SCREENING AND ISOLATION OF ACTINOMYCETESS FROM RIVER SEDIMENTS

Sahil Abbas[1]*, Girendra Gautam[1,2], Pawan Kumar Gautam[3]

[1] Department of Pharmacy, Bhagwant University, Ajmer, Raj., India,

[2] Shri Ram College of Pharmacy, Muzaffarnagar, U.P., India,

[3] Departments of Pharmacy, Sarojini Naidu Medical College, Agra, U.P., India.

ABSTRACT

There is an increase or discovery of different diseases which causing great harm to society. The past researches indicated that huge number of antibiotics was produced by Gram +ve ike bacteria known as Actinomycetes. Among all more than 50% of the known antimicrobial compounds were produced by Actinomycetes only. These are a specific type of class of prokaryotes forming thread like structure at some stage of their growth, so refereed as filamentous prokaryotes. This class or group is an actively produce of different types of enzymes, enzyme inhibitors, growth promoter and antibiotics etc. In our study screening of Actinomycetes was performed by using different river sediments. Soil samples was collected from river Godaveri and Krishna and stored in the UV and alcohol sterilized Poly bags. Soil samples was serially diluted upto 10^{-6} and 1 ml from each dilution was placed on different isolation media like starch Casein agar, Albumin media and YMA media, consisting of antifungal agent Nystatin 50 µg/ ml, by pour plate technique. The plates were incubated at different temperature ranges 18^0C to 28^0C upto 7-14 days. There were 3 actinomycetes were isolated and these were streak on solidified Bennet agar media at straight line and plates were kept for incubation in incubator at 37^0C for nearly about 3 days. Identification of actinomycetes was performed using Gram's staining.

Keywords: Actinomycetes, River sediments, Nutrient media, Identification.

INTRODUCTION

Actinomycetes [1] are a widely distributed and successful group of bacteria which have a number of properties which favor them in competition with other saprophytic microorganisms and ensure their survival under unfavorable environmental conditions. Actinomycetes form an integral part of any balanced microbial community in soil, the majority of isolates being streptomycetes which manly exist in the form of dormant spores. These spores germinate in presence of suitable plant and animal remains to form a limited branching mycelium bearing short chain of spores. The spores are continuously washed into aquatic habitats where they accumulate in sediments. The past researches indicated that huge numbers of antibiotics were produced by Gram +ve like bacteria known as Actinomycetes. So we can say that among all microbes more than 50% of the known antimicrobial compounds were produced by Actinomycetes only. These are a specific type of class of prokaryotes forming thread like structure at some stage of their growth, so refereed as filamentous prokaryotes.

Actinomycetes production was almost exclusively confirmed to the group of streptomyces. In modem days human efforts are being generated to broad and performing research about rare actinomycetes which belong to different group's like-

Actinomadura

Actinoplanes

Actinosynnema

Dactylosporangium

Kibdilosporangium etc.

Number of Antibiotics Produced By Major Group Of Microorganisms [2]

Taxonomic groups	Number of antibiotics
Bacteria other than *actinomycetes*	950
Actinomycetes	4600
Fungi	1600

Important Microbes Producing Antibiotics[3]

S. No.	Name of microorganism	Name of antibiotics
1	*P. notatum*	Penicillin Griseofulvin
2	*P.griseofulvum*	Penicillin
3	*P. chrysogenum*	Streptomycin
4	*S. griseus*	Chloramphenicol
5	*S. venezuelae*	Chlortetracycline
6	*S. aureofacns*	Aureomycin
7	*S. virdofaciens*	Oxytetracycline
8	*S. rimosus*	Tetracycline
9	*S. texas*	Dimethyl-chlortetracycline
10	*S. aureofaciens*	
11	*S. erythricas*	Erythromycin
12	*S. halstedii*	Carbamycin
13	*S. ambofaciens*	Ravomycin
14	*S. noursei*	Nystatin
15	*S. griseus*	Cycloheximide

Actinomycetes are a special group of heterotrophic prokaryotes forming hyphae at some stage of their growth hence refereed as filamentous prokaryotes. They have been specialized and different morphological, cultural, biochemical and physiological characters. This group is a potential producer of different enzymes, enzyme inhibitors, growth promoter and antibiotics etc. Actinomycetes are gram +ve bacteria belonging to the order of actinomycetales. Actinomycetes are characterized by the formation of normally branching threads or rods, frequently giving rise to a typical mycelium which is unicellular, especially during the early stages of growth. Actinomycetes are heterotrophic group in nature. Most of them are strict saprophytes, while some parasitic or mutualistic association with plants and animals. They are aerobic and most of them readily grow on the common bacteriological media like

Nutrient Agar

Trypticase Agar

Blood Agar

Starch Casein Agar

Albumin Agar etc

NEEDS FOR NEW MEDICINES [2]

Now day's human is facing great harming due to different diseases because a number of microbes got resistance against the available drugs. These products have been exploited for human use for thousands of years, and plants have been the chief source of compounds used for medicine. Even today the largest users of traditional medicines are the Chinese, with more than 5,000 plants and plant products in their pharmacopoeia In fact, the world's best known and most universally used medicine is aspirin (salicylic acid), which has its natural origins from the glycoside salicin which is found in many species of the plant genera *Salix* and *Populus*. Examples abound of natural-product use, especially in small native populations in a myriad of remote locations on Earth. For instance, certain tribal groups in the Amazon basin, the highland peoples of Papua New Guinea, and the Aborigines of Australia each has identified More recently, the Benedictine monks (800 AD) began to apply *Papaver somniferum* as an anesthetic and pain reliever as the Greeks had done for years before Many people, in past times, realized that leaf, root, and stem concoctions had the potential to help them. These plant products, in general, enhanced the quality of life, reduced pain and suffering, and provided relief, even though an understanding of the chemical nature of bioactive compounds in these complex mixtures and how they functioned remained a mystery.

SCOPE OF HERBAL DRUGS:

India can play a major role in the coming years in the global market for herbal products based medicines, since there is a growing demand for plant based medicines and cosmetics, since pharmaceutical industry is plagued with increased cost of new drug development coupled with low serum rate. Scientific validation quality, quantity, consistency and good marketing network are quite essential for the growth of herbal plant industry in India. The absence of these in the country has affected growth of medicinal plant industry in the country. India has a big potential for the cultivation of herbal plants.

- In some Asian and African countries, 80% of the population depends on traditional medicine for primary health care.
- Herbal medicines are the most lucrative form of traditional medicine, generating billions of dollars in revenue. Traditional medicine can treat various infectious and chronic conditions: new antimalarial drugs were developed from the discovery and isolation of artemisinin from *Artemisia annua* L., a plant used in China for almost 2000 years.
- Counterfeit, poor quality or adulterated herbal products in international markets are serious patient safety threats.
- More than 100 countries have regulations for herbal medicines.

Traditional medicine is the sum, total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses. Traditional medicine that has been adopted by other populations (outside its indigenous culture) is often termed alternative or complementary medicine. Herbal medicines include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant materials as active ingredients.

Who uses traditional medicine?

In some Asian and African countries, 80% of the population depends on traditional medicine for primary health care. In many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine (e.g. acupuncture). Herbal treatments are the most popular form of traditional medicine, and are highly lucrative in the international marketplace. Annual revenues in Western Europe reached US$ 5 billion in 2003-2004. In China sales of products totaled US$ 14 billion in 2005. Herbal medicine revenue in Brazil was US$ 160 million in 2007.

Challenges

Traditional medicine has been used in some communities for thousands of years. As traditional medicine practices are adopted by new populations there are challenges. The possibilities for developing new drugs from forest resources should figure heavily in any calculation of the forests true worth. All 119 plants derived drugs, used worldwide in 1991, came from fever than go of the 250, 000 plants species that have been identified, each such plant is a unique chemical factory as correctly mentioned by Norman R. Faransworth of the university of Illinois at Chicago, that are capable of synthesizing unlimited numbers of highly complex and unusual chemical substances whose structures could otherwise escape the imagination, scientist may be able to synthesize, these plants compounds in the laboratory, but dreaming them up, rather than plucking then from the forest and then replicating them is quite different. The credit for having first recognized the ability of actinomycetes to destroy microbial cells is generally given to Gasperini (1890), who observed, in the course of his classical researches on streptothrix foersteri Cohn, that the filaments of this organism may destroy the cell-membrane of several bacteria and fungi.

MATERIALS AND METHODS

Collection of sample

The different samples of soil sediments were collected from river Krishna and Godaveri after making 2 cm depth and stored in sterile polybags.

Sterilization of polybags

Airtight polybags were purchased from market and these were sterilized after application of ethyl alcohol and keeping into UV light for 5 minutes.

Storing of sediments

Collected soil sediments first about 20 gm were kept in polybags and stored in refrigerator.

Preparation of samples for isolation of actinomycetes

The different soil samples were taken and these were serially diluted upto 10^{-6}. Each of samples was prepared using different test tube. Isolation of actinomycetes by using different nutrient media

The ingredients of media were accurately weighed for the each 500 ml of the three type's media i.e.

- Starch Casein Agar Media
- YMA Media
- Albumin Media

Weighed ingredient were dissolved in required quantity of distilled water and sterilized at 121 °C (15 lbs) for 15 min by using autoclave. After sterilization the antifungal Nystatin was added (50 µg/ml) then media were poured into Petri dishes under sterile condition (laminar air flow) and allow cooling for sufficient time for the solidification of media.

The surface sterilized plant parts were taken and crushed using sterile pestle and mortar and spread on the three of the media and kept at 28 °C for 2-3 weeks, growth of microbes were observed each day and produced actinomycetes colony were purified on the Petri dishes using streak methods on the same media.

Growth on different ISP media

Media composition were weighed and dissolved in water and sterilized at 121°C (15 lbs) for 15 min by using autoclave. After sterilization the media were poured into Petri-dishes under sterile condition (laminar air flow) and allow cooling for sufficient time for the solidification of media and after solidification isolated microbes were streaked on solidified media in zigzag fashion and kept for incubation in incubator at 37°C for about 24 hrs.

Gram's staining

a) The microbes' smears were taken on glass slide.
b) The smears were air dried.
c) Smears were covered with crystal violet for 30 seconds.
d) Covered each smear with Gram's Iodine solution for 60 seconds.
e) Washed off Iodine solution with 95% ethyl alcohol, ethyl alcohol was added drop by drop until no more colour flows from the smear.
f) The slides were washed with distilled water and drain.
g) Safranin was applied to smears for 30 seconds (counter staining).
h) The slides were washed with distilled water and blot dried with absorbent paper.
i) Let the stained slides air dry.
j) The slides were examined under microscope.

RESULTS AND DISCUSSION

RESULTS:

Table 1 Actinomycetes isolated from different sediments of different rivers on different media

S. No.	Name of Sediments	Nutrient Media	No. of Actinomycetes Isolated
1.	Ganga G1 G2 G3 G4 G5	Albumin	3 Actinomycetes
2.	Yamuna Y1 Y2 Y3 Y4 Y5	Albumin	2 Actinomycetes
3.	Godavari GD1 GD2 GD3 GD4 GD5	Albumin	3 Actinomycetes
4.	Krishna K1 K2 K3 K4 K5	Albumin	1 Actinomycetes
5.	Ganga G1 G2 G3 G4 G5	Starch Casein Agar Media	2 Actinomycetes
6.	Yamuna Y1 Y2 Y3 Y4 Y5	Starch Casein Agar Media	1 Actinomycetes
7.	Godavari GD1 GD2 GD3	Starch Casein Agar Media	4 Actinomycetes

	GD4 GD5		
8.	Krishna K1 K2 K3 K4 K5	Starch Casein Agar Media	2 Actinomycetes
9.	Ganga G1 G2 G3 G4 G5	YMA Media	No Actinomycetes 1 Actinomycetes
10.	Yamuna Y1 Y2 Y3 Y4 Y5	YMA Media	No Actinomycetes
11.	Godavari GD1 GD2 GD3 GD4 GD5	YMA Media	
12.	Krishna K1 K2 K3 K4 K5	YMA Media	No Actinomycetes

Out of 12 sediments sample total 19 Actinomycetes were isolated. Out of 19 sediments sample only 4 showed antibacterial activities. These 4 Actinomycetes further allow growing on ISP media. Out of 4 Actinomycetes 2 were isolated on Starch Casein Media from river sediments and named as S1 and S2, one was isolated from Godavari river sediment on Starch Casein Media named as S3 and 4th Actinomyctes was isolated from Yamuna river sediment on Starch Casein Media. The Actinomycetes isolated from sediments were shown in Figure 1-3.

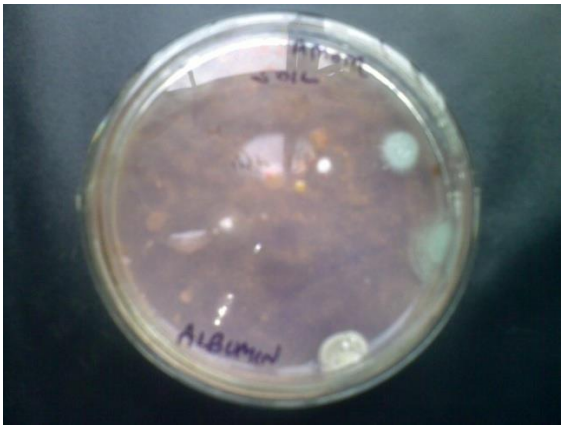

Figure 1: Actinomycetes growth on albumin media

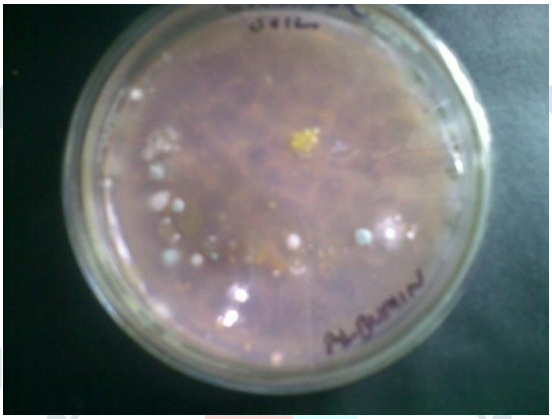

Figure 2: Actinomycetes growth on YMA media

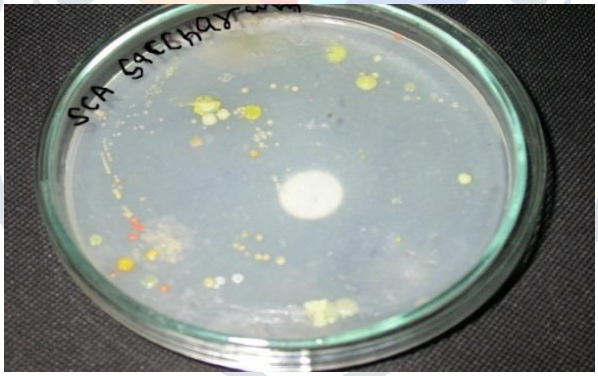

Figure 3: Actinomycetes growth on starch casein agar media

Morphological study:

The actinomycetes strains which showing anti-bacterial activity isolated from different river sediments samples (S1, S2, S3, and S4) were allowed to grow on the different types of ISP (International *Streptomycin* Project) media as follows.

a. ISP-2

b. ISP-4

c. ISP-5

d. ISP-6

e. ISP-7

Culture characteristics of actinomycetes strain on ISP-2 and ISP-4 Media

The colour of mycelium above the Petri dishes and colour of media were shown in Table 2.

Culture characteristics of actinomycetes strain on ISP-5 and ISP-6 media

The colour of mycelium above the petri dishes and colour of media were shown in Table 3.

Culture characteristics of actinomycetes strain on ISP-7 media

The colour of mycelium above the petri dishes and colour of media were shown in given Table 4.

GRAM'S STAINING

All the eight actinomycetes were found Gram's positive and showed the violet colour on the slide, isolated from different river sediments samples. i.e. S1, S2, S3 and S4.

BIOCHEMICAL TEST

There are certain biochemical test were performed for the identification of 4 strains of actinomycetes producing anti-bacterial compound given as-

MELANOID FORMATION

The actinomycetes producing anti-microbial compound isolated from different soil samples showed different results, given in Table 5

TEST FOR NITRATE REDUCTION

The actinomycetes producing anti-microbial compound isolated from different soil samples showed different results, given in Table 6

TEST FOR ACID PRODUCTION

The actinomycetes producing anti-microbial compound isolated from different soil samples showed different results, given in Table 7.

Table 2: Shows the color of mycelium and culture media on ISP-2 and ISP-4 media

Actinomycetes strain	ISP-2		ISP-4	
	Color of mycelium	Reverse side color	Color of mycelium	Reverse side color
S1	- ve	- ve	- ve	- ve
S2	- ve	- ve	- ve	- ve
S3	- ve	- ve	Yellow	Dark Brown
S4	- ve	- ve	- ve	- ve

The –ve sign indicate that actinomycetes are unable to grow on the ISP media because of their culture characteristics of growth on different media

Table 3: Shows the color of mycelium and culture media on ISP-5 and ISP-6 media.

Actinomycetes strain	ISP-5		ISP-6	
	Color of mycelium	Reverse Side Color	Color of mycelium	Reverse Side Color
S1	- ve	- ve	- ve	- ve
S2	White	Brown	Orange white	Orange
S3	White	Dark brown	- ve	- ve
S4	- ve	- ve	- ve	- ve

The –ve sign indicate that actinomycetes are unable to grow on the ISP media because of their culture characteristics of growth on different media

Table 4: Shows the color of mycelium and culture of media on ISP-7

Actinomycetes strain	ISP-7	
	Color of mycelium	Reverse Side Color
S1	White	Brown
S2	- ve	- ve
S3	- ve	- ve
S4	Orange	Brown

The -ve sign indicate that actinomycetes are unable to grow on the ISP media because of their culture characteristics of growth on different media

Table 5: Shows the melanoid formation test

Actinomycetes strain	Reaction
S1	+ve
S2	+ve
S3	+ve
S4	-ve

Table 6: Shows nitrate reduction test

Actinomycetes strain	Reaction
S1	-ve
S2	-ve
S3	+ ve
S4	-ve

Table 7: Shows acid production test

Actinomycetes strain	Reaction
S1	-ve
S2	-ve
S3	-ve
S4	-ve

Table 8: Shows hydrogen sulphide test

Actinomycetes strain	Reaction
S1	-
S2	+
S3	+
S4	+

ISOLATION OF ACTINOMYCETES PRODUCING ANTIBACTERIAL COMPOUND:[5]

Determination of antibacterial activities of pure actinomycetes cultures will be perfomered by using streak -plate method. Mueller hinton agar plates will prepared and inoculated with actinomycetes cultures by a single streak of inoculums in the center of the Petri dish and will incubated at 27^0C for 4 days. Later, the plates will seeded with test organisms by a single streak at a 90^0 angle to actinomycetes strains. Antagonism will measured by the determination of the size of the inhibition zone.

PRODUCTION OF ANTIMICROBIAL COMPOUND USING DIFFERENT MEDIA LIKE STARCH CASEIN BROTH AND L.B. BROTH:[4]

Actinomycetes showing antibacterial activity will be then Inoculated into different media like L.B. broth and Starch Casein Broth and Albumin Broth etc and will be kept for incubation at different Temperatures ranging from 28^0C- 40^0C for the production of antibacterial compound upto18 days.

EXTRACTION OF ANTIMICROBIAL COMPOUND USING DIFFERENT SOLVENTS [6]

All of above Broth of Actinomycetes will be taken at the end of 7^{th} day and centrifuged at 10,000 rpm for 15 min to separate the mycelial biomass; the supernatant will obtained separated by filtration using Whatman filter paper. Certain solvents used for extraction of antibacterial compound like butanol, n-hexane, ethyl acetate**,** petroleum ether**,** chloroform, ethanol (1:1) ratio. Supernatant mixture was agitated for 50 min. with homogenizer and the solvent will separated from broth by separating funnel, Solvent present in the broth will be separated by centrifugation at 5000 rpm for 15 min to remove traces of fermentive broth. All extracts obtained through this method will be assayed for antibacterial study against different microbes using respective solvents as control by agar well diffusion method.

ANTIBACTERIAL SCREENING [6]

The antibacterial activity of compound will tested against different gram +ve and Gram –ve and Certain Fungus by the standard disc diffusion method and cup plate method. Standard Kanamycin, streptomycin etc. will be used for comparison of the antibacterial activity. Nutrient agar was used as a bacteriological media. The minimum inhibitory concentration (MIC) will be calculated.

PURIFICATION OF ANTIBACTERIAL COMPOUND:[7]

Purification of the compound was performed using TLC and Column chromatography using different solvent composition.

IDENTIFICATION OF ANTBACTERAL COMPOUNDS USING FOLLOWING TECHNIQUE [7,8]

- Calculation of λ max of the antibacterial compound. .
- Using FTIR SPECTROSCOPY.
- Using NMR SPECTROSCOPY.

Using MASS SPECTROSCOPY

DISCUSSION:

There were only two actinomycetes S1 and S3 showed strong antibacterial activity. S1 was isolated from Godavari river sediment on Starch Casein Media named as S3 Actinomyctes was isolated from Yamuna river sediment on Starch Casein Media. There were two compounds were extracted in Starch Casein Broth and named as A1 and A2.

Calculation of λ max of the antibacterial compound

The λ max of two compounds A1, A2, was calculated using methanol and ethanol respectively.

λ max of A1 was 306 and A2 was 324.

Analysis of the antibacterial compound

The physical analysis of the compound was performed as follows

Physical analysis

Color of the compound

The color of the compound were

1. A1 Compound - white
2. A2 Sample-Brownish in colour

State

Solid in nature (powder)

The compound A1 and A2 were given for elucidation of structure

Antimicrobial Assay

S. No.	Name of microorganisms	Zone of inhibition (mm) C1
1.	*E. coli* ATCC 8739	3.9 mm
2.	*S. typhi* ATCC 23564	0 mm
3.	*S. aureus* ATCC 29736	4.0 mm
4	*M. luteus ATCC11880*	3.9mm
5.	*K. pneumoniae ATCC 10031*	0 mm
6.	*S. fecalis ATCC 8043*	3.5 mm
7.	*B. subtilis ATCC 6633*	6.3mm
8.	*S. boydi ATCC 9207*	3.9 mm
9.	*P. mirabilis ATCC 2124*	2.2 mm

S. No.	Name of microorganisms	Zone of inhibition (mm)C2
1.	*E. coli* ATCC 8739	4.2 mm
2.	*S. typhi* ATCC 23564	3.9 mm
3.	*S. aureus* ATCC 29736	4.4 mm
4	*M. luteus ATCC11880*	0 mm
5.	*K. pneumoniae ATCC 10031*	4.3 mm
6.	*S. fecalis ATCC 8043*	0 mm
7.	*B. subtilis ATCC 6633*	5.6 mm
8.	*S. boydi ATCC 9207*	4.4 mm
9.	*P. mirabilis ATCC 2124*	2.4 mm

REFERENCES

1. Librado OO, Luis FB, Veronica Y. Biological, Biochemical and Biomedical Aspects of Actinomycetes. Academic Press New York; 1984; p. 453.
2. Hung PQ, Kumar SM, Govindsamy V, Annapurna K. Isolation and characterization of Endophytic bacteria from wild and cultivated soybean varieties. *J Biol Fertil Soils*, 2007; 44: 155-162.
3. Sivakumar K. Centre of advanced study in marine biology. Annamalai University: 197-204.
4. Silambarasan S, Praveen Kumar E, Murugan T, Saravanan D, Balagurunathan R. Antibacterial and antifungal activities of actinobacteria isolated from Rathnagiri hills. *J Appl Pharm Sci*, 2012; 2: 099-103.
5. Kaur S, Kaur HP, Kaur G. Isolation and characterization of antibiotic producing *actinomycetes* from agriculture soil. *World J Pharm Pharml Sci*, 2016; 5: 1109-1117.
6. Gebreselema G, Feleke M, Samuel S, Nagappan R. Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of Lake Tana, Ethiopia. *Asian Pac J Trop Biomed*, 2013; 3: 426-435.
7. Pradhan S, Mishra BB, Rout S. Screening of novel actinomycetes from near lake shore sediment of the chilika lake, Odisha India. *Int J Curr Micobiol Appl Sci*, 2015; 4:66-82.
8. Lisboa MP, Bonatto D, Bizani D, Henriques J and Brandelli AP. Characterization of a Bacteriocin- like Substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic Forest. *J Int Microbiol*, 2006; 9:111-118.