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NUMERICAL STUDY ON SOME ACTINOMYCETES ISOLATED FROM BURULLOS LAKE IN EGYPT

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ABSTRACT

Twenty nine actinomycetes isolates were isolated from Burullos Lake and characterized taxonomically for 62 phenotypic traits including morphological; biochemical, nutritional, substrate utilization and anti-microbial activities. The results were analyzed by numerical techniques using the simple matching coefficient (S_SM) and UPGMA clustering. At 54% similarity level, the majority of the isolates were grouped into six phena (A, B, C, D, E and F). Only two isolates were grouped separately and formed two single clusters at the same level of similarity. A representative isolate from each phenon was identified. The isolates were found to be *Streptoverticillum morookaense*, *Nocardia brasiliensis, Streptomyces alanosinicus*, *Streptomyces globosus* and *Streptomyces gancidicus*

Keywords: Burullos Lake, Actinobacteria, Numerical taxonomy, Fresh water habitats

INTRODUCTION

Class Actinobacteria (high G+C content, gram positive bacteria) has been proposed by **Stackebrandt *et al* (1997)** and includes members with unparalleled ability to produce diverse secondary metabolites **(Mellouli *et al* 2003)**. They represent an ubiquitous group of microbes widely distributed in natural ecosystem including soils **(Xu *et al* 1998)**, marine sediments **(Ghanem *et al* 2000, Sabry *et al* 2004)** and fresh water **(Hahn *et al* 2003)**. Actinomycetes are not adapted for growth in aquatic habitats but can nevertheless be recovered readily from fresh water, sea water and sediments samples **(McCarthy and Williams, 1990)**.

Several studies demonstrated by cultivation independent methods revealed that the freshwater actinobacteria are present in a wide spectrum of ecologically different and globally distributed freshwater habitats **(Glöckner *et al* 2000; Lindström and Leskinen, 2002; Hahn *et al* 2003)**. **Zwart *et al* (2002)** recently identified 34 putative phylogenetic clusters of bacteria which seem to contain typical freshwater

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inhabitants, 5 of the 34 clusters were affiliated with the class Actinobacteria.

Distribution of Actinobacteria in fresh water has been reported by **Glöckner *et al* (2000)** who performed in situ hybridization. On the other hand, **Pernthaler *et al* (2001)** successfully enriched a phylotype of one freshwater cluster of actinobacteria in a continuous culture system. Thus, despite the high number of cells of actinobacteria observed in fresh water samples no representatives of the freshwater actinobacterial lineages have been isolated so far **(Zwart *et al* 2002). Zwart *et al* (2003)** found that actinobacterial cluster ACK-M1 is being well represented in most fresh water habitats. They detected this cluster in all of the 81 lakes screened (from Belgium, The Netherlands, Denmark, Sweden and Norway), using reverse line blot hybridization. In the most recent published data **(Van der Gucht *et al* 2005)** the ACK-M1 (Actinobacteria) was the most representative in each of the studied lakes. These data confirm those previously reported **(Crump *et al* 1999; Urbach, *et al* 2001).**

Up to our Knowledge, few studies dealt with the distribution of class Actinobacteria in aquatic habitats in Egypt **(Al-Diwany and Cross 1978; Ghanem *et al* 2000).** Lake Burullos is shallow brackish water that serves as fishery resources and reservoirs for drainage water. It is contaminated with anthropogenic materials **(Abu-Elela *et al* 2004).** Therefore, this study is an attempt to evaluate the distribution of members of class Actinobacteria in Lake Burullous using phenotypic, physiological and biochemical data, depending on numerical taxonomic procedures.

MATERIAL AND METHODS

Actinomycetes isolates

Twenty nine actinomycetes isolates were selected from a total of 130 isolates previously isolated from sediments and water samples of Lake Burullous **(Abu-Elela *et al* 2004)**. They were maintained on a basal medium prepared with 1 liter of Lake water of the following composition (g/l): soluble starch, 10.0; KNO_3, 2.0; K_2HPO_4, 2.0; $MgSO_4$.$7H_2O$, 0.05; $CaCO_2$, 0.02; $FeSO_4$, 0.01 and agar, 20.0 at 28 ºC **(Küster and Williams, 1964)**. Culture stocks were maintained at -20 ºC in starch nitrate broth with 20% glycerol **(Kieser *et al* 2000)**.

Characterization and numerical analysis

Numerical analysis was not used for a direct taxonomical purpose but also to facilitate data handling and strains grouping. A total of 62 characters were coded as negative (0) or positive (1). The simple matching coefficient (SsM) **(Sokal and Michener, 1958)** and the Jaccard coefficient (Sj) **(Sneath, 1957)** were used and clustering was achieved by unweighted pair group average linkage (UPGMA), **Sneath and Sokal, (1973)**

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and **Sneath, (1979)**. The computations were performed by using SYSTAT-PC program V 7 **(Wilkinson *et al* 1992)** on an IBM computer.

The selected isolates were phenotypically characterized as described by **Pridham and Gottlieb (1948); Shirling and Gottlieb, (1966) and Tresner *et al* (1968)**. All tests were carried out at 28ºC for up to 3 weeks.

For morphological characterization, the following media were used: Krasslinikov SRL agar, Glycerol- nitrate agar, Czapek- Dox agar after slight modification, Glycerol- asparagine agar and Inorganic salts- starch agar **(Shirling and Gottlieb, 1966).**

Additional phenotypic characterization was performed using the standard procedures, catalase and urease production were detected, melanin production according to **Shirling and Gottlieb, (1966)**, nitrate reduction **(Williams *et al* 1983)**, sulphide precipitation **(Cowan, 1974)** and indole production **(Molin and Trenstrom, 1986).** Lecithinase was performed on egg-yolk medium according to the method of **Nitsch and Kutzner, (1969)**, Lipase **(Elwan *et al* 1977)**, protease **(Ammar *et al* 1991),** pectinase, amylase **(Ammar *et al* 1998)** production was investigated. Also Degradation activities of some substance such as starch, cellulose, chitin, gelatin and tyrosine were performed according to **Nonomura and Ohara (1969).**

The antagonistic activity of the tested isolates against *Escherichia coli* HP101, *Staphylococcus aureus* ATTC 29523 and *Bacillus subtilis* was determined as described by **Goodfellow *et al* (1990)**. Zones of inhibition were scored as positive results after 24 h at 28C°. Resistance against phenol (0.002, 0.01 w/v), crystal violet (0.0001, 0.001 w/v), and sodium azide (0.01w/v) was tested. Antibiotic resistance was examined as the ability to grow on medium supplemented with antibiotics one at a time using gentamycin (998 $\mu g.ml^{-1}$), erythromycin (804 $\mu g.ml^{-1}$), tetracycline (990 $\mu g.ml^{-1}$) and cefixine (997 $\mu g.ml^{-1}$) according to **Williams *et al* (1983).**

Chemotaxonomic study was performed by the detection of diaminopimelic acid (DAP) isomers and diagnostic sugars according to **Becker *et al* (1964)** and **Lechevalier *et al* (1977)** in AL-Azhar University Fermentation Biotechnology and Applied Microbiology Center. Identification was carried out in comparison to reference strains.

Scanning electron microscopy

Electron micrographs were made with Jeol model ISM 5300 scanning electron microscope (SEM) operating at 15 KeV **(Molitoris *et al* 1996).**

RESULTS AND DISCUSSION

In a previous survey on the distribution of *Actinobacteria* in Lake Burullos, 130 isolates were obtained **(Abu-Elela *et al* 2004)**. Based on colony morphology, 29 isolates were selected which represent different morphotypes. In this study, we aimed to cluster those isolates for the purpose of identification and classification.

Actinomycetes isolates were studied for 62 characters listed in Table (1). Analysis of the 62 characters using the simple matching coefficient (S_SM) and UPGMA clustering yielded the dendogram in Fig. (1). Data showed that the majority of the isolates were grouped at 54% similarity level, into six phena (A, B, C, D, E and F). Only two isolates were grouped separately and formed two single clusters at this level.

Phenon A: The four isolates grouped in phenon A possess several features that are common to members of genus *Streptoverticillium*. It is thus suggested that the

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Table 1. Comparison of the frequencies of positive and negative characters for the six clusters of actinomycetes obtained by numerical taxonomy analysis

Character	Phenon	A	B	C	D	E	F
	No. of isolates	4	3	6	5	3	6
Growth on							
Krasslinikov SRL agar		100	100	100	0	33	83
Glycerol-asparagine agar		100	100	100	100	100	100
Glycerol –nitrate agar		100	100	100	100	100	100
Czapek-Dox agar		100	100	100	100	100	100
Inorganic salts –starch agar		75	100	100	100	100	100
Substrate mycelium							
Yellow brown		50	67	33	80	0	33
Reddish brown		25	0	50	0	67	50
Blue		25	0	0	0	0	0
Violet		0	33	17	20	33	17
Aerial mycelium							
Gray yellowish pink		0	33	0	0	67	0
Pink gray		0	67	17	0	0	0
Gray		0	0	67	80	0	50
Whitish gray		50	0	0	0	0	0
Blue		25	0	0	0	0	0
Violet		0	0	17	20	0	17
Yellow		25	0	0	0	33	33
Diffusible pigment							
Unpigmented		0	100	100	80	67	50
Reddish brown		0	0	0	20	33	0
Yellowish brown		100	0	0	0	0	50
Growth at ºC							
20		50	100	100	100	100	100
30		100	100	100	100	100	100
40		100	100	100	100	100	100
50		25	0	0	0	0	17
Growth at pH							

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9	75	67	100	100	100	100

Table 1. continued

Character	Phenon	A	B	C	D	E	F
	No. of isolates	4	3	6	5	3	6
Utilization of							
D-Glucose		75	100	83	80	100	100
D-Fructose		75	67	100	40	33	100
L-Arabinose		0	0	50	0	67	50
Lactose		75	0	100	100	100	50
Raffinose		50	0	67	20	0	50
Rhamnose		50	33	67	40	67	83
Mannitol		25	33	67	80	67	67
Galactose		25	33	50	60	33	50
Xylose		25	33	67	80	67	100
Growth in presence of NaCl %							
0		100	100	100	100	100	100
4		100	100	100	100	100	100
7		75	33	50	20	33	50
10		0	0	0	0	0	0
Degredation of							
Starch 1%		100	100	100	100	100	100
Cellulose 1%		50	67	100	0	0	100
Chitin 0.25%		0	0	33	0	0	0
Gelatin 0.4%		0	0	0	0	0	67
Tyrosine 0.1%		100	67	100	100	67	100
Enzyme activities							
Catalase		25	67	33	100	100	50
Urease		50	67	0	40	0	50
Nitrate reduction		50	100	17	40	33	67
Sulfide precipitation		25	0	0	0	33	50
Melanin production		0	0	0	100	33	0
Antibiosis against							
E.coli HP101		0	33	17	0	33	50
S.aureus ATCC 29523		25	0	100	20	33	83
B.subtilis		0	0	17	0	67	100

Table 1. continued

Character	Phenon	A	B	C	D	E	F
	No. of isolates	4	3	6	5	3	6
Resistance to							
Phenol (0.002 w/v)		100	100	100	100	100	100
Phenol (0.01w/v)		0	0	0	0	0	0
Crystal violet (0.0001 w/v)		50	100	100	80	100	100
Crystal violet (0.001 w/v)		25	67	33	40	100	100
Gentamycin at (998 µg.ml^{-}1)		0	67	0	0	0	17
Erythromycin (804 µg.ml^{-}1)		0	0	0	0	0	17
Tetracycline (990 µg.ml^{-}1)		0	0	0	0	0	0
Cefixine (997 µg.ml^{-}1)		0	0	0	0	0	0

isolates are different species of this genus (Table 1). SEM observation of the representative strain (Fig. 2A) showed phenotypic features clearly related to the reference strain *Streptoverticillium morookaense* **(Hensyl, 1994)** the characters shared with both strains are shown in (Table 2) aerial mycelia are gray while substrate mycelia are yellow brown, the aerial mycelia consisted of long, straight filaments of conidia showing side branches at regular intervals and whitish to gray spore masses. The cell wall hydrolysates contained LL-DAP with no diagnostic sugars. Spores are not motile. Absence of melanin pigments but gray yellowish brown diffusible pigments was produced. Several carbon sources were utilized (Table 2). Positive results were recorded with urease, nitrate reductase and capable degrading starch, tyrosine, xanthine and esculin .Our isolate differs only from the reference strain in the fact that it could not utilize raffinose and in this respect, it was suggested that the isolate is closely related to *Streptoverticillium morookaense* as recommended by the international Keys **(Williams *et al* 1989 and Hensyl, 1994).**

Phenon B: This phenon contained three isolates grouped at 55% similarity level. They had morphological and biochemical characteristics similar to members of nocardioform actinomycetes **(Lechevalier, 1989).**

Selected isolate from phenon B (Fig.2 B) was similar to the reference strain *Nocardia brasiliensis* in having pink gray aerial mycelium and the aerial mycelium moderately fragmented into a chain of conidia. The cell wall hydrolysate contained meso-DAP with arabinose and galactose as diagnostic sugars. The experimental strain shared some

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properties with *N. brasiliens in being* non motile,

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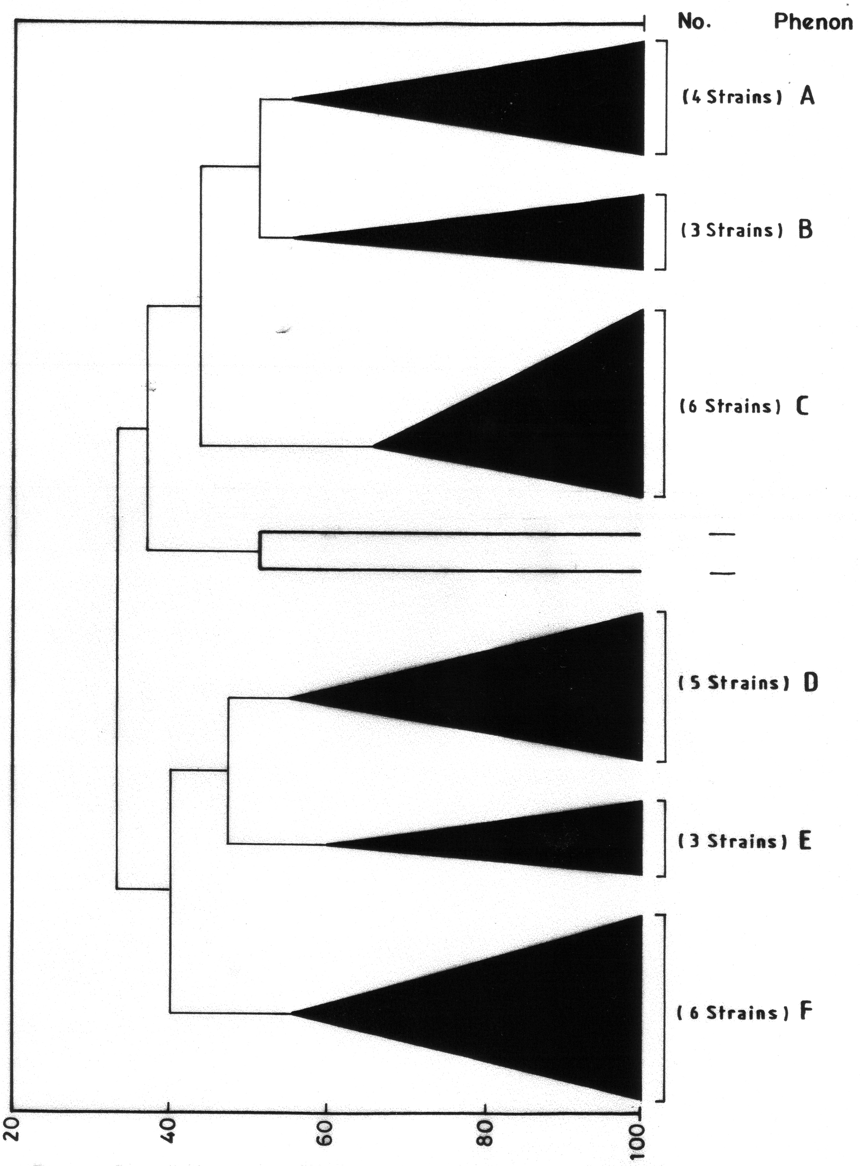

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Fig. 1. Simplified dendrogram showing the relationships among phena based on the Ss μ - UPGMA analysis.

Table 2. Characteristic features of the identified actinomycetes isolates

S.gn	S.r	S.g	S.a	N.b	St.m	Characteristic
						Morphological characters
gray yellowish brown	reddish brown	yellowish brown	reddish brown	Light yellow brown	yellow brown	Substrate mycelium
gray	gray yellowish pink	gray	gray	Pink gray	gray	Aerial mycelium
spiral	spiral	spiral and rectiflexible	spiral	Aerial conidial chain	Long straight filament of conidia showing at regular intervals side branches	Spore chain /conidia
-	-	-	-	-	gray yellowish brown	Diffusible pigments
-	-	-	+	-	-	Melanin production
						Cell wall hydrolysate
LL-DAP	LL-DAP	LL-DAP	LL-DAP	meso-DAP	LL-DAP	Diaminopimelic acid
nd	nd	nd	nd	arabinose & galactose	nd	Sugar pattern
						Utilization of carbohydrates
+	+	+	+	+	+	D-glucose
+	+	-	+	+	+	D-fructose
+	+	+	+	-	-	L-arabinose
-	+	+	+	-	-	Lactose
-	-	-	+	-	-	Raffinose
+	+	-	+	-	+	Rhamnose
+	+	-	+	-	+	Mannitol
+	+	-	+	+	+	Galactose
+	+	+	+	+	+	Xylose
-	-	-	-	-	+	Sucrose
+	+	nd	+	+	nd	Trehalose
-	+	+	+	-	+	Maltose
nd	nd	-	nd	nd	+	Cellobiose
+	-	-	-	+	+	Sodium citrate
nd	nd	+	nd	nd	+	Sodium acetate
-	-	nd	-	-	-	Ribose

+	+	nd	nd	+	nd	Mannose

Table 2. Continued

S.gn	S.r	S.g	S.a	N.b	St.m	Characteristic
						Utilization of amino-acids
+	-	+	-	-	+	L-cycteine
+	+	-	-	+	+	L-valine
-	+	+	+	+	+	L-alanine
-	-	-	-	-	+	L-phenyl-alanine
+	+	+	+	+	+	L-arginine
-	-	+	-	-	+	L-tryptophane
						Biochemical activities
-	-	+	-	-	-	Catalase
+	+	+	+	+	+	Urease
+	+	+	+	+	+	Nitrate reductase
-	+	-	-	-	+	Sulfide precipitation
-	-	-	-	-	-	Indole test
						Degradation of
+	+	+	+	+	+	Starch
+	-	-	+	-	-	Cellulose
-	-	-	-	-	-	Chitin
+	-	-	-	-	-	Gelatin
+	+	+	-	+	+	Tyrosine
+	+	+	+	+	+	Xanthin
+	-	+	+	+	+	Esculin
						Degradation of
+	+	-	+	+	+	Protein
+	+	-	+	+	+	Lipid
nd	nd	-	+	nd	+	Pectin
-	+	nd	+	+	+	Lecithin
						Antibiosis against
+	+	-	+	-	-	E.coli
+	+	-	-	-	-	S.aureus
+	-	-	+	-	-	B.subtilis

Table 2. Continued

S.gn	S.r	S.g	S.a	N.b	St.m	Characteristic
						Resistance to
-	-	-	-	-	-	Phenol (0.01w/v)
+	+	+	+	+	+	Phenol (0.002 w/v)
+	+	-	-	+	-	Crystal violet (0.001 w/v)
+	+	+	+	+	+	Crystal violet (0.0001 w/v)
+	-	-	-	-	-	Sodium azide (0.01 w/v)
-	-	-	-	+	-	Gentamycin (998 µg.ml-1)
-	-	-	-	-	-	Erythromycin (804 µg.ml-1)
-	-	-	-	-	-	Tetracycline (990 µg.ml-1)
-	-	-	-	-	-	Cefixine (997 µg.ml-1)

St.m : Streptoverticillum morookaense
N.b : Nocardia brasiliensis
S.a : Streptomyces alanosinicus
S.g : Streptomyces globosus
S.r : Streptomyces rubber
S.gn : Streptomyces gancidicus
nd : not detected
DAP : Diaminopimelic acid

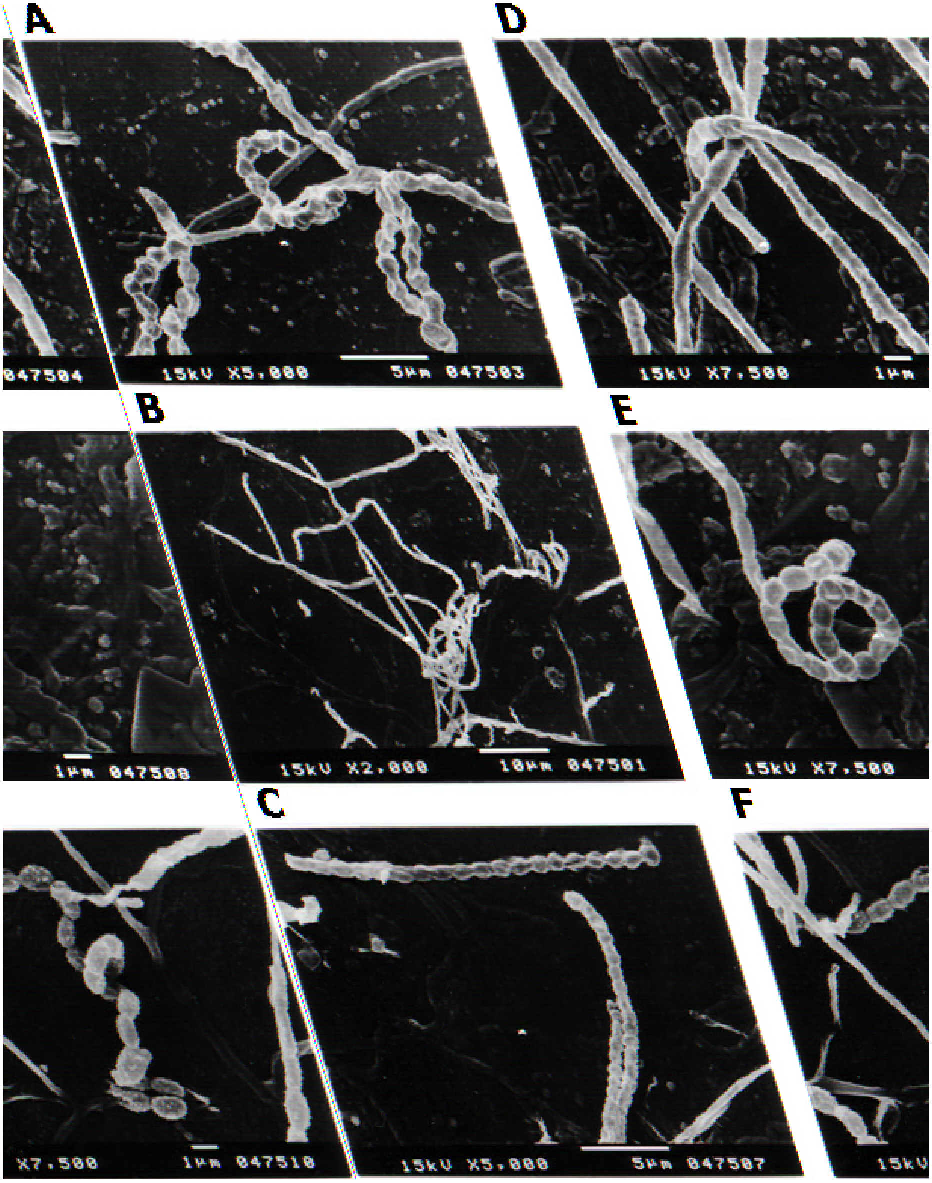

Fig. 2. Scanning electron micrographs of representative strains from each phena *Streptoverticillum morookaense* (A); *Nocardia brasiliensis* (B); *Streptomyces alanosinicus* (C); *Streptomyces globosus* (D); *Streptomyces ruber* (E); *Streptomyces gancidicus* (F)

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lacking melanin pigments, positive for urease and nitrate reductase, degraded starch, tyrosine, xanthine and esculin, in addition to the number of carbon sources utilized (Table 2). So it could be stated that actinomycete members of this phenon are closely related to *Nocardia brasiliensis*.

Phena C, D, E, and F: It is widely accepted that the genera *Streptomyces* and *Streptoverticillium* are closely related. Both have cell-wall I (**Lechevalier 1989**), a high content of GC in their DNA (**Pridham and Tresner 1974)** and high similarities in DNA homology (**Kroppenstedt *et al* 1981**), and are lysed by the same phages (**Wellington and Williams 1981**). Additionally, they contain similar menaquinones, fatty acids and polar lipids (**Lechevalier 1989**). The only support for distinguishing between *Streptomyces* and *Streptoverticillum* has come from DNA-DNA pairing studies (**Gladek *et al* 1985**).

The presence of LL-diaminopimelic acid in the cell wall of isolates belonging to phena C, D, E and F with no diagnostic sugars is consistent with the proposal that these strains belong to the genus *Streptomyces* and related genera (**Williams *et al* 1989; Hensyl 1994**). As pointed out by **Williams *et al* (1983),** there is no simple, rapid procedure for objective identification of streptomycetes. The problems of streptomycetes identification are largely a reflexion of the difficulties in streptomycetes taxonomy. Morphological or pigmentation characteristics were not included in streptomycetes taxonomy by **Kampfer *et al* (1991).** It is generally accepted that more than one category of spore chains can be observed in the same species and the distinction between rectiflexibles and spirals is not clear (**Williams and Wellington 1980; Williams *et al* 1989**). The determination of the color of the spore mass is also not easy **(Kutzner, 1981),** especially with respect to spore color and other morphological criteria. These traditional characters, which are often difficult to determine (**Williams and Wellington 1980; Kutzner, 1981**), are inadequate for classification and identification. Carbon source utilization tests have been found in earlier studies to be characters with great differentiation potential. The utilization of sucrose, L-arabinose, inositol, mannitol, rhamnose and raffinose was recommended as an aid to species differentiation in the International *Streptomyces* Project **(Shirling and Gottlieb, 1972)** and these tests have been used in several identification schemes **(Nonomura, 1974; Szabo *et al* 1975).** The phenetic and diversity of streptomycetes are major problem and as pointed out by **Kampfer and Kroppenstedt (1991),** streptomycetes taxonomy is still developing.

Phenon C: The 6 isolates grouped in phenon C were clustered at 65% similarity level (Fig.1).The chemotaxonomic properties and

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morphological characteristics of our experimental isolates are consistent with the reference strain *Streptomyces alanosinicus* (gray series) **Williams *et al* (1989)**. Both possess the same cell wall chemotype; produce gray aerial mycelia carrying gray spiral and rectiflexible spore chains (Fig. 3C). No observed motility and melanin pigments not produced. Both utilized arabinose, fructose, galactose, glucose, mannitol, raffinose and xylose but no utility to sucrose (Table 2).

Phenon D: Five isolates are grouped in this phenon at similarity level 55%. The strain selected from this phenon showed phenotypic characteristics (Fig. 2D) closely related to the reference strain *Streptomyces globosus*. Their aerial mycelia bear spiral and rectiflexible (straight to flexuous) spore chains forming gray spore masses. The cell wall contained LL-DAP with no sugars; absence of motility; glucose, L-arabinose, lactose and xylose were utilized by the selected strain but can not utilized fructose, galactose, mannitol, raffinose, and sucrose. (Table 2). On the basis of the previous collected data and in view of the comparative study of the recorded properties of the tested strain in relation to closet strain, the recommended identification may be closely related to *S. globosus*..

Phenon E: At 60% similarity level this phenon included three isolates. As reported in Table (1) the representative isolate (Fig. 2 E) was closer to the reference strain *Streptomyces rubber*, having grayish-yellowish pink spore masses, the spore chains were spiral, and the substrate mycelia were reddish brown. Both strains have the same cell wall chemotype. No detection to motility or melanin production. Both of them utilized glucose, D- fructose, L-arabinose, mannitol, rhamnose and xylose (Table 2).

Phenon F: At 55% similarity level, six isolates constituted this phenon, the selected isolate and the reference strain *Streptomyces gancidicus* shared in most characters. Their spore masses were in the gray series with gray yellowish brown substrate mycelia. Spore chains are spirals (Fig. 2F). Their cell hydrolysates contained LL-DAP with no diagnostic sugars. No motility. Absence of melanin. Both of them were able to use arabinose, fructose, glucose, mannitol, galactose, and xylose where as they were unable to utilize raffinose and sucrose (Table 2).

Conclusion

The data obtained from examined fresh water habitat in Burullous Lake clearly showed that the Burullous Lake has its own distinct actinobacteria community. The lake is characterized by high representation of Streptomycetes species from different series, and fewer numbers of nocardioforms were observed.

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رصم سلربلا ةريحب نم ةلوزعملا تيسيموينيتكالا ضعب نع ةيددع ةسارد

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