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# *ANTAGONISM AND SEASONAL FLUCTUATIONS OF SOME MARINE ISOLATES OF BACILLACEAE IN PORT SAED HARBOUR, EGYPT.*

*BY*

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Key words: Antagonism- Bacillus sp.- Distribution-Diffusion- Inhibition.

## *ABSTRACT*

*Eighty-nine bacterial isolates related to Bacillaceae were collected from Port Saed harbour during 2001. These isolates were examined for bacterium bacterium antagonism using tooth picking technique and Bulkholder diffusion technique. It was found that, more than 50% of these tested isolates showed positive antagonism and the most potential producers for antagonistic agents in such marine environments were Bacillus cereus, B. megaterium, B. licheniformis, B. lentus and B. amyloliquefaciens. Moreover, the effect of the antagonistic activity on the ecological distribution of these bacteria was investigated using agar well diffusion technique. The results indicated that, the presence of B. megaterium highly affected the presence of B. circulans, B. cereus and B. licheniformis specially in winter and autumn (193.7, 134 and 207 absolute activity unit (Au)/ml, respectively), also the appearance of B. amyloliquefacenies in spring showed a great inhibition activity against B. cereus, B. licheniformis, B. lentus and B. megaterium (198.0, 126.3, 143.7 and 207.0 Au/ml, respectively). While B. licheniformis affected in a clear vision the presence of B. subtilis (286 Au/ml).*

## *INTRODUCTION*

Many investigators focused their view on producing several antibiotics and other antagonistic agents from different marine microorganisms specially marine bacteria to control different types of pathogens even the human pathogens (Jensen and Fenical 1994,1996 and 2000), regardless the effect of such antibiotic producers on the other bacterial isolates present at the same environmental marine habitat. Moreover, Okami (1986), found that the frequency of the antagonistic interactions between marine bacterial isolates did not exceed 5-8%.

However, on studying the *Bacillaceae* group it was believed that *Bacillus* species act friendly in the environment, but some *Bacillus* species showed to be pathogenic for man Example, *B. cereus* which is very related to *B. anthracis*. Also, *B. megaterium* may cause pharyngilis and psoriasis and *B. pumilus* causing rectal abscess or they may be food poisoning bacteria like *B. licheniformis* and *B. subtilis* (Sneath, 1986).

This investigation aimed to study the effect of the antagonistic activity on the ecological distribution of different *Bacillus* sp. isolated from Port Saed harbour during 2001 with special interest to the pathogenic species. Also to estimate the antagonistic frequency between this group of bacteria under such marine environment, since there is a lack in the information concerning interactions between marine bacteria and their potential antagonistic products.

## *MATERIALS AND METHODS*

### Sampling site

The seawater samples were collected seasonally during 2001 with a permission of the authority of the Suez canal at Port Saed site. Seven locations were chosen (Fig. 1), they extended from the northern opening of the Suez canal at the link with the Mediterranean sea (location 1) to the opening of the Suez canal (location 7).

### Isolation and Identification

For isolating marine *Bacillus sp.* seawater samples were heated for 10-15 min using a water bath adjusted at (70-80°C). Then serial dilutions ($10^{-1}$-$10^{-5}$) for each sample were made using sterile seawater and speeded separately on plates containing a marine agar (MA) medium (5g Peptone, 1g Yeast extract, Boric acid 0.022g, Sodium silico-fluoride 0.005g, Di-sodium phosphate 0.008g, Ammonium nitrate 0.0016g, and 20g Agar, dissolved in 1L seawater). The inoculated plates were incubated at 37°C for 24h. A biochemical identification process for these *Bacillus* isolates was carried out using the API-50CHB strips in addition to the API-20E strips (BioMerieux – France). The distribution of each strain was seasonally detected in three replicate samples and counted as cfu/100ml.

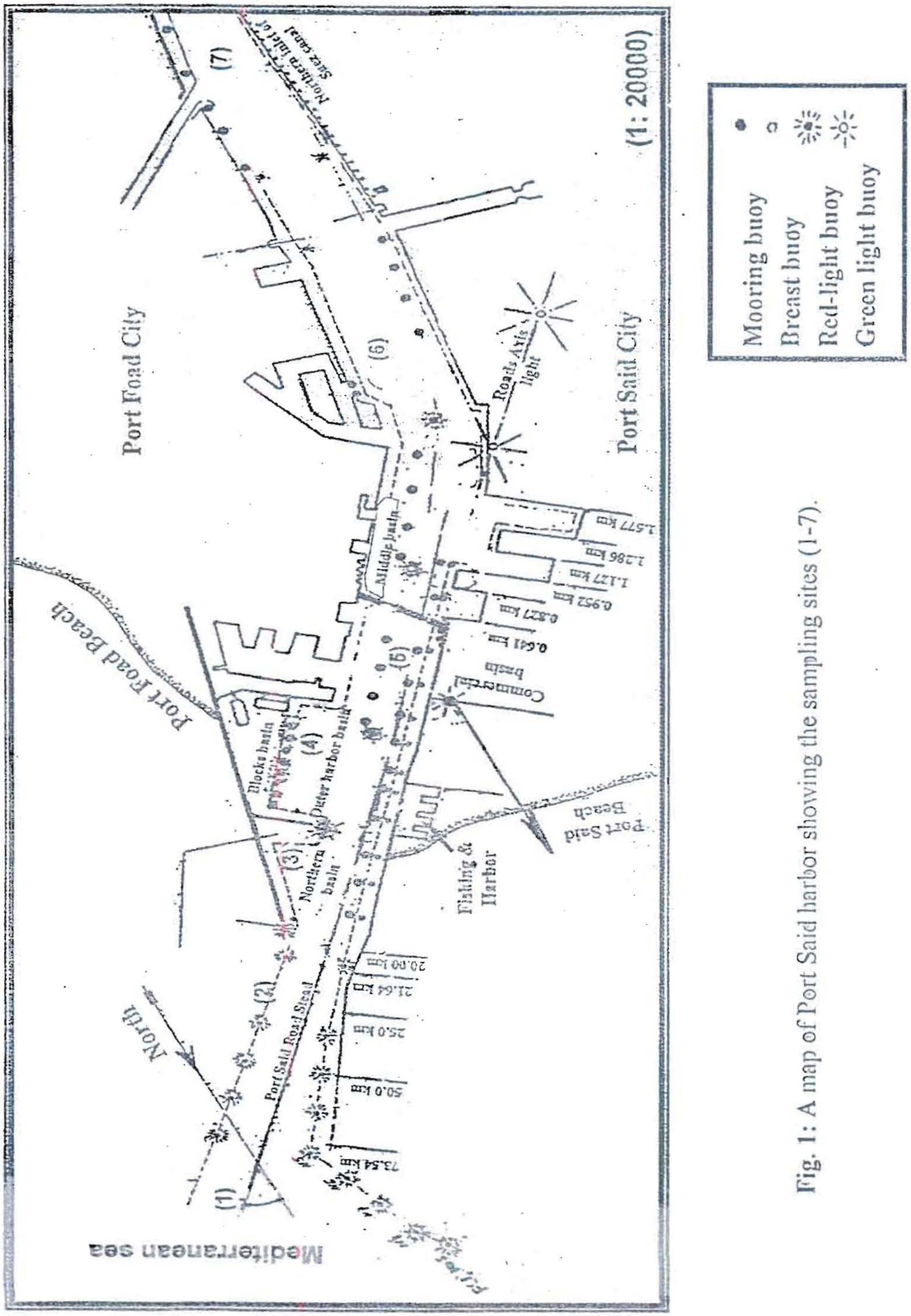



Fig. 1: A map of Port Said harbor showing the sampling sites (1-7).

After incubation, the clear zones were detected in mm around each well. An absolute unit of the antagonistic activity /µl of the tested producer was calculated by dividing the volume of the clear zone (has a cylindrical shape) by the volume of the well (has a cylindrical shape), according to the following equation:

$$\text{Absolute activity unit (AU)} = \pi x^2 h / \pi y^2 h$$

Where: $\pi = 3.14$ and h = the cylinder height, that can be deduced from both sides.. Therefore, the antagonistic activity can be estimated for one µl of the producer in relation to the square value of the inhibition zone radius ($x^2$) and the square value of the well radius ($y^2$).

### Statistical analysis

Statistical analysis was carried out according to Steel and Torrie (1980), using ANOVA, F-test, DUNCAN and L.S.D (least significant differences), procedures available with the MSTAT-C software package (Version 1.2, 1991). Histogram graphs were produced using Harvard graphic software (H.G, Version 4.0, 1998).

## *RESULTS*

Eighty-nine bacterial isolates were examined for antagonism, one isolate against the other eighty–eight isolates using the tooth picking technique and the Bulkholder diffusion technique. The data presented in Table (1) showed that, the bacterium bacterium interaction has great frequency between the members of this *Bacillaceae* group (55.1%).. The maximum antagonistic frequency was obtained by *B. circulans* followed by *B. cereus* and *B. licheniformis*, where, the frequency percentage for each was 86.7%, 81.8% and 80.0%, respectively. On the other hand, *B. amyloliquefaciens* showed the greatest frequency percentage (100%), but it appeared just for one time along the collection period.

**Table 1** : The antagonistic frequency of the bacterial specimens related to Bacillaceae that isolated from Port Saed harbour during 2001,using Bulkholder diffusion technique.

| *Bacillus Species* | The frequency of | | | The frequency (%) |
|---|---|---|---|---|
| | Total tested specimens | Non antagonistic specimens | Antagonistic specimens | |
| *B. megaterium* | 11 | 4 | 7 | 63.6 |
| *B. cereus* | 11 | 2 | 9 | 81.8 |
| *B. licheniformis* | 5 | 1 | 4 | 80 |
| *B. lentus* | 23 | 8 | 15 | 65.2 |
| *B. circulans* | 15 | 2 | 13 | 86.7 |
| *B. mycoides* | 6 | 6 | 0 | 0 |
| *B. subtilis* | 4 | 4 | 0 | 0 |
| *B. amyloliquefaciens* | 1 | 0 | 1 | 100 |
| *B. pumilus* | 11 | 11 | 0 | 0 |
| *B. spharicus* | 2 | 2 | 0 | 0 |
| Total | 89 | 40 | 49 | |
| % | 100 | 44.9 | 55.1 | |

The bacterial distribution illustrated in Fig. (2,3 and 4) showed that, *B. lentus* was the most presented species along the collection period at all tested locations, with an approximately presence percentage of 25% except in spring at location (7), it was less than 10%. While, the distribution of *B. pumilus* indicated that, this bacterium tend to appear in summer with presence % ranging from 100% to 40%, this was followed by autumn especially in locations (7) and (3), the presence % were 100% and 60%, respectively. Moreover, the same trend of appearance was obtained in case of *B. circulans* where the majority of this bacterium was observed in summer at the southern part of the harbour from location (5) to (7) with a 100% presence. While the northern locations (1) -(4) showed a maximum presence for this bacterium in spring (~100%).

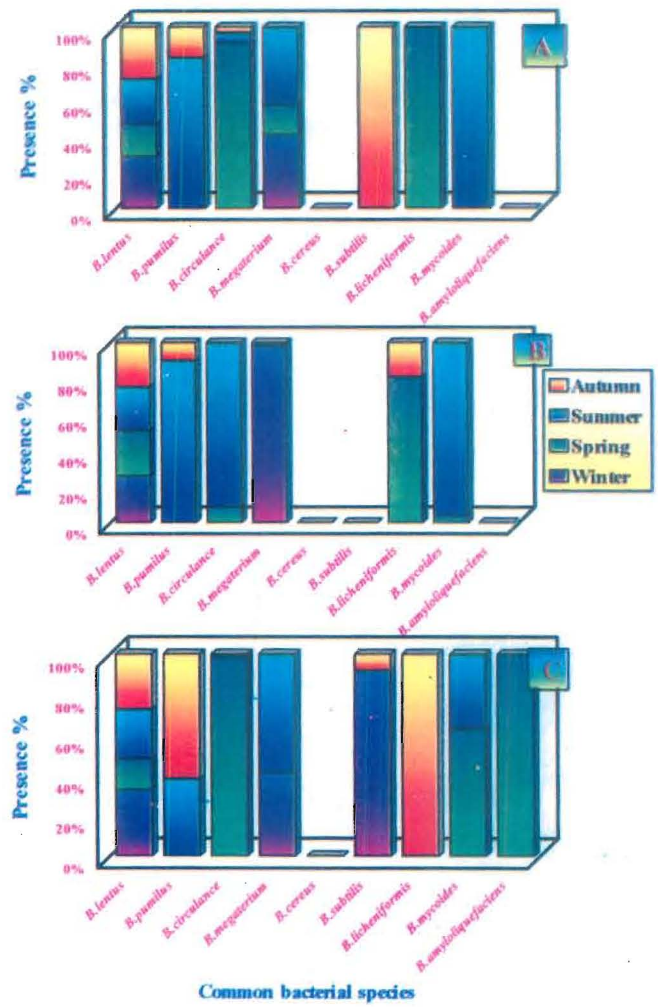



Fig. 2: The presence percentage of the common Bacillus species at the collection sites 1 (A), 2 (B) and 3 (C) of the Port Said harbour during 2001.

On the other hand, B. megaterium was generally observed in winter at six of the seven tested locations with presence % ranging from 45% to 100%. This was followed by spring at three locations (1), (5), and (7) (20%, 30%, 100%, respectively), then autumn at locations (1) and (3) with presence percentage of 40% and 60%, respectively. While, B. cereus was rarely presented in the tested harbour, where, it appeared only at locations (4), (5) and (6), in spring with presence percentage of 100%, 70 and 85%, respectively, and in summer at locations (5) and (6) with presence % of 35% and 20%, respectively

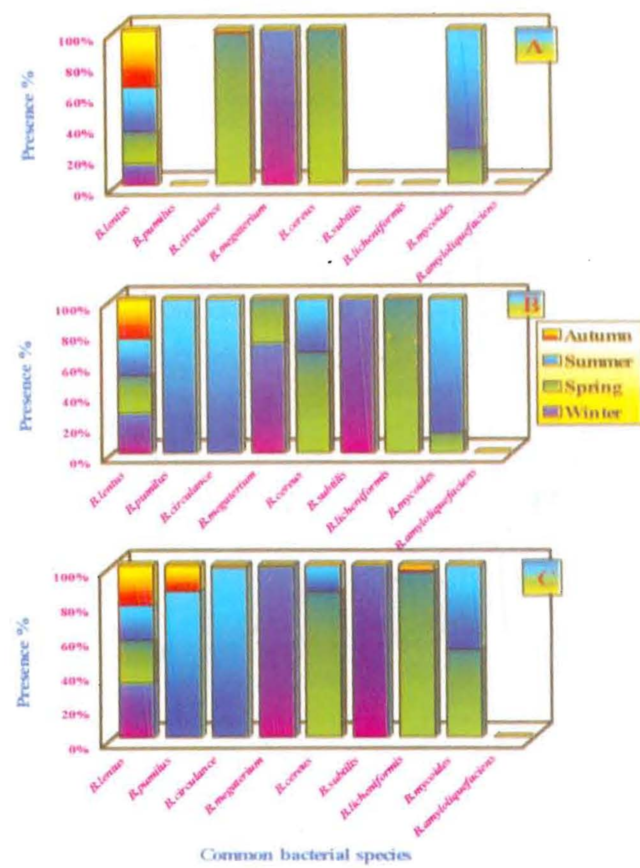
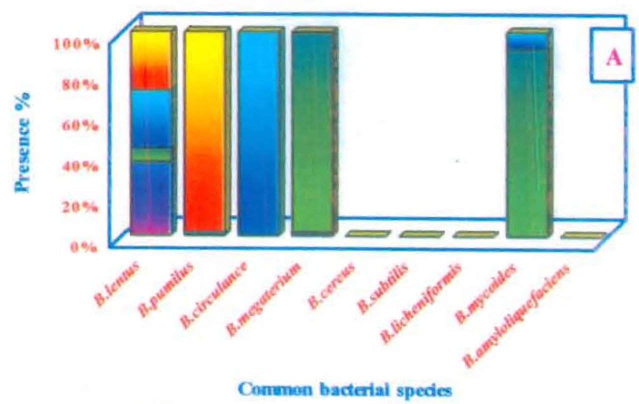



Fig.3: The presence percentage of the common Bacillus species at the collection sites 4 (A), 5 (B) and 6 (C) of the Port Said harbour during 2001.

Moreover, B. subtilis' was affected with the changes in the sea level in this area (high at north in autumn and high at south in winter), where it appeared in autumn at the northern location (1) with a presence percentage of 100% and disappeared at the following locations, then it appeared in winter at the southern locations (5) and (6) with presence % of 100%. While B. licheniformis can be harvested in spring at locations (1), (2), (5) and (6) where the presence % was more than 80% and in autumn the presence % was 100 at location (3).

The distribution of B. mycoides appeared to follow certain trend in Port Said harbour, where, at the northern locations (1)-(2) it appeared only in summer with more than 90% presence. While, at the middle locations (3)-(6) it appeared in summer and spring with average presence % of 59 and 41, respectively. Meanwhile, at the southern location (7) it appeared only in spring with 90% presence. On the other hand, B. amyloliquefaciens was only detected in spring at location 3.



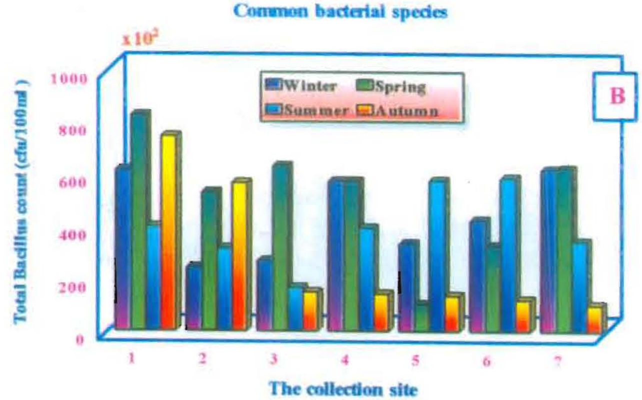




Fig. 4: The presence percentage of the common Bacillus species at the collection site 7 (A) and the total Bacillus counts at all collection sites (B) of Port Said harbour during 2001.

The antagonistic activity of these bacterial species were studied in three successive experiments using agar well diffusion technique (Fig. 5). The photographs represent the inhibition zones obtained from the interaction between *B. cereus*, which is considered as the most problematic *Bacillus* sp. and the antagonistic agents producers which were isolated from the same marine environment *B. megaterium*, (Fig. 1-A) and *B. amyloliquefaciens* (Fig. 1-B), compared with the untreated control, (Fig. 1-C).

The data were statistically analyzed using F-test, also the least significant difference values (LSD) were detected for each indicator and tested bacterium, in order to study the effect of this antagonism on the distribution of these bacterial isolates in Port Saed harbour. The data in Table (2) was interpreted with the representation of these isolates in Fig. (2), (3) and (4). It was found that, *B. lentus* proved to be the least affected *Bacillus* species by the action of the presence of such antagonistic producers in the surrounding environments. While, the presence of *B. megaterium* can affect the distribution of *B. circulans, B. cereus and B. licheniformis*, especially, in winter and autumn at all tested locations. Its antagonistic activity against these bacteria ranged from 130 to 200 Au/ml. Meanwhile, the presence of *B. licheniformis*, which was found to be an antagonistic agent producer, affected in a clear vision the presence of *B. subtilis*, where the antagonistic activity was 320 Au/ml.

On the other hand, *B. amyloliquefaciens* (when collected from location-3 in spring, Fig.1-C, and examined for antagonism using Bulkholder diffusion technique and the agar diffusion technique) showed a wide antagonistic activity against several examined *Bacillus* species (*B. cereus, B. licheniformis, B. megaterium, B. mycoides, B. lentus*, and *B. circulans*), where the activity ranged from 126.3 to 207 Au/ml. But on studying the environmental distribution of these bacteria in spring, it was noticed that the distribution of only *B. cereus, B. licheniformis* and *B. megaterium* was highly affected by this tested bacterium.

However, the total count of the isolated *Bacillus* species was illustrated in Figure (4-B). It was observed that the most countable location was the location–1 ($2.6 X10^5$), while the least countable location was location-5 ($1.2X10^5$). Moreover, in autumn the count showed certain trend, where it gradually decreased from location–1 ($7.9X10^4$ cfu/100ml) to location-7 ($1.0X 10^4$ cfu/100ml).

**Table (2): The cross interaction and the antagonistic activty ($Au^{\#}$ /ml) of the most potential *Bacillus* speices presented in Port Said harbor during 2001,using the Agar well diffusion technique.**

| Indicator bacteria | Tested bacteria | | | | | F values | P values | L.S.D values |
|---|---|---|---|---|---|---|---|---|
| | *B. megaterium* | *B. cereus* | *B. licheniformis* | *B. lentus* | *B. amyloliquefaciens* | | | |
| ***B. megaterium*** | — | 126 | 239 | 82 | 207 | 51.3 | 0.0001** | 68.5 |
| ***B. cereus*** | 134 | — | 0 | 0 | 198 | 36.3 | 0.0001** | 44.3 |
| ***B. licheniformis*** | 207 | 169 | — | 126 | 126 | 18.2 | 0.002** | 52.6 |
| ***B. lentus*** | 0 | 178 | 365 | — | 144 | 22.8 | 0..0003** | 89.6 |
| ***B. circulans*** | 144 | 261 | 309 | 0 | 169 | 28.3 | 0.0001** | 104.3 |
| ***B. mycoides*** | 0 | 126 | 0 | 0 | 178 | 19.6 | 0.002** | 72.5 |
| ***B. subtilus*** | 0 | 0 | 286 | 151 | 0 | 12.4 | 0.005** | 62.3 |
| **F-values** | 19 | 14.2 | 9 | 9.7 | 7.3 | | | |
| **P-values** | 0.002* | 0.01* | 0.02* | 0.02* | 0.03* | | | |
| **L.S.D.- values** | 82.3 | 62.3 | 45.2 | 41.3 | 62.3 | | | |

* There was a significant difference between the antagonistic activities of this tested bacterium when used against these indicators except *B. megaterium* it was a highly significant different. ** There was a highly significant difference between the antagonistic activities of the tested bacteria used against this indicator. # $Au = (y^2/x^2)$, where, y- is the well radius (3mm) and x- is the radius of the inhibition zone in mm.

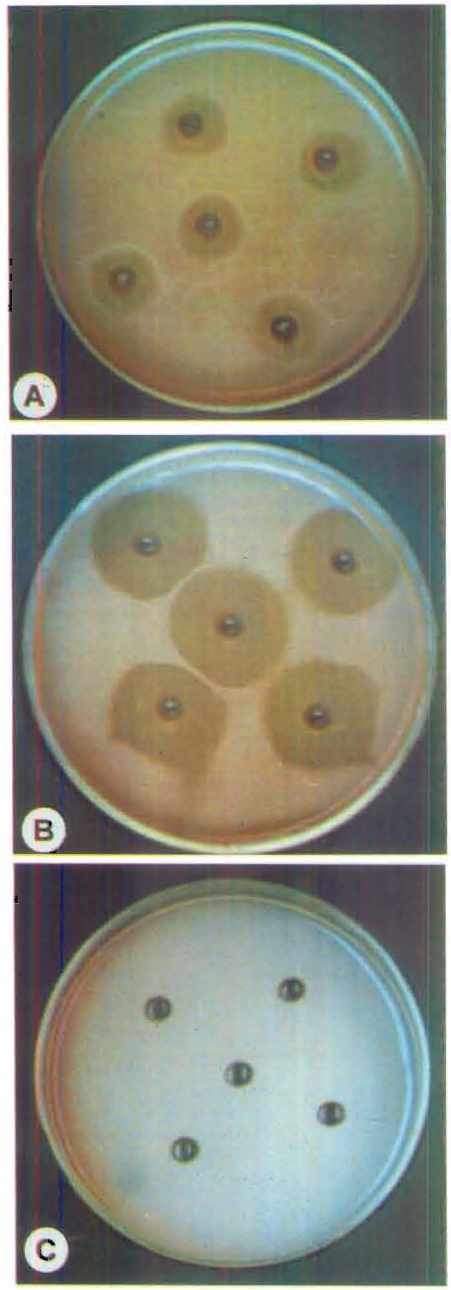



Figure (5): Photographs show the inhibition zones of B. cereus by the action of B. megaterium (A), and B. amyloliquefaceins (B) Compared with the control (C), using Agar- well diffusion technique.

## *DISCUSSION*

Port Saed harbour was chosen according to its strategic position where two different marine environmental conditions are joined together, the Mediterranean Sea and the Red Sea through the Suez canal. The climate at this area is subtropical, the tidal effect is small (0.4- 0.8 m) and the waves range is from 0.2 to 0.4 m. Also the sea level varies from season to the other. In winter it shows a higher level at the south compared with that at the north, this fact is reversed in summer (Morcos and Gerges, 1974). However, the most variable factor in this area was the current that varies from time to time along the year (Banssan, 1983, Morcos, 1974). All these factors may affect the biological communities especially the bacterial communities in such examined area.

However, it was believed that the distribution of the bacterial communities in the seawater are variable at millimeter scale, in response to the heterogeneity in the distribution of the organic matter, regardless the bacterium bacterium antagonistic interactions which may contribute to variations in the community structure at micrometer scale, (Long and Azam, 2001).

Therefore, this study aimed to estimate the antagonistic interactions and its frequencies between different *Bacillus* species that is commonly presented in Port Saed harbour. In order to establish if these interactions affect the seasonal distribution of such marine bacterial isolates, regardless the characterization or identification of these inhibitory substances. Since single or multiple inhibitory substances may be produced by a single bacterial isolate (Wratten, *et al.*, 1977).

The antagonistic feature was examined *in vitro* using Burkholder agar diffusion technique, where, a remarkably high percentage (55.1%) of the tested marine bacterial isolates exhibited antagonistic activity against the other collected bacteria. This result was in agreement with that obtained by Long and Azam (2001), who found such great percentage of antagonism between other groups of marine pelagic bacteria ( 53.5 %). On the other hand, other investigators showed much lower percentage (5-8%) of bacterial antagonism in such marine environments (Krasilnokova, 1961; Nair and Simidu, 1987).

Unlike the previous studies the effect of such antagonistic interactions was estimated in relation to the seasonal distribution of the most represented bacterial isolates in Port Saed harbour. The obtained data indicated that, *B. cereus, B. megaterium, B. licheniformis* and *B. amyloliquefaciens* were the most potential *Bacillus* species in producing antagonistic agents against other examined bacterial species. This was in agreement with the results obtained by several investigators who isolated many *Bacillus* species (*B. lentus, B. amyloliquefaciens, B. cereus* and *B. circulans*) from a variety of environmental and food sources and found that, these bacterial isolates have the ability to produce enterotoxins in their habitat as a result of the presence of five gene targets (hblc, hblD, hblA, nheA and nheB) in two enterotoxin operons (HBL and NHE), that traditionally harboured by *B. cereus*, (Phelps and Mckillip, 2002).

Moreover, many investigators found that *B. amyloliquefaciens, B. licheniformis, B. megaterium, B. cereus* and *B. circulans* were able to produce several enzymes (proteases, amylases and chitinnases) that allow them to degrade extracellular macromolecules. This fact may play an important role in the survival of other bacterial species present in their surrounding environments ( Priest, 1985 ; Vihinen and Mäntsälä, 1989 ; Ito, 1997 ; Sabry, 1997).

On the other hand, the nucleic acid based detection assays designed to target enterotoxin genes may not always provide a positive signal when in fact the gene product is expressed and vice versa (Jensen and Fenical, 1996). This may explain the case of *B. amyloliquefaciens* that exhibit wide antagonistic activity against the majority of the tested *Bacillus* species *in vitro*, but *in situ* only *B. cereus, B. licheniformis* and *B. megaterium* were inhibited.

However, the results and the analyzed data in this study indicated that, the bacterium bacterium interaction may highly affect the seasonal distribution of the bacterial community of *Bacillaceae* group under such marine conditions and vice versa, since the antagonistic percentage between such a group was found to reach more than 50%. This does not agree with the results observed by Okami (1986), who mentioned that the antagonism between the bacterial isolates under the marine conditions did not exceed 5-8 %.

Moreover, on studying the antagonistic feature of the bacterial isolates, it showed great variations according to several conditions (temperature changes, starvation, mineralization, sporulation,.....), that play an important role in the production of the antagonistic agents even from the same species and also affect the bacterium strategy ( Labbe and Rodriguez ,1992 ; Preyer and Oliver, 1993 ; Igura *et al.*, 2003 ).This may explain the variations in the ability of the majority of the examined species in this investigation to produce such antagonistic agents along the collection period, where, the temperature changed in this studying area from 14°C (in winter) to 30°C (in summer) So, more investigations concerning the action of these antagonistic producers *in situ* are recommended. Finally, it may be useful to use *B. amyloliquefaciens* or *B. megaterium* for bio-controlling *B. cereus*, that is considered as the most problematic *Bacillus* species in food industries.

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