# REVIEW

# 2- REVIEW

## Isolation of actinomycetes from natural habitat.

***Williams* and *Cross, (1971)*** described the methods for the isolation of streptomycets. ***Ahmad, (1990)*** isolated 87 actinomycetes isolates, 34 of them exhibited anti-microbial activities. He illustrated the method and media used for such purpose. ***Daqun et al., (1996)*** isolated 93 *Streptomyces* isolates, twenty-two strains showed more antibiotic activity against virulent *Streptomyces scabies* RB 311 than the standard pathogen-suppressive strains.

## Causal organism of chocolate spot diseases.

***Naguib, (1948)*** demonstrated that, chocolate spot disease of faba bean in Egypt can be caused by either *Botrytis fabae* or *B.cinerea*, while spots caused by the *B.cinerea* were inconspicuous compared with those by *B.fabae*.

***Leach, (1955)*** illustrated that, leaf spots caused by *B.cinerea* effect only on epidermal cells where as *B.fabae* nearly always causes necrosis of the mesophyl.

***Myra* and *preece, (1968)*** showed that, the addition of pollen grains or orange juice to the spores on the leaf surface improved spore germination and germ tube elongation and induced the development of the aggressive phase of chocolate spot, which is normally caused by *B.fabae*.

***Mansfield* and *Deverall, (1974)*** found that Conidia of *Botrytis fabae* produced spreading lesions at nearly all inoculation sites on leaves

grown in green houses. The development of lesions produced by *Botrytis cinerae* was more variable.

***Harrison, (1980)*** found that, under conditions of continuous high humidity, limited lesions become aggressive darkening and rapidly increasing in size, often causing defoliation and eventually killing the entire shoot system.

***Abou-Zeid* and *Le Normand ,(1981)*** mentioned that, the most effective inoculum concentration of *B.fabae* suspension was $250x10^3$ spore / ml which used for artificial inoculation.

***Mohamed et al., (1981)*** studied the range of variability within the fungus *B.fabae*, they tested five isolates from different area, Sakha, Nubaria, Ismalia, Gemiza and Alexandria, on different cultivars from faba bean, they found that, isolate from Nubaria is the most virulent than the rest.

***Abau-Zeid* and *Mohamed (1987)*** demonstrated that, the optimum concentration for inoculation of faba bean differed according to *B.fabae* isolate, the faba bean cultivar and the method of inoculation used. The investigators found that, the reaction of the detached leaflets from different nodes of Giza 3 and Rebaya 40 faba bean plants to *B.fabae* isolated from Nubaria differed significantly, the newest node ($5^{th}$) were more resistant than the older one ($1^{st}$).

***Harrison, (1988)*** reported that, both *Botrytis* sp. could cause chocolate spot disease on faba bean in the field, but *Botrytis fabae* was the more important pathogen because it was more aggressive than

*B.cinerae.* in the laboratory, however, the pathogenicity of *B.cinerea* could be manipulated by the addition of certain compounds to the inoculum droplets, or by using very young conidia, to be similar to *B.fabae* in inducing infection and development.

***Hassanein et al., (1990)*** reported that, *B. fabae* isolates which showed the least amount of growth and spore production were the most virulent on the twice tested faba bean entries while isolates of vigorous growth and high spore production (*B. cinerea*) were less virulent

***Abou-Zeid et al., (1998)*** isolated 40 isolates of *Botrytis* sp. from different growing area of faba been in Egypt during 1994 / 1995 season and they identified 40 isolates to the species level based up on the key of ***Ellis (1976)*** as *B.cinerea* (16 isolate ) and *B.fabae* (24 isolate).

***Kuti* and *Nawer, (1999)*** demonstrated that, in pathogenicity studies of five isolates of chocolate spot fungi (3 *B.cinerea* and 2 *B.fabae*), one isolate of *B.cinerea* originally isolated from faba bean was more pathogenic to faba bean than the two *B.fabae*, also originally isolated from faba bean, while the other two isolates of *B.cinerea* isolated from graps and eggplant respectively were moderately pathogenic to faba bean.

## Biological control of chocolate spot disease.

***Forbes* and *Bretag, (1991)*** reported that, a streptomycin was applied for control of bacterial blight of peas. The crop losses by the diseased were estimated in field trials by using streptomycin sprays, which decrease the disease by 62%.

***Jackson et al., (1991)*** have been studied the potential of both bacterial and fungal isolates, isolated from soil and leaves to using

significant control leaves against downy mildew of lettuce (*Bremia lactucae*), powdery mildew (*Uncinula necator*) and *Botrytis cinerea* of grape, late blight (*phytophthora infestans*) and several other host pathogen combinations. The resulting product is long shelf life, efficacious and easy to use.

***Abou-Zeid* and *Hassanein, (2000)*** reported that, some isolates of *Bacillus* spp. isolated from phylloplane had an antagonistic effect against *B.fabae* on PDA medium. *In vivo*, data indicated that, the isolates No. 1,2,3, and 4 were more effective than the other isolates, till the end of experiment, while the isolates No. 5,6,and 7 were less effective after 7 days from incubation. This indicates the inhibitory effect is not persistent with the same efficacy or otherwise the pathogen growth overcomes the activity of the antagonist under the experimental conditions. Also, it may indicate that the operating inhibitory agent is not always the same as that operating on leaf surface. Other factors such as the inoculum potential of the antagonistic bacteria, the nature of interaction of antagonist with others and/or the variability within the tested isolates of the pathogen can also play a role in such situation.

## Identification of streptomycetes and their anti-microbial activities.

***Goodfellow & Board, (1980)* and *Goodfellow & Cross, (1984a&b)*** reported that, the, application of new and reliable biochemical, chemical, genetical numerical and molecular biological techniques is the resason of the revolutionary change in bacterial systematic in the last 20 years and the rapidly changing view on having

**The actinomycetes as antibiotic producing microorganisms.**

Actinomycetes are a successful and widely distributed group of bacteria which have number of properties that favor them in competition with other saprophytic microorganisms. Most of the known antibiotics were isolated from various species belonging to actinomycetes.

**What's antibiotic?**

Antibiotic was first used by Waksman in 1942 to describe a chemical substance produced by a microorganism and inhibit other microorganisms Waksman in 1947 have been define antibiotic as a chemical substance produced by microorganism which has the ability to inhibit the growth and even to destroy bacteria and other microorganisms ***(Betina, 1983).*** Also, the problem of bacterial resistance to antibiotics had evolved, and new compounds or derivatives of known antibiotics had to be found to replace existing ones.

The isolation of (6-APA) 6-amino-penicillin acid in the late of 1950s opened the filed to the semi-synthetic penicillin and provided an example for development of semi-synthetic antibiotics in general, ***(Rolinson, 1979* and *Vandame, 1980).***

At the end of 1950s, arrival of antibiotic discovery occurred owing to the application of novel screening programs, supersensitive test organisms, new antibiotics sources and the broading of the search for novel microbial products to include agents with pesticidial, anti tumor, insticidial, herbicidal, anticoccidal, cytotaxic, anthelminthic hormonal immunoregulatory, food preserving, growth promoting and enzyme inhibiting activities, as well as products with pharmacological activity ***(Lwai & Omura, 1982; Miller et al., 1983* and *Demain, 1983).***

Most of the antibiotics were isolated from species that belong to genus *Streptomyces*. The best known antibiotics produced by the genus *Streptomyces* include **aminoglycosidies** (streptomycins, olendomycins, spirmaycines, *etc.*) **polyenemarcolides** (nystatin, Amphatracin, B.filipin, etc.) **Tetracyclines, anthracyclines** (chloramphenicol) **heterocylic** (polymixin), **alicyclic** (cyclohexi-mide), polypeptides (viamycin and actinomycins).

*Micromonospora* is anther important genus show the ability to produce several antibiotics as **gentamycins** and other antibacteria, the most recent description of antibiotics from *Micromonospora* was reported by ***Wagman* and *Weinstein, (1980).***

**The antibiotics functions in the nature.**

Antibiotic function in the metabolism of the producing organisms has been the subject of considerable speculation and discussion. However, we still in need to understand more about the role they actually do. Some ideas were discarded which involved antibiotics as evolutionary relics, waste products of cellular metabolism, reserve food materials, spore coat components, or break down produces derived from cellular macromolecules. Other classical hypothesis are,

(1) Secondary metabolism serves to maintain the enzymatic machinery of the cell in working order until conditions favorable for growth are found.

(2) Antibiotics are detoxification products one possibility still being considered that, antibiotic function is to kill or inhibit the growth of other organisms in nature by providing a competitive advantage to the producing species ***(Demain 1980 a).***

***Makato et al., (1995 I&II)*** they isolated three new macrolide antibiotics from *S.violaceusninger* which isolated from a soil sample. These antibiotics had anti-microbial activities against fungi, and Gram positive bacteria. They described the taxonomy, production, isolation ,biological activities, physicochemical properties and their structure.

***Sugata et al., 1995*** reported that, the *Streptomyces* spp. produce phencomycin which was active against Gram positive bacteria..

***Ogawa et al., (1995)*** during their screening of endo-thelin antagonists they have isolated a novel cyclic peptide RES-701-1 from the fermentation broth of *Streptomyces* sp. RE-701. They found that many strains of *Streptomyces* produce RES-701-1 related compounds and isolated three novel compounds from the culture broth. In their work they, describe taxonomy of the producing strains, fermentation, isolation and biological properties of RES-701, 1, 2, 3 and 4.

Strain HILY-9120362 isolated from soil, produced a new members of a Zalomycin class, 2-demethylazalomycin F4a 1 and 2-demethyl-azalomycin F5a (2). Both the compounds 1 and 2 exhibited *in vitro* and *in vivo* activity against a wide range of fungal strains, but they didn't exhibited any antibacterial activity, ***(Mukhopadhay et al., 1995).***

***Baker et al., (1996)*** they discovered the potent anthelmintic activity exhibited by the milbemycins and reported the isolation and structure elucidation of the milbemycins, also they have been describe the fermentation and isolation.

***Burkhardt*** **and** ***Hans-Peter,(1996)*** demonstrated the taxonomical characterization, fermentation as well as the isolation and purification procedures studies of *Streptomyces griseoviridis* (FH-S 1832). ***Hanafi et***

Although microorganisms perform most of their growth processes before they produce antibiotics they might be killed by their own antibiotic during production, we known however that, industrial fermentions are usually conducted for many days after the onset of antibiotic production. Thus the synthesizing organism develops resistance during production ***(Demain, 1974).***

The resistance mechanisms developed by antibiotic producing microorganisms against their own antibiotic are not different from those in clinically resistant bacteria. Permeability modifications are involved in many cases, antibiotics are pumped out of the cells against it's concentration gradient, permeability, during the idiophase, protects the organisms from high extracellular concentration of its own antibiotic. Additional mechanisms exist to protect the cells from internal antibiotic that is not excreted, or from antibiotic that escapes from an antibiotic production compartment ***(Krassilnikov, 1960).***

One of such mechanisms is the synthesis of enzyme that modify the antibiotic and many antibiotic producers possess enzyme capable of converting their antibiotic into inactive or less active derivatives. Another mechanism involves a modification in the machinery of the producer, such as, in ribosome which serve as targets of the particular antibiotic. Another means by which antibiotic producers protect them selves is by feed back inhibition of repression of antibiotic production ***(Vining, 1979)***

**Factors affecting on antibiotic Biosynthesis.**

***Effect of different incubation periods.***

The determination of incubation period on the production of antibiotic is quite important, there's a relation between productivity of antibiotic and the time of incubation. Time required for high yield

production of antibiotic by microorganisms varied greatly from one to another. For example corminomicin biosynthesis depends on the culture growth time, the maximum antibiotic production was obtained on the 7th to 8th day. With an increased incubation time a relative content of long chained components of phytobacteriomycin increased in *Actinomyces lavendulae* culture (***Samoilov et al., 1967).***

***Juslen et al., (1982)*** reported that, the maximum yield of tetracycline, isolated from a strain of *Streptomyces* spp. was obtained after 4 days incubation at 27°C. ***Evans et al, (1983)*** mentioned that, a good result was obtained with incubation time from two to four days for the production of a new clavamantibiotic, produced by *Streptomyces clavuligerous.*

***Ahmad, (1990)*** found that the maximum AZ- $B_{316}$ biosynthesis and mycelial formation were obtained after 5 days. Also ***Baker et al., (1996)*** isolated novel milbemycin from *Streplomyces* sp. after incubation time of 48 hours. ***Nabuo et al., (1996)*** reported that , a new ansomycins designated hydroxymoctirenins A and B were isolated from *Bacillus* spp. fermentation broth after 2 days incubation.

***Effect of different hydrogen ion concentrations (pHvalues).***

***Ketoki*** **and** ***Majumdar, (1973)*** found that, the pH value of medium might be an important factor for kanamycin formation by *Streptomyces kamamyceticus,* which give high production at alkaline pH.

***Takiguchi et al., (1981)*** reported that, milbemycin biosynthesis produced by *Streptomyces hydrosopius* was detected at pH 7.2.

The initial pH value of the growth medium is important for antibiotics production by certain actinomycetes. The optimum pH values for antibiotics biosynthesis varied, an acidic, neutral and alkaline environments were reported to have a great effect on both growth and metabolic activities, some bacteria *e.g* lactic acid *Streptococci* grow and produce Nisin at pH 5.5-6.0 ***(Egorov,1985).*** He added that most antagonistic *Streptomyces* grew well at pH values 6.7 to 7.8 but not at pH below 4.5-4.0 However acidophilus streptomycetes have been isolated at pH 3.5 to 6.5. ***Razak et al., (1994)*** found that ,pH 5.0 was the best initial medium pH for the protease production.

***Effect of different incubation temperature.***

This can be explained by substantial effect of temperature on the activity of the enzymes, the activity of the transport systems, and other important physiological and biochemical function of the microbial cell. When a penicillin producing strain was grown at 30°C and then shifted to 20°C for the antibiotic production, a highly effective process was obtained ***(Owen & Johnson, 1955).*** Streptomycetes produced antibiotics when cultivated at temperature ranges from 26 °C to 30°C, although some streptomycetes grow at lower temperature from (0 to 18°C) and also elevated temperature (55 °C to 60 °C)( ***Johnson, 1955).***

*Streptomyces* spp. No.81 strain, produce the antibiotic M-81 at 27°C and forms of Gryomycin at low cultivating temperature of 12°C ***(Yashida et al., 1974). Sidler and Zuber ,(1980)*** reported that, the optimum cultivation temperature for *B.stearothermophilus* strains NCI B – 8924 and NRRL-3880 was at 55°C and 37°C for strain A TCC-7954.

The growth temperature has an important effect on both growth and metabolic activities of microorganisms. The optimum temperature

differs for different groups of microorganisms. Most bacteria develop at incubation temperature ranges from 30 to 37°C. The optimum temperature for culture producing gramicidin (*Bacillus brevis*) is 40°C and the some microorganisms can also develop normally and synthesize the antibiotic at 28°C, but the maximum amounts of gramicidin was produced at incubation temperature 40°C ***(Egorov,1985).***

***Razak et al., (1994)*** reported that, optimum protease production by *Bacillus stearothermophilus* was occurred at 60°C. New ansomycins designated hydroxymcotrienins A and B were isolated from culture broth of *Bacillus* sp. BMJ958 –624 at 27°C. ***(Nobuo et al., 1996).***

**Extraction and purification of the anti-microbial product.**

***El-Gamal, (1985)*** reported that, *S.violochromagens* produce deep yellow antibiotic in starch nitrate media which was moved as one single clear zone eluted and precipitated by petroleum ether.

***Ahmad, (1990)*** studied the extraction and purification of the anti-microbial product obtained from *S.nogalater Az-B*$_{316}$. The product showed only yellow fluorescence band at Rf 0.8. ***Hussein et al., (1998)*** found that, n-butanol succeeded to extract the antibiotic from the broth. The extract resulted in a yellow substance with high Rf value with butanol pyridine water.

***Yassin, (1998)*** reported that, *Streptomyces violaceus* produce a yellow cryslalline antibiotic which was extracted from broth using n.butanol at pH 2.0 ***Yuji et al., (1998)***. mentioned that, the crude powder obtained from strain ku-4. 486 was chromatographed on silica gel column (ϕ 29 x 100 mm, Wako gel C. 200) resulted in three products I, II and III with Rf 0.71, 0.45 and 0.12 respectively.

# MATRIALS AND METHODS

# 3-MATERIALS AND METHODS

## 3.1. Isolation of Microorganisms.

### *3.1.1. Soil samples.*

The used soil samples were taken from 3 different cultivated areas, sample No. 1 (T) from Toukh, (Kalubia), sample No.2 (N) from Nubaria (El-Beheria) and sample No. 3 (H) from El-Hamol (Kaffer El-Sheikh). Samples were taken from five different parts of a plot, remove about 1cm of the soil surface, and take the sample with clean sterilized long spatula at a depth of 6 .0 to 6.5 inches. The samples were placed in a sterile clean polyethylene bags about 1 kg, for each sample.

### *3.1.2. Treatments of soil samples.*

Each bulked sample was sieved through a set of sieves of pore size 1.0, 0.5 and 0.25 mm in diameter to get ride of plant residue as well as other impurities. To obtain as many streptomycetes as possible from each sample, the soil samples were enriched by:

(a) Addition of $CaCO_3$, 0.1 gm to each 1 gm air dried soil samples and incubated at 28 –30°C for 7-9 days at moist chamber, ***(Tsao et al., 1960).***

(b) Heating, drying and storage of soil samples before isolation ***(Nüesch,1965).***

### *3.1.3. Isolation of actinomycetes from natural habitat.*

The media recommended by the society of actinomycetes, Japan, January 1975, were used for such a purpose *i.e.*, glucose-starch aspragine agar[1], glycerol-starch peptone agar[2], starch casein agar[3] and starch nitrate agar[4] (Index of media and reagents).

The used media were sterilized and poured into Perti-dishes 20 ml/ plate under aseptic conditions, the plates were left for few minuets uncovered to prevent water condensation on plate cover, after solidification the plates covered and kept in incubator for 24h. at 28-30°C. Soil suspension was prepared by mixing 10 gm of each soil sample completed into 100 ml sterile de-ionized water, shaken by hand for five seconds, then mechanically shaken ($175_{RPM}$) for one hour at 28-30°C.

Each sample was serially diluted (10 fold), to cover the range of $10^{-1}$ to $10^{-8}$ in sterile water, 100 µl of each dilution was spread onto each plate surface containing the agar media *i.e.*, glucose-starch aspragine[1], glycerol-starch peptone[2], starch casein[3] and starch nitrate[4] by using glass spreader. Three replicates of each dilution were used . The plates were incubated at 28-30°C for 7 to 10 days, colonies having actinomycetes morphology were selected from the second, third and fourth dilutions, and transferred to agar medium and purified by streaking technique several times on the medium, and maintained on agar slants at 4 °C until used .

The selected actinomycetes isolates were re-inoculated on the previous media amended with (w/v) 0.05%,0.10%,0.15%,0.20%and 0.25% of Kocide 101 (used as chemical control for some plant diseases, active ingredient Copper Hydroxide 77.0%, inert ingredients 33%) and incubate at 28-30°C.

The colonies that developed on these plates were subcultured several times on medium containing fungicide, Yeast extract-Malt extract agar[5] (ISP2), but each time the fungicide concentration was increased to be higher than that of the previous medium ,this was repeated until the organisms became adapted on growing on fungicide in high concentration. The colonies that developed on these plates were isolated

and mentioned on subculture of the same medium ***(Mukher et al., 1995 and Hassaballa, 2000).***

A spore suspension of each isolate was made by adding 10 ml sterilized glycerol (20%)to the culture and scraping the colony surface with a sterile swab; the resulting spore suspension was poured into micro-centrifuge tube and stored at-20°C for further studies.

## 3.2. Isolation from faba bean and pathogencity test.

### *3.2.1. Isolation of pathogenic fungi from faba bean leaves.*

The course of this part was related to one of the most important disease which attack the susceptible faba bean plants under the local environmental conditions. The concerned disease was chocolate spot of faba bean caused by *Botrytis fabae* Sard. Samples of naturally infected leaves of faba bean (*vicia faba* L.) were collected from three different areas Toukh, (Kalubeia), Nubaria (El-Beheria) and El-Hamol (Kafr El-Sheikh),infected leaflets presented symptoms of chocolate spot disease were cut into small pieces, each with single lesion of the concerned disease. The infected tissues were sterilized by immersing in 2% sodium hyphochlorite for two to three minutes, rinsed with distilled water, dried on sterilized filter paper and then placed on Potato Dextrose Agar[(6)] (PDA) plates. The plates were incubated at 20°C for 7 days. Suspected isolates were purified on PDA plates and re-incubated at 20°C of 12 days. Cultures were then single spored before identification and kept on PDA slants for further studies ***(Abou-Zeid et al., 1998).***

### 3.2.2 *Identification of the causal agent of chocolate spot disease on faba bean.*

*Botrytis* sp. isolates from infected faba bean leaves were identified to species level according to ***Abou-Zeid, ( 1978 )***based up on, growth rate, spore production, sclerotia production, their number and size using two different media, Potato Dextrose Agar[6] (PDA) medium and Faba Bean Leaves Agar[7] (FBLA) medium.

### 3.2.3. *Pathogencity test of Botryts sp.*

Three faba bean cultivares (c.v) *i.e.* ,Giza 716, Giza 3 and Giza 40 were planted in 2-liter polyethylene pots filled with field soil (5 replicates for each c.v.). Five seeds were sown in each pot and irrigated with water. The pots were grown in natural environmental conditions, after five to six weeks the pots were transferred in the green house at 20 to 25°C.

- ***Detached leaves.***

Leaves were taken at the fifth node and incubated horizontally (five replicates for each treatment) in polyethylene boxes on a polyethylene grid overlaid with water soaked filter paper in order to obtain high relative humidity ***(Abou-Zeid, 1985).*** Seven isolates of the causal organism were used, two isolates from Kalubia, three isolates from El-Beheira and the rest isolates from Kaffer El-Sheikh. The incolum was prepared by culturing the isolates on Faba Bean Leaves Agar(FBLA)[7] medium. A concentration of 2.5 x $10^5$ spores / ml was prepared from each isolate of the pathogen. 10 µl of each isolate were placed on each leaflet of the tested three cultivares, then cover these boxes with transparent polyethylene bags to maintain high humidity ***(Abou-Zeid et al., 1998).*** Data were recorded on the type of infection (disease sevirty) after 48 h. using scale (0-9) depending on the extent of lesions Table (1).

**Table (1):** Rating scale for disease assessment of chocolate spot disease caused by *B.fabae* ***(Abou-Zeid; 1985).***

| Score | Leaves | Flowers | Stems |
|---|---|---|---|
| 0 | No infection | No infection | No infection |
| 1 | Few localized lesions on some leaves. Percentage of infected leaf area (1-5%) | Few lesions on some flowers | No lesions |
| 2 | Some few lesions on 1 /2- 3/4 of infected leaves. Percentage of infected leaf area (5-10%) | Some lesions on 1 /2- ¾ of flowers | Few lesions on lower part of stem |
| 3 | Large lesions on more than ¾ of infected leaves. Infected leaf area is less than 25% | Striped flowers | Many lesions on steam |
| 4 | Some coalseed lesions on infected leaves. Percentage of infected leaf area is larger than 25% | Lower flowers turned to black colour | Coalseed lesions on stem. |
| 5 | Coaleseed lesions on 1 /2 of the infected leaves. Drop of the lower leaves. Percentage of infected leaf area ranged from 25-50%. | Drop of earlier flowers | Spreading lesions on stem. |
| 6 | Coaleseed lesions on 1 /2 – 3 /4 of the leaves. Percentage of infected leaf area is more than 50% | Drop 1/ 4 of the flowers | Mottled stems |
| 7 | All leaves infected by large couleseed lesions. Percentage of infected leaf area reached to 75% | Drop > 3 /4 of the flowers | Blackness of lower part of stem. |
| 8 | Whole plant died except the apex | | |
| 9 | Death of the whole plant. | | |

- ***Plants in pots (under greenhouse conditions)***

The plants were misted with water and covered by polyethyelen bags to obtain high relative humidity. The spore suspension was sprayed on normal plants with an outomyzer to obtain fine mist on the inoculated plants. Plants were recovered by polyethylene bags and kept for 24 to 48 hours at20-25°C in the greenhouse, the bags was removed and the plants were kept in the greenhouse, the diseased plants were scored as infection type and severity of leaf area damage using scale (0-9) ***(Abou-Zeid, 1985).***

## 3.2. The anti-microbial potentialities of actinomycetes isolates.

### *3.3.1. Anti-microbial activities of the used actinomycetes isolates.*

#### *3.3.1.1.Media used for investigating antimicrobial activity.*

Beside the isolation media described before, the following media were used for survey the anti-microbial activities of the actinomycetes isolates *i.e.* Yeast extract Dextrose[8], Yeast extract-Malt extract[5], soybean meal[9], Modified Bennet agar[10](MBA)and starch Nitrate[4] while nutrient agar[11] and Czapex-Dox agar[12] were used for anti-microbial activity assay (Index of media and reagents).

The isolates were inoculated into Yeast extract Dextrose[8] (YD) medium, then incubated at30°C for 3 days on a shaking incubator at $180_{RPM}$ and 30°C, 5 ml of each culture was transferred to Yeast extract-Malt extract[5] (YM) and Soybean Meal[9] (SBM) medium separately.

After incubation period (3 days) on a rotary shaker at $180_{RPM}$ and 30°C, anti-microbial activity was tested using classical diffusion method (using filter glass disks 4 mm in diameter), and /or wells agar ***(Cooper,***

***1972 and Leifert,1995).*** The result was based on occurrence of inhibition zone of microbial growth.

Anti-microbial activities were tested against several microbial cultures as: Gram positive bacteria *eg. Bacillus subtilis* NCIB 3610;*Micrococcus leteus* NCIB 190 and *Rhadococcus equi* ATCC 6939; Gram negative bacteria *eg. Pseudomanas aeruginosa* ATCC10145; *Eschericha coli* NCIB9132; *Salmonella typhie* NCTC4111 and *Staphylococcus epidermidis* ATCC12228 ;some fungal isolates were used as test organism **(a)** unicellular as *Condida albicans* ATCC10231; *Geatrichum condium* ATCC34614 and *Saccharomycs cervisiae* ATCC 2601 **(b)** filamentous fungi as *Batrytis fabae*; *B.cenirea; Fusarum oxysporium* f.sp. *faba;m F.solani* and *F.moniliforme.*

For testing the anti-microbial activity all the cultures of the used test organisms must be fresh. The test carry out as the following:

Five ml. distilled sterile water was added to each refreshed test organism subculture and shacked well by a vortex for 3-5 min., each 100 ml of the substrate medium was inoculated with 5 drops of the test organism spacmence at worming (45°C) and poured into the plates, 3-replicates of each one, the plates mixed well handily in 8-figure movement***( Ahmad ,1990).***

The filter glass disks were loaded by the fermentation broth and placed over-side the plate surface seeded media. In case of the tested fungi, a spore suspension was made, then the media were seeded with the tested fungi spore suspension (0.2 ml) and poured into Petri-dishes. After the agar had set, 4mm wells were cut into the agar with cork borer and filled with 30 µl of the cell-free culture filtrates ***(Hussein, et al., 1998).*** Keep the plates for 6 h. in a refrigerator to allow the anti-microbial

substance diffused through the seeded medium, these plates were incubated at 28-30°C for over night in case of bacteria and 48-72h. in case of fungi at 20-25°C. The results were scored as inhibition zone of growth for the test organism(***Yassin & Swelim,1999***) .

### *3.3.1.2. Estimation of antifungal producing actinomycetes.*

The anti-fungal producing actinomycetes were estimated by methods according to ***Newhook (1951).*** The antagonist and test fungi are placed on media opposite each other at the periphery of Petri dishes,(Test fungi was applied 2 days after the antagonist).

Mycelial discs (5 mm diameter) cute from seven days old culture of *Botrytis fabae* and then transferred aseptically to the surface of Potato-Dextrose Agar plates previously seeded with actinomycetes isolate. The inoculated plates were incubated at 20-22°C for 5-6 days and examined for inhibition zones. Agar plates without the actinomycetes isolates were used as control ***(Clarkson & Lucas,1993).***

- *Effect of culture filtrate of isolate $T_{118}$ on percentage of spore germination and lengths of germ tubes of B. fabae.*

A spore germination bioassay similar to that of Spurr's was used . Spores of *B.fabae* were collected from agar plates in sterile tap water, a different concentrations (V/V) of the culture filtrate of isolate $T_{118}$ were made ( 100% , 75% , 50% and 25%) . Each assay consisted of three replicates including spores in sterile tap water (control ) with and without the used concentration of culture flitrate (free from spore or cells) . Approximately 150 µl of the used treatment were added to depression well of 96 well micro - plate and incubated for 24 hr . in sterile petri dish containing filter paper moistned with distilled water . 100µl were added to depression well of microscope slides , the

percentage of spore germination and lengths of germ tubes were recorded .

### 3.3.2.Taxonomical and biological studies of the selective actinomycete isolate.

#### *3.3.2.1. Identification of genera.*

The identification of actinomycetes in the last period show light changes, this may be due to the improvement of criteria used for the characterization of genera and species of actinomycetes *(Cross,1989)*. The identification methods include ;morphological, physiological and cell wall hydrolysate analysis which illustrate the type of Di-Amino-Pimelic acid (DAP), LL-, DL – or meso –DAP *( Shash, 1992).*

##### *3.3.2.1.1. Methods used for morphological studies.*

Culture morphological characteristics of actinomycete isolate were determined by the methods of the International Streptomycetes Project (ISP) *(Shirling & Gottlieb,1966),* in addition to the cover-slip method *(Kawato & Shinobu,1959).* The agar medium ISP-4 was used for such purpose, the medium was poured into Pyrex Petri-dishes, after solidify a sterilized cover slip was inserted vertically in a few milliliters of the agar medium. The actinomycete isolates was then inculcated on the agar along with the line where the medium meets the cover slip edge. Then plates incubated at 30°C for 10-14 days.

After incubation time remove the cover slips were removed and fixed on glass slides by Canda balsam droplets with upper surface up wards, washed by methanol 99% for one minute and then by sterilized water and dried. The slips were examined microscopically and photomicrographs were taken.

*3.3.2.1.2.Analysis of cell wall hydrolysate.*

Based on methods maintained by ***Becker et al., (1964)*** and ***lechevalier & lechevalier (1970),*** the obtained organisms were grown on Trypton Yeast medium[13] (ISP-1) ***(Pridham & Gottlieb,1948)*** at 30°C for 3-10 days, then the growth was collected by filtration and washed by ethyl alcohol and distilled water, then air dried.

- ***The detection of Diamino Pimelic Acid (DAP)and sugars.***

The dried cells (10 mg) were hydrolyzed with 1 ml 6N HCl in a sealed Pyrex tube, held at 100°C for 18 h., after cooling the sample was filtered through filter paper, then washed by 1 ml distilled water and the filtrate was dried two or three consecutive times on a steam bath to remove the most of HCl. The residue is taken up in 0.3 ml $H_2O$, five µl. was loaded on Whatman No. 1 filter paper, also 10 µl of 0.01 M. mixture of meso and LL – DAP were loaded side by side the sample as a reference.

For separation the amino acids the following solvents mixture wasused. Methanol-water-10N HCl-Pyridine (80:17.5: 2.5:10 v/v). Amino acids were detected by spraying with acetonic ninhydrine (0.1 % w/v), followed by heating for 2 min. at 100°C. DAP spots are olive-green fading yellow, where as the other amino acids give purple spots, also LL-DAP migrate faster than meso – or DL- form.

- ***Detection of sugars.***

Dried cells (50 mg) are hydrolyzed in 1 ml of 2 N $H_2SO_4$ in a sealed Pyrex tube at 100°C for 2h. the hydrolysate was neutralized to pH 5.0 to 5.5 and the solution was evaporated at 40°C on a rotary evaporator.

The residue was taken up in 0.4 ml $H_2O$. Five µl were then spotted on thin layer plate coated with cellulose (***Becker et al., 1964***). For separation of sugars the following solvent mixture was used; ethyl acetate-Pyridine-water (10: 3.5: 2.5). The sugars are detected by spraying with the following reagent, 2 ml of aniline; 3.3 g of phathalic acid and 100 ml of water saturated n-butanol, after the plates had dried it heated at 100°C for 2.5 min. The mono-saccharids appear as differently colored spots.

### *3.3.2.2. The methods used for the classification of genus Streptomyces.*

The direct microscopic examination of agar cultures as described by the International Streptomycetes Project (ISP) ***(Shirling & Gottlieb, 1968 a & b and 1972)*** was followed for studying the classification of genus *Streptomyces.*

#### *3.3.2.2.1. Macroscopic characteristics and pigmentation of culture media.*

To study the culture characteristics of streptomycetes the following media are used; *i.e.* Yeast extract-Malt agar[5], Oat-meal agar[14], inorganic salts starch agar[15] and glycerol-aspragine agar[16] (Index of media and reagents).

- ***Additional characterization media (Waksman ,1961).***

In addition to the above mentioned media there are others have been described by Waksman which are used to detect the colour of sporulating arial and substrate mycelia, diffusible pigments and melanin production. *I.e.* sucrose-nitrate agar [19], Potato-Dextrose Agar (PDA) [6], nutrient agar [11],glucose-digested casein yeast extract beef extract agar [20] and glycerol-nitrate agar [18], also fish meal agar[30],Czepeak 's -Dox solution agar[12] and starch nitrate agar[4] were used (Index of media and reagents).