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Full Length Research Paper

Antibiotic produced by streptomycetes associated with rhizosphere of purple nut sedge (*Cyperus rotundus* L.) in Surakarta, Indonesia

Ambarwati Ambarwati[1], Langkah Sembiring[2] and C. J. Soegihardjo[3]

[1]Department of Public Health, Faculty of Health Science, Muhammadiyah University of Surakarta
A. Yani Street, Portal Box 1, Pabelan, Surakarta, 57102. Indonesia.
[2]Faculty of Biology, Gadjah Mada University, Yogyakarta 55281, Indonesia.
[3]Faculty of Pharmacy, Gadjah Mada University, Yogyakarta 55281, Indonesia.

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In attempt to understand the diversity of actinomycetes that have the potential of producing antibiotics, Streptomycetes were isolated from soil sample taken from rhizosphere and non rhizosphere of purple nut sedge (*Cyperus rotundus* L.). Isolates were screened for the ability to produce antibiotics by using agar-block method. The best antibiotics producers were then identified by Scanning Electron Microscopy (SEM) analysis. Finally the identification of antibiotics was conducted by using Thin layer chromatography (TLC) analysis. The results of the study showed that 45 isolates were assigned to 18 colour groups. 12 isolates among the representatives of 18 colour groups were found to be potential antibiotics producers. Out of 12 isolates, 8 isolates could inhibit not only *Staphylococcus aureus* ATCC 25923 but also *Bacillus subtilis* FNCC 0060. However only one could inhibit *S. aureus* ATCC 25923 (SR17) and *B. subtilis* FNCC 0060 (RR7). And only 2 isolates could strongly inhibit *B. subtilis* FNCC 0060, namely: NR19 (inhibition zone diameter of 31.33 mm) and RNR25 (33.33 mm). Based on their spore chain morphology and spore surface ornamentation, isolate SNR19 was putatively identified to be a member of *Streptomyces albovinaceus* or *Streptomyces niveus*. On the bases of TLC analysis, the antibiotic produced by the isolate RNR25 was identified as lincomycin.

Key words: *Streptomycetes*, rhizosphere, purple nut sedge, antibiotic.

INTRODUCTION

The discovery of penicilin from *Penicillium notatum* by Alexander Fleming in 1929, appearing of various new infection diseases and resistantly pathogenic microorganism to old antibiotic, have motivated another antibiotic discovery from bacteria, fungus and actinomycetes (Oskay et al., 2004; Nedialkova and Naidenova, 2005). Now, some researchers have focused on actinomycetes, especially *Streptomycetes* that is, is indicated as the bacteria which produces the biggest number of antibiotics. Holt et al. (1994) stated that characteristics of Streptomycetes have vegetative hyphae and aerial mycelium and the vegetative hyphae (0, 5 to 2, 0 µm in diameter) produce an extensively branched mycelium that rarely fragments. The aerial mycelium at maturity form chain of three to many spores (more than 50 spores) (Holt et al., 1994; Prescott et al., 1999). A few species bear short chains of spores on the substrate mycelium. Spores are non motile. Colonies are relatively smooth surfaced, but later they develop a weft of aerial mycelium that may appear floccose, granular, powdery, or velvety (Holt et al., 1994; Korn-Wendisch and Kutzner, 1992).

Gram- positive bacteria and many strains produce one

*Corresponding author. E-mail: ambarwati7@yahoo.com. Tel: 0271717417. Fax: 0271715448.

Table 1. Density of isolates *Streptomycetes* in rhizosphere and non rhizosfer of purple nut sedge (*C. rotundus* L.).

Sample site	Density of the member of genus *Streptomyces* (Cfu/g-dw) in medium	
	Strach casein agar	Raffinose histidine agar
Rhizosphere	15,81 X 10^4	10,72 X 10^4
Non rhizosphere	9,95 X 10^4	7,80 X10^4

or more antibiotics (Holt et al., 1994; Madigan et al., 2003). Streptomycetes produces more than 500 antibiotics (Madigan et al., 2003). Based on the data from Antibiotic Literature Database (ABL), among 8000 biological antimicrobial products, 45,6% are produced by Streptomycetes, 21,5% are produced by fungi, 16,9% are produced by other bacteria and 16% are produced by strains belonging to rare genera of actinomycetes (Lazzarini et al., 2000). Oskay et al. (2004), found 50 different actinomycetes strain from farming soil samples in Manisa Province, and 34% of all isolates were potential as antibiotic producer. Nedialkova and Naidenova (2005) had isolated Actinomycetes from Antarctica, about 34 to 60% isolates were potential to be antibiotics producer and 20% had broad spectrum of antibiotic.

Microorganism, included Streptomycetes can be found in soil and rhizosphere. Microorganism activity is suspected to be high in the rhizosphere, where demodable materials are exuded from plant (Bais et al., 2006). The aim of this research was to know the diversity of Streptomyces that have the potential of producing antibiotic; Streptomycetes could be isolated from soil sample taken from rhizosphere and non rhizosphere of purple nut sedge (*Cyperus rotundus* L.). Isolates were screened for the ability to produce antibiotic by using agar-block method, with four tested bacteria (*Escherichia coli* ATCC 35218, *Salmonella typhimurium* FNCC 0164, *Staphylococcus aureus* ATCC 25923 and *Bacilus subtilis* FNCC 0060).

MATERIALS AND METHODS

Sampling site and preparation of samples

Rhizosphere and non rhizosphere samples were collected from the root system of purple nut sedge (*C. rotundus* L.) in Menwa's office yard of Muhammadiyah University of Surakarta, Indonesia.

Isolation and purification

Isolation of Streptomycetes were done by pour plate technique using Starch casein agar (SCA) and Raffinose histidin agar (RHA) complemented with cyclohexamide. 1 g of dried soil was taken in 9 ml of ¼ strength ringer's solution, agitated vigorously and preheated at 50°C for 10 min. The dilutions (10^{-1} until 10^{-5}) of the suspension were applied onto plates and 20 ml of melted medium at 50°C was added to it. After gently rotaring, the plates were incubated at 25°C for 4 to 14 days (Sembiring et al., 2000). Selected colonies of Streptomycetes were transferred from mixed culture of the plate onto Kenkninght and Munaier agar (Rao, 2001) plate and incubated at 25°C for 4 to 14 days.

Colour grouping and gram-stain

The representative isolates were inoculated onto oatmeal agar which were incubated at 25°C for 14 days. The oatmeal agar plates were examined by eye and aerial spore mass colour, substrate mycelium pigmentation and colour of any diffusible pigments. The Gram-stain procedure adopted was according to that illustrated by Prescott et al. (1999).

Screening for antibiotic ability

The pure isolates of Streptomycetes were screened for the ability to produce antibiotic by using agar-block method (Nedialkova and Naidenova, 2005). Nutrient agar (oxoid) plates (which had been inoculated with tested bacteria by using pour plate method) and were prepared and inoculated with Streptomycetes cultures by a block of inoculum (diameter 8 mm) in the center of the petri dish and incubated at 30°C for 4 days. Four tested bacteria were used in this research: *E. coli* ATCC 35218 and *S. typhimurium* FNCC 0164 (Gram negative bacteria), *S. aureus* ATCC 25923 and *B. subtilis* FNCC 0060 (Gram positive bacteria).

Identification of spores using SEM

The isolates which could strongly inhibit the tested bacteria (indicating by producing more than 25 mm of inhibition zone diameter) was selected as the best antibiotics producer. The best antibiotics producers were then identified based on the spore chain morphology and spore surface ornamentation obtained from SEM analysis. SEM analysis was done in Airlangga University, Surabaya, Indonesia.

Identification of antibiotics produced

The identification of antibiotics produced by the best producers were conducted by using TLC analysis and visualized by ultraviolet (UV) illumination at 254 nm. Five standard antibiotics were used in this research, namely: tetracycline, lincomycin, erythromycin, kanamycin and chloramphenicole.

RESULTS

Density of streptomycetes

The population of member of the genus *Streptomyces* is described in Table 1.

Colour grouping

The result of colour grouping of isolates is illustrated in Table 2 and Figure 1.

Table 2. Colour grouping of isolates *Streptomycetes* that is isolated from rhizosphre and non rhzosfer of purple nut sedge (*C. rotundus* L.)

Group	Group Code	Aerial spore mass colour	Reverse colour	Soluble pigment colour	Total of isolates	Representative member
Rhizosphere of purple nut sedge						
1	GR1	Whitish-orange	Light brown	None	2	RR1* and SR3
2	GR2	Whitish-brown	Blackish-brown	Light brown	3	RR4, RR5 and SR17*
3	GR3	Light yellow	Light yellow	None	2	SR2 and RR6*
4	GR4	Yellow	Yellow	None	3	RR7* and RR12, RR15
5	GR5	Light red	Brown	Yellow	2	SR8* and SR9
6	GR6	Gray	Redish-brown	Brown	2	RR10* and RR14
7	GR7	Greenish-gray	Light brown	none	2	RR11* and RR13
8	GR8	Whitish-red	Brown	Light Brown	2	RR16* and SR19
9	GR9	Purplish-red	Brown	Light brown	2	SR18* and SR20
Non rhizosphere of purple nut sedge						
10	GNR1	Gray	Brown	Light brown	3	RNR1*, SNR3 and SNR5
11	GNR2	Greenish-gray	Brown	Light brown	4	SNR2, RNR4*, SNR8 and SNR10
12	GNR3	Whitish-orange	Light brown	None	3	SNR12,RNR13* and RNR15
13	GNR4	Greenish-gray	Light brown	None	4	SNR6, SNR9, SNR11 and RNR14*
14	GNR5	Whitish-gray	White	None	3	RNR16*, SNR18 and SNR22
15	GNR6	Grayish-green	Light brown	None	2	SNR21* and RNR24
16	GNR7	Whitish-black	Black	None	1	SNR19*
17	GNR8	Grayish-green	Yellowish-green	Yellow	4	SNR7, SNR17, RNR20*and RNR23
18	GNR9	White	Brownish-white-orange	None	1	RNR25
Total of Isolates					45	

Note * = representative isolate of group.

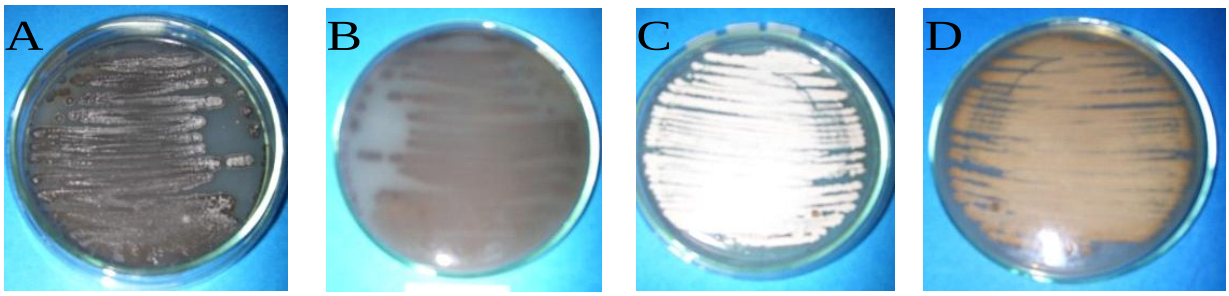

Figure 1. Colour grouping of the best isolates *Streptomycetes;* A, aerial mycelium colour of isolate SNR19; B, reverse colour of isolate SNR19; C, aerial mycelium colour of isolate RNR25; D, reverse colour of isolate RNR25.

Antibiotics produced

The result of screening for the ability of isolates to produce antibiotic is showed in Table 3 and Figure 2.

Gram-stain result

The result of Gram-stain of the best isolates is illustrated in Figure 3.

Isolates' spore

The result of the spore chain morphology of the best isolates is described in Figure 4.

Rf best isolates and standard antibiotics

The result of the Rf of the best isolates and standard antibiotics is showed in Table 4.

Table 3. Screening for ability of isolates Streptomycetes to produce antibiotic.

No	Group	Isolate code	Inhibition zone diameter (mm) of isolate toward tested bacteria			
			E. coli ATCC 35218	*S. typhimurium* FNCC 0164	*S. aureus* ATCC 25923	*B. subtilis* FNCC 0060
1	GR2	SR17	0.00	0.00	16.67**	0.00
2	GR4	RR7	0.00	0.00	0.00	12.33*
3	GR5	SR8	0.00	0.00	18.33**	12.00*
4	GR7	RR11	0.00	0.00	13.67*	12.33*
5	GNR1	RNR1	0.00	0.00	12.67*	15.33**
6	GNR2	RNR4	0.00	0.00	11.67*	11.67*
7	GNR4	RNR14	0.00	0.00	13.33*	12.00*
8	GNR5	RNR16	0.00	0.00	13.67*	8.00*
9	GNR6	SNR21	0.00	0.00	12.00*	9.67*
10	GNR7	SNR19	15.33**i	11.00*i	12.33*i	31.33***
11	GNR8	RNR20	0.00	0.00	11.67*	9.33*
12	GNR9	RNR25	12.67*	15.33**	11.67*	33.33***

Note: the isolate potentially as antibiotic producer, without diameter of agar block, 8 mm (Nedialkova and Naidenova, 2005). *, inhibition zone is categorized weak (7 to 15 mm); **, inhibition zone is categorized moderate (16 to 25 mm); ***, inhibition zone is categorized strong (more than 25 mm); I, iradikal inhibition.

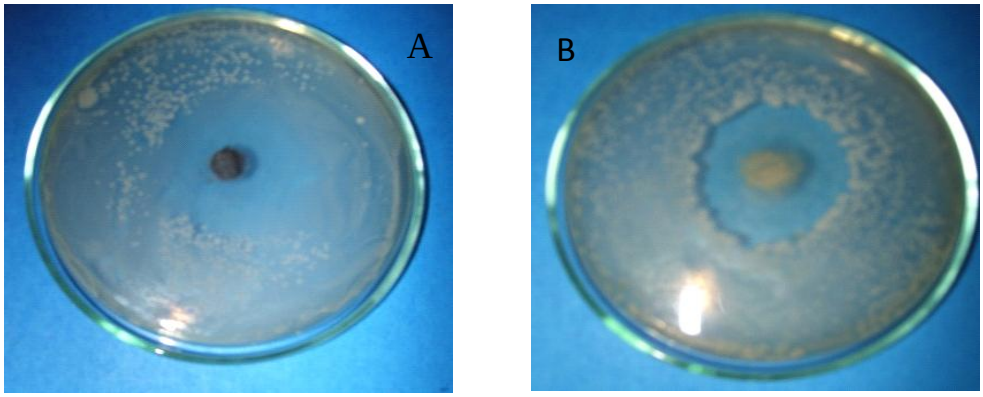

Figure 2. Inhibition zone of the best isolates Streptomycetes toward tested bacteria; **A,** inhibition zone of isolate SNR19 toward *B. Subtilis* FNCC 0060; **B**, inhibition zone of isolate RNR25 toward *B. Subtilis* FNCC 0060.

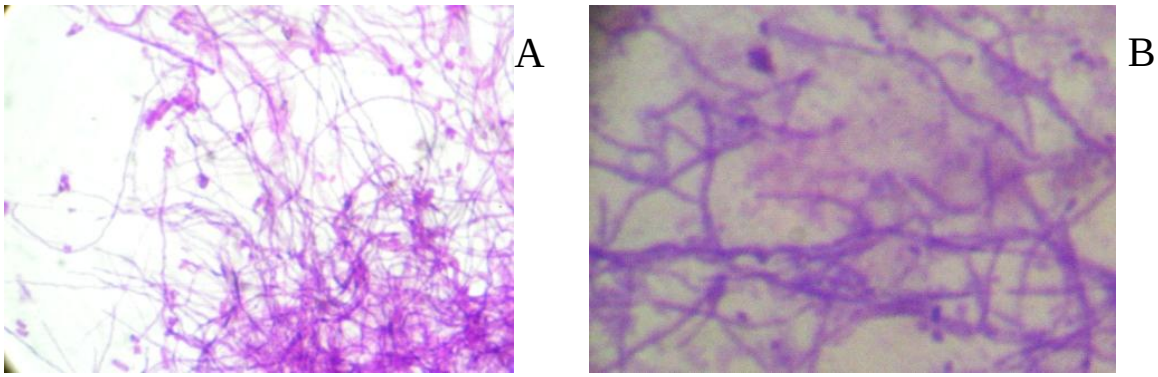

Figure 3. Cell morphology of the best isolates Streptomycetes; A, cell morphology of isolate SNR19; B, cell morphology of isolate RNR25.

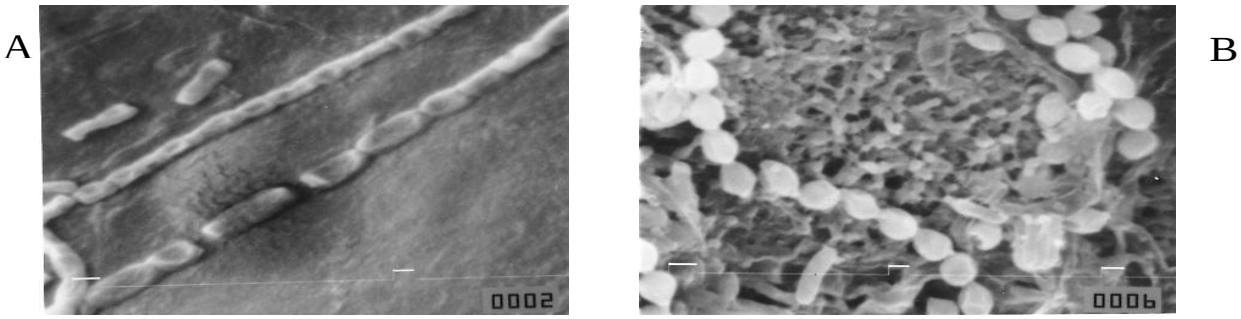

Figure 4. Spore chain morphology of the best isolates Streptomycetes; A, spore chain morphology of isolate SNR19 (7500X); B, spore chain morphology of isolate RNR25 (5000X).

Table 4. Rf of the best isolates Streptomycetes and standard antibiotics.

Isolate code/antibiotic	Rf	UV_{254} nm illumination
SNR19	0.70	Dark blue
RNR25	0.54	Dark blue
Tetracycline	0.28	Dark brown
Lincomycin	0.68	Dark blue
Erythromycin	0.36	Dark blue
Kanamycin	0.44	Dark brown
Chloramphenycol	0.82	Dark blue

DISCUSSION

The sample in this research was taken from rhizosphere and non rhizosphere of purple nut sedge (*C. rotundus* L.). Two selective isolation media were used in this research (SCA and RHA). The same medium was used by Sembiring et al. (2000) for isolating Streptomycetes from rhizosphere of *Paraserianthes falcataria*. Raffinose, starch and casein can be used as carbon source by microorganism, include Streptomycetes (Antonova-Nikolova et al., 2005; Korn-Wendisch and Kutzner, 1992). Cyclohexamide was added onto medium as antifungal, it was done to inhibit the growth of fungal (Korn-Wendisch and Kutzner, 1992; Sembiring, 2000; de Araujol et al., 2000). Based on Table 1, it could be known that the population of member of the genus *Streptomyces* in plant root systems was higher than in soil, not only onto SCA, but also onto RHA. This result supported the Sembiring research (2000) that concluded the density of Sreptomycetes in the rhizosphere of *P. falcataria* was about three times higher than in the non rhizosphere. It happen because the roots of plants have ability to produce exudates (Sorensen, 1997; Rao, 2001) that contains several amino acids and sugars (Bolton et al., 1992; Rao, 2001), organic acids, vitamins, nucleotides and other substances (Rao, 2001). Bais et al. (2006) added the exudates also contain phenolic, mucigel and protein that can be used as carbon and energy sources for microorganism growth. The result of the study showed that 45 isolates could be obtained from the soil sample taken from the rhizosphere and non rhizosphere of purple nut sedge (*C. rotundus* L.).

Based on the colonies morphology, colour grouping and gram-stain procedure, all isolates were assigned to be member of the genus *Streptomyces*. 20 isolates from rhizosphere were assigned to nine multi-membered colour groups. Whereas 25 isolates from non rhizosphere were assigned to seven multi-membered (23 isolates) and two single-membered colour groups. 12 isolates among the representatives of 18 colour groups were found potential as antibiotic producer. Two isolates out of 12 isolates could inhibit both Gram negative and positive bacteria (*E. coli* ATCC 35218, *S. typhimurium* FNCC 0164, *S. aureus* ATCC 25923 and *B. subtilis* FNCC 0060). Among 10 isolates which inhibited Gram positive bacteria, 8 isolates could inhibit not only *S. aureus* ATCC 25923 but also *B. subtilis* FNCC 0060. However only one could inhibit *S. aureus* ATCC 25923 and *B. subtilis* FNCC 0060, namely: SR17 and RR7, respectively. Among 12 isolates of antibiotics producer it was found that only 2 isolates, namely: SNR19 and RNR25 could strongly inhibit *B. subtilis* FNCC 0060 with inhibition zone diameter of 31.33 and 33.33 mm, respectively.

Based on their spore chain morphology and spore surface ornamentation, 1 isolate (SNR19) was putatively identified to be member of *S. albovinaceus*

(Antonova-Nikolova et al., 2005) or *S. niveus* (Korn-Wendisch and Kutzner, 1992) whereas the other one (RNR25) could not be identified up to species yet. On bases of TLC analysis, the antibiotic produced by the isolate RNR25 was identified to be lincomycin whereas the antibiotic produced by SNR19 could not be identified yet. The mechanism of action of lincomycin is inhibits the protein synthesis by binding on the 50S subunit of ribosome and affecting the process of peptide chain initiation (Arai et al., 2003). The description of isolate SNR19: it was discovered from non rhizosphere of purple nut sedge (*C. rotundus* L.), which was isolated in SCA medium, the aerial mycelium colour is grayish-black, reverse colour is black and none soluble pigment colour. The cell morphology is rod with branched mycelium, the colour is purple, Gram positive, the spore chain morphology is straight rod, total of spore chain more than 10 and the spore surface ornamentation is smooth. The illustration of isolate RNR25: was found from non rhizosphere of purple nut sedge (*C. rotundus* L.), which was isolated in RHA medium, the aerial mycelium colour is white, reverse colour is brownish-orange white and none soluble pigment colour. The cell morphology is rod with branched mycelium, the colour is purple, Gram positive, the spore chain morphology is circle, total of spore chain more than 10 and the spore surface ornamentation is smooth.

In conclusion, two isolates of Streptomycetes that are isolated from non rhizosphere of purple nut sedge (*C. rotundus* L.) can strongly inhibit *B. subtilis* FNCC 0060 with inhibition zone diameter of 31.33 mm (SNR19) and 33.33 mm (RNR25). Isolate SNR19 is putatively recognized to be member of *Streptomyces albovinaceus* or *Streptomyces niveus* and isolate RNR25 is known as lincomycin producer.

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REFERENCES

Antonova-Nikolova S, Tzekova N, Yocheva L (2005). Taxonomy of *Streptomyces* sp. strain 3B, J. Cult. Collect., 4: 36-42.

Arai A, Kawachi E, Hata M, Ogura M, Tanak T (2003). Inhibition of *Bacillus subtilis* apr expression by Lincomycin at the posttranscriptional level through inhibition of ppGpp synthesis, J Biochem., 134(5): 691-697.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006). The role of exudates in rizosfer Interactions with plants and other organisms. Ann. Rev. Plant Biol., 57: 233-266.

Bolton HJr, Fredrickson JK, Elliott LF (1992). Microbial ecology of the rizosfer. In Soil microbial ecology. (FB Metting Jr, Ed). Marcel Dekker, Inc. New York.

de Araújol JM, Silva AC, da Azevedo JL (2000). Isolation of endophytic Actinomycetes from roots and leaves of Maize (*Zea mays*. L.), Braz. Arch. Biol. Technol., 43(4): 52-58

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994). Bergey's manual of determinative bacteriology. Ninth Edition. Lippincott Williams and Wilkins. USA.

Korn-Wendisch F, Kutzner HJ (1992). The family Streptomycetaceae. In: A. Balows HG, Truper M, Dworkin, W. Harder, and Karl-Heinz Schleifer. (Eds). The prokaryotes, Second Edition. A handbook on the biology of bacteria : ecophysiology, isolation, identification, aplications. Springer-Verlag, New York.

Lazzarini A, Cavaletti L, Toppo G, Marinelli F (2000). Rare genera of Actinomycetes as potential producer of new antibiotics. ANTON LEEUW INT J G., 78(3-4): 399-405.

Madigan MT, Martinko JM, Parker J (2003). Brock biology of microorganisms. Tent Edition. Prentice Hall, USA.

Nedialkova D, Naidenova M (2005). Screening the antimicrobial activity of Actinomycetes strains isolated from antarctica. J. Cult. Collect., 4: 29-35.

Oskay M, Tamer AU, Azeri C (2004). Antibacterial activity of some Actinomycetes isiolated from farming soil of Turkey. Afr. J. Biotechnol., 3(9): 441-446.

Prescott LM, Harley JP, Klein DA (1999). Microbiology. Fourth Edition. WCB McGraw-Hill, Boston.

Rao NSS (2001). Soil microbiology. Soil microorganism and plant growth. Fourth Edition. Science Publishers, Inc. Enfield (NH), USA

Sembiring L, Ward AC, Goodfellow M (2000). Selective isolation and characterisation of members of the *Sreptomyces violaceusniger* clade associated with the roots of *Paraserianthes falcataria*., ANTON LEUWAN INT J G., 78(3-4): 353-366.

Sorensen J (1997). The rizosfer as a habitat for soil microorganisms. In Modern soil microbiology. (JVD. Dirk JT, Trevors and EMH. Wellington, Eds.). Marcel Dekker, Inc. New York.