autumn Beldi (2007).

The biochemical analysis shows that carbohydrates are the main component of gonad. The changes in biochemical composition of gonads are related to the reproductive cycle of *D. trunculus* in the gulf of Annaba confirming previous studies showing an elevation in protein and lipid amounts during the gametogenesis, and a decrease of these components related to spawning phase, while a decrease in carbohydrate essentially glycogen during the reproductive period (Ojea *et al.*, 2004; Dridi *et al.*, 2006; Mladinea *et al.*, 2007; Joaquim *et al.*, 2008). The changes in carbohydrates and lipids recorded in the present study are in accordance with previous observations made in the same species in the Algiers bay (Ansell *et al.*, 1980) or in other mollusc species (Zandee *et al.*, 1980; Polak *et al.*, 1987; Pazos *et al.*, 1997). Moreover, our results indicated higher amounts of biochemical components in females of *D. trunculus*. This could be explained by a difference in the energy demand between male and female gametes (Beninger *et al.*, 2003). In addition, triacylglycerols and acylglycerols were the principal constituents of the oocytes (Napolitano *et al.*, 1992) and spermatozoids do not store lipid reserves (Soudant *et al.*, 1996). Lastly, male bivalves produce small spermatozoa with few energy reserves by comparison with females which elaborate vitellin for developing oocytes (Beninger and Le Pennec, 1997).

The lowest values in carbohydrates and proteins, and the highest values in lipids recorded in individuals sampled at Sidi Salem are probably related to the level of exposition of this site. Indeed, this site is affected by several pollutants such as heavy metals released from

factories and port activities. This is supported by our previous findings since Beldi *et al.* (2006) reported significant amounts of cadmium in tissues of *D. trunculus* collected from Sidi Salem in comparison with individuals from El Battah. More recently, Sifi *et al.* (2007), in a biomonitoring study on the gulf of Annaba, observed a significant effect on AChE and GST activities in *D. trunculus* collected from Sidi Salem as compared to individuals from El Battah.

It has been shown that Cd caused a decrease in the storage of glycogen in *Mya arenaria* (Gauthier-Clerc *et al.*, 2002), while Viarengo (1989) found that this metal inhibited oxidative phosphorylation and reduced protein synthesis. This supported our observations concerning the reduction of biochemical amounts in *D. trunculus* from Sidi Salem. Indeed, Sidi Salem was more exposed to metallic pollution as compared to El Battah (Beldi *et al.*, 2006).

5. Conclusion

Our present study on the main components in gonads of *Donax trunculus* during the reproductive period revealed changes correlated to the reproductive events in the gulf of Annaba. Moreover, there are differences between sex and site. The difference between the two sites was related to their level of exposition to pollution.

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Storage Effects on the Quality of Sachet Water Produced within Port Harcourt Metropolis, Nigeria

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Abstract

The present investigation highlights the effect of storage on the physico-chemical status and bacteriological quality of sachet water produced in Port Harcourt, Nigeria for a period of four months. Ten brands of sachet water were collected within 24 hours of production and stored at ambient temperature. Sub-samples were drawn from the stock samples on monthly basis for physico-chemical measurement and on weekly basis for enumeration of total aerobic heterotrophic bacteria and indicator organisms using ASTM, APHA and WHO analytical methods. pH values increased in all brands to acceptable WHO limits within 8 weeks of storage and gradually decreased toward the end of the experiment. Dissolved oxygen, volatile organic matter and nitrate values decreased throughout the investigation period while phosphate and potassium values increased throughout the investigation period in all brands tested. Total aerobic heterotrophic bacterial count increased gradually in all brands to unacceptable limit within four weeks of storage and gradually diminished to zero level by the end of experiment. Total and faecal coliform appeared in 40% of sachet water samples analyzed within the first three weeks and were no longer detected throughout the investigation period. *Escherichia coli* was isolated in one brand at the onset while faecal *Streptococci* were absent throughout the investigation period. Results of the experiment indicate that 60% of the brands analyzed met the WHO guideline limit for drinking when stored at ambient temperature within four week period. However, storage beyond this period led to diminished aesthetic quality of sachet water and increased proliferation of bacteria to a level deleterious to human health.

Keywords: Sachet water, storage effects, quality, Nigeria.

1. Introduction

Most people living in the major cities of Nigeria do not have access to pipe borne water, probably due to unavailability or inadequacy where obtainable (Omalu *et al*., 2010). People, therefore, resort to the more costly alternative of buying water from vendors; sachet or bottled water became a major source of drinking water.

Sachet water, a brand of packaged water has, therefore, gradually become the most widely consumed liquid for both the rich and the poor in Nigeria. It is the brand of choice to everyone because it is a cheaper alternative to the bottled brand, considered to be the refreshment of the affluent. Hygiene, purity, tastes, and, most importantly, safety is probably amongst various reasons for sachet water consumption. Unfortunately, the problems of its purity and health concerns have begun to manifest (Oladipo *et al*., 2009).

Sachet water is regulated as a food product in Nigeria by National Agency for Foods Drugs Administration and Control (NAFDAC). The agency relies on World Health Organization (WHO) standards for the product regulation, registration and certification. There has been a tremendous improvement in sachet water regulations by NAFDAC as the number of illegal producers has drastically reduced and most brands on sale now have NAFDAC registration.

Sachet water is not completely sterile; it may not be entirely free of all infectious microorganisms. The potential danger associated with sachet water is contamination, which is a factor of the source of the water itself, treatment, packaging materials, dispensing into packaging materials and closure (Omalu *et al*., 2010). Under prolonged storage of packaged water at favorable environmental conditions, total aerobic heterotrophic bacteria can grow to levels that may be harmful to humans (Warburton *et al*., 1992). Total aerobic heterotrophic bacterial counts are sensitive and practical indicators of water treatment efficiency as well as after-growth and biofilm formation. Some of the total aerobic heterotrophic bacteria have been identified as opportunistic pathogens (Rusin *et al*., 1997). These microorganisms can be found in source waters and in treated drinking water. Thus, consumption of water containing large numbers of total aerobic heterotrophic bacteria can lead to diseases such as gastroenteritis and mucous membrane infections particularly in persons whose immune systems are

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compromised by AIDS, organ transplantation or chemotherapy (Grabow, 1996; Rusin *et al*., 1997).

The objective of this study, therefore, is to examine the effect of prolonged storage on the physico-chemical status and bacteriological quality of sachet water that has been hitherto certified for consumption by appropriate authority.

2. Materials and Methods

Ten brands of sachet water with NAFDAC certification were randomly collected in different parts of Port Harcourt metropolis, Nigeria in bags within 24hours of production and stored in a room at ambient temperature. Sub-samples were drawn from the stock samples in triplicates for physico-chemical characterization and bacteriological assay using ASTM, APHA and EPA analytical methods (EPA, 1996; APHA, 1998; ASTM, 1999).

Physico-chemical parameters of the sachet water were analyzed immediately after collection and subsequently thereafter on a monthly basis. pH, temperature and dissolved oxygen of the sachet water were determined electrometrically with a multi-parameter data logger (Hanna model HI991300, Hanna Instruments, Singapore). The meter was calibrated prior to use with 0.01N and 0.1N standard potassium chloride solutions (according to the manufacturer's specifications), and buffer standards (obtained from AccuStandard Europe) of pH 4 and 7 at room temperature. Volatile organic matter was determined by weight lost on ignition method at 550±50^{o}C in accordance with APHA 2540E. Nitrate and phosphate in sachet water samples were determined colorimetrically by UV/Visible spectrophotometer in accordance with EPA 352.1 and APHA 4500. Potassium was analyzed with Flame Atomic Absorption Spectrophotometer (FAAS) in accordance with APHA 20th edition 3111B. Samples were analyzed by direct aspiration in an acidic medium into an Air/Acetylene flame at specified wavelength for potassium.

Bacteriological analysis of the sachet water was carried out immediately after collection and subsequently thereafter on weekly basis. Total aerobic heterotrophic bacterial count was determined following the heterotrophic plate count method, using spread plate technique in accordance with ASTM D5465-93 and APHA 9215. 1ml of the sample or 0.1ml of final dilution of the sample in sterile Ringer's solution where necessary was aseptically introduced onto dry nutrient agar surface in triplicates spread plated with a glass spreader and incubated in an inverted position at 35±2^{o}C for 18-24 hours. Plates containing 30 - 300 were counted at the end of the incubation period. Total coliform, faecal coliform, *Escherichia coli* and faecal *Streptococci* were determined by using Membrane Filtration Technique in accordance with ASTM D5392-93, APHA 9222B and WHO Guidelines for Drinking Water Quality (2001, Volume 3). Filtration unit comprising of Erlenmeyer flask, vacuum source and porous support were assembled and with the aid of a flame sterilized forceps, a sterile membrane filter (0.45µm Millipore) was placed on the porous support. The upper funnel was placed in position and secured with appropriate clamps. 100ml of sachet water sample was aseptically poured into the upper funnel and suction applied to create a vacuum. After the sample was passed through the membrane filter, the filtration unit was taken apart and with the aid of a sterile forceps. The membrane filter was placed in the Perti dish on the pad that had been saturated with McConkey broth for total and faecal coliforms, Eosine Methylene Blue agar for *Escherichia coli* as well as Slanetz and Bartley agar for faecal *Streptococci*. The upper funnel was then removed and rinsed with 200ml of sterile Ringer's solution prior to use for the next sample. All plates were incubated in inverted position at 37±2^{o}C (total coliforms) and 44±2^{o}C (faecal coliforms, *Escherichia coli* and faecal *Streptococci*) for 18-24 hours.

3. Results and Discussion

The pH value of the sachet water samples is presented in Fig. 1. The pH values varied from 4.43 to 7.71 averaging 6.07 throughout the investigation period. pH was outside WHO limits in all the 10 samples analyzed at the onset of the investigation (Week 0) while an increase in pH was observed in all the samples up to week 8 with about 40 to 100% falling within WHO limit followed by a decline between Week 8 and Week 16.

pH is one of the parameters that addresses the aesthetic quality of water such as taste which has no serious health significance (WHO, 1996). However, pH played a significant role in determining the bacterial population growth and diversity in sachet water. Increase, observed in pH, could be attributed to the production of basic metabolic waste products by increasing bacterial population. In their review, Prescott *et al.* (1999) stated that microorganisms frequently change the pH of their own habitat by producing acidic or basic metabolic waste products.

The temperature readings of the sachet water samples are as presented in Table 1. The temperature values ranged from 27.1 to 28.5^{o}C averaging 27.9^{o}C. The temperature values obtained throughout the investigation period fall within the optimal growth range for mesophilic bacteria including human pathogens. Prescott *et al.* (1999) reported 20-45^{o}C as optimal growth temperature for mesophilic microorganisms. According to WHO report (1996), the microbiological characteristics of drinking water are related to temperature through its effects on water-treatment processes and its effects on both growth and survival of microorganisms. Consequently, growth of nuisance microorganisms is enhanced by warm water conditions and could lead to the development of unpleasant tastes and odors.

The dissolved oxygen values obtained for the sachet water samples are as presented in Fig. 2. The dissolved oxygen values ranged from 4.10-7.85 mg/l averaging 4.92 mg/l. Growth of aerobic and facultative anaerobic bacteria will be enhanced by the presence of dissolved oxygen in sachet water. A decrease in dissolved oxygen was generally observed in all sachet water samples throughout the investigation period; an indication of possible bacterial respiration of organic materials by the bacterial flora of the sachet water samples tested. WHO (1996) reported that there is tendency for the level of dissolved oxygen to fall with time indicating possible microbial respiration of organic materials amongst other reasons.

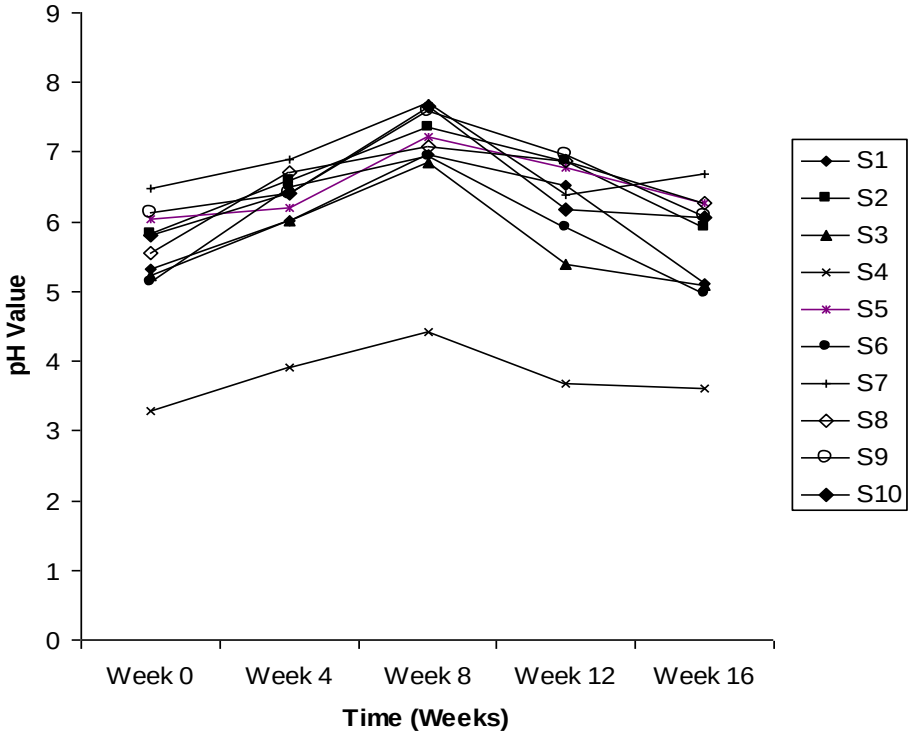

Figure 1. pH Values of Sachet Water in 16 Week Period.

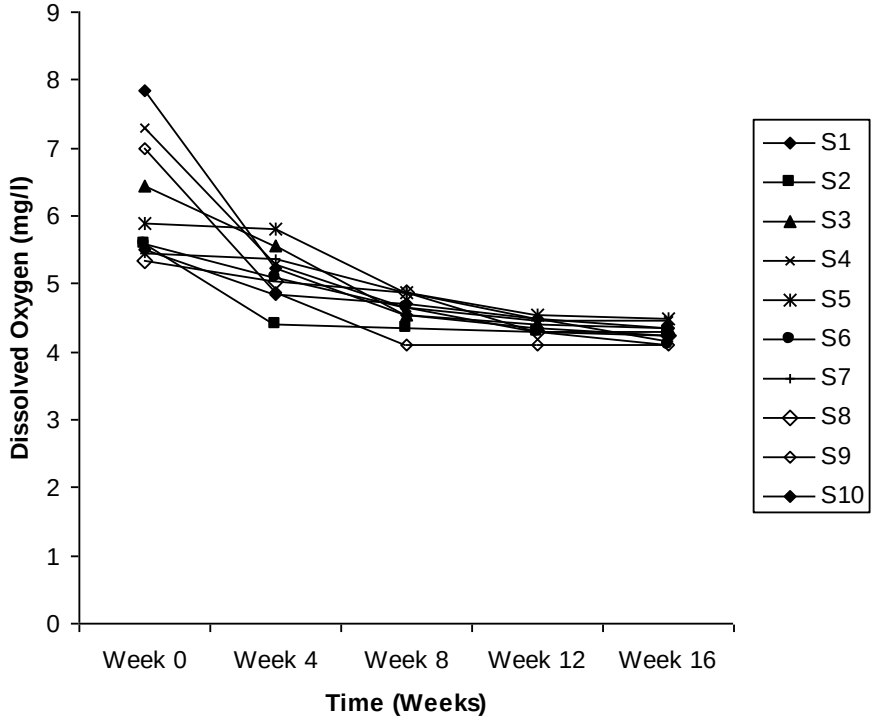

Figure 2. Dissolved Oxygen Values of Sachet Water in 16 Week Period.

Table 1. Temperature (^oC) of Sachet Water in 16 Week Period

Sample code / Period	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Week 0	27.7	27.5	27.1	27.7	27.6	27.3	27.3	27.1	27.1	27.6
Week 4	27.8	27.5	27.7	28.0	27.6	27.8	27.6	27.5	27.6	27.5
Week 8	28.3	28.3	28.3	28.1	28.2	28.3	28.3	28.3	28.4	28.3
Week 12	27.6	27.5	27.3	27.6	27.3	28.0	28.0	27.6	27.6	27.6
Week 16	28.4	28.3	28.3	28.3	28.4	28.5	28.3	28.3	28.3	28.4

Volatile organic matter values obtained for the sachet water samples are as presented in Fig. 3. The values of volatile organic matter ranged from 1.0-2.5 mg/l. Volatile organic matter values decreased throughout the investigation period. Decrease in volatile organic matter could be attributed to their uptake as substrate for energy production and growth (Pelczar *et al*., 1993).

Nitrate values obtained for the sachet water samples are as presented in Fig. 4. The values of nitrate ranged from 0.54-1.09 mg/l. Nitrate values decreased throughout the investigation period. Decrease in nitrate values could be attributed to their utilization by microorganisms for growth and reproduction (Prescott *et al*., 1999)

Phosphate and potassium values are also presented in Fig. 5 and 6, respectively. The values of phosphate and potassium ranged from 0.62-3.48 mg/l and 0.14-2.98 mg/l, respectively. Phosphate and potassium values increased throughout the investigation period. This could be attributed to microbial death and accumulation of metabolic waste (Prescott *et al*., 1999).

Total aerobic heterotrophic bacterial counts obtained from sachet water samples are as presented in Table 2. The counts of total aerobic heterotrophic bacteria ranged from 0.0 to 2.7×10^4 cfu/ml of the sachet water samples. This is in agreement with Olaoye and Onilude (2009) who observed that varying levels of microbial contamination were recorded in samples from the different sampling locations. A gradual increase in total aerobic heterotrophic bacterial counts was observed in all the brands tested up to week 8 followed by decease in counts up to the end of the experiment, a growth pattern typical of microorganisms growing in closed system (Brock and Madigan, 1988).

The total aerobic heterotrophic bacterial count method determines the general microbiological quality of treated drinking water (Allen *et al*., 2002) WHO drinking water quality specifications allow total aerobic heterotrophic bacterial counts of 100 cfu/ml (Allen *et al*., 2002). However, this limit was exceeded by all the sachet water samples tested after four weeks of storage.

The result of total aerobic heterotrophic bacterial count obtained in this study is in agreement with the findings of Warburton *et al*. (1992). In a study involving the determination of the microbiological safety of bottled water stored for 30 days, Warburton *et al*. (1992) reported that the total aerobic heterotrophic bacterial counts increased considerably to a level detrimental to human health when the water was stored at room temperature.

Total coliform bacteria (Table 2) were detected in 40% of the brands of sachet water analyzed within the first three weeks while faecal coliform were detected in 10% of the brands of sachet water analyzed at the onset of the investigation. However, they were not detected after this period. Indicator organisms loose viability in freshwater environment with time (WHO, 2001).

Total coliforms are widely used as indicators of the general sanitary quality of treated drinking water while faecal coliforms give a much closer indication of faecal pollution (Ashbolt *et al*, 2001). WHO limit is that none should be detected. Unlike total aerobic heterotrophic bacteria, total and faecal coliform counts did not increase in sachet water samples tested. Among the criteria for indicator organisms, Prescott *et al*. (1999) stated that indicator bacterium should not reproduce in the contaminated water and produce an inflated value. This justified the choice of coliform bacteria by WHO as indicator organisms (WHO, 2001).

The counts of faecal *Streptococci* (Table 2) remained 0.0 cfu/ml in all brands tested throughout the investigation period while *Escherichia coli* was detected in one brand at the onset of the investigation (Week 0). The presence of faecal S*treptococci* in potable water is an additional or a secondary indicator of faecal pollution while the presence of *Escherichia coli* confirms faecal pollution of potable water, which is not acceptable (Ashbolt *et al*, 2001; WHO, 1996). Detection of *Escherichia coli* in one brand therefore is a confirmation of its faecal contamination.

4. Conclusion

The objective of this work was to evaluate the effect of prolonged storage on the physico-chemical status and bacteriological quality of sachet water samples collected within Port Harcourt metropolis, Nigeria.

The analytical results revealed that prolonged storage caused an increase in pH up to week 8 followed by a decrease up to the end of the experiment in all pure water samples tested. The presence of dissolved oxygen coupled with availability of organic material and nutrients aided continuous and rapid proliferation of bacteria in sachet water tested over time.

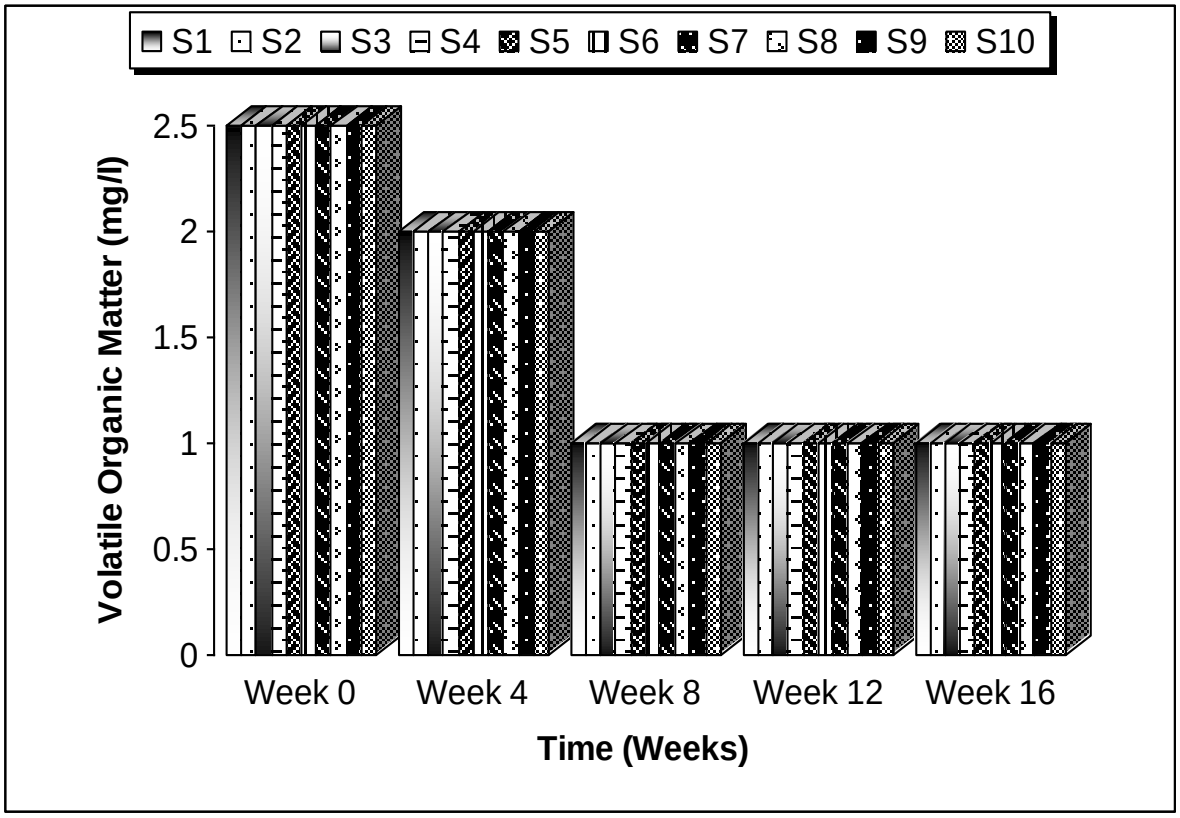

Figure 3. Volatile Organic Matter Values of Sachet Water in 16 Week Period.

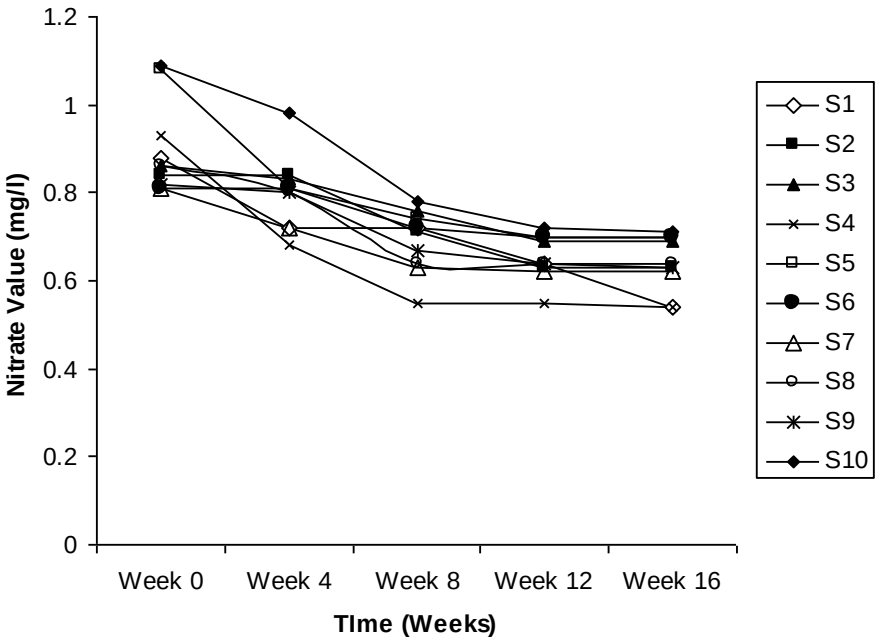

Figure 4. Nitrate Values of Sachet Water in 16 Week Period.

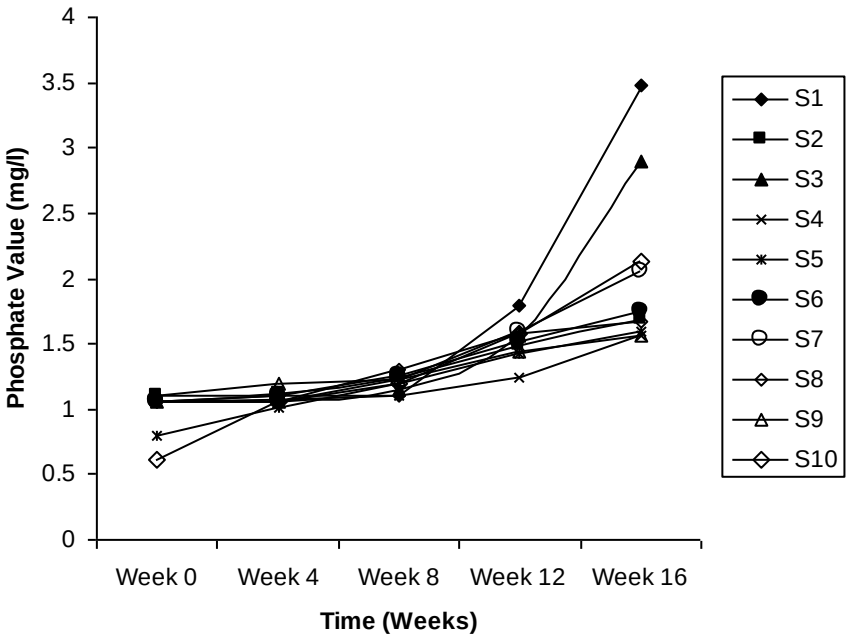

Figure 5. Phosphate Values of Sachet Water in 16 Week Period.

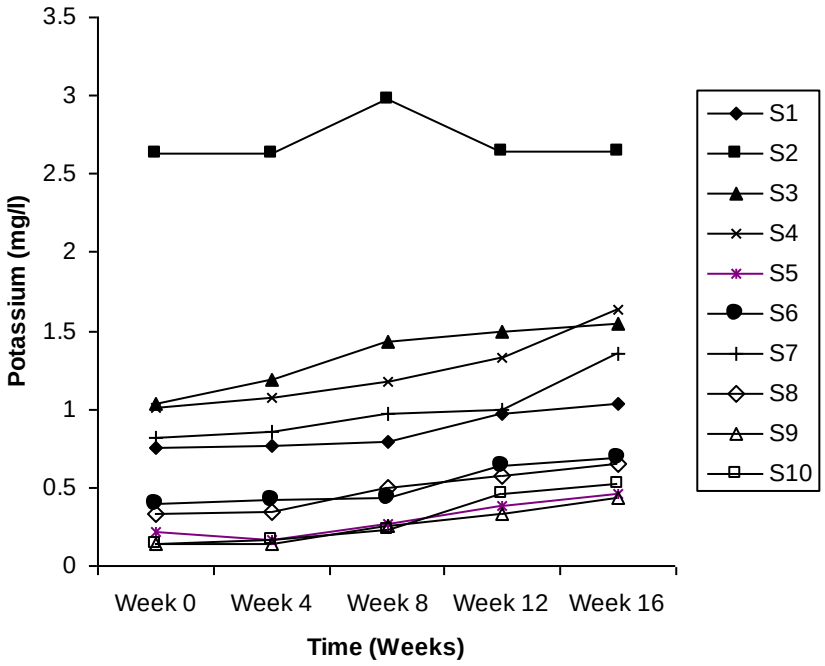

Figure 6. Potassium Values of Sachet Water in 16 Week Period.

Table 2. Total Aerobic Heterotrophic and Pathogenic Bacterial Count (cfu/ml)

Period	Bacteria Type	Sample Code S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Wk 0	THB	1.0	37.0	12.0	4.0	1.0	1.0	1.0	4.0	2.0	2.0
	TC	0.0	4.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0
	FC/EC	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 1	THB	5.0	40.0	48.0	12.0	6.0	1.0	6.0	14.0	2.0	11.0
	TC	0.0	2.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	0.0
	FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 2	THB	10.0	46.0	54.0	22.0	65.0	2.0	25.0	32.0	3.0	12.0
	TC	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 3	THB	13.0	97.0	94.0	53.0	71.0	5.0	28.0	42.0	5.0	22.0
	TC	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 4	THB	23.0	127.0	97.0	72.0	88.0	7.0	40.0	50.0	12.0	5.0×10^2
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 5	THB	3.9×10^3	146.0	4.9×10^3	1.0×10^3	1.0×10^3	1.3×10^2	3.0×10^3	4.0×10^2	1.5×10^3	1.8×10^3
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 6	THB	5.4×10^3	1.4×10^4	6.6×10^3	3.4×10^3	1.4×10^3	2.6×10^2	4.1×10^3	1.2×10^3	2.8×10^3	1.0×10^2
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 7	THB	8.4×10^3	2.7×10^4	4.0×10^2	7.3×10^2	2.8×10^3	4.0×10^2	5.4×10^3	3.2×10^2	5.6×10^2	1.0×10^2
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 8	THB	2.5×10^3	6.0×10^3	1.7×10^2	1.2×10^2	4.0×10^2	1.9×10^3	9.7×10^2	1.4×10^2	2.1×10^2	1.0×10^2
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 9	THB	2.5×10^3	1.2×10^3	96.0	1.2×10^2	1.0×10^2	2.0×10^3	9.6×10^2	52.0	1.7×10^2	1.0×10^2
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 10	THB	2.5×10^3	1.1×10^3	82.0	1.2×10^2	26.0	122.0	1.1×10^2	21.0	35.0	1.0×10^2
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 11	THB	1.6×10^3	3.6×10^2	80.0	56.0	4.0	42.0	56.0	20.0	33.0	1.0×10^2
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 12	THB	1.1×10^3	12.0	20.0	11.0	1.0	42.0	11.0	20.0	21.0	2.0
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 13	THB	16.0	12.0	1.0	2.0	1.0	2.0	1.0	2.0	2.0	1.0
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 14	THB	3.0	1.0	1.0	2.0	0.0	2.0	0.0	1.0	1.0	1.0
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 15	THB	2.0	0.0	1.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wk 16	THB	2.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
	TC/FC/FS/EC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

THB: Total heterotrophic bacteria, TC: Total coliform, Faecal colifirm, FS: Faecal *Streptococci*, EC: *Escherichia coli*

All pure water samples analyzed exceeded WHO limit of 100 cfu/ml for total aerobic heterotrophic bacteria within weeks 4 and 8. Total and faecal coliform appeared in 40% of sachet water samples analyzed within first three weeks and died off. They lost viability in freshwater environment with time. No faecal *Streptococci* was detected throughout the investigation period while *Escherichia coli* was detected in one brand.

The present study has revealed that sachet water when stored at room temperature for a long period can increase total aerobic heterotrophic bacteria to a level that may be harmful to human health.

5. Recommendations

Expiry date of sachet water produced in Nigeria should not exceed four weeks from the date of production. The public should be sensitized not to drink sachet water that has exceeded four weeks from the date of manufacture. The regulatory body should promulgate standardized method of storage of sachet water in order to increase its shelf life. Periodic sanitary inspection of sachet water factories by the regulatory body is absolutely necessary to ensure conformity.

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The Role *of milleri Streptococci* in the Formation of Cariogenic Biofilm: Bacteriological Aspects

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Abstract

It is remarkable that the biofilm (adherence) mode of growth of bacteria on the tooth surfaces in the oral cavity has been well documented as one of the important causes of dental caries. This study has been undertaken to detect the ability of *milleri Streptococci* in the formation of bacterial cariogenic biofilm qualitatively and quantitatively by spectrophotometric assay with ELISA reader, and to detect, in terms of BICs and MBECs, biofilm antimicrobial susceptibility test for biofilm producer isolates of *milleri Streptococci* to ciprofloxacin. Forty swabs obtained from patients admitted to Operative Dental Clinic in the College of Dentistry in Al-Ramadi City were studied during the period from December 2009 to March 2010. Lancefield's group sero-grouping was done. Quantitative biofilm formation by spectrophotometric method was achieved. Antimicrobial susceptibility test for the study isolates at two physiological stages planktonic and sessile states was done. Out of 40 study specimens, 25 (62.5%) were culture positive cases. Among culture positive cases, 33 mixed bacterial infection cases were observed consist of both of *Staphylococcus* spp. and *Streptococcus* spp. while α-hemolytic *Streptococci* were identified as pure single culture in 25 (100%) cases. α-hemolytic *Streptococci* isolates which were submitted to Lancefield's group classification were identified as group F *Streptococci*. Out of 25 bacterial study isolates, 20 (80%) were biofilm producers distributed into 17 (68%) as strong biofilm producer and 3 (12%) as weak producer. In biofilm antimicrobial susceptibility test for sessile cells of Group F *Streptococci* against ciprofloxacin, the biofilm inhibitory concentration required were from (10X - 100X MIC)µg/ml of this antimicrobial agent to inhibit bacterial biofilm. The minimal biofilm inhibitory concentrations from (100 X to 1000 X MIC) MICs µg/ml were enough to eradicate bacterial biofilm.It is concluded that most of group F *milleri Streptococci* isolated from patients with dental caries produced cariogenic biofilm. Also, in term of BICs and MBECs, the biofilm producer isolates were required 10-50 X MICs of ciprofloxacin to inhibit bacterial biofilm and 100-1000 X MICs to remove of bacterial biofilm in patients with dental caries.

Keywords: Cariogenic biofilm, dental caries, *milleri Streptococci*.

1. Introduction

It is well realized that bacterial biofilm is an aggregate of microorganisms in which cells are stick to each other and/or to a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) (Hall-Stoodley *et al.,* 2004). On the other hand, dental caries is an infectious and transmissible disease, and the primary infection can often come from family members or caregivers (Florio *et al.*, 2004). There is a significant challenge for many patients to be able to modify their risk factors in order to create an oral environment that will lead to a re-establishment of a healthy bacterial population within the oral biofilm (Featherstone, 2003).

It is well known that dental plaque is the material that adheres to the teeth and consists of bacterial cells (mainly *Streptococcus mutans* and *Streptococcus sanguis*), salivary polymers and bacterial extracellular products and it is consider a form of biofilm on the surfaces of the teeth (Rogers, 2008). It is well known that *S. mutans* is an "obligate" biofilm-forming bacterium (Burne, 1998a) and the primary etiological agent of human dental caries. This bacterium has also a primitive role in infective endocarditis (IE) (Gauduchon, 2000). It has a variety of mechanisms to colonize the tooth surfaces to become a corner stone bacterial species in cariogenic biofilms (Burne,1998b). The production of acids by *S. mutans* causes dissolution of minerals in tooth enamel and formation of dental caries. The bacteria in biofilm can produce lactic acid through the fermentation of dietary sugars such as sucrose and carbohydrates. In addition, oral streptococci can metabolize sucrose to produce insoluble glucans that promote the formation of biofilm extracellular polymeric slime (EPS) matrix (PaesLeme *et al.*, 2006).

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Thus, sucrose has a negative synergy with respect to caries in that it promotes both biofilm formation and acid production by cariogenic bacteria such as *S. mutans* (Paul Stoodley *et al.,* 2008). .

In fact, depending on the organism and type of antimicrobial and experimental system, biofilm bacteria can be up to thousand times more resistant to antimicrobial stress than free-swimming bacteria of the same species (Amy, 2008). Thus, this study has been undertaken to detect the ability of *milleri Streptococcus* in the formation of bacterial cariogenic biofilm, and, furthermore, in terms of MICs and MBECs, to determine antimicrobial activity of ciprofloxacin against study sessile cells.

2. Patients and Methods

Forty swabs obtained from patients admitted to Operative Dental Clinic in the College of Dentistry in Al-Ramadi City were studied during the period from December 2009 to March 2010. The swab was taken from inside each badly carious tooth to specify the tested sample and to confine the causative cariogenic bacteria as much as possible. The patients were distributed into 14 male and 11 female with male to female ratio1: 1.27and the age range between 17-64 years old with mean 37.48 ±12.3. Full informative history had been taken directly from patients and the information was arranged in an informative clearly detailed formula sheet. The study on the colonial morphology of grown bacteria on culture media (Blood agar, Chocolate agar and Nutrient agar) was done. Isolation of *mutans Streptococcus*, pigments and other characteristics and all biochemical tests were done according to Baron *et al.,* 1994).

2.1. Lancefield's Grouping Identification

This test was intended to be used for the identification of Lancefield groups A, B, C, D, F, and G by agglutination of specific antibody-coated latex particles in the presence of enzymatically extracted antigen. The principle of the test was based on streptococci carry group specific carbohydrate antigens in their cell walls. After extraction by especially developed enzyme preparation, these antigens will agglutinate latex particles coated with the corresponding antibody. The latex remains in smooth suspension in the absence of group specific antigen. Any colony which has the following characteristic was submitted to α-hemolysis on blood agar, Gram stain, catalase and oxidase tests.

This test was performed by using a kit of Streptococcal grouping latex test (Plasmatic, UK) as following: Two-Six colonies of streptococci were picked by using a sterile bacteriological loop and were emulsified them in 0.4 ml of extraction enzyme. The mixture was incubated in a water bath at 37°C for 10 minutes and the tubes were shaken vigorously after 5 minutes of incubation. The latex reagents were re-suspended by gently agitation, and one drop was delivered of each latex on to a circle on the test slide. One drop of the extract was added by micropipette tip to each drop of latex on to a circle on the test slide. Finally, the slide was rotated for no longer than 1 minute, and then observed for agglutination. A positive result was indicated by the visible agglutination of the latex particles, while a negative result was indicated by a milky appearance without any visible agglutination of the latex particles (Brooks *et al.,* 2004**).**

2.2. Qualitative assay of biofilm formation: Tube Method (Adhesion assay)

Glycocalyx production was determined, as described by Yassien and Khardori (2001) and Mathur *et al.* (2006) , as follows: Two to three colonies of study isolates were inoculated into 5 ml of brain heart infusion broth supplemented with 2% glucose in plastic conical tubes. After that, cultures were incubated at37° C for 18-24 hours and the contents were aspirated; one tube was examined unstained and other stained with crystal violet. Finally, slime positivity was judged by the presence of visible stained or unstained film lining the wall of the tube.

2.3. Quantitative assay of biofilm formation: Micro titer plate assay

Adhesion and biofilm formation was determined by using a spectrophotometric method, described by Yassien and Khardori (2001) and Stepanovic *et al*. (2003) as follows: working cultures were prepared by inoculation study isolate on Columbia agar supplemented with 5% blood and incubated aerobically at 35°C for 24 hrs. The cultures were used to prepare standardized bacterial suspension in sterile distilled water adjusted to a 0.5 McFarland turbidity standard to reach 10^5CFU/ml and the suspensions obtained were inoculated into a brain heart infusion broth with glucose (glucose supplemented medium) and without glucose.200 µL of standardized cultures were added to each well of sterile polystyrene Microtiter plate and incubated at 37°C for 18 hrs. Following incubation, the content of each well was aspirated, and each well was washed 3-4 times with sterile distilled water and the remaining attached bacteria were fixed with 200 µL of methanol per well. After 15 minutes, the plate wells were emptied and left to air dry. After wards the plates were stained for 5 minutes with 160 µL per well of crystal violet (0.25%) and the excess stain was rinsed off by placing the Micro titer plate under the running tap water. The plates were air-dried, and the dye, which was bound to the adherent cells, was re-solubilized with 160 µL of 33% glacial acetic acid per well. Finally, the optical density (OD) of each well was measured at 570 and 630 nm by using Stat Fax 3200 ELISA Reader. The isolates were classified according to biofilm production depending on the criteria laid down by Christensen *et al.* (1985) as follows: Strong producer more than 0.240; weak producer between 0.120-0.240 while non-producer less than 0.120.

2.4. Antimicrobial susceptibility for planktonic cells: Broth macrodilution method (MIC method

The antimicrobial susceptibility test for seventeen strong biofilm isolates was done against ciprofloxacin. The bacterial standardization was performed according to 0.5 McFarland turbidity standards **(**Vandepitte *et. al*., 1996; Al-Ouqaili, 2002).

Antimicrobial agents stock solutions were filter sterilized and prepared at concentration (1000µg/ml). Different antibiotic concentrations (0.5-32µg/ml) were prepared in 5 ml of Mueller-Hinton broth and transferred to sterile capped tubes. At least 4-5 morphologically similar colonies were inoculated into Mueller-Hinton broth

and incubated at 37 ^{o}C until the viable turbidity was equal to the 0.5 McFarland, (about 10^8 CFU/ml). After that, the suspension was diluted 1:100 and certain volumes transferred to the tubes containing antibiotic dilutions, to reach a final cell concentration of (about 10^5 CFU/ml). Controls were represented by two tubes; one of them contained broth only and the other contained broth plus microorganism. Then, the tubes were incubated overnight at 37 ^{o}C. The result of minimal inhibitory concentration (MIC) was interpreted as the lowest concentration of antimicrobial agents which inhibits visible bacterial growth after overnight incubation (Ferraro *et al.*, 2002).

2.5. In vitro *biofilm antimicrobial susceptibility test: Biofilm formation by study isolates on catheter segments*

The method used for bacterial biofilm formation on catheter segments was described by Ishida *et al*.(1998). Briefly, the tested bacteria incubated in brain heart infusion broth overnight at 37 ^{o}C. Then, 10 µl of overnight culture was added to 500 µl of sterile media in which catheter segments ($1cm^2$) were inoculated, and subsequently incubated overnight at 37 ^{o}C. After that washing of the segments was achieved by sterile media (3-4) times to remove weakly attached bacteria. Then, segments were resuspended with sterile media and vortexed vigorously for 2 min which was considered as controls.

2.6. Bactericidal activity of antibacterial against biofilm forming sessile cells

To determine the bactericidal activity of selected antibiotics against the sessile cells, the catheter segments were incubated with the organism as described above, were taken out, washed gently with sterile media or saline and, subsequently, transferred to saline containing a given antibiotic with distinct concentrations (10X, 50X, 100X, 500X and 1000X) in which X represented the minimal inhibitory concentration of mentioned antimicrobial agents against planktonic cells which was previously detected. After that, the tubes were incubated for 24 hr. at 37 ^{o}C**.** After exposure of tested organisms to the desired concentrations of antibiotic, they were transferred to 10 ml of fresh brain heart infusion broth and stirred vigorously with a vortex mixer for 2 min. for dispersion sessile or adherent cells. Then, the suspension was diluted and plated on nutrient agar plates for bacterial colony counting and compared with original bacterial count before exposing to antimicrobial agents (Ishida *et al*., 1998).

2.7. Detection of biofilm inhibitory concentrations (BICs) and minimal biofilm eradication concentration (MBECs)

After incubation, the tubes for 24hr. at 37 ^{o}C, the biofilm inhibitory concentration was detected and defined as the lowest concentration of antimicrobial agents which inhibits bacterial biofilm growth on a surface of catheter. It was represented by the clearance of broth medium consisting (1cm) catheters and the required concentrations of antimicrobial agents. After plating the diluted suspension into agar plates and counting the number of bacterial colonies, minimal biofilm eradication concentration (MBEC) was determined. MBEC was defined as the lowest concentration of antibiotic or biocide capable of killing biofilm producer bacteria. It was represented by disappearing of colonies of biofilm producer organisms on the culture plates (Ceri *et al.*, 2006).

2.8. Statistical analysis

All data were analyzed using the SPSS statistical program (statistical Package for the Social Science) version 14.0 and ANOVA test. Statistical significance was taken with p value < 0.05 and 0.001. The significant differences were detected by using either the goodness fit test within chi-square test or independent sample-test. All the study graphics (bar chart, scatter diagram or dot chart) were done by using Microsoft Excel XP (Simon, 2006).

3. Results

3.1. Bacteriological and Lancefield's grouping Identification

Out of 40 study specimens, 25 (62.5) were culture positive cases while 15 (37.5) were culture negative cases. Among culture positive cases, 33 mixed bacterial infection cases were observed consist of both of *Staphylococcus* and *Streptococcus* while *Streptococcus* were identified as pure single culture in 25 (100%) cases (table 1). All α-haemolytic streptococcal isolates 25 (100%) which were submitted to Lancefield's group classification were identified as group F *Streptococcus*.

Table 1. Culture positive and negative cases among study specimens obtained from patients with dental caries.

No. of specimens	Culture positive cases no. %)	Culture negative cases no. (%)	Microbial isolates *Streptococcus* and *Staphylococcus spp.* no. (%)	Group F. *Streptococcus* no. (%)
40	25 (62.5%)	15 (37.5%)	33 (82.5%)	25 (100%)

Out of 25 bacterial study isolates, 20(80%) were biofilm producers distributed into 17 (68%) as strong biofilm producer (OD more than 0.25) and 3 (12%) as weak producer (OD between 0.125-0.25 while 5 (20%) were non-biofilm producer isolates.

Under the field of biofilm production particularly tube method, our results showed that out of 20 isolates (80%)of *mutans Streptococcus* were biofilm producer on the inner lining of the tubes. On the other hand this phenomenon was not observed in 5 isolates (20%).

Furthermore, in the quantitative biofilm formation assay, our results showed that out of 20 (80%) of biofilm producer isolates, 17 (68%) were strong biofilm producer while 3 (12%) were weak producers. All of these events were under the presence of glucose in the experiment.

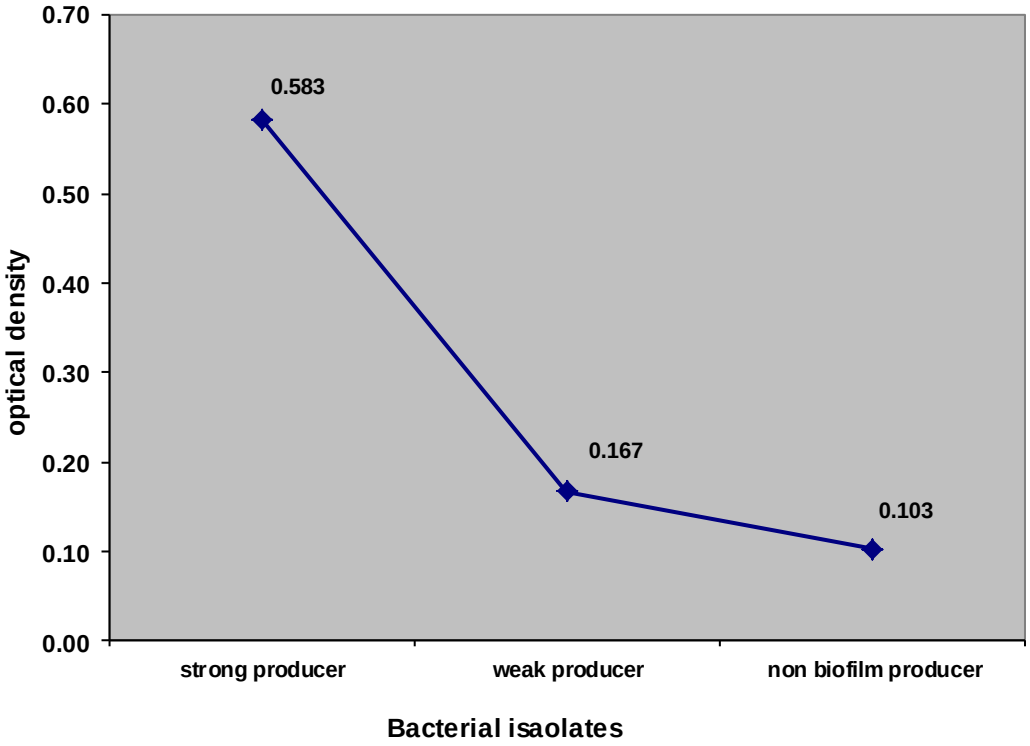

Figure 1. Biofilm production phenomenon regarding isolates potency.

Our results showed that the strong biofilm producer isolates have the highest mean of 0.583in comparison with the non-biofilm producer isolates which have the lowest mean of 0.103 (see Fig. 1). The study revealed that there is a highly significant difference between strong, weak and non-biofilm producer isolates of *mutans Streptococcus* (F-value was 74.882). Further, the age effect on bacterial isolates reveal highly significant difference between them and the F-value was 6.941 (Fig. 2).

Regarding sex differences between volunteers and its relation with biofilm phenomenon, our results showed that male isolates (14 isolates) had the highest mean in biofilm production which was 0.46829 ±0.187714 and for female the mean was 0.40027 ±0.283325.

The result of ciprofloxacin, against sessile cell of *milleri Streptococcus* obtained from patients with dental caries, the BIC of ciprofloxacin against strong biofilm study isolates S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, S16, and S17 were 50 (50X MIC), 800 (50X MIC), 80 (10X MIC), 100 (100X MIC), 160 (10X MIC), 800 (50X MIC), 200 (50X MIC), 100 (100X MIC), 800 (50X MIC), 400 (50X MIC), 400 (50X MIC), 200 (50X MIC), 50 (50X MIC), 800 (100X MIC), 100 (50X MIC), 1600 (100X MIC), and 1600 (50X MIC) µg/ml, respectively. These isolates have shown clearance of broth (inhibition of biofilm) and reduced the viable count of bacterial biofilm from 20 $\times 10^{5,}$ 68×10^5, 75×10^5, 62×10^5, 45×10^5, 37×10^6, 81×10^5, 45×10^5, 43×10^6, 45×10^5, 50×10^5, 61×10^5, 53×10^5, 77×10^5, 120×10^4, 53×10^5, 88×10^5, respectively. On the other hand, the MBEC of 500 (500X MIC), 8000 (500X MIC), 800 (100X MIC), 1000 (1000X MIC), 16000 (1000X MIC), 8000 (500X MIC), 2000 (500X MIC), 1000 (1000X MIC), 8000 (500X MIC), 5000 (500X MIC), 4000(500X MIC), 4000 (1000X MIC), 100 (100X MIC), 4000 (500X MIC),1000 (500X MIC), 16000 (1000X MIC), 32000(1000X MIC) µg/ml were enough to eradicate biofilm of above mentioned isolates respectively (Table 2).

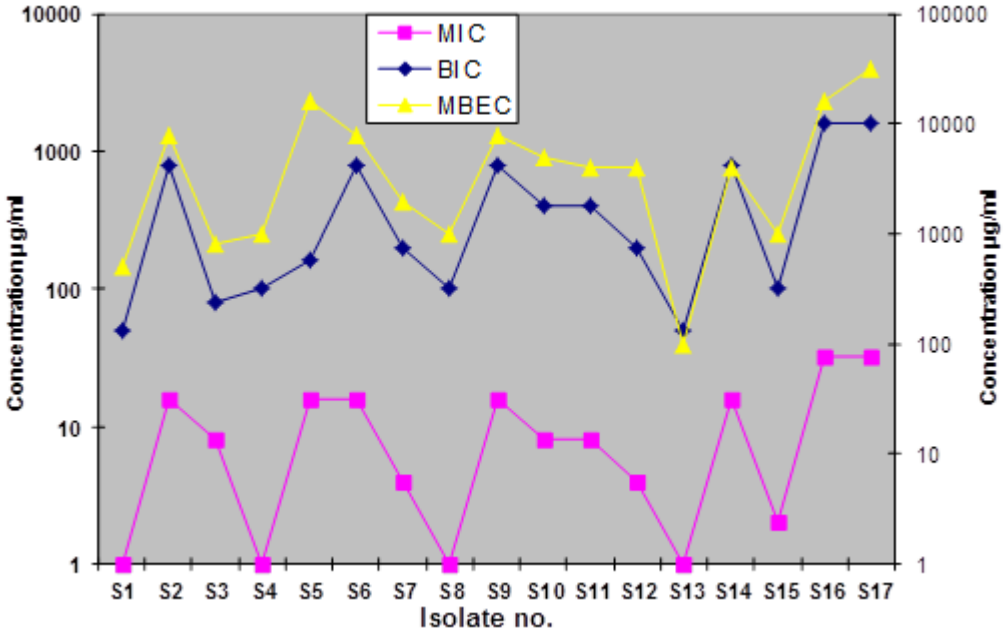

Figure 2. Relationship between of Minimal inhibitory concentration, biofilm inhibitory concentration and minimal biofilm eradication concentrations (µg/ml)

Table 2. The minimal inhibitory concentration (MIC), the biofilm inhibitory concentration (BICs) and minimal biofilm eradication concentration (MBECs) for ciprofloxacin against biofilm producing isolates among study specimens.

Isolate no.	MIC µg/ml	Colony count for control (CFU/ml)	Biofilm Inhibitory Concentration (BIC)			Minimal Biofilm Eradication Concentration (MBEC)		
			No. of folds higher than MIC	Conc. mg/ml	Colony count CFU/ml	No. of folds higher than MIC	Conc. mg/ml	Colony count CFU/ml
S1	1	20×10^5	50 x	50	45	500x	500	zero
S2	16	68×10^5	50 x	800	29×10^2	500x	8000	zero
S3	8	75×10^5	10 x	80	25×10^2	100x	800	zero
S4	1	62×10^5	100x	100	30×10^2	1000x	1000	2
S5	16	45×10^5	10x	160	75	1000x	16000	1
S6	16	37×10^6	50x	800	45×10^2	500x	8000	1
S7	4	81×10^5	50x	200	30×10^2	500x	2000	3
S8	1	45×10^5	100x	100	43	1000x	1000	1
S9	16	43×10^6	50x	800	43×10^2	500x	8000	2
S10	8	45×10^5	50x	400	33	500x	5000	zero
S11	8	50×10^5	50x	400	49×10^2	500x	4000	2
S12	4	61×10^5	50x	200	48	1000x	4000	zero
S13	1	53×10^5	50x	50	33×10^2	100x	100	zero

4. Discussion

As a biofilm, dental plaque exhibits an open architecture much like that of other biofilms. The open architecture, which consists of channels and voids, helps to achieve the flow of nutrients waste products metabolites enzymes and oxygen through the biofilm (Overman, 2000). Because of this structure a variety of microbial organisms can make up biofilms including both aerobic and anaerobic bacteria. Dental plaque biofilms are responsible for many of diseases common to the oral cavity including dental caries, periodontitis, gingivitis and the less common peri-implantitis (similar to periodontitis but with dental implants); however biofilms are present on healthy teeth as well (Sbordone and Bortolaia, 2003).

Bacteria living in a biofilm usually have significantly different properties from free-floating bacteria of the same species, as the dense and protected environment of the film allows them to cooperate and interact in various ways. One benefit of this environment is increased resistance to detergents and antibiotics, as the dense extracellular matrix and the outer layer of cells protect the interior of the community. In some cases antibiotic resistance can be increased 1000 fold (Stewart and Costerton, 2001). The concept that biofilms are more resistant to antimicrobials is not completely accurate. For instance, the biofilm form of *Pseudomonas aeruginosa* has no greater resistance to antimicrobials, when compared to stationary phase planktonic cells. However, when the biofilm is compared to logarithmic phase planktonic cells, the biofilm does have greater resistance to antimicrobials. This resistance to antibiotics in both stationary phase cells and biofilms may be due to the presence of persister cells (Spoering and Lewis, 2001).

Technological progress in microscopy, molecular genetics and genome analysis has significantly advanced our understanding of the structural and molecular aspects of biofilms, especially of extensively studied model organisms such as *Pseudomonas aeruginosa*. Biofilm development can be divided into several key steps including attachment, microcolony formation, biofilm maturation and dispersion; and in each step bacteria may recruit different components and molecules including flagellae, type IV Pili, DNA and exopolysaccharide (Jarrell, 2009; Ullrich, 2009). The rapid progress in biofilm research has also unveiled several genetic regulation mechanisms implicated in biofilm regulation such as quorum sensing and the novel secondary messenger cyclic-di-GMP. Understanding the molecular mechanisms of biofilm formation has facilitated the exploration of novel strategies to control bacterial biofilms (An, 2010).

Biofilms, by their nature, are very resistant to change, and when they do change, it usually takes time for evolution of the bacterial species to occur. Modifying pressures can cause a change from constant overload of pathogenic organisms, external risk factors and risk behavior. These can all lead to environmental changes within the biofilm, which favor the proliferation of aciduric and acidogenic pathogenic species like *mutans Streptococcus* and *Lactobacilli*, that help them to take over the biofilm (Busscher and Evans, 1998). A cariogenic biofilm may consist of over 96% acidogenic/aciduric, pathological bacteria, compared to less than 1% in a healthy biofilm. When all the factors that may contribute to a biofilm evolution are examined, it appears the primary driver is an acidic pH shift that can be either extrinsic or intrinsic to the dental biofilm or both (Marsh, 2006).

It is remarkable that the biofilm (adherence) mode of growth of bacteria on the tooth surfaces in the oral cavity has been well documented as one of the important causes of dental caries. Numbers of tests are available to detect slime production by bacteria, including tissue culture plates or spectrophotometric assay tube method (Mathur *et al*., 2006). Under the field of biofilm production, particularly qualitative adhesion assay, tube method, our results showed that out of 20 isolates (80%) of *mutans Streptococcus* were biofilm producer in the inner lining of the tubes; this phenomenon was not observed in 5 isolates (20%). These results were in agreement with Zezhang and Robert (2002) who found the genes required for biofilm development by *S. mutans* isolated from the oral cavity. In the quantitative biofilm formation assay, spectrophotometric method with ELISA reader was achieved under the presence of glucose and the crystal violet stain was dependent in this technique. Because crystal violet uniformly stains bacterial cells regardless of the presence or absence of slimy materials, properly speaking, the optical densities of bacterial films stained with crystal violet indicate the concentration of bacteria on the surface of the plates, not the presence of the slime. Thus, researchers considered these readings as index of the adherence of an organism to a surface and not a measure of slime production. Also the same researchers used weight of bacterial biofilm through counting the chromosomes by measuring the DNA content as an index to the number of bacterial cells per gram of film. They concluded that the weight of bacterial cells in biofilm was relatively constant among study isolates and eventually indicated that measuring of optical densities by spectrophotometric assay is a reasonable method for comparing the adherence phenomenon to plastic surfaces.

In order to enable easier study of bacterial attachment and colonization, a variety of experimental, direct and indirect observation methods have been developed. Microtiter plate assay (spectrophotometric assay) is the most frequently used techniques for quantifying biofilm formation (Stepanovic *et al.*, 2003). Microtiter plate assay is an indirect method for estimation of bacteria in situ, it has the advantage of enabling researcher to rapidly analyze adhesion of multiple bacterial strains or growth conditions within each experiment, easy technique and used widely for antimicrobial agents susceptibility of biofilm. In this technique our results showed that among17 isolates (68%) of bacterial isolates were strong biofilm producer and 3 (12%) were weak biofilm producer isolates and highly significant difference was observed between groups of isolates in their ability of biofilm production. Our results were in coincidence with the observation laid down by Christensen and co-workers (1985) that bacterial adherence and glycocalyx formation was enhanced with the supplementation of glucose in the culture media.

It is well authorized that biofilms are considered to be highly resistant to antimicrobial agents. Generally speaking, this is not the case – biofilms that grow in the presence of antimicrobials better than planktonic cells do.

Biofilm is indeed highly resistant to killing by bactericidal antimicrobials, compared to logarithmic-phase planktonic cells. This should properly be referred to as phenotypic tolerance or tolerance. Several factors have been suggested to account for biofilm tolerance, slow growth, the presence of an exopolysaccharide matrix that can slow the diffusion of antimicrobials; multiple resistance pumps represent a generalized resistance mechanism and have been considered as candidates for a biofilm resistance mechanism (Spoering and Lewis, 2001). Antimicrobial susceptibility test for sessile cells of strong biofilm study bacterial isolates was achieved by detecting minimal biofilm eradication concentration (MBEC) was based on minimal inhibitory concentrations obtained by broth macrodilution technique achieved logarithmic phase planktonic cells of these bacteria. Regarding to ciprofloxacin our result revealed that the biofilm cells were required 10-100 times the MIC values for inhibition of bacterial biofilm while 100-1000 times the MIC values were needed to remove bacterial biofilm.

Virtually all antimicrobials are more effective in killing rapidly growing cells. Some antibiotics have an absolute requirement for cell growth in order to kill some of the more advanced β-lactams, flouroquinolones can kill non growing cells, but they are distinctly more effective in killing rapidly dividing cells. Slow growth undoubtedly contributes to biofilm resistance to killing (Al-Doori, 2009).

The study concluded that group F *milleri Streptococcus* isolated from patients with dental caries produced cariogenic biofilm qualitatively and quantitatively. Also, in terms of BICs and MBECs, the biofilm producer isolates were required 10-50 X MICs of ciprofloxacin to inhibit bacterial biofilm and 100-1000 X MICs to remove of bacterial biofilm in patients with dental caries.

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Determination of Larval Instars of Black Cutworm *Agrotis ipsilon* (Hufnagel) (Lepidoptera, Noctuidae)

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Abstract

The numbers of larval instars of *Agrotis ipsilon* (Hufnagel) were determined using measurements of larval head-capsule width. The obtained results emphasized that the head capsule width recorded was 0.28 mm for the 1st instar (newly hatched larvae) and 3.42 mm for the 6th instar (taken to pupation). The overall results fit in with Dyar's rule.

Keywords: *Agrotis ipsilon*, head-capsule width, larval instars, Dyar's Rule, number of instars.

1. Introduction

The larvae of *Agrotis ipsilon* (Hufnagel) (Lepidoptera, Noctuidae) is more commonly known as the black or greasy cutworm. It is a polyphagous serious pest of different economic plants found throughout the world (Rings and Johnson, 1975; El-Salamouny *et al*., 2003). Moreover, it is a serious pest of golf courses as well as vegetables and field corps (Hong and Williamson, 2004). The feeding habits vary across the six larval instars (Allan, 1975; Clement and McCartney, 1982).

There are several studies that apply Dyar's rule to other insect species. According to Dyar's rule, which is used for determining the instars and stages of many insect species, Moser *et al.* (1991) found that the width of the head capsules of *Ctenocephalides felis* (Bouche) of different instars had geometrically progressing growth. Goldson *et al.* (2001) found larval instar's distribution of the Argentina stem weevil by using head capsule width. Lauzon and Harper (1993) followed the growth of aquatic snipe fly, *Atherix lantha* Webb (Diptera, Athericidae), depending on measurements of second antennal segment of the larvae.

Rodriguez-Loeches and Barro (2008) found that the measurement of head capsule of *Phoenicoprocta capistrata* (Lepidoptera, Arctiidae) larvae followed a geometric pattern consistent with Dyar's rule. Moreover, Shashi and Singh (2009) determined the exact nymphal stages of *Chrotogonus trachypteras* (Blanchard).

In the present study, it was attempted to find out the number of larval instars depending on the measurement of larval head capsule width, and applying Dyar's rule to the results.

2. Material and Method

By using light traps, the moths were caught then placed in plastic boxes. They were fed with 10% honey water. The collection of eggs was facilitated by muslin cloth. The eggs were placed in an incubator (25°C) to hatch. The larvae were separated by plastic grids to form individual chambers and were kept at 25°C, 60-70% relative humidity. The larvae were reared on an artificial diet (Oxford recipe). The diet consisted of caseine, wheat germ, dry yeast, cholesterol, agar, wessons salt, vitamins and antibiotics mixture, fungicide and water (Hansen and Zethner, 1979). The experiment started with approximately 1000 newly hatched larvae which were reared as discussed above. Widths of larval head capsules were measured to determine the larval instars according to Dyar's rule (Gullan and Cranston, 2005).

The width of the head capsule of each larva was measured using a calibrated ocular micrometer in a binocular dissecting microscope. For each instar, 100 larvae were randomly selected and their head capsules measured in interval periods. The rest were kept rearing on the diet until the last instar measurement of head capsule took place. Since it was difficult to measure a live larval head capsule, 70% alcohol was used to kill the larvae before measuring the head capsule. The larvae which were deemed to be in their final instar were not killed; instead, their head capsule was measured while they were alive. They were then kept until pupation to ensure that further molting did not occur.

3. Results and Discussion

The figures obtained from measuring the head capsule width were plotted on diagram paper against the number of larvae. In this way, a histogram (Figure 1) was produced, with six different groups of larvae.

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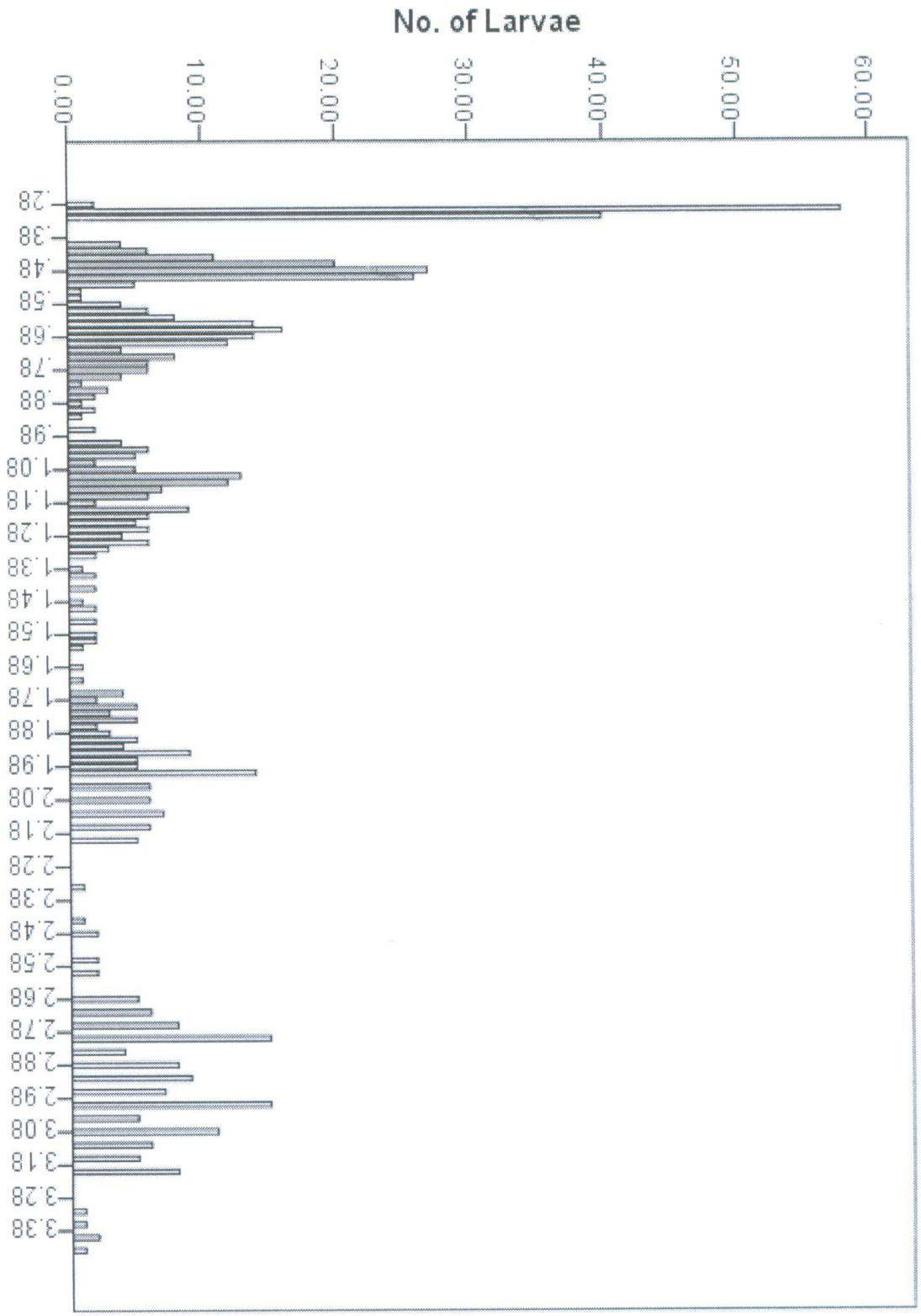

Figure 1: The measurement of larval head capsule width plotted against the number of larvae producing 6 different groups

The 1st group included only newly hatched larvae which were consequently representative of the 1st stage instar. On the other hand, the 6th group included the larvae which were pupated without further molting, so it is regarded as the final instars.

For many population studies, it is important to know the number of larval or nymphet instars within a species in order to be able to recognize the instars to which any immature individual belongs. More frequently, the only obvious difference between successive larval or nymphal instars is the increase in size that occurs after each molt. Thus, it should be possible to determine the actual number of instars in the life history of a species from a frequency histogram of the measurements of a sclerotized body part such as the width of the head capsule or the length of the mandible. Dyar's rule developed from his observation of the measurement of the larval head capsule width of some Lepidoptera species (Gullan and Cranston, 2005). Dyar's rule states that if the logarithms of the measurements of some sclerotized body part in different instars is plotted against the instars number, a straight line should result; any deviation from a straight line indicates a missing instars. In figure 2, one can notice that a straight line is produced, which indicates that there is no missing instars, fitting in with Dyar's rule. In figures 1 and 2 along with Table 1, we can see that there are six larval instars of *A. ipsilon.* These results agree with Zaz (1999), and Alnaji and Ghafoor (1988) who found that the larvae have six instars. On the other hand, Luckman *et al.* (1976) reported that larvae have seven instars. But Allan (1975) stated that the black cutworm has five instars only.

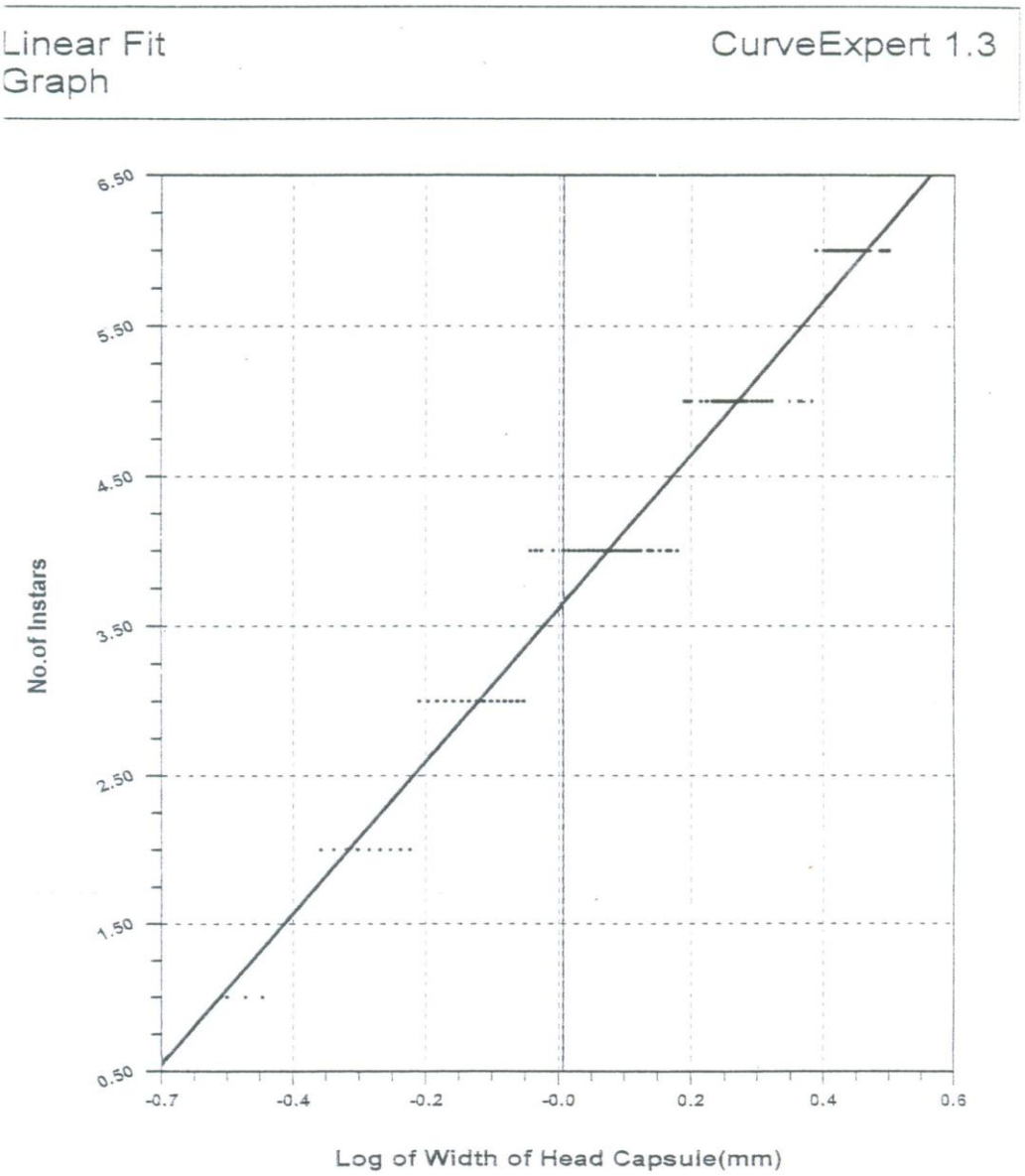

Figure 2. A linear regression shows a straight line which fitted with Dyar's rule

Table 1. Mean measurements width (mm) of larval head capsules of the *Agrotis ipsilon*

Larval instars	No. of larvae	Average of head capsule width (mm)± S.E
1st	100	0.307±0.0015
2nd	100	0.4726±0.0030
3rd	102	0.681±0.056
4th	108	1.16129±0.009
5th	98	1.96816±0.012
6th	117	2.95982±0.012

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In vitro Differentiation of Embryonic Stem Cells to Muscle like Cells after Treatment with Muscle Extract

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Abstract

One goal of embryonic stem cells (ESCs) research is the development of specialized cells such as neurons, heart muscle cells, endothelial cells of blood vessels, etc. Thus, the directed derivation of ESCs is vital to the ultimate use of such cells in the development of new therapies. This study was conducted to describe the conditions that induce differentiation of inner cell mass (ICM) derived ESCs into muscle like cells. Blastocysts were recovered from female's albino mice of strain Balb \c at day 3.5 after natural mating had taken place, cultured in MEM medium supplemented with 20% FCS in the presence of mitotically inactivated feeder layer attached to gelatin layer as a substrate at 37C° and 5% CO2. Within two days of the culture, the blastocyst were erupted from the Zona Pellucid and attached by their equatorial pole to the underlying substrate and the ICM, visible as a round dense cell mass in the center of the outgrowth of the trophoblastic cells. Within seven days, the ICM proliferated and increased in their girth, and numbers of ESCs were observed to be librated from the ICM to the surrounding area. When these ESCs were treated with mouse embryonic skeletal muscle extract (ESME) for twelve days, they differentiated and appeared as smooth muscle like cells and expressed positive response against primary monoclonal antibody anti myosin. We can conclude that mouse ESME plays an important role to direct differentiation of ESCs to the muscular pathway.

Keywords: Inner cell mass, embryonic stem cell, myogenic differentiation, anti-myosin. .

1. Introduction

Stem cells are unique cell populations with the ability to undergo both self-renewal and differentiation. This fate choice is highly regulated by intrinsic signals and the external microenvironment, the elements of which are being rapidly elucidated (Watt and Hogan, 2000).

A wide variety of adult mammalian tissues harbors stem cells "adult stem cells" which may be capable of developing into only a limited number of cell types. In contrast, embryonic stem cells (ESCs), derived from a group of cells called Inner Cell Mass (ICM), which is part of the blastocyst stage early mammalian embryos have the ability to form any fully differentiated cell of the body (Drukker, 2004; Snykers *et al.*, 2009).

Stem cells of the ICM promptly differentiate to generate primitive ectoderm, which ultimately differentiates during gastrulation into the three embryonic germ layers. When removed from their normal embryonic environment and cultured under appropriate conditions, ICM cells give rise to cells that proliferate and replace themselves indefinitely. ICM cells are the source cells from which pluripotent mouse, nonhuman primate, and human ESCs are generally derived (Evans and Kaufman, 1981; Brook and Gardner, 1997).

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It is widely accepted, in the resent few years, that ESCs are promising source of transplantable cells to be used in regulative medicine. These cells can be maintained in culture conditions for long periods of times without losing their pluripotency. Understanding how to control both proliferation and differentiation of stem cells and their progeny is a challenge in many fields, going from preclinical drug discovery and development to clinical therapy (Snykers *et al.*, 2009).

Directing the rout of differentiation can be carried out for example by addition of growth factors to the cells undergoing differentiation for specific cell types such as neurons (Carpenter *et al.*, 2001), cardiac-like muscle cells (Waheed *et al*., 2010), renal lineage (Stephan *et al*., 2007), hepatocytes (Snykers *et al.*, 2009), etc.

The present study aims at describing the conditions that induce differentiation of ICM-derived ESCs reliably and high efficiency into muscle like cells.

2. Materials and Methods

2.1. Harvesting and culturing of balstocysts

Blastocysts were recovered from female's albino mice of strain Balb\c by flushing the uterus at day 3.5 after natural mating had taken place (Evans and Kaufman, 1981; Pollard and Walker, 1997). Blastocysts were cultured in Minimum Essential Medium (MEM) Eagle modified (Sigma) supplemented with 20% Fetal Calf

Serum (FCS) in the presence of mitotically inactivated mouse embryonic fibroblast cells (Feeder layer), which were attached to gelatin layer as substrate. The cultures were maintained at 37C°, 5% CO_2 (Evans and Kaufman, 1981; Pollard and Walker, 1997; Waheed and Hammash, 2008).

2.2. Preparation of mouse embryonic fibroblast cultures (Feeder layer)

The feeder layer was prepared according to the method of Pollard and Walker (1997). Briefly, 16-18 days old mouse embryos of strain Balb/c were trypsinised (0.125% Trypsin in Phosphate Buffer Saline without Ca & Mg (CMF free PBS)) and cultivated in MEM medium plus 15% FCS. After confluence, the monolayer of the cells was treated with mitomycin – C (10 µg/ml) for two hours then seeded at a density of $5X10^6$ cells per each well of the 4-well plate precoated with gelatin (0.01% solution of gelatin in H_2O). The feeder layer was prepared fresh (one day before use).

2.3. Induction of differentiation

To initiate directed differentiation, the attached and proliferated ICM derived ESCs were cultured with addition of mouse embryonic skeletal muscle extract (E SME) to the culture medium (1:3) for two days. Skeletal muscle extract was prepared from the skeletal muscles of the legs and hands of 12-14 days aged mouse embryos following the general principles of embryo extract preparation as set by (New, 1966).

2.4. Immunocytochemical detection of differentiation of ICM-ESCs in vitro

The cells that differentiated from treated ICM-derived ESCs with ESME and the cells of un treated (as a control group) were fixed with 4% Phosphate buffered formalin for 10 minutes, then detected by immunocytochemistry method, which was performed with primary monoclonal antibodies against anti-myosin (Antimyosin Kit of Sigma – Germany) according to the method of (Pochampally *et al.*, 2004; Xu *et al.*, 2004).

3. Results

Mouse blastocysts, recovered 3.5 days after fertilization, have two distinct populations of cells (trophoblast cells and ICM cells) distinguished by their position within the non cellular spongy fibers Zona Pellucida (Z. P.) (Fig. 1).

During the first day of culture, most blastocysts began to shed the Z.P., and within two days a flat sheet of trophoblastic cells was formed. The embryo was visible as a round dense cell mass in the center of the outgrowth of trophoblast. Until this stage the total embryonic mass did not increase significantly, although division continued and resulted in an increased number of small and smaller cells. Within the time of culture, these ICMs continued to increase in girth and in day seven we observed a large number of ESCs which have a high ratio of nucleus to the cytoplasm are librated from the ICM and occupied the surrounding area (Fig. 2 A and B).

The ICM-derived ESCs were treated with ESME, and certain morphological changes were observed and recorded in these cells in day six after treatment (Fig. 3). After twelve days of treatment, these cells appeared fusiform in shape, largest at their midpoints and taper toward their end. Each cell has a single nucleus located in the central the broadest part of the cell. The narrow part of one cell lies adjacent to the broad parts of neighboring cells. Such an arrangement is characteristic of smooth muscle cells (Fig. 4 A and B).

3.1. Immunocytochemical detection for differentiation of ICM-ESCs to muscle like cells

The immunocytochemical detection for differentiated ESCs to muscle like cells was performed using monoclonal antibody against myosin, the contracting protein in muscle cells. Cells expressed positive response which appeared as brown color (Fig.5). While in control group, the cells showed negative response.

4. Discussion

The ESC is defined by its origin that is from one of the earliest stages of the development of the embryo, called the blastocyst. At this stage, the preimplantation embryo of the mouse is made up of 150 cells and consists of a sphere made up of an outer layer of cells (the trophectoderm), a fluid-filled cavity (the blastocoel) and a cluster of cells on the interior the ICM (Slack, 2000). Specifically, ESCs are derived from the ICM of the blastocyst at a stage before it would implant in the uterine wall. When these cells maintained in cultures and as described in our results and as demonstrated by other reports (Ledermann, 1997; Waheed and Hammash, 2008), the ICMs continued its division and resulted in an increased number of small and smaller cells of ESCs. The resulted ESCs have a high ratio of nucleus to the cytoplasm are librated from the ICM in the day seven from initial culture.

The ESCs are pluripotent; it can give rise to cells derived from all three germ layers (ectoderm, endoderm and mesoderm). These three germ layers are the embryonic source of cells of the body. Mouse ESCs can be maintained in a proliferative, undifferentiated state *in vitro* by growing them on feeder layer (embryonic fibroblasts). As demonstrated by Smith (2001), feeder layer produces differentiation inhibitory factor (DIF)\Leukemia inhibitory factor (LIF) . These factors allow mouse ESCs *in vitro* to continue proliferating without differentiating (Rathjen *et al.*, 1990). LIF exerts its effects by binding to a two-part receptor complex that consists of the LIF receptor and the gp130 receptor. The binding of LIF trigger the activation of the latent transcription factor STAT3, a necessary event *in vitro* for the continued proliferation of mouse ESCs (Burdon *et al.*, 1999).

4.1. Directed differentiation of ICM-derived ESCs into muscle like cells in vitro

Embryonic stem cells have the capacity to differentiate into all cells of the developing embryo and may provide a renewable source for future cell replacement therapies (Stephan *et al.*, 2007).

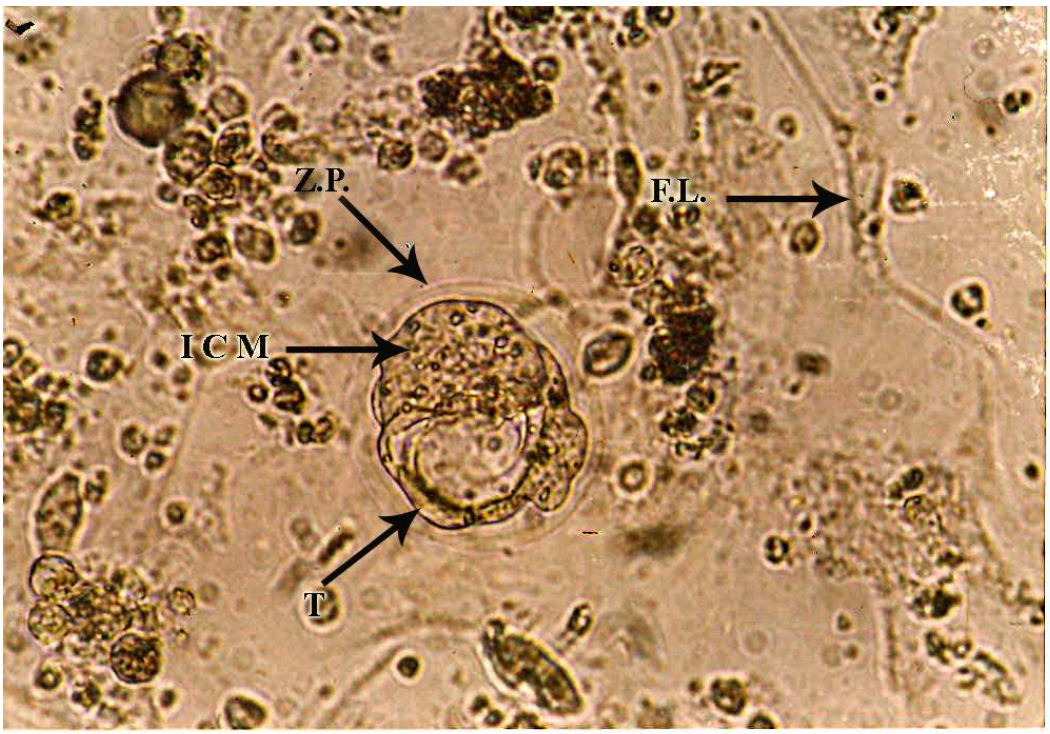


Figure 1. Mouse Blastocyst 3.5 days after fertilization, have two distinct populations of cells (trophoblast (T) cells and ICM cells) distinguished by their position within the non cellular spongy fibers Zona Pellucida (Z. P.), cultured on feeder layer (F.L.), (X160) .

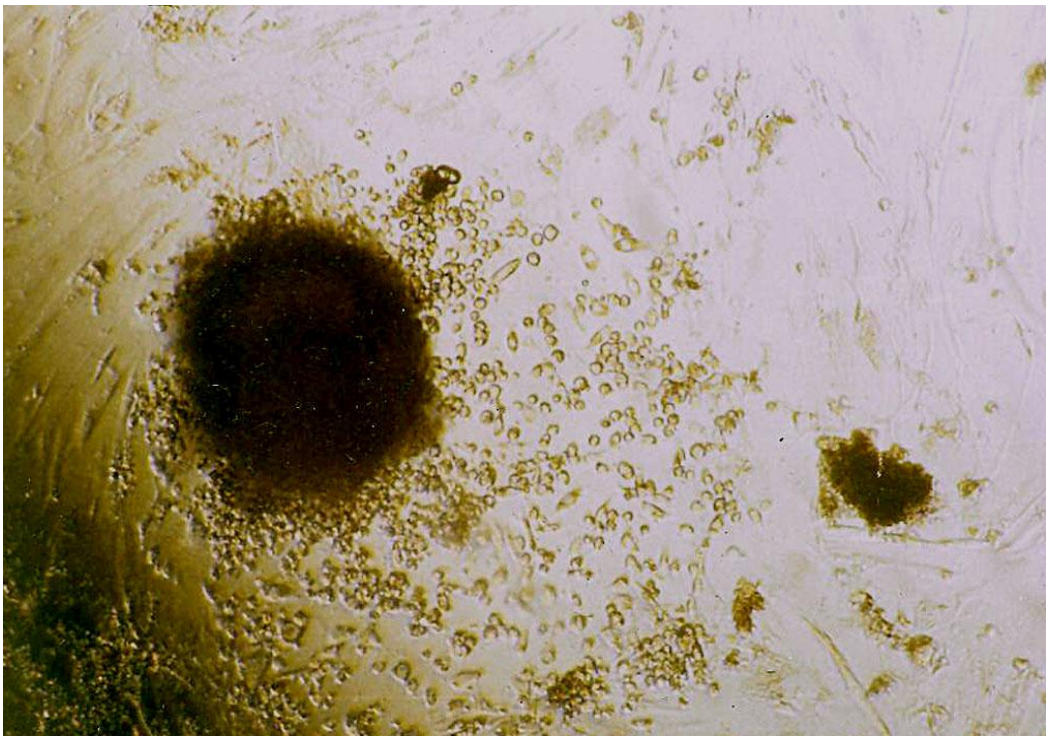


Figure 2.A.

Figure 2 continues next page……

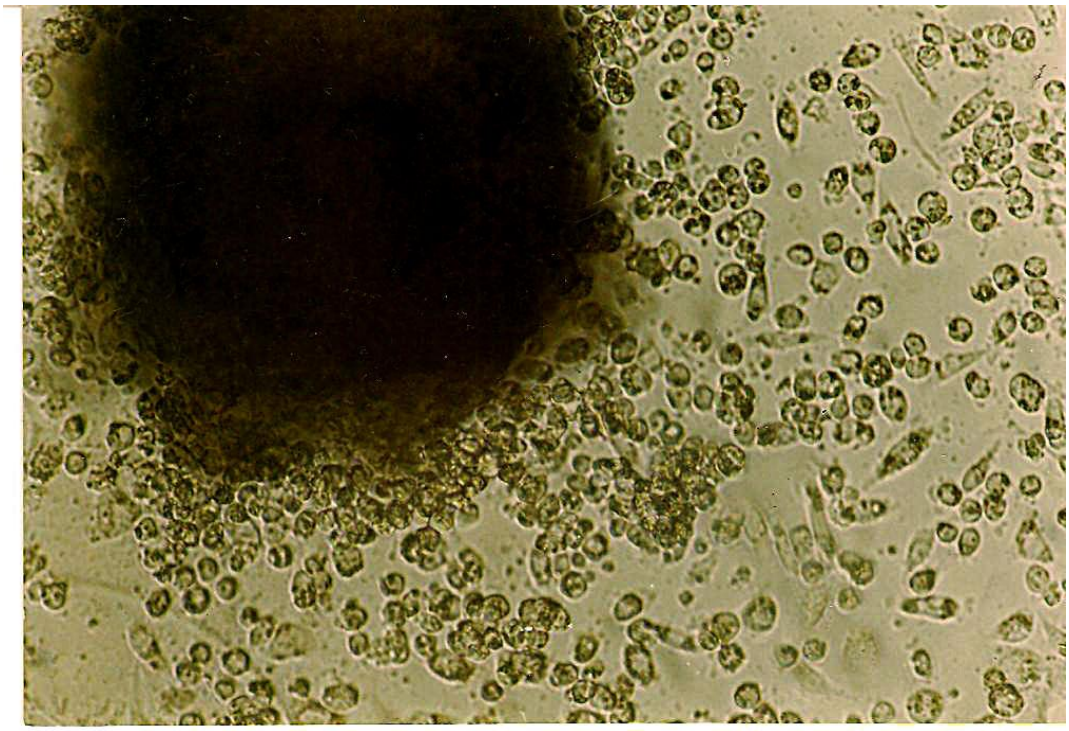


Figure 2.**B**.

Figure 2. (A and B) Seven days of culture. The ICMs continued to increase in girth and number of ESCs have a high ratio of nucleus to the cytoplasm are librated from the ICM. A (X 40), B (X160), (B is a higher magnification of A)

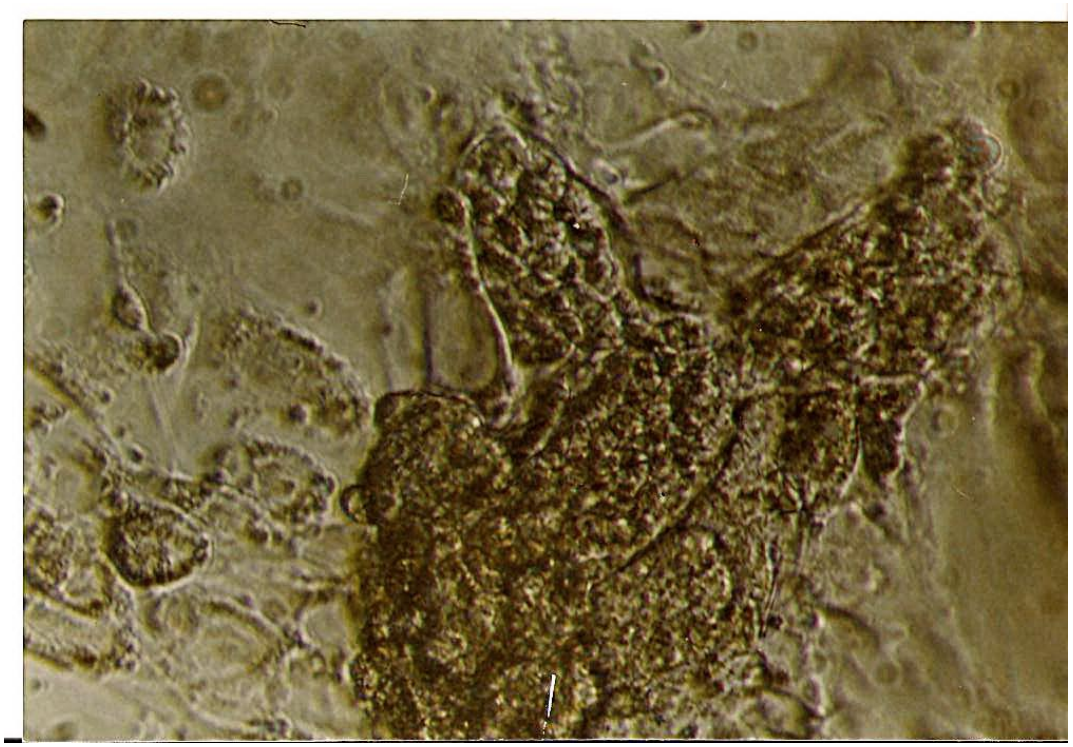
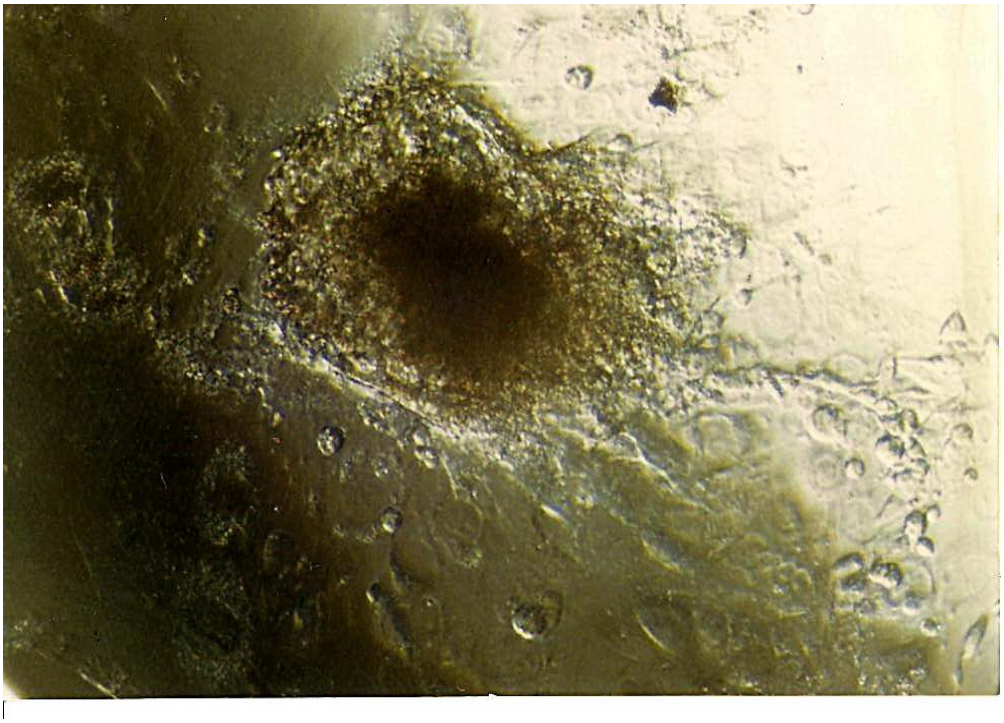
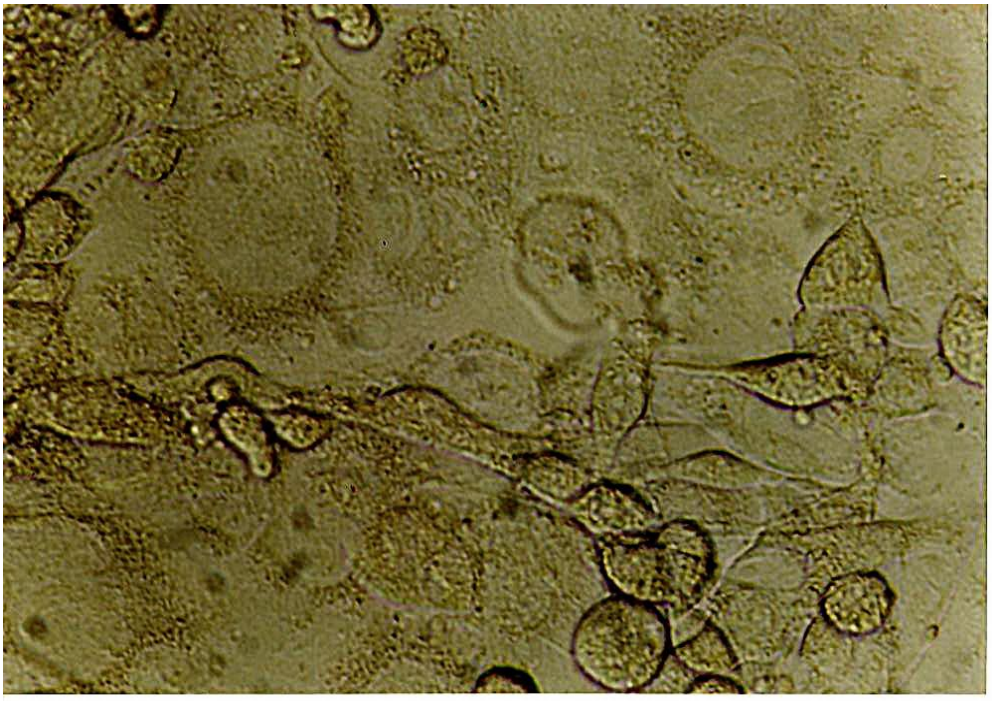


Figure 3. Six days in culture after treatment with ESME the cells began to differentiate into muscular pathway (X160).



A



B

Figure 4. (A and B) (B is a higher magnification of A): After 12 day from treatment with ESME these cells appeared as a fusiform in shape, they are largest at their midpoints and taper toward their end. The narrow part of one cell lies adjacent to the broad parts of neighboring cells. Such an arrangement is characteristic of smooth muscle cells, A (X 100.8), B (X 160).

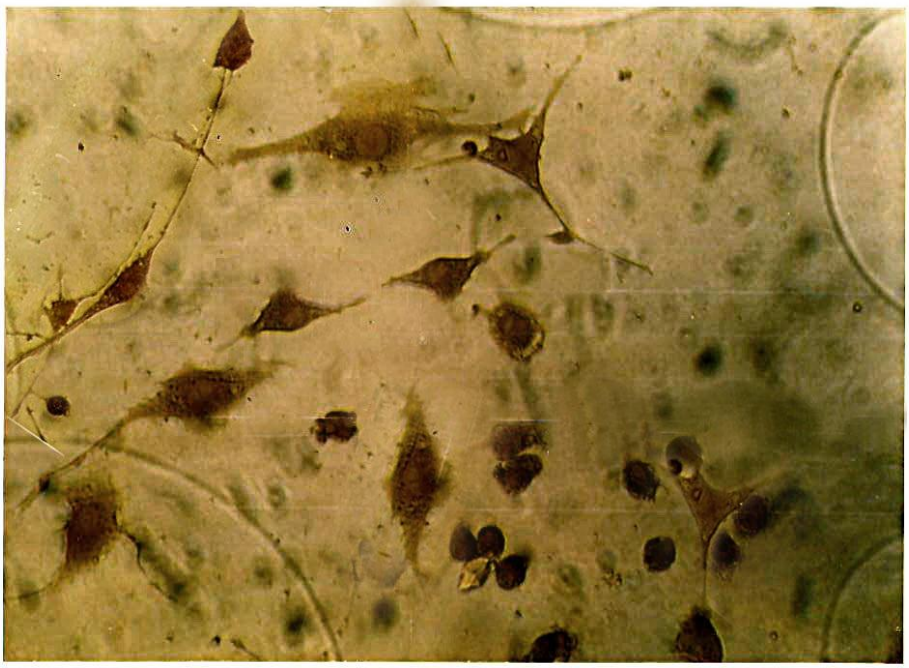


Figure 5. Immunocytochemical detection for differentiation of ESCs *in vitro* in treated groups with mouse ESME, The differentiated cells was expressed positive response for anti-myosin marker and stained with brown color (DAB) stain (X160).

In the present study, first, the ICM-derived ESCs is stimulated to differentiate into muscle like cells by treating them with muscle extract (in the present study is a crude source of stimulating factor) for directing differentiation of ICM-ESCs *in vitro*; secondly, these cells were recognized by their morphological characteristic. By using inverted microscope, we observed the differentiation of ICM-derived ESCs into muscle like cells in treated cells compared with untreated cells. These results are consistent with other reports (Drab *et al.*, 1997; Grove *et al.*, 2004) which suggest that retinoic acid (RA) and db-cAMP induce ICM-derived ESCs to differentiate into myogenic cells *in vitro*.

The precise mechanism of induced ICM-derived ESCs to differentiate into muscle like cells is unknown. In studies on myogenic differentiation of the mouse embryonic cell line after treated with 5-azacytidine" (which is a DNA demethylating chemical compound used to induce cardiomyogenic differentiation), Konieczy *et al.*, (1984) proposed that these cells contain a myogenic determination locus in a methylated state with a transcriptionally in active phase, which become demethylated and transcriptionally active with 5-azacytidine, causing the cells to differentiate into myogenic cells. The role of muscle extract as shown in this study and as mentioned by Leor *et al.*(1996) which indicated that specially most embryonic extract are regarded as an important source of extracting factors that stimulate the growth and differentiation of stem cells into special direction, then the use of these cells in the therapeutic angiogenesis. The results in the present study is in agreement with the results obtained by Al-Jumely (2006) which demonstrated that differentiation of the mouse Bone marrow hematopoietic stem cells (HSCs) into muscle like cells *in vitro* after treatment with heart muscle extract of new born mice. The present results are also in agreement with the results of Waheed *et al.* (2010) demonstrate that the edition of rat embryonic heart muscle extract to the culture medium induced the differentiation of the rat mesenchymal stem cells (MSCs) into cardiac like muscle cells, which then detected by using specific markers such as anti-myosin and anti cardotin.

The results of the immunocytochemistry examination showed that the detection by using anti-myosin marker express positive response of the differentiated cells which accepted chromogen DAB stain. The results correspond to different studies (Makine *et al.*, 1999; Sherwood *et al.*, 2004; Al-Jumely, 2006) which suggests that the expression may be associated with the activation of the myosin gene. Myosin is one of the major proteins of muscle, and makes up thick myofilamants in myofibrils of muscle cells that play an important role in contraction of muscle cells (Pochampally *et al.*, 2004).

We can conclude that the success in stimulating the differentiation of ICM-derived ESCs into myogenic cells is attributed to the effect of muscle extract which contain certain growth factors that stimulate differentiation and it may play an important role in the directing differentiation of these cells to the muscular pathway.

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Short Communication

The Sesarmid crab *Parasesarma persicum* Naderloo and Schubart, 2010 (Crustacea: Decapoda: Brachyura: Sesarmidae), New to the Iraqi Coastal Waters of Khor Al-Zubair and Shatt Al-Basrah Canal, Basrah, Iraq

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Abstract

Specimens of the sesarmid crab *Parasesarma persicum* Naderloo and Schubart, 2010 were collected from the intertidal zones of Khor Al-Zubair and Shatt Al-Basrah Canal, Basrah, Iraq during the period from June 2009 to May 2010. A note on the morphological features and photographs of the species are provided to confirm its identification. *Parasesarma persicum* is the only species of the genus known in Iraqi coastal water.

Keywords: *Parasesarma persicum*, Khor Al-Zubair, Shatt Al-Basrah canal, Basrah, Iraq.

1. Introduction

The sesarmid genus *Parasesarma* De Man, 1895 (type species *Cancer quadratus* Fabricius, 1798) currently contains 33 species (Ng *et al.*, 2008, Rahayu and Ng 2009, Naderloo and Schubart 2010, Koller *et al.*, 2010). As discussed by Rahayu and Ng (2010), the taxonomy of the genus is one of the most problematic issues in the Sesarmidae, with many of the species, now included in it, and is not well known. Thus, a revision of *Parasesarma* is clearly necessary. One of the most frequently reported species in the Indo-West Pacific is *P. plicatum* (Latreille, 1803). This species has long been believed to have a wide distribution with records from East Africa (Lenz and Richters, 1881; De Man, 1889; Borradaile, 1907; Crosnier, 1965), the Arabian Gulf (Apel, 2001), the Gulf of Bengal and Andaman Sea (its type locality) (Latreille, 1803), and East Asia (as *P. affine*) (Tesch, 1917). Only recently, the validity of the originally described species has been accepted, and this way the presumed range of *P. plicatum* becomes more restricted (Rahayu and Ng, 2010). Naderloo and Schubart (2010) recently described *P. persicum*, a species that resembles *P. plicatum*, from different localities in Persian-Arabian Gulf based on the morphological and molecular characters. This paper is to report the occurrence of *P. persicum* in Iraqi coastal water for the first time.

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2. Materials and Methods

Specimens reported in this study were collected during a biodiversity monitoring project on the brachyuran fauna of the intertidal zones of Khor Al-Zubair and Shatt Al-Basrah during the period from June 2009 to May 2010. The material was mainly collected by hand and preserved in 70% ethanol. They are deposited in the Marine Science Centre (MSC, 22), University of Basrah, and the Zoological Reference Collection (ZRC) of the Raffles Museum of Biodiversity Research, the National University of Singapore. The abbreviations CL and CW indicate the carapace length and carapace width, respectively. Measurements given in text are given in millimeters.

2.1. Study area

Khur Al-Zubair is an extension of the Persian-Arabian Gulf waters in the lower reaches of Mesopotamia (Fig 1). It has a length of approximate 42 km, and a width of 1km at low tide, with an average depth of 10-20 m. In 1983, this water body was connected to an oligohaline marsh (Hor Al-Hammar,), by the Shatt Al-Basrah Canal, changing the environment of lagoon of the Khor from a hypersaline to an estuary (Hussain and Ahmed, 1999). The topography of the Khor Al-Zubair looks like a spindle with tapering ends at the northern and southern ends. The northern end receives fresh water influx of average 700 m^3/sec throughout the tidal cycle. The current in the Khor is characterized by one direction throughout the tidal cycle towards the southern end (Arabian Gulf), with velocity exceeding 2m/sec during ebb tide and 0.66 m/sec in flood tide. At the southern end, the water discharge reaches 10000 m^3/sec with velocity range of 0.8-5.78 m/sec and

with big tidal range at the Umm-Qasar reaching 4.3m. (Al-Badran *et al.*, 1996).

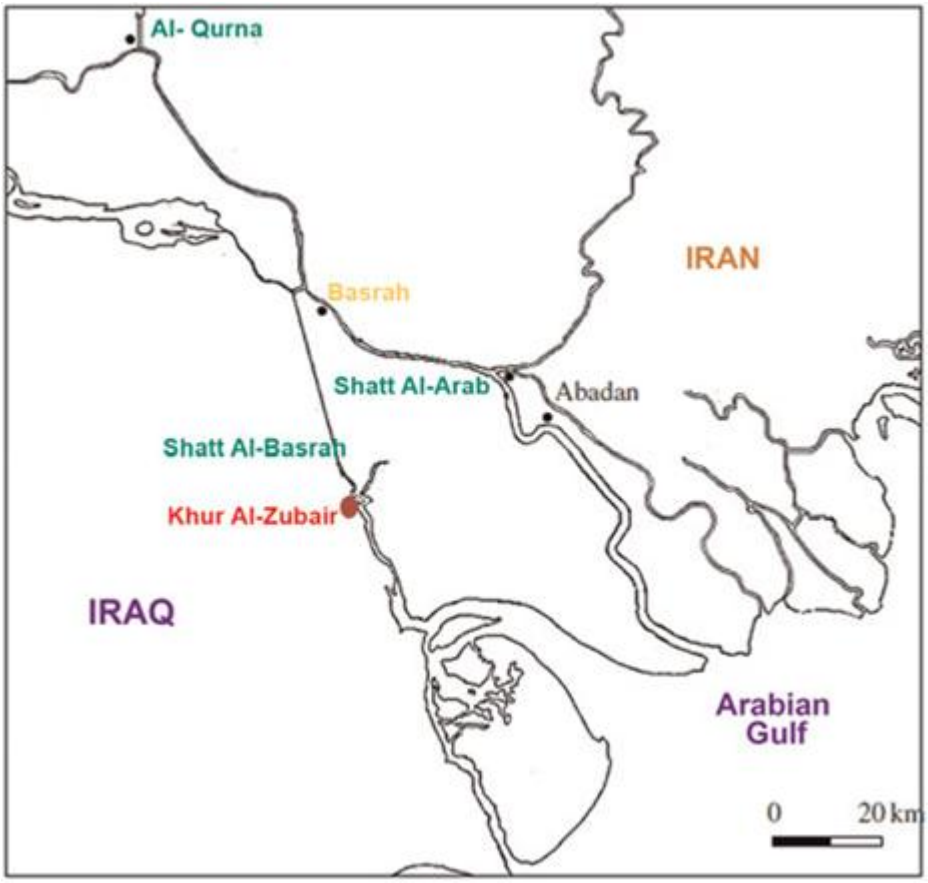

Fig. 1. Sampling sites.

3. Results

3.1. Systematic account

Family Sesarmidae

Genus *Parasesarma* De Man, 1895

Parasesarma persicum (Figs. 1a, b, 2a–g, 3a–d, 5a, 6)

Sesarma plicata – Clayton, 1986: 86. *Sesarma (Parasesarma) plicata* – Jones, 1986: 160-161, pl. 46. *Sesarma (Parasesarma) plicatum* – Al-Ghais and Cooper, 1996: 414-415, (Fig. 4).

Parasesarma plicatum – Tirmizi and Ghani, 1996: 151–153, (Fig. 58); Apel and Türkay, 1999: 133; Apel, 2001: 119; Naderloo and Schubart, 2009: 61 (not *Sesarma plicata* Latreille, 1803).

Parasesarma persicum – Naderloo and Schubart, 2010: 33-43, (Figs, 1-4).

3.2. Material examined

2 males (CL 26.4× CW 33.0, CL 25.2×CW 32.3), 2 females (CL 18.4× CW 24.4, CL 17.8× CW 23.7) from Shatt Al-Basrah Canal; 2 males (CL 23.4× CW 29.5, CL 22.4× CW 28.6) from Khor Al-Zubair; (all measurements in mm).

Fig. 2. *Parasesarma persicum*, male (CL 23.4× CW 29.5 mm) collected from Khor Al-Zubair : (a) dorsal view; (b) ventral view.

4. Diagsosis

Carapace squarish (Figs. 2a and 3a) is about 1.3 times as broad as long; maximum width is slightly behind external orbital angle; dorsal surface of carapace is relatively convex, smooth, with sparse tufts of short setae (especially distinct on frontal and branchial regions); carapace regions are mostly well defined; frontal region is sharply deflexed, four-lobed, nearly equal-sized, with narrow and deep groove between median lobes, extending onto gastric region, separated from laterals by shallow groove; gastric region is well defined, a pair of round depressions on anterior portion, a pair of depressions on posterior portion, two very small linear depressions on middle part of posterior portion; cardiac region is separated from intestinal region by low depression; small transverse depression is immediately behind supra-orbital margin; six ridges are on posterolateral region, various-sized, smooth, curved, beset with short setae.

Abdomen of male (Figs. 2b, .3b) is relatively short triangular, third segment widest; the sixth segment is slightly longer than the fifth, gently converging distally; telson is small, slightly longer than the sixth segment; margins of segments are all with short setae.

Figure 3. *Parasesarma persicum*, male (CL 26.4× CW 33) collected from Shatt Al- Basrah Canal: (a) dorsal surface of carapace; (b) abdomen and telson; (c) right

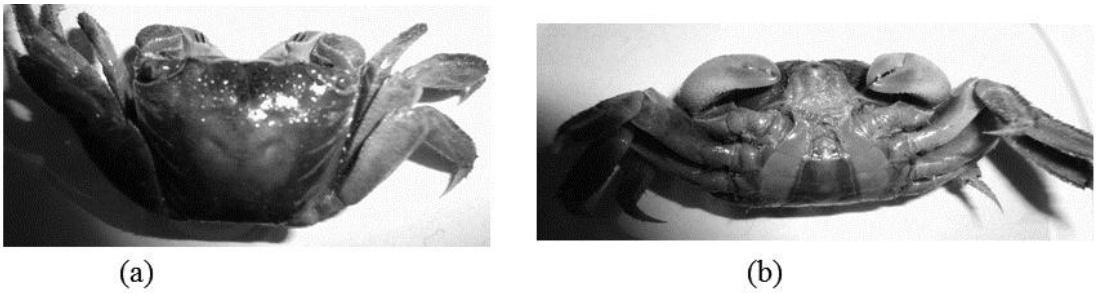

Figure 2. *Parasesarma persicum*, male (CL 23.4× CW 29.5) collected from Khor Al-Zubair: (a) dorsal view; (b) ventral view

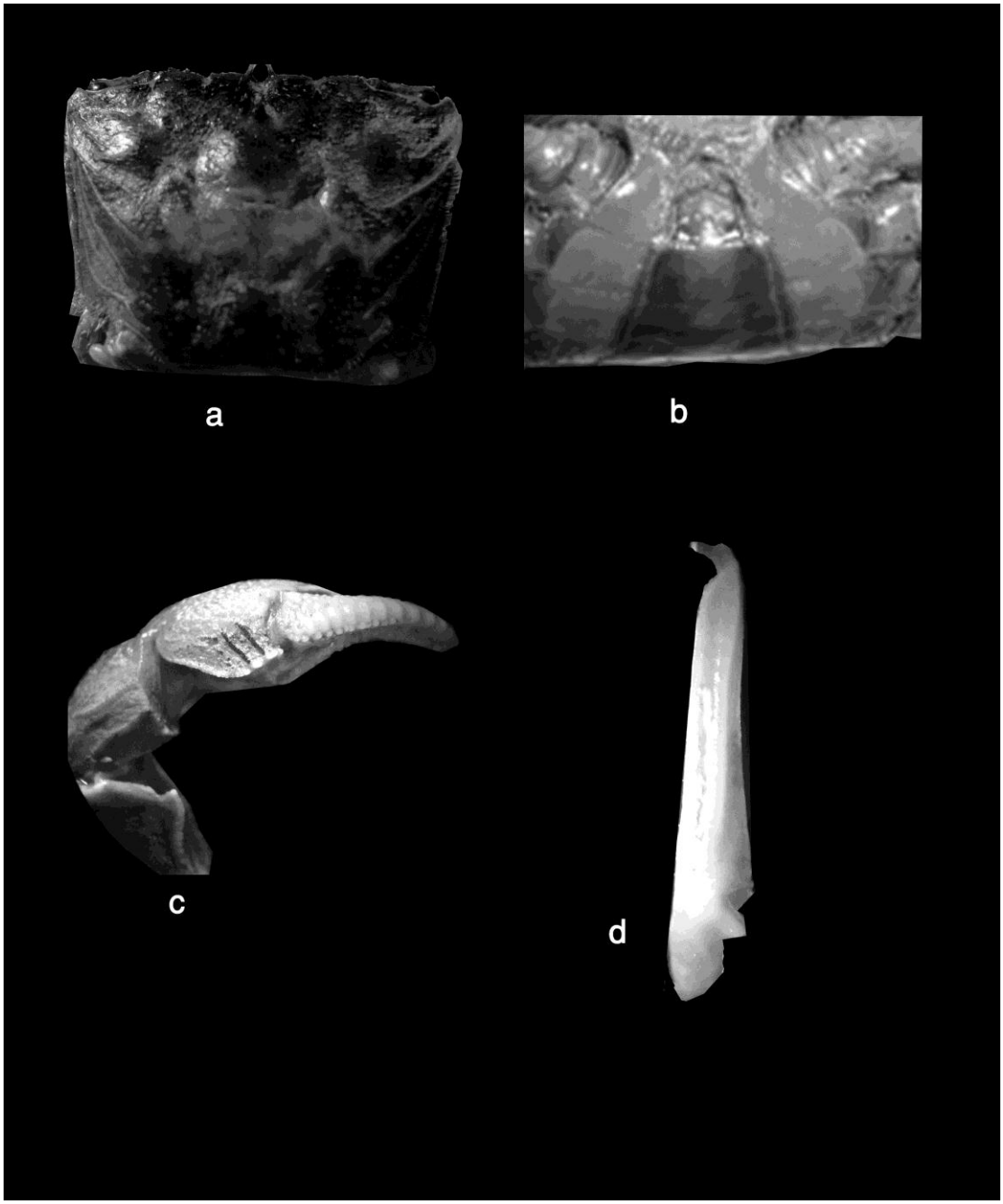

Figure 3. *Parasesarma persicum*, male (CL 26.4× CW 33.0) collected from Shatt Al-Basrah Canal: (a) dorsal surface of carapace; (b) abdomen and telson; (c) right major chela, outer view; (d) right first male gonopod, ventral view

major chela, outer view; (d) right first male gonopod, ventral view.

Chelipeds (Fig. 3c) are equal to subequal to each other or unequal, relatively large. Ischium is with small granules on anterior margin. Merus is with finely granulated transverse ridges on outer surface; inner surface is smooth. Carpus is with anterior angle produced; outer surface is with transverse line of small granules; inner surface is with longitudinal line of small granules, bundle of relatively long setae proximally. Manus is granulated on outer surface; proximal lower portion is with small granules and oblique granulated ridges; two prominent pectinate crests are on upper surface, sometimes additional small one behind them; two transverse rows of granules are on proximal part; inner surface is granulated on lower half; a transverse row of granules is near base of movable finger; lower margin is densely granulated, slightly concave near base of immovable finger; upper margin is proximally arched, irregularly granulate, distally with two large tubercles. Movable finger is gently arched downwards, markedly curved inwards distally, with 11 or 12 large tubercles along upper surface, proximally oval, distally becoming small, round, and low; 12 or 13 small tubercles are on proximal half of movable finger.

Walking legs are medium-sized, flattened; merus of second walking leg is relatively wide, about 1.9 times as long as wide; that of last leg is about 1.8 times as long as wide. Merus of second walking leg is serrated, with acute subdistal spine on anterior margin; posterior margin is smooth, distally slightly serrated; posterior surface is with transverse ridges, minutely granulated; posterior surface of merus of last walking leg is smooth, with very few ridges on anterior part, curved smooth ridge on posterior part. Carpus is with one and two carinae on anterior and posterior surfaces of second leg, respectively; carpus of last leg is with one carina on posterior surface. Propodus of second leg is with carina on posterior and anterior surfaces; propodus of last legs is without carina on anterior surface.

First male gonopod (Fig. 3d) is relatively stout, straight; stem is triangular in cross section, distinct longitudinal depression along ventrolateral surface; sperm channel is along dorsal face, curving outwards dorsally, gonopore on dorsal margin of chitinous process, subterminal; chitinous process is long, curved outwards, at angle of about 45º, slightly concave basally on mesial surface, with distinct depression on lateral surface; long setae is on distal ventromesial border and around terminal process, few short setae along stem.

5. Remarks

The specimens from Iraq agree well with the original description of *P. persicum*, based on the material from the Arabian Gulf. *Parasesarma persicum* was very often misidentified as *Chiromantes boulengeri*, a native freshwater species that is confined in its distribution to the upper part of Shatt Al-Arab River. For *P. persicum*, the distribution is restricted in the Arabian Gulf, from lower reaches of Shatt Al-Arab River, Fao region, mouth of the NW of the Arabian Gulf, Khor Al-Zubair, and Shatt Al-Basrah Canal. *Parasesarma persicum* is the only species of the genus known in Iraqi coastal water. The habitat of *P. persicum* in Khor Al-Zubair differs from that of Shatt Al-Basrah Canal. In Khor Al-Zubair, the crabs live on muddy-silty substrata at the upper intertidal zone, some living on decayed leave materials, others live under old boats. Whereas, in Shatt Al-Basrah Canal, in front of a dam, the crabs live under stones (artificial stones) and among the aquatic plants *Phragmites australis*.

The examination of the present specimens shows that the crabs from Shatt Al-Basrah Canal are obviously larger in size than those collected from Khor Al-Zubair. Some physical-chemical characteristics were recorded for both regions, as follow:

Shatt Al-Basrah: May 2010, air temperature, 39°C; water temperature, 29°C; pH, 8.46; salinity, 8 psu; dissolved oxygen (DO), 11.86 mg/L; total dissolved solids (TDS), 9.106 g/L. Khor Al-Zubair: May, 2010, air temperature, 39◦C; water temperature, 28°C; pH, 8.46; salinity, 22 psu; dissolved oxygen (DO), 8 mg/L; total dissolved solids (TDS), 11 g/L.

The differences in sizes of *P. persicum* at both sites may be assigned to the differences of the food items, optimum salinity requirements for the growth, and to the effect of high pollution on the growth of the crab by the crude oil at Khor Al-Zubair.

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I thank Dr. P.K.L. Ng (Raffles Museum of Biodiversity Research, Department of Biological Sciences, National University of Singapore) for confirming the identity of *Parasesarma persicum*, and Dr. D.L. Rahayu (Marine Bio-industry Implementation Unit-Research Centre for Oceanography, Indonesian Institute of Sciences) for her reading and editing the first draft of the manuscript and to the anonymous reviewers for their comments and corrections on the MS.

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المجلة الأردنية للعلوم الحياتية : مجلة علمية عالمية محكمة ومفهرسة ومصنفة، تصدر عن الجامعة الهاشمية و بدعم من صندوق دعم البحث العلمي- وزارة التعليم العالي و البحث العلمي .

هيئة التحرير

فريق الدعم:

ترسل البحوث إلى العنوان التالي :

رئيس تحرير المجلة الأردنية للعلوم الحياتية
عمادة البحث العلمي و الدراسات العليا
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