ISOLATION OF ACTINOMYCETES FROM SOIL SAMPLE USING DIFFERENT PRETREATMENT METHODS AND ITS COMPARATIVE STUDY

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Received: February 16, 2019 Accepted: March 23, 2019

ABSTRACT: *Actinomycetes are potential sources of pharmaceutically important intermediates, drugs, enzymes, amino acids & vitamins etc. Actinomycetes are the most widely distributed group of microorganisms in nature which are primarily presence in the soil. The main focus of the present study was to isolate the actinomycetes strains from different habitat especially from the soil samples. Soil samples from different habitat were collected and were subjected to different pre-treatments and its enrichment. Different isolation medium with various antibiotic concentration were also tried for selective isolation of the strains. Upon comparative analysis of the treatments, it was found that treatment with Calcium Carbonate, Phenol and with combination of antibiotic medium gave higher number of actinomycetes isolates with lesser bacterial contamination. The isolated actinomycetes strains were screened for their ability to produce bioactive compounds.*

Key Words: *Actinomycetes, Streptomyces, Phenol, Bioactive.*

INTRODUCTION

Actinomycetes are undoubtedly the interesting organism in many aspects for basic microbial research. Actinomycetes have been commercially exploited for the production of pharmaceuticals, neutraceuticals, enzymes, antitumor agents, enzyme inhibitors,and so forth (Remya M.*et. al,*2008). These bioactive compounds are of high commercial value, and hence actinomycetes are regularly screened for the production of novel bioactive compounds.

Actinomycetes long been recognized asmainproducers of enzymes, antibiotics,amino acid, anti-cancerous agents, anti-diabetic drugs, anti-obesity drugs, pharmaceutically and industrially important chemicals. Microbial secondary metabolites continue to be a chemically diverse source for the discovery and development of pharmaceutical agents and also biochemical probes to study human disease processes. Several enzyme-inhibitor-producing actinomycetes were isolated from various samples collected from the marine environment and characterized. Most of them produced novel compounds that are useful in medicine and agriculture (ImadaC,2005). Actinomycetes are the most widely distributed group of microorganisms in nature which primarily inhabit in soil (Oskay *et. al,* 2004). They have provided many important bioactive compounds of high commercial value and are routinely screened for new bioactive compounds. These searches have been remarkably successful and approximately two thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes. The knowledge regarding emergence of new diseases and multiple drug resistance to human pathogenic bacteria and fungi, toxic nature of reactive oxygen species (ROS), harmful side effects associated with available synthetic drugs and complications raised due to diabetes, have opened a new vista for the search of new antiradical, antidiabetic and antimicrobial agents from natural sources. Successful commercialization of enzymes is an important step towards revolutionizing "green technology." Reduction in the cost of bioactive compound production on low-cost substrates using different actinomycetes *spp*. is really challenging. Such low-cost production initiatives can be extended to byproducts and metabolites. Novel properties like thermal and ionic stabilities and a better turnover make these systems infallible and regenerative (Divya P. *et. al*, 2013)

Actinomycetes can also produce enzyme inhibitors of pancreatic lipase and α amylase to treat obesity which is a risk factor for hypercholesterolemia, hypertension and diabetes. In addition actinomycetes are still the richest source for enzyme inhibitors that have a main role in cancer treatment like inhibitors of reverse transcriptase, glyoxalase, and adenosine deaminase and tyrosine protein kinase, in addition to glutathione-S-transferase.

Isolation of actinomycetes can fulfill all the novel essentialities associated with actinomycetes. Actinomycetes are widely distributed in the natural habitats, hence various methods like pretreatments,

enrichment, combinations of antibiotics, specific isolation media and some novel methods has been adapted for isolation. Marine actinomycetes were also isolated by various methods and found to be important source for novel secondary metabolites.

The richness and diversity of actinomycetes present in any specific soil, is greatly influenced by the soil type, geographical location, cultivation and organic matteramongst other factors. Novel microbial metabolites can be discovered from actinomycetes by isolating diverse group of actinomycetes. Isolation of a new strain with desired activity is very challenging process. Therefore, selection of novel separation methods is important. Various isolation methods have been used earlier for different actinomycetes and some novel methods has been applied in recent year. Hayakawa and Nonomura developed various methods for isolating desirable rare actinomycetes genera from natural habitats. These methods include a variety of pretreatment techniques in combination with enrichment techniques that appropriately supplement agar media with selective antibacterial agents (Hayakawa *et. al.,* 2007). Further novel methods had been applied for isolation of rare and uncommon actinomycetes. In continuation of referencehere in we tried to use improved methodologies for isolation of various genera of actinomycetes from soil. Some of the methods are described in material and methods.

Isolation of Actinomycetes:-

Novel microbial metabolites can be discovered from actinomycetes by isolating diverse group of actinomycetes. Isolation of a new strain with desired activity is very challenging process. Therefore, selection of novel separation method is important. Various isolation methods have been used earlier for different actinomycetes and some novel methods has been applied in recent year. Hayakawa and Nonomura developed various methods for isolating desirable rare actinomycetes genera from natural habitats. These methods include a variety of pretreatment techniques in combination with enrichment techniques that appropriately supplement agar media with selective antibacterial agents. Further novel methods had been applied for isolation of rare and uncommon actinomycetes. Herein we tried to use improved methodologies for isolation of various genera of actinomycetes from soil.Some of the methods are described as follows-

Pretreatment of soil samples:-

Pre-treatment of soil can stimulates the isolation of actinomycetes by either promoting growth of actinomycetes or eliminating most unwanted gram negative bacteria. Various pretreatment techniques have been developed for different genera of actinomycetes. Physical, chemical and combination of both methods can be used for selective isolation of actinomycetes species.

Physical Treatments

Physical treatment such as moist incubation use of radiation, glycerol treatment, air dry, dry heat, centrifugation, cellulose infiltration, baiting technique followed by drying are commonly used for different genera of actinomycetes.

Spores of actinomycetes are more resistance to desiccation as compared to gram negative bacteria and hence, heat dry of soil sample at 120°C for 1 hour favors growth of *Streptomyces* and other rare genera on humic acid vitamin (HV) agar (Hayakawa *et.al*., 1991, Tamura *et. al.,* 1997). Agate &Bhat (1963) attempted suppression of bacteria and fungi by preincubation of soil at 110ºc for 10 min.

Isolation of motile actinomycetes can be done by centrifugation which eliminates Streptomycetes and other non-motile actinomycetes and facilitates motile actinomycetes, retained in the supernatant and can be spread on appropriate medium containing nalidixic acid and trimethoprim (Hayakawa *et. al*., 2008).

For selective isolation of filamentous actinomycetes from mixed microbial populations without relying upon specific media and antibiotics membrane baiting technique can be used. Nutrient agar medium supports the growth of both bacterial cultures and also it is suitable for actinomycetes isolation. Nutrient agar medium is overlayed with a 0.22 to 0.45µm pore size cellulose ester membrane filter followed by inoculation of filter surface with mix cultures and incubation allows the growth of bacteria. Actinomycetes have highly branched mycelial networks and hence they have ability to penetrate the pores of membrane filter. During incubation, mycelium of actinomycetes penetrates the filter pores to the underlying agar medium, whereas growth of other bacteria is restricted to the filter surface. Removal of membrane filter and incubation of agar medium allow the development of the isolated actinomycetes colonies (Hirsch *et. al.,* 1983)

Selective isolation of actinomycetes was also favored by radiation (Bredholdt*et. al*., 2008). Super-high frequency irradiation favors isolation of *Streptosporangium* and *Rhodococcus* species, Extremely high

frequency irradiation was effective for *Streptosporangium* spp., *Nocardiopsis*, *Nocardia* and UV-irradiation is also suitable for isolation of some species.

Chemical Treatments

Use of chemicals treatments such as Calcium carbonate and chitin treatment, calcium chloride, Phenol, SDS, yeast extract, Germicide, Chemotactic agents, and Chloramine-T can be used for selective isolation of actinomycetes (Ravi Rajan*et. al*,2016). Calcium carbonate and chitin acts as carbon and nitrogen source, support growth of actinomycetes.

Tsao, Leben&Keitt (1960) reported increased selective development of actinomycetes when air-dried soil was remoistened, mixed with calcium carbonate and incubated at 28ºC temp. Lechevalier (1963) found that the calcium carbonate treatment gave highest colony counts, while the centrifugation and phenol treatments gave counts lower than those from untreated suspensions.

Treatment of soil suspensions with a 1.4 % (w/v) phenol solution was recommended by Lawrence (1956). Soil sample treated with ChloramineT was found to promote the growth of some species on nalidixic acid contain HV agar (Hayakawa *et. al*., 1997).

Soil sample treated with SDS 0.05% and yeast extract 5% favors growth of *Streptomycetes* and other genera on nalidixic acid contain HV agar (Hayakawa *et. al*., 1989).

Both combination physical and chemical techniques:-

The combination of physical and chemical treatment was also found to be more suitable method effectively isolate various genera of actinomycetes.

Dry heating followed by phenol treatment, calcium chloride treatment followed by centrifugation were found to be suitable for effective isolation of actinomycetes.

Dry heating of soil sample at 110ºC for 1 hour and treatment with 1% phenol favors isolation of *Actinomaduraspp.*on kanamycin, Josamycin, lysozymeand nalidixic acid containing HV agar (Hayakawa *et al.,* 1995). Dry heat at 120°C to the soil sample along with 0.01% benzethonium chloride favors Streptosporangium or *Dectylosporangium* genera on nalidixic acid and leucomycin contain HV agar (Hayakawa *et al.,* 1991). Sucrose gradient method followed by HV agar enriched with nalidixic acid supports growth of Nocardia.

Enrichment Method

Various genera of actinomycetes can be isolated by addition of nutritional or non-nutritional ingredients in media for the purpose of selective isolation. Enrichment is one of the successful methods in terms of diversity and abundance of culturablebacteria.The pretreatment of soil suspension with peptone (6%) and lauryl sulfate (0.05%) at 50°C for 10 min, also greatly increased the number of actinomycetes from soil prior to incubation with new medium. Antonie Van Leeuwenhoek used differential centrifugation in 2000 for selective isolation of motile actinomycetes in soil and plant source. Rehydration and centrifugation (RC) method was developed by Hayakawa*et al.,* for selective isolation of diverse and rare zoosporic/motile actinomycetes genera.

Selective inhibition

Use of antibiotics in media to get selective inhibition of various undesirableorganisms helps to get growth of desired species of actinomycetes. The use of antifungal antibiotics in media for selective isolation of bacteria has already been tested and reported. Use of different potent antibiotics like nystatin and pimaricinby Porter *et al.,* (1960) as well as Actidione (cycloheximide.

Nutritional selection

Some of the carbon, nitrogen and other complex substances have been considered for selective substrates for isolation of actinomycetes. Use of selective media is always preferred for isolation of actinomycetes eliminating other microorganism. Designing the isolation media by adding selective substrates so as to reduce the growth of undesirable microorganisms and allowing selective growth of actinomycetes spp. is considered to be important step (Cross *et. al.,* 1982,). Nitrogen sources such as proteins and amino acids play important role in selective isolation of actinomycetes. Use of L-arginine and L-aspargine as a nitrogen sourcefavoursactinomycetes over bacteria was reported by Porter *et.al.(*1960) .Glycine can also be used for selective isolation of some spp of actinomycetes. Use of some other substrates as starch, glycerol, nitrate, casein, etc. were most selective is Chitin as a source of carbon and nitrogen source.Some of the selective media reported for isolation of actinomycetes; glycerol-aspargine agar, chitin agar, starch-nitrate agar, humic acid vitamin actinomycetes isolation agar, starch casein agar, inorganic salt starch agar, glycerol-glycinagar,Gause's no-1 medium, Zhang's starch soil extract agar, etc.

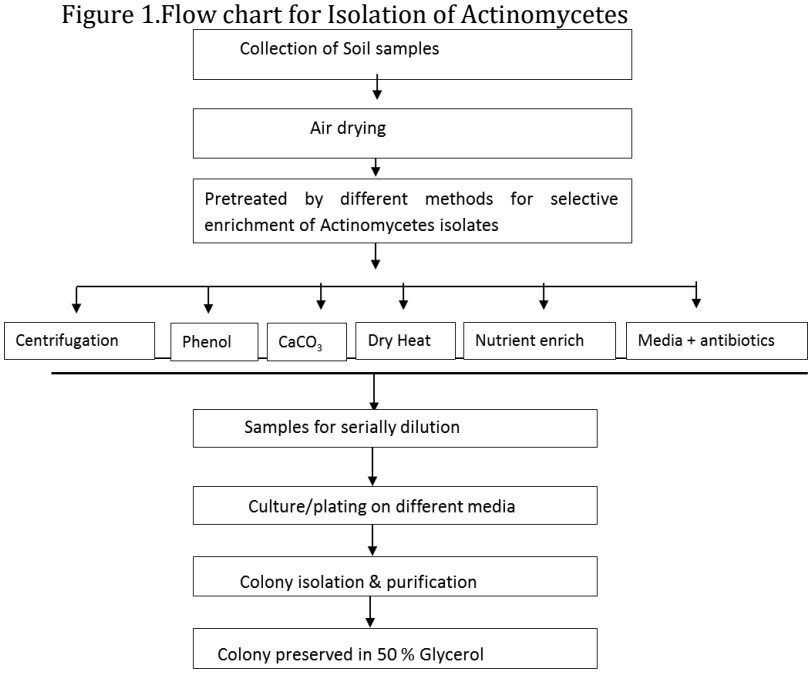
Isolates of Actinomycetes from different habitat were isolated and stored on Glycerol-aspargine agar slants for further studies.

The present research works describes and focus on isolation of actinomycetes from different habitat soil by using different techniques as well as combination of techniques.

MATERIALS AND METHODS:-

1. **Soil sampling and processing**: Seven soil samples were collected from different locations like agricultural field, milk industry, oil industry and hill station nearby region of Solapur. Soil sample and location details as Agriculture land (Bopale),Sugarcane Industry(Lokmagal),Oil Mill Industry(MIDC Pakani),Milk Industry (Dudhpandhari),Forest area (Ramling),Black fertile soil(Yellamwadi- Grape Garden), Livestock keeping area soil (Yellamwadi)The surface layer of soil was removed and the central portion was collected in sterile plastic bags with the help of spatula. Soil samples were air dried in laminar airflow unit for 20 hrs. at room temperature and samples were stored at 4°C until processed.
2. **Isolation of Actinomycetes:-** Various pretreatments, physical and chemical treatment methods used for the isolation of actinomycetes from the soil samples. The flow chart of the process described in the Figure 1.

Figure 1.Flow chart for Isolation of Actinomycetes



3. **Common Media for Isolation** Yeast Extracts Malt Extract Dextrose Agar, Glycerol Aspargine Agar, Starch Aspargine Agar. These media with and without antibiotics and antibiotic concentration Cycloheximide (50 µg/mL) and Nystatin (50 µg/mL)
4. **Treatments**

4.1. Centrifugation

1 gram soil sample added in 9 ml 10mM phosphate buffer and kept for settling. Fluid centrifuged at 4000 rpm for 20 minutes. Supernatant serial diluted and plated on various medium as given above.

4.2. Phenol treatment

Air-dried soil sample suspended in 1.5 % (w/v) phenol solution was kept at 30^0C for 30 minutes, and subsequently serial diluted and cultured on different medium plates as described earlier.

4.3. Calcium Carbonate treatment

Air dried soil sample (10 g) was taken in a sterile plate and was mixed with 1% $CaCO_3$ and was incubated at 30°C for 3 days in a closed sterile petridish. A high relative humidity maintained during incubation andsupernatant serial diluted and plated on various medium as described earlier.

4.4. Dry heat treatment:

1 gram soil sample heated in the oven at 60^0C for 15 min, soil sample diluted and serial dilution plated on the various medium as mentioned earlier.

4.5. Nutrient enrichment:

1gram soil sample treated with the 0.05 SDS and 5 % Yeast Extract and incubated at 30^{0}C for 48 hrs. Nutrient enriched sample serial diluted and plated on the medium as described earlier.

4.6. Selective antibiotics in medium:

The air dried sample serial diluted and plated on the various medium containingantibiotics. We have selected different concentration of antibiotic in the medium,Cycloheximide (50 µg/mL) and Nystatin (50 µg/mL).

5. RESULTS AND DISCUSSIONS:-

Actinomycetes Isolation results:

A total of 220 actinomycetes strains were isolated from different soil sample by using the different treatments and methods. It was observed that calcium carbonate, phenol and selective antibiotic are the most suitable treatments for isolation of the actinomycetes. The results of actinomycetes isolation are shown in the Table 1. The percentages of isolates by treatment are shown in Table 2. It was also observed that each treatment werehaving different nature of colonies.It was also observed that black soil was more enriched of actinomycetes and details shown in Graph no.1 and Table 1.

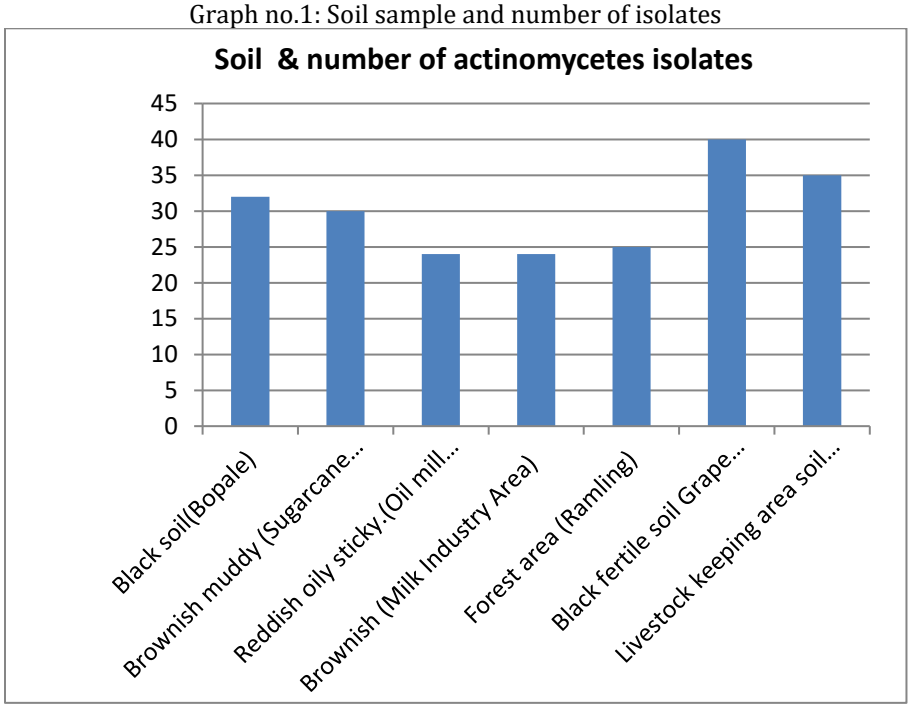
Table no. 1. Soil sample and actinomycetes isolation details.

Sr.no.	Soil Sample	No. of Isolates
1	Black Soil (Agriculture Land, Bopale)	32
2	Brownish muddy (Sugarcane Factory)	30
3	Reddish Oily sticky.(Oil mill Industry)	24
4	Brownish (Milk Industry Area)	24
5	Forest area (Ramling)	35
6	Black fertile soil Grape Garden.(Yellamwadi)	40
7	Livestock keeping area soil (Yellamwadi)	35

Table no.2.Treatment and isolated actinomycetes % with respective total actinomycetes isolates.

Treatment	% of total isolates
Phenol	33.3
Heat treatment	13.3
Calcium Carbonate	53.3
Without treatment	26.6
Selective antibiotics	40.0

Graph no.1: Soil sample and number of isolates



6. Conclusions: From ongoing research it is confirmed that the soil samples are rich of actinomycetes particularly Black soil sample and it was observed that calcium carbonate, phenol and selective antibiotic medium are most effective treatment for isolation of actinomycetes.

REFERENCES:-

1. Remya, Mohan & Vijayakumar, Ramasamy. (2008). Isolation and characterization of marine antagonistic actinomycetes from west coast of India. Medicine and Biology. 15466. 13-19.
2. Imada C.(2005) Enzyme inhibitors and other bioactive compounds from marine actinomycetes.Antonie Van Leeuwenhoek. 2005 Jan;87(1):59-63.
3. Oskay, Mustafa & Tamer A., Uuml;Same&Cem, Azeri. (2004). Antibacterial activity of some Actinomycetes isolated from farming soils of Turkey.African Journal of Biotechnology. 3. 441-446.
4. DivyaPrakash, Neelu Nawani, Mansi Prakash, Manish Bodas, AbulMandal, MadhukarKhetmalas, and Balasaheb Kapadnis.(2013). Actinomycetes: A Repertory of Green Catalysts with a Potential Revenue Resource. BioMed Research International Volume 2013, Article ID 264020, 8 pages
5. Hayakawa M., Nakagawa, Y., Limura, Y,.2007. A New Enrichment Method for the Selective Isolation of Streptomycetes from the Root Surfaces of Herbaceous Plants. Actinomycetologica, 21: 66-69.
6. Hayakawa, M., Tamura, T., Iino, H., Nonomura, H. 1991. Pollen-baiting and drying method for highly selective isolation of Actinoplanes spp. from soil. J. Ferment. Bioeng., 72: 433-438.
7. Tamura, T., Hayakawa, M., Hatano, K. 1997. A new genus of the order Actinomycetales, Spirilliplanes gen. nov., with description of Spirilliplanesyamanashiensis sp. nov. Int. J.Syst. Bacteriol., 47:97-102.
8. Hayakawa, M. 2008. Studies on the isolation and distribution of rare actinomycetes in soil. Actinomycetologica, 22:12– 19.
9. Agate, A.D. &Bhat,(1963). A method for the preferential isolation of actinomycetes from soilsJ.V. Antonie van Leeuwenhoek (1963) 29: 297.
10. Hirsch, C., Christensen, D. 1983. Novel Method for Selective Isolation of Actinomycetes. Appl. Environ. Microbiol., 46: 925-929.
11. Bredholt, H., Fjaervik, E., Jhonsen, G., Zotechev, S.B. 2008. Actinomycetes from sediments in the TrondheinFjrod, Norway: Diversity and biological activity. Mar Drugs, 6:12-24
12. Ravi Ranjan Kumar and Vasantba J. Jadeja.(2016). Isolation of Actinomycetes: A Complete Approach. Int.J.Curr.Microbiol.App.Sci. 5(5): 606-618.
13. Tsao, P., Leben, C., Keitt, G. 1960. An enrichment method for isolating actinomycetes that produce diffusible antifungal antibiotic. Pytopathol., 50:88-89.
14. El-Nakeeb, M., Lechevalier, H. 1963. Selective isolation of aerobic actinomycetes. Appl. Microbiol., 11: 75-77.
15. Lawrence, C. 1956. A method of isolating actinomycetes from scabby potato tissue and soil with minimal contamination. Can J. Bot., 34:44-47.
16. Hayakawa, M., Iino, H., Takeuchi, S., Yamazaki, T. 1997. Application of a method incorporating treatment with Chloramine-T for the selective isolation of Streptosporangiaceae from soil. J. Ferment. Bioeng., 84:599-602.
17. Hayakawa, M., Sadakata, T., Kajiura, T., Nonomura, H. (1991). New methods for the highly selective isolation of Micromonospora and Microbispora from soil. J. Ferment Bioeng., 72: 320-326.
18. Hayakawa, M., Yoshida, Y., Iimura, Y. (2004). Selective isolation of bioactive soil actinomycetes belonging to the Streptomyces violaceusniger phenotypic cluster. J. Appl. Microbiol., 96: 973-981.
19. Mukesh Sharma, PinkiDangi, MeenakshiChoudhary. (2014). Actinomycetes: Source, Identification, and Their Applications Int.J.Curr.Microbiol.App.Sci (2014) 3(2): 801-832
20. Hayakawa, M., Tamura, T., Iino, H., Nonomura, H. (1991). New methods for the highly selective isolation of Streptosporangium and Dactylosporangium from soil. J.Ferment. Bioeng.,72: 327-333.
21. Yamamura, H., Hayakawa, M., Iimura, Y. 2003a. Application of sucrose-gradient centrifugation for selective isolation of Nocardia spp. from soil. J. Appl. Microbial., 95: 677-685.
22. Yamamura, H., Hayakawa, M., Nakagawa, Y., Iimura, Y. (2003). Species diversity of Nocardiae isolated from Lake and Moat sediment samples. Actinomycetol., 17: 44-46.
23. Porter, J., Wilhelm, J,.Tresner, H. 1960. Method for the Preferential Isolation of Actinomycetes from Soils. Appl.Microbial., 8:174-178.
24. Cross, T. 1982. Actinomycetes: a continuing source of new metabolites. Dev.Indust. Microbiol., 23: 1-18.
25. Tamotsu, F., Yasuhiro, I., Takako, I., Tomomitsu, S., Noriko, S., Ryuji, Y. 2002. Isolation of Actinomycetes from Live Plants and Evaluation of Antiphytopathogenic Activity of Their Metabolites. Actinomycetol., 16:9-13.