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Full Length Research Paper

Optimization of fermentation conditions for actinomycin D production by a newly isolated *Streptomyces sp.* AH 11.4

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A biologically active streptomyces strain was isolated from soil samples collected from different geographical areas in Sudan. This isolate was taxonomically characterized on the basis of morphological and biochemical properties along with 16S rRNA partial sequence analysis. One major active compound was extracted from the purified fermented broth and chemically characterized as actinomycin-D, based on the spectroscopic data obtained from the application of MS, UV, ^{1}H NMR, ^{13}C NMR and by comparison with published data. A number of carbon sources were tested for their effect on growth of *Streptomyces* sp. AH11.4 and actinoomycin D production. Arabinose, glucose, glycerol, maltose, manitol, raffinose sucrose, starch, and xylose were excellent carbon sources for actinomycin-D production, TSB medium containing 1.0 % starch gave the maximum actinomycin-D production (306 mg/L), as compared to the production medium with other different carbon sources.

Key words: Streptomyces *sp. AH 11.4,* actinomycin *D, 16S rRNA*

INTRODUCTION

Actinomycetes are reported to be the major producers of biologically active compounds. Two-thirds of microbially derived antibiotics are obtained from this source, especially *Streptomyces* spp. (Kieser et al., 2000). The production of microbial metabolites can be substantially increased by manipulating the nutritional conditions. The nature and concentration of some components of the fermentation medium also have a marked effect on antibiotic production (Parekh et al., 2000; Sanchez and Demain, 2002)**.** The actinomycins are chromopeptide lactone antibiotics, and important class of natural products that, despite their first discovery more than 70 years ago, continue to be a focus of many research areas, especially in the biological and medicinal sciences. Among the actinomycins, actinomycin D has been studied most extensively and is widely used for treatment of malignant tumors, such as Wilms' tumor (Farber et al., 2002), and childhood rhabdomyosarcoma (Womer, 1997). Recently, actinomycin D has been proposed as a therapeutic agent for AIDS, because it is a potent inhibitor of HIV-1 minus-strand transfer (Guo et al., 1998). The present paper describes the identification of *Streptomyces* sp. AH11.4 as well as optimizing medium components in order to enhance actinomycin D production.

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MATERIALS AND METHODS

Isolation, characterization and identification of *Streptomyces sp.*

The producer organism, designated as AH 11.4, was isolated from a soil sample collected from different geographical areas in the Sudan. A morphological, cultural, and physiological characteristic of the isolate were studied using the International

Streptomyces Project (ISP) media recommended by Shirling and Gottlieb (1966), Bergey's Manual of Systematic Bacteriology, and on the basis of 16S rRNA homology. The isolate was grown in nutrient broth for the preparation of genomic DNA which was extracted according to methods described by (Nikodinovic et al., 2003). PCR amplification and sequencing of 16S rRNA gene was carried out as described previously (Stackebrandt et al., 1997) using a Peltier thermal cycler (BIO-RAD). The reaction mixture included the universal primers 27f (5'-CCG TCG ACG AGC TCA GAG TTT GAT CCT GGC TCA G-3') and 1392r (5'-CCC GGG TAC CAA GCT TAA GGA GGT GAT CCA GCC GCA-3'). To improve the denaturation of the genomic DNA, 5 µl DMSO was added to the reaction mixture. Amplification of the 16S rRNA gene was performed according to the following temperature profile: 95 °C for 2 min, followed by 30 cycles consisting of denaturing (40 sec), primer annealing at 50 °C (40 sec) and at 70 °C extension (1 min). A final extension step at 70 °C was included (10 min). Amplified DNA was detected by electrophoresis on a 1% agarose gel and visualized by UV fluorescence after ethidium bromide staining. Amplified fragments were purified using Qiaquick PCR cleanup kit (Qiagen) according to the manufacturer's instructions, and sequenced commercially by MWG. Trees were generated using CLUSTAL X programme (Larkin et al., 2007).

For Scanning Electron Microscopy ISP4 agar plate was inoculated with spores of the AH 11.4 isolate and incubated for 10 days at 30^{o} C. A plug of the culture was removed using a boring tool with a diameter of 5 mm. The sample was fixed in glutaraldehyde (2.5 % v/v), washed with water and post fixed in osmium tetroxide (1 % w/v) for 1 h. The sample was washed twice with water and dehydrated in ascending ethanol before drying in a critical point drying apparatus (Polaron E3000) and finally coated in gold and examined in a JEOLJSM 5410LV Scanning Electron Microscope at 15KV.

Extraction, purification and production of actinomycin D

The production of antibiotic by the strain AH 11.4 was examined in different broth media, Tryptone Soya Broth (TSB), Yeast Extract Malt Extract Broth (YEMEB), Starch Casein Nitrate Broth (SCNB), and Starch-Glycerol Nitrate Broth (SGNB), Nutrient Broth (NB) and Streptomyces Antibiotic Medium (SAM): all media were purchased from Oxoid and from fluka Chemie GmbH. Baffled Erlenmeyer flasks (250 ml), containing 50 ml of medium, were inoculated from a spore suspension, and incubated on a rotary shaker (200 rpm) at 30 °C for 48 h, Coiled springs (Shannon Coiled Springs Ltd., Ireland) were used as baffles to aerate liquid cultures.

The mycelium and supernatant were separated by centrifugation at 13,000 rpm for 15 minutes, and the mycelium extracted with ethanol-acetone (1:4, 25 ml). Activities against test organisms *(Staphylococcus aureus, Bacillus subtilis, Streptococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger*, and *A. flavus*) were monitored during the isolation, using agar well diffusion assay method as described by (Holder and Boyce, 1994). Extracts from both supernatant and mycelium were combined and concentrated under vacuum to give a red-orange powder.

The crude organic extract was separated by solid phase extraction (SPE) on a Hypersil C18 column, and eluted with a stepwise gradient of methanol (20-100 %). Fractions containing highest antibiotic activity were purified further by HPLC/DAD (Varian Prostar system) using an isocratic elution (80% methanol-water) on a Zorbax StableBond column. Peak purity was assessed by analytical HPLC/DAD (gradient elution from 10% acetonitrile-H2O to 100% acetonitrile over 20 minutes, flow rate 0.8 ml/min and UV 441 nm using a Varian Microsorb-MV 100-5 C8 250 x 4.6 mm column). Purified compound was analysed by ESI MS, ^{1}H and ^{13}C NMR.

Actinomycin D production levels using different carbon sources were assessed as follows: baffled Erlenmeyer flasks (250 ml), containing 50 ml of TSB medium (without glucose) plus the carbon source of interest (1%), Actinomycin D concentration was determined by comparison of peak areas at 440 nm with an actinomycin D standard (Sigma-Aldrich). The values were combined to give overall production levels. Experiments were conducted in duplicate and the values given are averages of the two results.

RESULTS AND DISCUSSION

In vitro production of antibiotic was first observed by soil microorganisms many decades ago when dilution plates for isolation of bacteria and fungi were prepared. The result of an extensive screening of bacteria and fungi for antibiotics have been lead to the discovery of about 4000 substances, many of which have found applications in medicine. Most of these antibiotics are produced by Streptomyces species (Saadoun and Muhana, 2008). Actinomycins are important old class of natural products that continue to be a focus of many research areas, especially in the biological and medicinal sciences. There is continuing interest in the actinomycins and their microbial producers.

The actinomycete strain in this study was isolated from soil samples collected from different geographical areas in Sudan, purified and screened for their antimicrobial activity against pathogenic microbes. On the bases of morphological, cultural, and physiological characteristics, AH 11.4 isolate seemed to be closely related to the genus Streptomyces (data not shown). Light and scan electron microscopy revealed that the strain is Gram positive, filamentous and spore forming. The spore chain with open loops, hooks and primitive spirals is shown on Figure 1. The strain was further identified on the bases of 16S rRNA homology studies. The partial sequences of 16S rRNA of the strain AH11.4 was compared with those found in databases based on Basic Local Alignment Search Tool (BLAST) analysis. The comparison of the 16S rRNA sequence of the AH 4.4 isolate with those sequences submitted to GenBank demonstrated that the AH 11.4 isolate was 94% similar to the 16S rRNA sequence to number of isolates all of which were *Streptomyces* species. According to the sequence alignment and phylogenetic tree based on the 16S rRNA genes, strain AH 11.4 is closest to *Streptomyces sp* EGT, *Streptomyces variabilis, Streptomyces sp*. ES (2011), *Streptomyces* sp. CPE236, and *Streptomyces* BDUKASO6. However, there are many differences between strain AH 4.4 and these

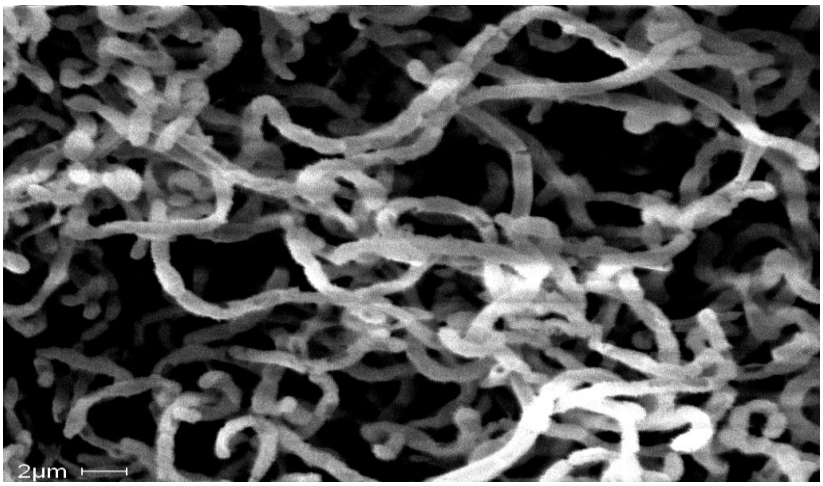

Figure1. Scanning electron micrographs of the spore chains of the bioactive *Streptomyces sp*. AH 11.4.

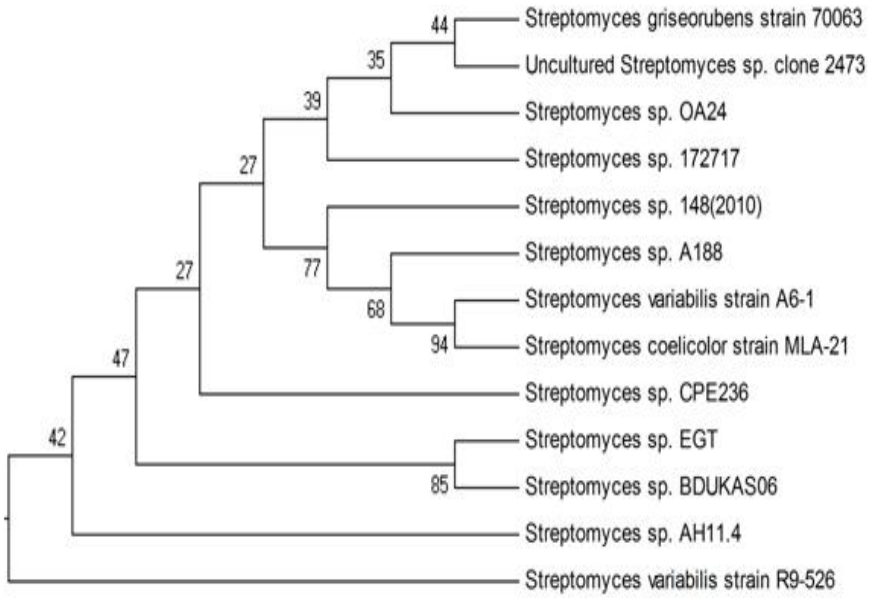

Figure 2. Neighbour-joining tree based on partial 165 rRNA gene sequences showing relationships between strain *Streptomyces sp*. AH 11.4 and related members of the genus Streptomyces.

strains according to the published data. The phylogenetic tree, suggested that strain AH 11.4 arose from different node which suggested that this organism was not a closely related species. AH 11.4 was proposed as a novel *Streptomyces sp.* AH 11.4 (Figure 2). The *Streptomyces sp*. 11.4 strain was deposited at the Industrial Microbiology Department (IMD) University College of Dublin (UCD) under the number (2731). The 16S rRNA sequences of the *Streptomyces sp.* AH 11.4 was submitted to GeneBank (accession numbers GU013557). The phylogenetically closest related streptomyces strains are not known to produce this antibiotic, thus this strain is a new source of this antibiotic.

Purification, identification and production of Actinomycin D.

The strain produced a red/orange colored active

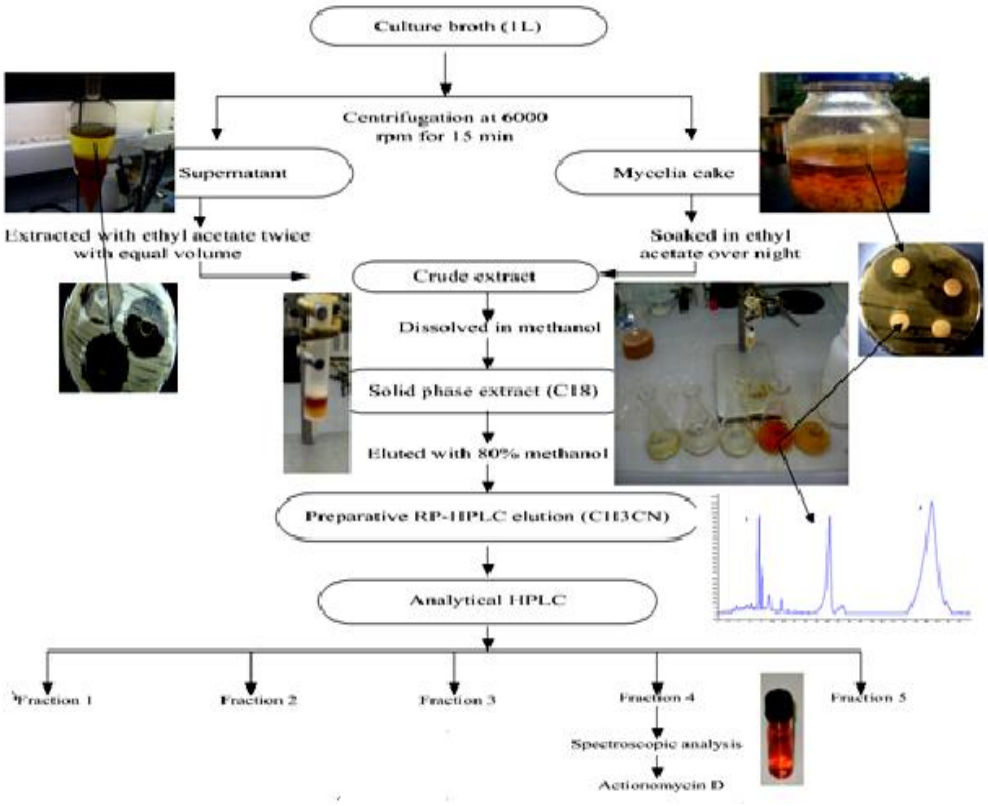

Figure 3. Extraction and purification of the actinomycin D from *Streptomyces* sp. AH 11.4.

compound. HPLC was the most successful strategy investigated and became the routine approach for identification of the active compounds contained in bioactive extracts. The correlation between peaks in the chromatogram and biological activity levels in the different HPLC fractions, allowed the identification of the active compound contained in the extract as shown in Figure 3.

Preliminary HPLC-DAD analysis of this extract identified an absorbance spectrum characteristic of actinomycin D. This tentative identification was supported by the observation of strong ions in the ESI (+) MS spectra of the extracts corresponding to the presence of actinomycin D (*m/z* 1255, [M+H] +). Purification of the active compound was carried out using reverse phase SPE and HPLC. NMR analysis (COSY, HSQC, and HMBC) was conducted, which was used to assign the ^{1}H and ^{13}C NMR resonances of actinomycin D.

Culture medium is a key factor for the growth as well as metabolites production by microorganisms. In shake flask study six broth media were examined for antibiotic production by the strain of *Streptomyces sp*. AH 11.4. This strain recovered the ability to produce antimicrobial secondary metabolites in three of the tested media. Among them culture supernatant of *Streptomyces sp*. AH 11.4 in tryptone soya broth (TSB) showed the highest activities against test organisms than starch casein nitrate broth and starch-glycerol nitrate broth, no activities was observed when used Yeast Extract Malt Extract Broth, Nutrient Broth and Streptomyces Antibiotic Medium, as production media (Figure 4).

Further study was also performed in order to increase the production of acinomycin-D. For this purpose, different carbohydrates were added to the selected TSB media in the shake flask at a concentration of 1% (w/v). *Streptomyces* sp. AH 11.4 was found to produce 110 mg /L actinomycin- D in the basal medium. It is well established that actinomycin production is affected by carbohydrate source (Katz et al., 1958). The carbon source needed for maximal yield of the antibiotic production seems to be different among bacterial strains. Glycerol supported better antibiotic production by *Streptomyces hygroscopicus* 1.5 (Bhattachryya et al., 1998). Glucose was also reported as the most suitable carbon source for maximum phenazine production by *Pseudomonas fluorescens* 2-79 (Slininger and Shea-wilbur, 1995).

Table 1 illustrates that the antibiotic production by the *Streptomyces sp.* AH 11.4 was strongly influenced by carbon source. The use of starch as a carbon source yielded much higher levels of actinomycin D (306 mg/l), which compared favorably to other values reported in the literature for shake-flask culture, though much higher levels have been reported using a bioreactor (Souza *et*

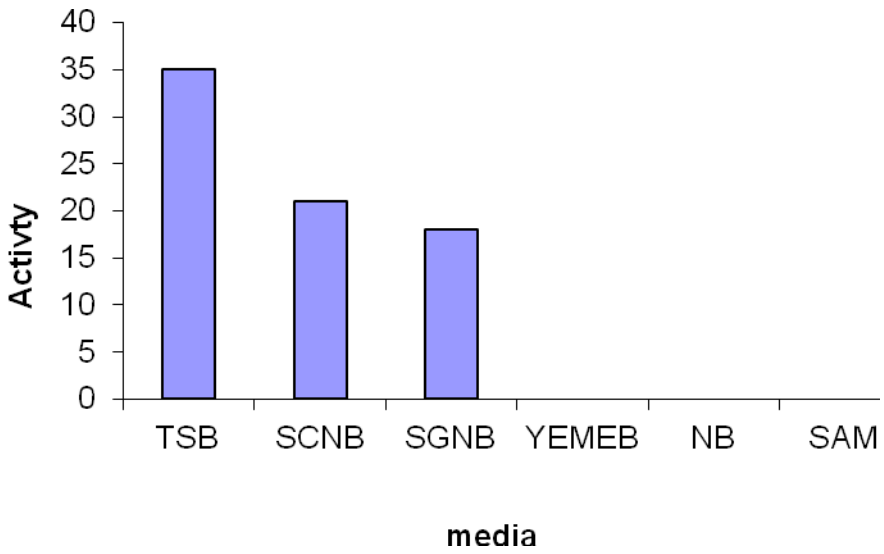

Figure 4. Production of Actinomycin-D by *Streptomyces* sp. AH 11.4**.**in different media. Tryptone Soya Broth (TSB), Starch Casein Nitrate Broth (SCNB), Starch-Glycerol Nitrate Broth(SGNB). Yeast Extract Malt Extract Broth (YEMEB). Nutrient Broth (NB) and SAM= Streptomyces Antibiotic Medium (SAM). Values are mean of three replicates

Table 1. Actinomycin D production in strain AH 11.4 when grown in TSB containing different carbohydrates.

Carbohydrate source	Acinomycin D production (mg/L)
Arabinose	185
Fructose	192
Glucose	110
Glycerol	99
Maltose	225
Mannitol	223
Raffinose	100
Starch	306
Sucrose	83
Xylose	16

al., 2002). In comparison to another actinomycin –D producer described in the literature, *Streptomyces sindenensis* (Vandana et al., 2008), was shown to produce 80 mg/L actinomycin-D. Sousa et al., (2002) reported that *S. parvulus* among three of the species of Streptomyces that produce actinomycins has the greatest antibiotic activity (152 mg/L), *S. felleus* 20 mg/L, and *S. regensis*, which did not exceed 12 mg/L. There is continuing interest in the actinomycins and their microbial producers; there are at least more than 20 species of Streptomyces capable of producing actinomycins. Examples of such strains include *S. antibioticus* (Waksman and Woodruff, 1940), *S. michiganensis (*Frommer*,* 1959*)*, *S. parvulus* (Williams and Katz, 1977), *S. nasri* (El-Naggar, 1998), *S. plicatus* (Lam et al., 2002), and *S. sindenensis* (Praveen et al., 2008).

These results suggest that *Streptomyces* sp .AH 11.4 is a comparable better producer of actinomycin D, and will be a new source of this important antibiotic.

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REFERENCES

Bhattacharyya BK, Sushil CP, Sukanta KS (1998). Antibiotic production by *Streptomyces hygroscopicus* D 1.5: cultural effect. Rev. Microbiol., 29(3).

El-Naggar MYM (1998). Synergistic effect of actinomycin X-2 produced by *Streptomyces nasri* strain YG62 with other antibiotics. Biomedical Lett., 58: 169-173.

Farber S, D'Angio G, Evans A, Mitus A (2002). Clinical studies of actinomycin D with special reference to Wilms' tumor in children. 1960. J. Urol., 168:2560-2562.

Frommer W (1959). Zur Systematik Der Actinomycin Bildenden Streptomyceten. Archiv Fur Mikrobiologie 32: 187-206.

Guo JWT, Bess J, Henderson LE, Levin JG (1998). Actinomycin D inhibits human immunodeficiency virus type 1 minus-strand transfer in in vitro and endogenous reverse transcriptase assays. J. Virol. 72: 6716-6724.

Holder IA, Boyce ST (1994). Agar well diffusion assay testing of bacterial susceptibility to various antimicrobials in concentrations non-toxic for human cells in culture. Burns 20: 426-429.

Katz E, Pienta P, Sivak A (1958). The role of nutrition in the synthesis of actinomycin. Appl. Microbiol., 6(4): 236-241.

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000). Practical Streptomyces Genetics, 2nd edn., Norwich, UK: John Innes Foundation. 3(4): 260-261.

Lam K, Gustavson DR, Pirnik DL, Pack E, Bulanhagui C, Mamber SW, Forenza S, Stodieck LS, Klaus DM (2002). The effect of space flight on the production of actinomycin D by *Streptomyces plicatus*. J. Ind. Microbiol. Biotechnol., 29: 299-302.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentinn F, Wallace IM, Wilm A, Lopez R, Thompson TJ, Gibson RA, Higgins DG (2007). Clustal W and clustal X version 2.0. Bioinformatics 23: 2947-2948.

Nikodinovic J, Barrow KD, Chuck JA (2003). High yield preparation of genomic DNA from Streptomyces. Biotechniques 35: 932.

Parekh S, Vinci VA, Strobel RJ (2000). Improvement of microbial strains and fermentation processes. Appl. Microbiol. Biotechnol., 54(3): 287-301.

Praveen V, Tripathi CKM, Bihari V, Srivastava SC (2008). Production of Actinomycin-D by the Mutant of a New Isolate of *Streptomyces Sindenensis*. Brazilian J. Microbiol., 39: 689-692.

Saadoun I, Muhana A (2008). Optimal production conditions, extraction, partial purification and characterization of inhibitory compound(s) produced by Streptomyces Ds-104 isolate against multi-drug resistant Candida albicans. Curr. Trends Biotechnol. Pharm., 2(2): 402-420.

Sanchez S, Demain AL (2002). Metabolic regulation of fermentation processes. Enz. Microb. Technol., 31:895-906.

Shirling EB, Gottlieb D (1966). Methods for characterization of Streptomyces species. Int. J. Syst. Bacteriol., 16: 313-340.

Slininger P, Shea-Wilbur M (1995). Liquid-culture pH, temperature, and carbon (not nitrogen) source regulate phenazine productivity of take- all biocontrol agent *Pseudomonas fluorescens* 2-79. Appl. Microbiol. Biotechnol. 43: 794-8000.

Sousa MFVQ, Lopes CE, Pereira Jr, N (2002). Development of A bioprocess for the production of Actinomycin-D. Brazilian J. Chem. Eng., 19(3): 277-285.

Stackebrandt E, Rainey FA, WardRainey NL (1997). Proposal for a new hierarchic classification system, Actinobacteria classis nov. Int. J. Syst. Bacteriol., 47: 479-491.

Vandana C, Praveen KM, Tripathi A, Vinod C, Bihari SC, Srivastava F (2008). Production of Actinomycin-D by the Mutant of a new isolate of *Streptomyces sindenensis.* Brazilian J. Microbiol., 39: 689-692.

Waksman SA, Woodruff HB (1940). Bacteriostatic and bacteriocidal substances produced by soil actinomycetes. Proc. Soc. Exp. Biol. Med. 45:609-614.

Williams WK, Katz E (1977). Development of a Chemically Defined Medium for Synthesis of Actinomycin-D by *Streptomyces Parvulus*. Antimicrob. Agents Chemother., 11:281-290.

Womer RB (1997). Soft tissue sarcomas. Eur. J. Cancer, 33: 2230-234; 2234-2236.