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# Noteworthy salt-loving actinomycetes from Kuwait

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## ABSTRACT

Two newly described actinomycetes, isolated from salt marsh soil in the southern part of Kuwait, are reported to degrade feather keratin. The first is a halotolerant *Nocardiopsis* sp. and the second is a halophilic *Saccharomonospora* sp.. Both species showed keratinase activities in the culture filtrate in the presence of feather meal as the sole source of carbon and nitrogen. The keratinase activities of both species were repressed when 1% glucose was included in the medium. These halophilic actinomycetes and their keratinolytic enzymes can be of great importance in understanding mechanisms of adaptation of microorganisms to saline environments. They may also be of some biotechnological application.

**Keywords:** ; Halophilic; halotolarant; keratinophilic actinomycetes.

## INTRODUCTION

Actinomycetes are a very important soil inhabiting microbial group, many of which are able to degrade complex organic compounds. Species of *Streptomyces* are the most common in soil and are known to play a major role in the mineralization process in soil, where several species are reported to degreade resistant substances such as keratin and lignin (Young & Smith 1975, Noval & Nickerson 1959, Mukhopadhyay & Chandra 1990, Bockle *et al*. 1995). Although there have been several reports on the existence of *Streptomyces* species in Kuwait's desert soils and rhizospheres (Al-Ghunaim 1976, Diab & Al-Zaidan 1976, Diab & Al-Ghunaim 1982) none were isolated from salt marsh soil as halophilic or halotolerant (Al-Oqaily 1980). In the present paper we report for the first time the isolation of salt-loving actinomycetes that also have kerationlytic activities. The two newly described slat-loving actinomycetes species that have been used in most of this study are *Saccharomonospora halophila* (#8) which is a halophilic nocardioform, and *Nocardiopsis halotolerans* (#F100) which is a halotolerant.

Morphological, physiological, chemotaxonomic properties as well as 16SrDNA sequence and phylogenetic analyses indicated that these actinomycetes are new species and were assigned the above names (Al-Zarban *et al*. 2002 a & b). They were deposited as *Saccharomonospora halophila* (DSM44411)$^T$ and *Nocardiopsis halotolerans* (DSM44410)$^T$ in the DSMZ culture collection in Germany.

## MATERIALS AND METHODS

### Isolation of Halophilic Actinomycetes

Thirty soil samples from the Al-Khiran region in the southern part of Kuwait were collected in aliquots of 2-3kg at depths of 0-10cm, and the samples placed in sterile plastic bags. Ten grams of air dry soil were transferred to a flask containing 100ml sterile water then shaken gently for 20 minute. Serial dilutions up to 1:1000 were performed and 0.1ml of the suspension from various dilutions spread on Petri plates containing starch nitrate medium (contents per liter: 20.0g starch, 1.0g $K_2HPO_4$, 2.0g $KNO_3$, 0.5g $MgSO_4$ .$7H_2O$, 3.0g $CaCO_3$ and 1ml of trace element solution composed of 0.1g each $FeSO_4.7H_2O$, $MnCl_2.4H_2O$ and $ZnSO_4.7H_2O$ per liter) fortified with 10% NaCl. The plates were incubated at 28°C for two weeks and the growing actinomycetes were transferred to starch nitrate/NaCl slants for further use.

### Determintation of salt requirement and salt tolerance

A number of the above isolated strains were checked for salt tolerance by incoculation on starch nitrate medium containing different percentages of sodium chloride in the following range: 0, 2, 5, 7, 10, 15, 20, 25, and 30% as described by Kushner (1993). According to the amount of growth on the different salt concentrations, the isolates have been grouped as halotolerant or halophilic.

### Determination of keratinophilic activities of salt-loving actinomycetes

*Screening for keratin degrading actinomycetes*

A basal medium, containing 0.5g $MgSO_4$. $7H_2O$, 1.0g $K_2HPO_4$, 3.0g $C_aCO_3$, 20g agar per liter and 1ml trace element solution (as described above) was used to test the ability of salt-loving actinomycetes to grow on sterile feathers. The feathers were supplied in small pieces on the surface of the solidified medium and were the sole source of carbon and nitrogen. The basal medium was fortified with 100 gm/L NaCl to adjust salinity to 10%, and the initial pH of the medium was adjusted to 7.5-7.6. The plates were seeded with spore suspensions of the actinomycete strains, and were incubated at 28°C. Plates were examined

for growth and colonization on feather pieces. Strains that showed visible growth on the feathers were considered to be potentially keratinophilic.

*Keratinolytic potential of selected salt-loving strains*

The keratinolytic potentials of the two newly described salt-loving actinomycete species, *Nocardiopsis halotolerans* (#F100) and *Saccharomonospora halophila* (#8) (Al-Zarban *et al*. 2002 a & b), were tested by a modification of the diffusion method of Wawarzkiewicz *et al*. (1987). A minimal agar medium containing 0.15% chicken feather flour (ball-milled) was poured in Petri plates. After solidification 4mm wells were cut in the agar, filled with culture fluid (initially grown in starch nitrate broth plus 10% NaCl) and incubated at 28°C. The positive utilization of the feather flour as the sole source of carbon and nitrogen was assessed by the formation of a clear zone around the wells.

Protease and keratinase activities of the two species were followed over a period of 12 days in a liquid medium containing 0.15% feather meal as the sole source of carbon and nitrogen supplemented with 10% NaCl. A modification of Apodaca & McKerrow (1989) and Bockle *et al*. (1995) methods, as described by Fasasi (1997), was used for the protease and keratinase assays using azocoll as the chromogenic proteolytic substrate, and the keratinazure as a dye-releasing keratinous substrate. In these assays, five milligrams of azocoll or keratinazure was suspended in 0.9ml of appropriate buffer containing 0.04% sodium azide, 0.1ml of culture supernatant was added and incubation was carried out at 37°C under shaking conditions. The assay was performed in triplicates per sample. Uninoculated medium was used as the control. The protease activity was expressed as $OD_{520}$ change of 0.01/m1/hour of the buffered culture supernatant, and the keratinase activity was expressed as $OD_{595}$ of 0.01/m1/hour of the culture supernatant. The protease and keratinase activity assays were repeated in the presence of 1% glucose in the assay medium throughout the sampling period.

## RESULTS

### Isolation and determination of salt tolerance

A number of salt-loving actinomycete isolates were recovered from the thirty salt marsh soil samples. Further analysis of the halotolerance of the isolates revealed two groups. Group 1 consisted of halotolerant actinomycetes that grew on starch-nitrate agar supplemented with 0, 2, 5, 10% and to a lesser extent, 12% NaCl. Three isolates are represented in this group (# F100, M5 & A5). Meanwhile, group 2 consisted of halophilic actinomycetes that failed to grow at low salt concentration, their growth beginning at 10% NaCl and continuing up to 30%. Three isolates are represented in this group (#8, #5 & #10). The results are summarized in the Table.

**Table:** Determination of salt tolerance and salt requirements for the growth of actinomycetes on starch nitrate agar at 28°C.

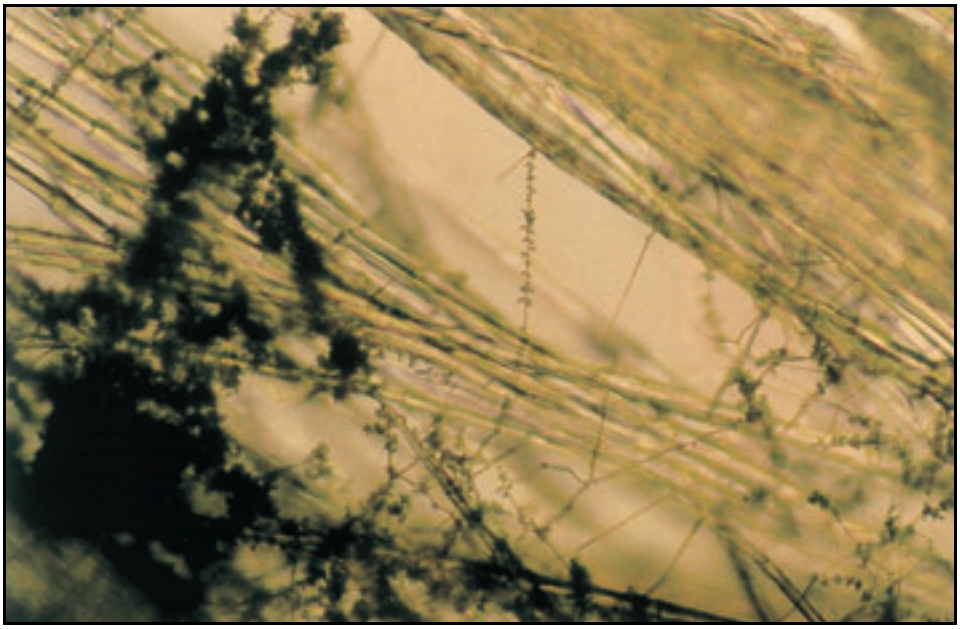
| Isolate # | Sodium chloride concentration (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **0** | **2** | **5** | **7** | **10** | **12** | **15** | **20** | **25** | **30** |
| F100 | ++ | ++ | ++ | ++ | ++ | + | - | - | - | - |
| M5 | ++ | ++ | ++ | ++ | ++ | + | - | - | - | - |
| A5 | ++ | ++ | ++ | ++ | ++ | + | - | - | - | - |
| 8 | - | - | - | - | ± | ++ | ++ | ++ | ++ | ± |
| 5 | - | - | - | - | ++ | ++ | ++ | ++ | + | + |
| 10 | - | - | - | - | ± | ++ | + | + | + | + |

(±): Minimal growth
(+): Growth
(++): very good growth
(-): No growth

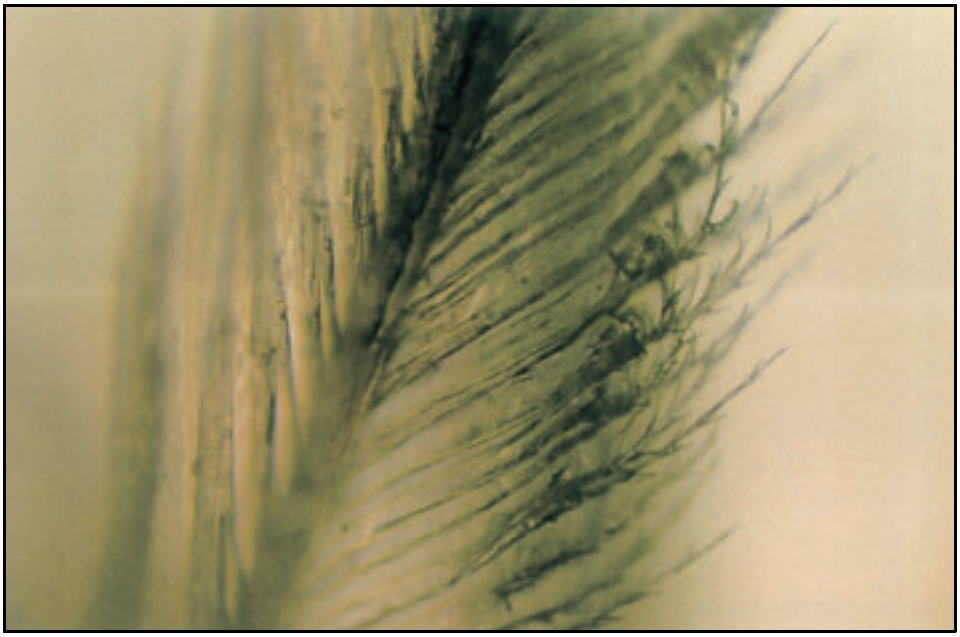
## Detection of keratin degrading activities by halotolerant and halophilic actinomycetes

The ability of halotolerant and halophilic strains to grow on and utilize feather pieces as the sole source of carbon and nitrogen is shown in Figs. 1 & 2. A solid medium supplemented with 10% NaCl seeded with feather pieces supported the growth of some of the isolates. The ability of some of the isolates to utilize


**Fig. 1.** (A). Growth of strain *Saccharomonospora halophila* (#8) on native feather as sole C & N sources. (B). Control medium (uninoculated).



A



B

**Fig. 2.** (A). A light micrograph of strain *Saccharomonospora halophila* (#8strain) colonizing native feather.
(B). A light micrograph of uninoculated feather.

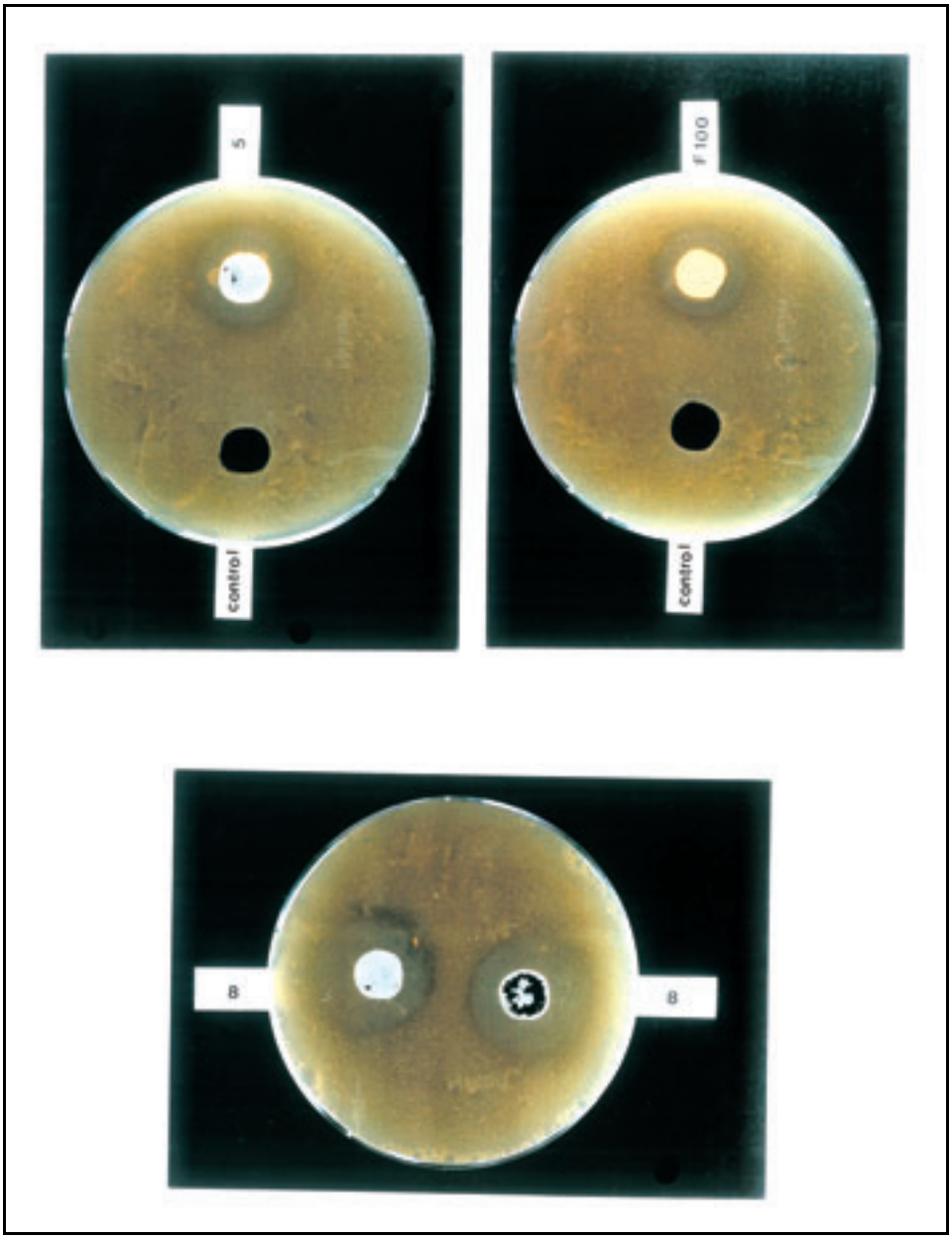



**Fig. (3)** Gel-diffusion method, showing clear zones produced by *Nocardiopsis halotolerans* (strains#F100&#5) and *Saccharomonospora halophila* (strain#8) as compared to uninoculated control wells.

feather powder is shown in Fig. 3. A clear zone around the inocula made by a spore suspension or culture fluid indicates the secretion of extracellular keratinolytic protease or keratinase.

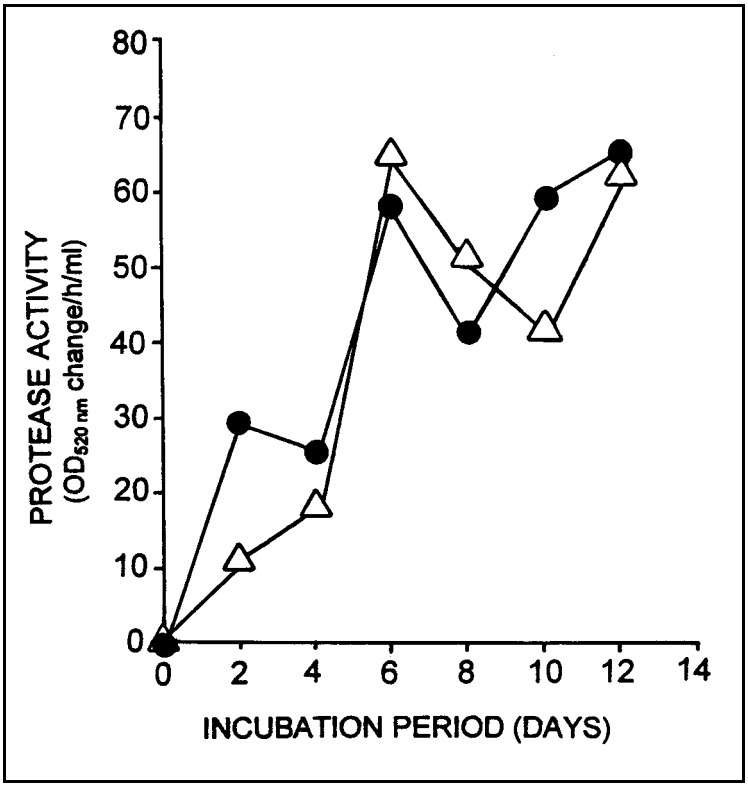



**Fig. (4).** Time course of proteolytic activity of isolates *Nocardiopsis halotolerans* (# F100). Δ, and *Saccharomonospora halophila* (#8), ●, in basal medium plus 10% NaCl and 0.15% feather powder.

Protease activities by *Nocardiopsis halotolerans* (# F100) and the halophilic *Saccharomonospora halophila* (#8) in the presence of 10% NaCl over a period of 12 days are shown in Fig. 4. Both species showed two activity peaks at days 6 and 12. The proteolytic activity was retained in the presence of 1% glucose throughout the 12 days.

The time course of keratinase activity in the presence of 10% NaCl and keratin as the sole source of carbon and nitrogen showed an activity peak at day 6 for the halophilic *Sacharomonospora,* and a wide activity peak at days 4 and 6 for the halotolerant *Nocardiopsis* (Fig. 5). Both species did not show any keratinase activity over the 12 day period in the presence of 1% glucose, indicating catabolite repression by glucose.

## DISCUSSION

This is the first report on the existence of salt-loving actinomycetes in the saline coastal soil in Kuwait, from which a large number of isolates have been

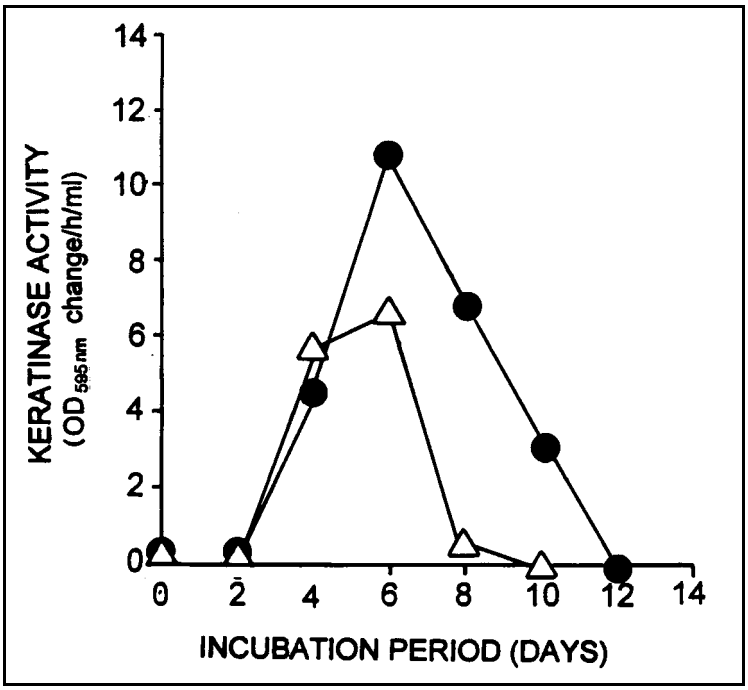



**Fig. (5).** Time course of keratinolytic activity of isolates *Nocardiopsis halotolerans* (#F100), Δ, and *Saccharomonospora halophila* (#8), ●, in basal medium plus 10% NaCl and 0.15% feather powder.

recovered on media with 10% NaCl. Several isolates were able to grow at high salt concentrations and failed to grow when NaCl was omitted, indicating a true halophilic species. Morphologically, these actinomycetes were divided into two groups: a monosporic halophillic species represented by the new species *Saccharomonspora halophila* (#8), and a polysporic halotolerant species represented by *Nocardiopsis halotolerans* (#F100) (Al-Zarban *et al.,* 2002 a & b). Two strains representing *Saccharomonospora* (#5 & #8) were able to colonize and digest feather as the sole source of carbon and nitrogen. They were also able to secrete extracellular enzymes that digest feather keratin in the presence of high salt concentrations (Fig. 3), indicating effective keratinolytic activity under saline conditions. Thus, they can be of potential importance for biotechnological purposes where they can perform keratin degradation in the presence of brackish water or seawater. To a lesser extent, the halotolerant strain, representing the new species *Nocardiopsis halotolerans* (#F100), was also able to grow on feather and produce a clear zone in the gel diffusion method of Wawrzkiewicz *et al.* (1987). This species also produced a remarkably high protease activity in the culture supernatant containing feather powder as the source of carbon and nitrogen and 10% NaCl (Fig. 4). On the other hand, *Saccharomonospora halophila* (# 8) expressed higher and longer lasting

keratinolytic activity than *Nocardiopsis halotolerans* (Fig. 5), indicating more specificity in the degradation of keratinous substrates.

Although keratin - degrading bacteria and actinomycetes have been reported from soil worldwide (Williams & Shih 1989, Mukhopadhyay & Chandra 1990, Ashour *et al*. 1992), no true halophilic keratin-degrading actinomycetes have been previously reported before, and thus we consider these newly described halotolerant and halophilic species as patent strains for keratinolytic activity under saline conditions.

## ACKNOWLEDGMENTS

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# أكتينومايسيتات ملحية جديرة بالملاحظة من الكويت

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## خلاصة

يسجل البحث ولأول مرة قدرة نوعين جديدين من الأكتينومايسيتات actinomycetes الملحية في النمو على كيراتين ريش الطيور وتكسيره إلى مكونات بسيطة. ومصدر هذين الميكروبين أراضي المسطحات الملحية في جنوب الكويت. وينتمي النوع الأول إلى جنس نوكارديوبسس *Nocardiopsis* sp.، ويتحمل درجات ملوحة عالية نسبياً. وينتمي النوع الثاني إلى جنس سكارومونوسبورا *Saccharomonospore* ويتطلب نسب عالية من كلوريد الصوديوم للنمو والتكاثر.

أظهر الميكروبين قدرة فائقة لإفراز إنزيم يحلل الكيراتين في مزرعة سائلة تحتوي على دقيق الريش كمصدر وحيد للكربون والنتروجين، وتنعدم قدرة الميكروبين على تكسير الكيراتين بإضافة جلوكوز بنسبة 1٪.

وترتكز الأهمية العلمية لهذين النوعين الجديدين من بكتيريا الأكتينومايستس على قابلية استخدامهما كأدوات لفهم ميكانيكية تأقلم الميكروبات على النمو في البيئات الملحية وتقييم النشاط الإنزيمي في مثل هذه البيئات المتطرفة والتي يمكن توظيفها في تقنيات تطبيقية.