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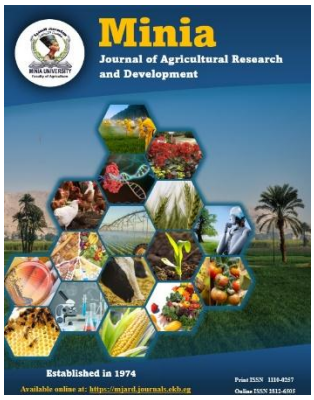

Actinophages of an actinomycete isolate of high antagonistic activity against certain pathogenic bacteria. (Isolation and characterization)

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ABSTRACT

Twelve isolates of actinomycetes were isolated from soil and compost samples collected from Minia Governorate Egypt and designated AC1, AC2,…upto AC12. Actinomycete isolate AC2 exhibited high antagonistic activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebselia sp.* and *Bacillus cereus*. actinophages specific to actinomycete AC2 were isolated from soil samples collected from Minia Governorate. Twelve single plaques ofactinomyceteviruses (actinophages), were isolated. Due to the similarity of the actinophage isolates in their optimum pH sensitivity to UV (260 nm), thermal inactivation point and host range theseactinomyceteviruses (phages) were classified into groups (group A and group B). On the basis of electron microscope study, micrographs of actinophages of every group were found to be similar in their size and morphology. Generally, the obtained results indicated that, the twelve isolates of actinophage were found to be belonging to two types of phages. These two phage types were named ØAC2a and ØAC2b.

Keywords: Actinomycetes, actinophages, thermal inactivation point, host range

INTRODUCTION

Actinomycetales is the order of actinomycetes. Actinomycetesare generally gram-positive and have mycelia in a filamentous and branching growth pattern. Some actinomycetes can form rod- or coccoid-shaped forms, while others can form spores on aerial hyphae. Actinomycetales have 2 main forms of reproduction: spore formation and hyphae fragmentation. Actinomycetes can be found mostly in soil and decaying organic matter,

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as well as in living organisms such as humans and animals **(Nonomura and Hayakawa, 1988)**. Many species of actinomycetes produce antimicrobial compounds under certain conditions and growth media. Streptomycin, actinomycin, and streptothricin are all medically important antibiotics isolated from actinomycetes (**Waksman *et al.*, 2010).** Almost two-thirds of the natural antimicrobial drug compounds used currently are produced by different species of actinomycetes.Actinomycetes can be infected by bacteriophages, which are called actinophages. All actinophages possess tails and contain double-strand DNA. They belong to three families of *Myoviridae*, *Siphoviridae* and *Podoviridae***(Ackermann and Prangishvili, 2012).** Actinophages are tailed bacteriophages in the order of *Caudovirales*and are characterized by the virion tail morphology. *Siphoviridae*have a long non-contractile tail, *Podoviridae*have a short non-contractile tail and Myoviridae have a contractile tail. Of the 2989 sequenced and annotated actinophage genomes, 92.9% are *Siphoviridae*, 6.2% are *Myoviridae*and 0.9% are of the *Podoviridae* morphology. This distribution is similar to other structurally characterized bacteriophages that infect awide range of bacterial hosts **(Ackermann, 2007 and 2012).**

This study aimed to isolate actinomycetes of high antagonistic activity against certain pathogenic bacteria from compost and soil. Moreover, this study aimed also to isolate actinophages specific to the isolated actinomycetes and the different characteristics of the isolated phages will be studied.

MATERIALS AND METHODS

Isolation of Actinomycetes: Twelve actinomycetes isolates were isolated from a compost sample and a soil sample collected from the Experimental Farm of Faculty of Agriculture – Minia University. One g of soil or compost was added to 9 ml of distilled water and serially diluted. One ml of each dilution was spread on Starch nitrate agar plates **(Waksaman and Leshevalier, 1961).** Plates were incubated for 7 days at 30°C. The plates were inspected for single colonies of actinomycetes (vegetative hyphae, aerial mycelium), and those colonies were selected and purified via streaking on Starch nitrate agar plates. The pure actinomycetes isolates were maintained at 4°C on test tubes containing slant surface of nutrient agar medium **(Dowson, 1957).**

The used pathogenic bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Klebselia sp.*, *Bacillus cereus* and *Pseudomonas aeruginosa* were kindly provided by Faculty of Pharmacy, El-Minia University, Egypt.

The antagonistic activity of actinomycetes isolates: The antagonistic activity of each actinomycete isolate against each pathogenic bacterium was tested as described by **Bauer (1966).** Each actinomycete isolate was intense streaked on plates containing nutrient agar medium and incubated for 7 days at 30°C. Using a sterile cork borer, 6 mm agar discs were removed from well-grown actinomycete plates and placed on nutrient agar plates, each seeded with a pathogenic bacterium. Plates were incubated for 18 to 24 hours at 35°C, and then plates were inspected for inhibition zones surrounding the actinomycetes discs. The inhibition zones were measured.

Actinophagesisolation: The liquid enrichment technique was used to isolate the bacteriophages of actinomycete isolate as mentioned by **Adams (1966).**

Phage detection: In agar double layer plats, actinophages were detected using spot test according to **Adams (1966).**

Purification of bacteriophages: To obtain pure single actinophage isolates, single plaques were isolated according to**Kiraly *et al*. (1970).** Each plaque was transferred into an eppendorf tube with 500 µl SM medium **(Maniatis *et al*., 1982)** and maintained at 4 °C over 200 µl of chloroform.

High titer phage suspension: Actinophage suspension of high titer was prepared using double layer agar plates technique according to**Maniatis *et al*. (1982)**

Titre estimation: Titer of actinophage suspensions was estimated according to**Kiraly *et al.* (1970),** andexpressed as pfu/ml (plaque forming unit /ml).

Characterization of isolated actinophages:

Effect of different pH levels: Actinophage isolates were exposed to different pH levels (i.e. 4.0, 5.0, 6.0, 7.0, …..upto 12) at 30°C for 60 min. The optimum pH for each isolate was determined as mentioned by **Hammad *et al.* (2018)**

Stability to ultraviolet irradiation: Petri plates containing 5 ml of each phage suspension were exposed to ultraviolet (260nm) at 20 cm distance from germicidal UV lamp. After 10, 20, 30, 40, 50, 60, 70, 80 and 90 min, the infectivity of every phage suspension was tested via spot test on double agar layer plates

Thermal stability: Thermal inactivation point of every actinophage isolate was determinedaccording to**Hammad *et al*. (2018).**

Host range of actinophage isolates: The infectivity of each actinophage isolate was tested for the 12 isolates of actiomycetes (as indicator bacteria) in separated plates via spot test as according to **Hammad *et al*. (2018).**

Electron microscopy: Grids of electron microscope were prepared for every actinophage isolate and 0.5% uranyl acetate pH 4.5 was used forstaining **(Stacey *et al*., 1984).**In transmission electron microscope (Joel, Model GEM 1010)the prepared grids were examined at 50 kv.

RESULTS AND DISCUSSION

The isolated actinomycetes

Since, actinomycetes can be found mostly in soil and decaying organic matter (**Waksman *et al.*, 2010),** twelve isolates of actinomycetes were obtained from compost and soil samples obtained from the Experimental Farm of Faculty of Agriculture, Minia University. The actinomycetes isolates were designated as AC1, AC2, AC3 …..upto AC12.

The antagonistic activity of actinomycetes against pathogenic bacteria:

Waksman *et al.* (2010) stated thatmany species of actinomycetes produce antimicrobial compounds. Streptomycin, actinomycin, and streptothricin are all medically antibiotics produced by actinomycetes. Almost two-thirds of the natural antimicrobial compounds used currently are produced by actinomycetes

The isolated actinomycetes (12 isolates) were tested for their antagonistic activities

against *Staphylococcus aureus*, *Escherichia coli*, *Klebselia sp.*, *Bacillus cereus* and *Pseudomonas aeruginosa*. The obtained results in **Table (1)** and **Figure (1)** indicated that just one isolate (AC2) among the 12 actinomycetes isolates, exhibited antagonistic activity against four bacterial isolates tested (*Staphylococcus aureus*, *Escherichia coli* ,*Klebseliasp.*and*Bacillus cereus*). On the other hand, actinomycete (AC2) was found to have no effect against *Pseudomonas aeruginosa* as shown in **Figure (2).**

Table (1): The antagonistic activity of actinomycete (AC2) against some pathogenic bacteria.

Actinomycete	Pathogenic bacteria				
	Staphylococcus aureus	***Escherichia coli***	***Klebselia sp.***	***Bacillus cereus***	***Pseudomonas aeruginosa***
	Inhibition zone (mm)				
AC2	**25**	**21**	**27**	**21**	**0**

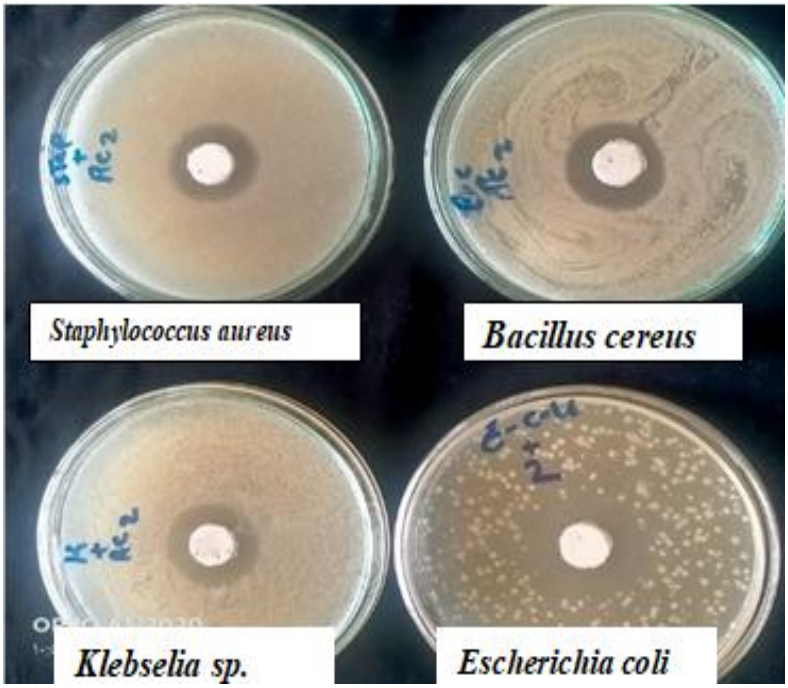

Figure (1): The antagonistic activity of actinomycte (AC2) against some pathogenic bacteria

Figure (2): Antigenicity test of actinomycete (AC2) against *Pseudomonas aeruginosa*

Isolation of actinophages:

Actinophages specific to actinomycete (AC2) were isolated from the compost and the soil samples collected from the Experimental farm, Faculty of Agriculture, Minia University. The isolated phages were detected via the spot test. As shown in **Figure (3)** actinophages specific to actinomycete (AC2) were found to be common in the locations from where the samples had been taken. It is well known that bacteriophages are widely distributed in locations which contain the suitable host.

Pringsulaka*et al.* (2004) reported that twenty-four actinophages were obtained by an enrichment procedure from soil samples collected from several parts of Thailand. Moreover, **Long*et al*. (1993)** isolatedactinophagesthat attack some actinomycetes from soil sample collected at a dry river bed on Sibuyan Island.

Figure (3): Detection of actinophages via spot test

Actinophages Purification:

Since it is known that the single plaque is developed by a single phage particle, purification of actinophages was carried out via isolation of single plaques. Twelve single plaques of actinophages specific to actinomycete (AC2) were picked and separately kept as single isolates of phages, each plaque was different morphologically **(Figure 4)**. **Barnet (1972)**; **Mohamed *et al.* (2023)**and **Osman *et al.* (2024)** reported that the bacteriophages of the same plaque morphology are belong to one phage type.

Figure (4): Single plaques of actinophages with different morphologies.

Since the twelve single plaque isolates of actinomyces phages were morphologically different, it is expected that every isolate of phage is considered one type. *i.e.* the isolated phages might be 12 phage types. To confirm this explanation, these phages were subjected to study their different characteristics.

Characterization of the isolated actinophages:

Determination of the optimum pH:

As shown in Table (2) at any pH level, lysed spots were formed bythe isolatedactinophages. These data showed that the actinophage isolates are tolerant to acidic and alkaline conditions. Similarly, **Roslycky *et al* . (1962); Challaghan*et al.* (1969); Hammad & Ali (1999)**and **Osman *et al.* (2024)** stated that bacterial viruses were stable at pH 5 to 12.

At pH 8, six actinophage isolates exhibited spots wider than those at any other pH.*i.e.* pH 8 is the optimum pH for the six actiophages (phage isolates No. 1, 2, 4, 7, 8 and 12). Whereas, pH 7 was found to be the optimum pH for actinophage isolates No. 3, 5, 6 , 9, 10 and 11.

Based on the obtained data different expectations are likely:

1) It is possible for the 12 isolated phages of actinophages to be two phage types. One type have the optimum pH 8 and classified in Group(A). In addition, the other type of the optimum pH 7 was classified in Group(B).

To accept or dismiss the hypotheses mentioned above, more characteristics were studied.

Table (2): Effect of pH levels on the isolated actinophages

Phage group	Phage No.	PH levels								
		4	5	6	7	8	9	10	11	12
		Diameter of the lysed spots (mm.)								
A	1	12.1	12.8	14.4	14.8	15.9	15.5	14.0	13.3	10.0
	2	11.1	13.2	14.6	15.0	16.4	14.1	13.4	12.4	11.0
	4	10.7	12.1	13.8	14.7	16.2	14.5	14.0	13.1	12.0
	7	13.0	13.8	14.2	14.8	15.8	13.9	13.0	12.1	11.7
	8	13.2	13.0	14.8	15.2	17.1	15.2	14.3	12.8	12.0
	12	13.5	14.3	15.0	15.7	16.8	14.8	13.5	12.9	11.5
B	3	13.4	14.1	16.9	19.3	16.5	15.7	15.0	14.5	13.2
	5	14.2	14.9	15.5	18.7	16.0	15.2	14.0	13.3	12.0
	6	16.5	17.5	18.0	19.5	17.1	16.5	16.0	15.3	14.3
	9	16.0	17.0	19.2	20.6	17.5	17.0	16.5	16.0	14.0
	10	13.8	14.5	16.8	18.9	15.7	14.5	14.1	13.5	12.9
	11	15.5	16.7	19.5	20.8	19.0	17.3	16.9	15.4	14.5

Effect of U.V. irradiation

Data in **Table (3)** showed that U.V. (260nm) inactivated the isolated actinophages at different times of exposure. Therefore, the isolated actinophages were divided into two groups (A and B). Every group contained phages that inactivated after the same time of exposure to U.V. Group A included isolates of phages No. 1, 2, 4, 7, 8 and 12 which inactivated after 90 min. exposure to U.V. at 260 nm. Group B contained isolates of phages No. 3, 5, 6 , 9, 10 and 11 that inactivated after exposure for 60 min.

Based on the obtained data, two hypotheses can be expected:

1) The twelve actinophages are likely to be two phage types. *i.e.* actinophages in group A represent only one type of phage since they exhibited the same response to U.V. radiation.

2) Since the isolated phages were morphologically different in their plaques, the phages of each group may be belong to different types of phages but exhibited the same response to U.V. radiation. Actually, further studies were conducted to confirm one of these hypotheses.

Thermal inactivation point

Hegazi *et al.* (1980) stated that incubation of two phages of Azotobacter at70°C and 80°C for 15 min. resulted in complete inactivation of phages.

Due to the similarity in the thermal stability (Table 4), actinophages were divided into two groups (A and B). Every group

contained the phages that showed the same thermal stability. Actinophages of group A (No. 1, 2, 4, 7, 8 and 12) after exposure to 90°C for 10 min. were completely inactivated. Whereas, actinophages of group B (isolates No. 3, 5, 6 , 9, 10 and 11) were inactivated at 90°C for 10 min..

Table (3): Effect of UV radiation (260 nm) on actinophage isolates.

Phage group	Phage No.	Exposure time (min.)								
		10	20	30	40	50	60	70	80	90
A	1	+	+	+	+	+	+	+	+	-
	2	+	+	+	+	+	+	+	+	-
	4	+	+	+	+	+	+	+	+	-
	7	+	+	+	+	+	+	+	+	-
	8	+	+	+	+	+	+	+	+	-
	12	+	+	+	+	+	+	+	+	-
B	3	+	+	+	+	+	-	-	-	-
	5	+	+	+	+	+	-	-	-	-
	6	+	+	+	+	+	-	-	-	-
	9	+	+	+	+	+	-	-	-	-
	10	+	+	+	+	+	-	-	-	-
	11	+	+	+	+	+	-	-	-	-

Table (4): Thermal inactivation point of the isolated actinophages

Phage group	Phage No.	Temperature (°C)					
		50	60	70	80	90	100
A	1	+	+	+	+	-	-
	2	+	+	+	+	-	-
	4	+	+	+	+	-	-
	7	+	+	+	+	-	-
	8	+	+	+	+	-	-
	12	+	+	+	+	-	-
B	3	+	+	+	+	+	-
	5	+	+	+	+	+	-
	6	+	+	+	+	+	-
	9	+	+	+	+	+	-
	10	+	+	+	+	+	-
	11	+	+	+	+	+	-

Interestingly, the two actinophage groups that classified based on optimum pH **(Table 2),**U.V sensitivity**(Table 3)** were found to be the same as those classified based on the thermal stability **(Table 4)**. Such results show that the phages of every group are belong to a single phage type. This is an expectation and it needs to be confirmed by additional characterization means.

Host range:

The host range of every isolate of actinophage was tested using spot test. The twelve actinophages of were tested against each of the twelve actinomycetes isolates. Data in **Table (5)** indicated that two host ranges for actinophages wereobserved. Therefore, the isolated phages were grouped into two groups (A and B). Every group comprised phages that showed the same host range. Actinophages in group A (No. 1, 2, 4, 7, 8 and 12) were infectious to four actinomycetes isolates (AC 2, AC 4, AC 6 and AC11) among twelve isolates tested .

Table (5): Host range of tewelve isolates of actinophages specific for actinomycete isolate (AC2).

Phage group	Phage No.	Actinomycetes isolates											
		1	2	3	4	5	6	7	8	9	10	11	12
A	1	-	+	-	+	-	+	-	-	-	-	+	-
	2	-	+	-	+	-	+	-	-	-	-	+	-
	4	-	+	-	+	-	+	-	-	-	-	+	-
	7	-	+	-	+	-	+	-	-	-	-	+	-
	8	-	+	-	+	-	+	-	-	-	-	+	-
	12	-	+	-	+	-	+	-	-	-	-	+	-
B	3	-	+	-	-	-	+	-	+	-	-	-	-
	5	-	+	-	-	-	+	-	+	-	-	-	-
	6	-	+	-	-	-	+	-	+	-	-	-	-
	9	-	+	-	-	-	+	-	+	-	-	-	-
	10	-	+	-	-	-	+	-	+	-	-	-	-
	11	-	+	-	-	-	+	-	+	-	-	-	-

In addition, phages in group B (phage isolates No. 3, 5, 6 , 9, 10 and 11) were infectious to three actinomycetes isolates (AC 2, AC 6 and AC 8), among the twelve isolates tested. *i.e.* any of the isolated actinophages was infectious to more than one actinomycete isolate. The capability of phage to lyse bacteria depends on the absence or presence of surface receptors on the bacterial cell for phages adsorption **(Barnet, 1972).** The two groups of actinophages that grouped based on hostspecificity were found to be the same as those divided on the basis of optimum pH, thermal stability and U.V sensitivity. These data indicate that the phages of every group belong to a single phage type.*i.e.* the twelve actinophages are two phagetypes. To confirm these results further characterizations were conducted.

Electron microscope study:

As shown in **Figure (4),** actinophage isolates of both groups (A and B) were of tail and head types . Actinophages of group A have long flexible non-contractile tail, whereas, actinophages of group B have short non-contractile tail.

Kowalski *et al.* (1974); Hammad (1989 & 1993); Mohamed *et al.* (2023)and**Osman *et al.* (2024)**isolated phages of the tail and head types specific to different bacterial hosts.As shown in **Table (6)** actinophage isolates showed some variation in their dimensions. actinophage isolates No. 1, 2, 4, 7, 8 and 12 that classified in group A were similar in their particle dimensions. Therefore, phages of group A were considered single phage type and named as ØAC2a **(Figure 5).** Whereas, actinophages of group B (No. 3, 5, 6 , 9, 10 and 11) exhibited similar dimensions, therefore they considered one type of actinophage and designated as ØAC2b. Accordingto ICTV, the actinophages of both groups A and B(*i.e.*ØAC2a and ØAC2b) were belonging to Order *Caudovirales* and classifiedunder Family *Siphoviridae* and family *Podoviridae*, respectively.

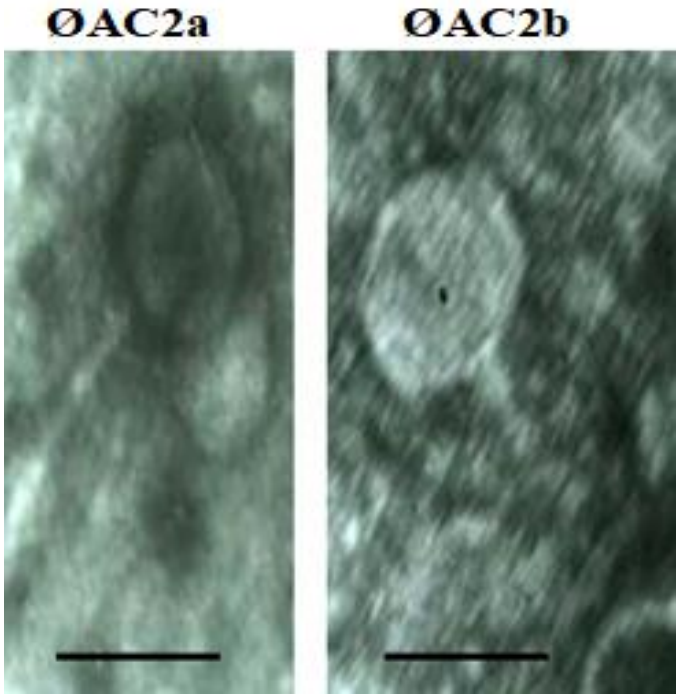

Figure (5): Electron micrographs of actinophages Magnification bar 50 nm

Table (6): Dimensions of actinophage particles.

Phage group	Phage No.	Particle dimensions (nm±SD)		
		Head Diameter	Tail	
			length	width
A	1	66±2	100±3	5±2
	2	67±1	99±2	4±2
	4	66±3	102±3	5±1
	7	65±2	100±2	4±1
	8	64±3	98±4	6±2
	12	67±2	102±4	5±2
B	3	70±3	36±2	6±1
	5	69±2	34±2	6±2
	6	72±3	35±1	5±3
	9	70±2	37±3	4±3
	10	69±3	35±3	6±2
	11	71±2	36±2	5±2

Generally, based on the obtained data it can be concluded that, the plaquemorphology is not an accurate technique to identify and classify the bacteriophages. This because in this study, the twelve actinophage isolates were found to be belong to two types of phages, although each of the twelve phage isolates was isolated with different plaque morphology. *i.e.* plaques of different morphologies can be formed by a single phage type. This is not surprising since, **Kowalski *et al.* (1963); Barnet and Vincent (1971)** reported that there are many factors which may affect the morphology of plaques of a single phage type. These factors are nutrient medium composition and agar concentration, temperature of incubation, age of theindicator bacteria, osmotic shock and presence of host debris. Therefore, theplaque morphology should not be used for phage identification and classification, whereas, it can be used as a rapid technique to purify the mixed phages.

Phages of each type exhibited the same response to U.V. at 260 nm. This may indicate that U.V. (260 nm) could be used to differentiate between the phage types. The thermal inactivation point of the phages revealed that the phage isolates which belonging to one type of phages had the same thermal stability. Such results may indicate that the thermal inactivation point isan accurate feature which could be used to differentiate between phage types. The thermal stability was used as a characteristic of phage isolates by many investigators **(Aharchi, 1992; Hammad, 1993; Hammad *et al.*, 1995; Othman, 1997; Hammad &Ali, 1999 and Osman *et al.* 2024).**

The host range of the isolated actinophages was found to be the same forphages of each type. *i.e* . the host range is a diagnostic character for each phage type, and it can be

used to identify and classify the phages. Many investigators used the host range to differentiate between phage types, *e.g.***Kankila and Lindstrom (1994);Hammad and Ali (1999) and Osman *et al.* (2024).**

Francki (1973) stated that because there are unknown factors which may affect dimensions of particle during preparative process, it is difficult to compare published morphometric data. Therefore, morphological similarities do not necessarily indicate a close relationshipbetween phages, but because in this study, phages of every type showed similarity in the other characteristics (thermal stability, U.V. sensitivity and host range), the electron microscope study can be used to confirm the results of the other characters.

Finally, based on the above mentioned information, different features were used all together in this work to compare and classify the actinophage isolates. No individual method for characterizing phages is in itself sufficient for complete classification or identification, but such characteristics (optimum pH, thermal stability, sensitivity to U.V., host range and electron micrographs) must be conducted all together to give clear differences between the phage isolates tested.

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الملخص العربى

الاكتينوفاجات المتخصصة على عزلة اكتينوميسيتات ذات النشاط المضاد لبعض البكتيريا الممرضة (العزل والخصائص)

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فى هذه الدراسة تم عزل 12 عزلة من الاكتينوميستات من عينات تربة وسماد عضوى تم جمعها من محافظة المنيا. تم تسمية العزلاتAC1وAC 2و.....AC12 . أظهرت العزلة AC2نشاط عالى مضاد لبكتيريا *Staphylococcus aureus, Escherichia coli, Klebselia sp.* وBacillus cereus . تم عزل 12 عزلة من الفاجات المتخصصة على AC2من عينات تربة جمعت من محافظة المنيا. وبناء على تشابه الفاجات المعزولة فى درجة الاس الهيدروجينى الامثل و الحساسية للاشعة فوق البنفسجية ذات طول موجى 260 نانوميترودرجة التثبيط الحرارى والمدى العوائلى ثم تقسيم الفاجات الى مجموعتين Aو B. وقد اظهرت صور الميكروسكوب الالكترونى للفاجات أن فاجات كل مجموعة متشابهة فى الشكل المورفولوجى والقياسات. ومن النتائج المتحصل عليها يتضح أن فاجات كل مجموعة تنتمى الى نوع واحد من الفاجات. أى أن 12 عزلة من الفاجات تنتمى الى نوعين فقط وقد اطلق عليهما اسماء ØAC2aوØAC2b.