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Di-(2-ethylhexyl) Phthalate, a Major Bioactive Metabolite with Antimicrobial and Cytotoxic Activity Isolated from the Culture Filtrate of Newly Isolated Soil Streptomyces (*Streptomyces mirabilis*, NSQu-25)

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Abstract: During the screening program for new specific bioactive substances, an actinomycete strain designated NSQu-25 was isolated from Egyptian soil and selected for its antimicrobial and/or cytotoxic activity. Identification of the producer strain was performed according to spore morphology and cell wall chemo-type, which suggested that this strain is a streptomycete. Further cultural, physiological characteristics and phylogenetic analysis of 16S rRNA gene of this isolate indicated that this strain is identical to *Streptomyces mirabilis* Uz1010 and then designated *Streptomyces mirabilis* strain NSQu-25. In its culture filtrate, this organism could produce one major compound separated by silica gel column chromatography and then purified on a sephadex LH-20 column. The isolated compound exhibited antimicrobial activities mainly against Gram-positive bacteria and yeasts but the inhibition of Gram negative bacteria was lower, also it showed potent cytotoxic activities against some human carcinoma cells. Fourier Transform Infrared (FTIR), Gas Liquid Chromatography (GLC), mass GC/MS and ^{1}H nuclear magnetic resonance (^{1}HNMR) spectroscopy studies suggested that the isolated compound (molecular formula of $C_{24}H_{38}O_4$; molecular weight of 390 Dalton) and found to be identical to di-(2-ethylhexyl) Phthalate. Although the compound is known, the newly isolated strain was able to produce the antibiotic as a major product providing an important biotechnological downstream advantage.

Key words: *Streptomyces mirabilis* NSQu-25 · Biological activity · Phenotypic and phylogenetic identification · Spectroscopic analysis · Di-(2-ethylhexyl) Phthalate

INTRODUCTION

Natural organic compounds produced by microorganisms are an important screening target for a variety of bioactive substances [1]. Compounds of actinomycete origin, in particular are valuable in the field of bioactive natural products [2]. However, the rate of discovery of novel substances from microorganisms, especially from actinomycetes of terrestrial origin, has recently decreased [3]. Microbial natural products are an important source of both existing and new drugs. Among the producers of commercially important metabolites, actinomycetes have proven to be a prolific source with a surprisingly small group of taxa accounting for the vast majority of compounds. Secondary metabolites produced by actinomycetes possess a wide range of biological activities such as antibacterial, antifungal, antioxidant, antitumor and antiviral [4]. Actinomycetes are the most significant group of microorganisms considered as an important producer of antibiotics and other important bioactive substances [5]. The filamentous Actinomycetales species produces over 10000 bioactive compounds. The most frequent producers, the *Streptomyces* species, produce 7600 compounds (74% of all Actinomycetales), while other actinomycetes represent 26%, altogether 2500 compounds [6]. These organisms produce perhaps the most diverse and most unique, unprecedented, sometimes very complicated compounds exhibiting excellent antibacterial potency and usually low toxicity [6]. Filamentous soil bacteria belonging to the genus *Streptomyces* is identified as a major source of bioactive natural products representing

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some 70-80% of the all isolated compounds [6-8]. The streptomycetes form a distinct clade within the radiation encompassed by the high-GC Gram-positive bacteria in the 16S rDNA tree. There is evidence that specific metabolites, such as clavulanic acid, may be synthesized by strains in a specific clade and that the ability to synthesize, for example, streptomycin and related metabolites appear to be randomly distributed across the whole genus [9]. The specific/infraspecific relationships in the streptomycetes and the way they are reflected in the biosynthetic potential to produce bioactive compounds could significantly influence strategies for search and discovery, screening and bioprocess development. To extending the whole-genomic studies of streptomycetes would reveal these relationships in a comprehensive way, which would enable validation of current methodologies (from 16S rDNA phylogenies to DNA-DNA pairing) and lead to new understanding of speciation, phylogenetic relationships and genome function in secondary metabolism [10, 11].

In the present study, we describe the polyphasic taxonomic characterization of actinomycete strain NSQu-25 isolated from El-Quseima region, North Sinai, Egypt, having antimicrobial and cytotoxic activities. The production, extraction, purification and partial characterization of active compound of this strain are reported as well.

MATERIALS AND METHODS

Samples Collection: Fifteen soil samples were collected at the period of February to April 2012 from four different regions (Wadi El-Gudirat, El-Quseima, Gebel El-Maghara and El-Hasana region) of North Sinai governorate. Soil samples were taken down to a 15-20 cm depth into the soil surface and were put into small pre-labeled sterile plastic bags and tightly sealed. The samples were then air-dried and pretreated with $CaCO_3$ (10:1 w/w) and incubated at 37°C for 4 days [12].

Isolation of Actinomycete Cultures from the Collected Soil Samples: The isolation and enumeration of actinomycete colonies were performed using a soil dilution plate technique on two different media. These media were; actinomycetes isolation agar medium (Difco, New Jersey (NJ), USA), composed of (g/L): glycerol, 5.0; sodium propionate, 4.0; sodium caseinate, 2.0; KH_2PO_4, 2.0; l-asparagine, 0.1; $MgSO_4.7H_2O$, 0.1 and $FeSO_4.7H_2O$ 1.0 mg; Agar, 15 and pH 7.0 [13] and starch nitrate agar medium composed of (g/L): Soluble starch, 20.0; $NaNO_3$, 2.0; K_2HPO_4 (anhydrous), 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $CaCO_3.2H_2O$, 2.0, Agar, 15 and pH 7.0 [14]. The isolation media were supplemented with $CaCO_3$ and cycloheximide (50µg/ml) to minimize the fungal contamination and incubated on 28°C for 7-10 days [13]. One gram of dried soil was suspended into test tube containing 9 ml sterile NaCl (0.85%) solution and one drop of Tween 80 and 0.05% lauryl sulfate (Sodium dodecyle sulfate, SDS) and heated at 50°C for 10 min [15]. Different dilutions 10^{-3}, 10^{-5} and 10^{-7} of the suspension were plated onto isolation media. The plates were incubated for 7 to 10 days at 28°C. Selected colonies were transferred from mixed culture of the plates onto respective agar plates and incubated at 28°C for another 7 days. Plates containing pure cultures were stored at 4°C until further examination.

Screening for Bioactive Compounds of the Isolated Actinomycetes

Screening for Antimicrobial Activity: The isolated actinomycete cultures were tested for their antimicrobial activity against the following test organisms; *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 7839, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404 and *Aspergillus flavus* ATCC 16883. The nutrient agar (Merck, Darmstadt, Germany) was used for bacteria at 37°C for 24 hours, Sabaroud's agar (Lab M., Bury, Lancashire, U.K.) for yeast at 30°C for 24 hours and PDA (Merck, Darmstadt, Germany) for fungi at 25°C for 48 hours. The bioassay method was carried out using the agar well diffusion method [16].

Screening for Cytotoxic Activity: The crude extracts of the bioactive isolates (exhibited highest antimicrobial activity) were tested in vitro for their cytotoxic activity against the following human tumor cell lines; human liver carcinoma cells (HEPG2), human colon carcinoma cells (HCT 116), human breast carcinoma cells (MCF7) and human cervix carcinoma cells (HELA). The tested cell lines were obtained frozen in liquid nitrogen (-180°C) from the American Type Culture Collection then maintained at the National Cancer Institute (NCI), Cairo, Egypt, by serial sub-culturing. Antitumor assay of the tested crude extracts was carried by MTT assay method [17].

Taxonomic Characterization of Actinomycete isolates, NSQu-25

Conventional Taxonomy: The characterization of the isolated strain followed the guidelines adopted by the International *Streptomyces* Project [18]. The electron microscope study was carried out using scanning electron microscope (JEOL JSM 5300, JEOL Technics Ltd., Japan).

Color characteristics were assessed on the scale developed by Kornerup and Wanscher [19]. Diaminopimelic acid isomers in the cell-wall were analyzed by using the methods of Becker *et al*. [20]. The melanin pigment production, nitrate reduction, utilization of C and N sources and the culture characteristics were studied in accordance with the guidelines established by the ISP [18]. The physiological and biochemical characteristics, such as the activities of lipase [21], protease [22], α-amylase [23] and catalase [24] were tested.

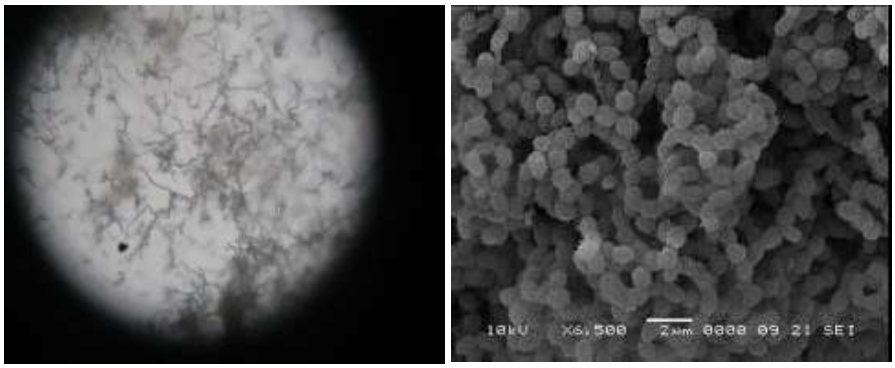
Molecular and Phylogenetic Identification: Strain NSQu-25 was used to inoculate 50 ml of ISP-2 broth and the culture was incubated at 200 rpm and 28°C for 24 h. The total genomic DNA was extracted according to the method of Sambrook *et al.* [25]. The 16S rRNA of the strain was amplified by PCR using a GeneAMP PCR System 9700 from PE Applied Biosystems (Perkin Elmer, Ohio, USA).

The Following Primers Were Used: F27, 5'-AGAGTTTGATCMTGGCTCAG-3' and R1492 5'-TACGGYTACCTTGTTACGACTT-3'usingBiolegioBV software (Biolegio, Nijmegen, Netherlands) [26]. The PCR mixture conditions were described by Awad *et al*. [13]. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and were detected using a gel documentation system, (Alpha-Imager 2200, CA, USA). The PCR products were sequenced using the Gene Analysis Unit in the genetics laboratories of an Egyptian company that produces vaccines, sera and drugs (VACSERA), Dokki, Cairo, Egypt. The DNA sequences were determined using an ABI PRISM 377 DNA sequencer and ABI PRISM Big Dye Terminator Cycle Sequencing (Perkin Elmer, Ohio, U.S.) at a sequencing facility at Cornell University in the U.S. BLAST (www.ncbi.nlm.gov) was used to assess the DNA similarities. A multiple sequence alignment and molecular phylogenetic analysis were performed using BioEdit software [27]. The phylogenetic tree was constructed using the TreeView program [28].

Fermentation Production of the Bioactive Metabolite: *Streptomyces mirabilis* strain NSQu-25 was cultivated at 28°C and 200 rpm on a rotary incubator shaker for 6 days at 30°C. The cultivation was performed in a 250 ml Erlenmeyer flask containing 50 ml fermentation medium. It contained (g/L): Starch; 20.0, $NaNO_3$; 2.0, K_2HPO_4; 1.0, KH_2PO_4; 0.5, $MgSO_4 \cdot 7H_2O$; 0.5, KCl; 0.5, trace salt solution 1.0 ml [$CuSO_4 \cdot 5H_2O$ (0.64 g/L), $FeSO_4 \cdot 7H_2O$ (0.11 g/L), $MnCl_2 \cdot 4H_2O$ (0.79 g/L) and $ZnSO_4 \cdot 7H_2O$ (0.15 g/L)]. The medium was adjusted to the initial pH 7.0. The inoculation was carried out using a spore suspension of the NSQu-25 culture slanted on ISP-2. Twenty seven-liters of culture broth were collected and filtered through Whatman No.1 filter paper, followed by centrifugation at 5000 rpm for 20 minutes. The clear filtrate was sterilized using 0.22 µm millipore filter and tested for its antimicrobial and cytotoxic activity.

Extraction and Purification of the Bioactive Compound: The culture broth (25 L) obtained after filtration was extracted twice with ethyl acetate and subsequently concentrated under reduced pressure using a rotary evaporator (Büchi, R-114, Switzerland).The temperature was maintained to be less than 50°C to give 3.6 g of crude extract. This crude extract was dissolved in 5 ml methanol and applied to a column chromatography (2.5 i.d. x 50 cm, silica gel 60; Merck) as stationary phase. The column was eluted using a gradient polarity of solvent system; ethyl acetate: methanol (10:1 to 1:10), fifty ml fractions were collected and the fractionation process was monitored using TLC analysis. The fractions that exhibited similar TLC profiles were combined to give a final total of 13 fractions (S_1-S_{13}) collected at 15, 80, 140, 75, 45, 130, 59, 150, 71, 96, 125, 215 and 520 mg, respectively. Bioactivity evaluations of the obtained fractions were tested for antimicrobial activity against *B. subtilus* (ATCC 6633) through the agar plate diffusion assay [16]. The bioactive fractions were tested again for their cytotoxic activity against human colon cancer cell line (HCT116) by MTT assay [17]. Fractions that exhibited cytotoxic activity were applied on sephadex LH-20 column (2 x 15 cm) using 100% methanol as the eluent solvent (500 ml) and lastly, 5 ml of the fractions were collected. The sub-fraction, 61 (190 mg), was separated as a single band to yield purified active compound.

Structural Elucidation of the Isolated Pure Compound: Structural elucidation of the obtained pure fraction (190 mg) was achieved via spectroscopic analyses such as FTIR spectra recorded using KBr method of Fourier Transform Infrared (JASCO FT/IR–6100) spectrophotometer. Other than that, its NMR spectra were also assessed and recorded on a Varian Mercury VX-300 NMR spectrometer operated at 300 MHz for ^1H in $CDCl_3$ using TMS as the internal standard. In addition to that, the EIMS spectra of the pure fraction were also taken into consideration and were obtained with a Direct Inlet part DI-50 connected to the mass analyzer Shimadzu GC/MS-QP5050, at the micro-analytical center of the Faculty of Science, Cairo University, Egypt.



(a) (b)

Fig. 1a: Light microscopy image of the aerial mycelium showing a spiral spore chain (G x400), (b) Scanning electron micrographs showing spiny spore surface of *Streptomyces mirabilis*, NSQu-25 (x6.500) grown on starch-nitrate agar medium for 10 days.

Table 1: Cultural characteristics of *Streptomyces mirabilis*, NSQu-25 on different culture media.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigments
Tryptone yeast extract broth (ISP-1)	Weak	White(ISCC-NBS 263)	Slightly yellow (ISCC-NBS 84)	None
Yeast -malt extract agar (ISP-2)	Good	Moderate gray(ISCC-NBS 265)	Dark yellow(ISCC-NBS 88)	None
Oat meal agar (ISP-3)	Good	Light Gray(ISCC-NBS 264)	Pale yellow(ISCC-NBS 89)	None
Inorganic-trace salt- starch agar (ISP-4)	Good	Moderate gray(ISCC-NBS 265)	Pale yellow (ISCC-NBS 89)	None
Glycerol asparagine agar (ISP-5)	Good	Light Gray(ISCC-NBS 264)	Pale greenish yellow(ISCC-NBS 104)	None
Peptone yeast extract iron agar (ISP-6)	No growth	--	--	None
Tyrosine agar (ISP-7)	Moderate	Deep gray(ISCC-NBS 266)	Dark yellow ISCC-NBS 88)	None
Starch nitrate agar	Good	Moderate gray(ISCC-NBS 265)	Pale greenish yellow(ISCC-NBS 104)	None
Nutrient agar	Weak	White	Colourless	None

RESULTS AND DISCUSSION

Taxonomic Characterization of the Actinomycete Isolate, NSQu-25

Conventional Taxonomy: Micro-morphological studies of actinomycete isolate, NSQu-25 through light microscopy (x400) and Scanning Electron Microscope (x6.500) revealed that, the spore chains of the strain were spirals with spiny spore surface (Fig. 1a, b). The entire hydrolysate cell of this strain contained LL-diaminopimelic acid (LL-DAP) and glycine indicating that, the strain has a chemo-type I cell wall but no characteristic sugars could be detected. Cell-wall composition analysis is one of the main methods that can be employed to identify the chemotaxonomic characteristics of *Streptomyces*; the presence of LL-DAP in the cell wall also signifies that this strain is *Streptomyces* [29]. The cultural characteristics of actinomycete isolate, NSQu-25 grown on different ISP and non-ISP media (Table 1) showed that the aerial hyphae of the strain was gray. Therefore, it was assigned to the gray series with slight yellow substrate mycelium also no diffused pigments were recorded for this isolate. The physiological and biochemical properties; C- and N-sources utilization, tolerance to NaCl, growth pH, growth temperature, growth inhibitors and sensitivity to antibiotics were studied (Table 2). The cultural and physiological properties of the isolated strain were compared to those reported for actinomycetes as described in Bergey's Manual of Determinative Bacteriology [30].

16S rRNA Gene Sequencing and Phylogenetic Analysis: To confirm the identification of the isolated strain, the 16S rRNA sequence of the local isolate, NSQu-25 was compared to sequences of 10 *Streptomyces* spp. through multiple sequence alignment. The primer pair, F27/R1492 was used to amplify the fragments of the genomic DNA's expected size (1500 bp); this DNA was isolated from the positive control strain *Streptomyces griseus* ATCC 10137. This primer pair was especially used to amplify the 27-bp and 1492-bp fragments. Experimental analysis of the PCR

Table 2: Morphological, physiological and biochemical characteristics of *Streptomyces mirabilis*, NSQu-25

Character	Results	Character	Results
Morphological characteristic	N-source utilization	--	
Spore chain	Spiral	L-Asparagine	++
Spore mass	Light gray	L-Glutamic	+
Spore surface	Spiny	L-lysine	++
Motility	Non motile	L-Ornithine	+
Color of substrate mycelium	Grayish	L-Tyrosine	+
Diffusible pigment	None	L-Valine	Wg[f]
Cell wall hydrolysis	--	L-Histidine	-
Diaminopimelic acid (DAP)	LL-DAP	Glycine	+++
Sugar pattern	ND[a]	L-leucine	+
Physiological characteristics	--	L-Phenyl alanine	-
Melanin pigment:	--	L-Serine	+++
Peptone-yeast extract iron agar	-[b]	L-Methionine	-
Tyrosine agar	-	L-Cysteine	++
Tryptone-yeast extract broth	-	Tolerance to NaCl (%)	
Hydrolysis of:	--	3:6	+++
Protein	+[c]	7:10	++
Starch	+	11	+
Lipid	+	12	Wg[f]
Catalase production	+	13	-
H_2S production	-	Growth inhibitors	
Nitrate reduction	+	Crystal violet (0.0001%)	-
Tyrosine degradation	+	Crystal violet (0.0002%)	+
Urea test	-	Sodium azide (0.02)	+
Utilisation of C-source		Phenol (0.1%)	+
D-Glucose	+++[d]	Growth temperature °C	
D-Mannose	++ [e]	10	-
D-Mannitol	++	25:37	+
D (+) trehalose	+	Growth pH	
L-Rhamnose	-	5:7	+++
D-Fructose	+	8	++
Meso-Inositol	+	9	Wg
D-Galactose	-	Sensitivity to antibiotic	--
Sucrose	+	Rifampicin (50 ig /mL)	-
Maltose	-	Erythromycin (15 ìg /mL)	+
Starch	+++	Penicillin (10 ìg/mL)	+
Cellulose	+	--	--
Salicine	-	--	--
L(+)Arabinose	++	--	--
Raffinose	-	--	--

[a]ND= not detected, [b](-) = negative, [c](+) = moderate, [d](+++) = abundant, [e](++) = good growth,,[f](wg)= weak growth

amplification was studied through agarose gel electrophoresis (Fig. 2). The results obtained are in agreement with those of Edwards [26] who found that these primers are specific for bacteria.

The alignment of the nucleotide sequences (540 bp) of *Streptomyces mirabilis*, NSQu-25 was done through matching with the 16S rRNA reported genes sequences in the gene bank. The phylogenetic tree was derived from the distance matrices using a neighbor-joining method (Fig. 3). Computer assisted DNA similarly searches against bacterial database revealed that, 16S rRNA sequence was 99% identical to *Streptomyces mirabilis* Uz1010 and then designated *Streptomyces mirabilis* strain NSQu-25. Modern *Streptomyces* identification systems are based on 16S rDNA sequence data, which have provided invaluable information about *Streptomyces* systematic and then have been used to identify several newly isolated *Streptomyces* [31].

In conclusion, the phylogenetic analysis coupled with a conventional identification of the local isolate, NSQu-25 indicated that, the most closely related strain is *Streptomyces mirabilis* Therefore, *Streptomyces mirabilis* NSQu-25 was proposed as its name.

Spectroscopic Studies on the Major Bioactive Compound: In the present work, the antibiotic produced by the local isolate, is a major bioactive constituent isolated from the culture filtrate and its structure was determined via spectroscopic techniques. The compound was isolated as a colorless oily liquid. It is soluble in ethanol, ethyl

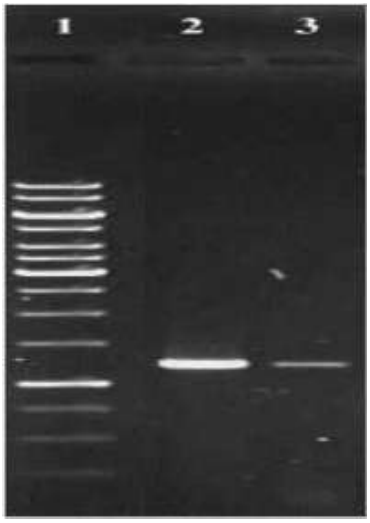


Fig. 2: Agarose gel electrophoresis of PCR products obtained by amplification of DNA mixtures isolated from 3 *Streptomyces* species. Lan 1: molecular weight marker 100 bp lader; Lan 2: Isolate NSQu-25; Lan 3: Positive control (*S. griseus* ATCC 10137).

acetate, chloroform and n-hexane, but insoluble in water. The isolated compound gave positive reactions (purple color) with concentrated H_2SO_4 and 0.5% solution of vanillin in methanol/sulphuric acid/acetic acid (2:1:1), but not with ninhydrin, $FeCl_3$ and $SbCl_3$ reagents on TLC. IR spectrum (Fig. 4) showed peaks at í 2981, 2936, 2868, 1731, 1124 and 1074 cm^{-1} which revealed presence of a carbonyl and strong C-O bands. The GLC analysis indicated the presence of a single major component; its retention time is 25.19 min (Fig. 5a). The molecular formula was established as $C_{24}H_{38}O_4$ based on EIMS (Fig. 5b) that showed the presence of molecular ion (M^+) at *m/z* 390 Dalton, other important ions were detected at *m/z* 279, 167 and 149 (base peak). The chemical formula of this compound suggested the presence of six double bond equivalents. The ^1H NMR spectrum (Fig. 6) showed aromatic protons at δ 7.68 (2 H, dd, *J*= 5.8, 3 Hz) and 7.54 (2 H, dd, *J*= 5.8, 3 Hz). The existence of two doublets, each representing two equivalent aromatic protons, suggested the presence of an *ortho*-disubstituted benzene ring bearing the same substituent in both positions. There was also 2 protons

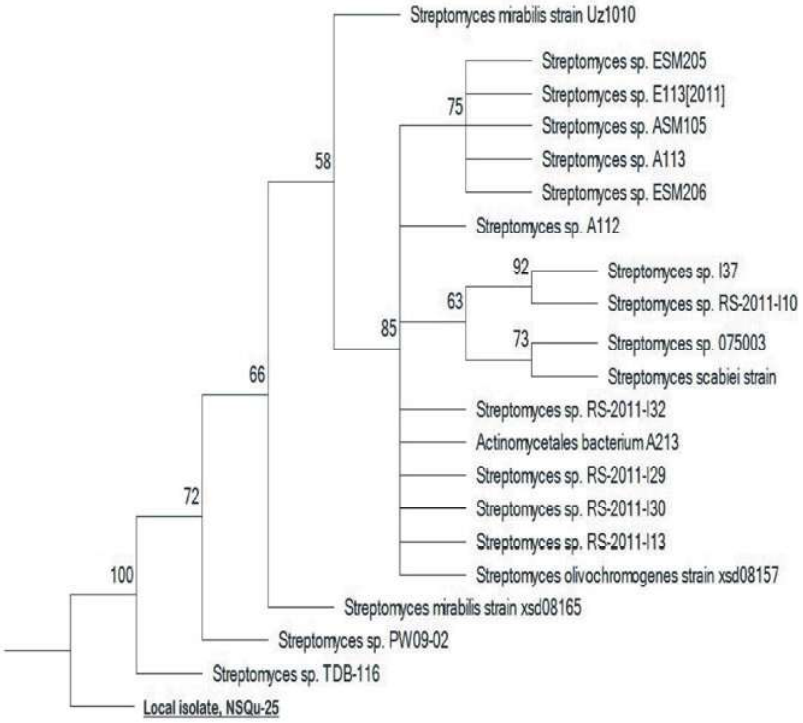
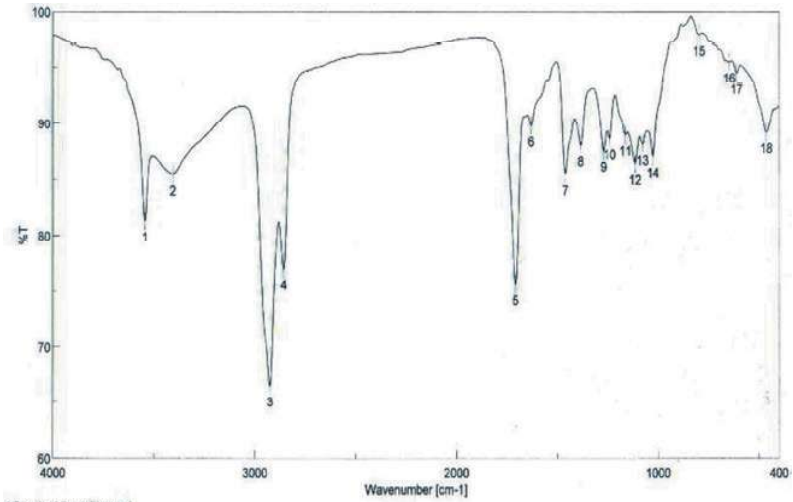


Fig. 3: The phylogenetic position of *Streptomyces mirabilis*, NSQu-25 among neighboring method showing 16S rDNA tree of the phylogenic similarity comparing with the sequences of other known *Streptomyces* species.



[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3544.52	81.3288	2	3405.67	85.4676	3	2925.48	66.4973	4	2857.02	76.9564
5	1708.62	75.5696	6	1631.48	99.7969	7	1461.78	85.5053	8	1384.64	88.0381
9	1270.86	87.5486	10	1241.93	88.6697	11	1162.87	88.9843	12	1115.62	86.4264
13	1078.01	88.1372	14	1026.91	87.025	15	798.385	97.9351	16	647.965	95.6028
17	611.324	94.5311	18	463.796	89.201						

Fig. 4: IR-Spectrum of di-(2-ethylhexyl) phthalate (DEHP) in Kbr.

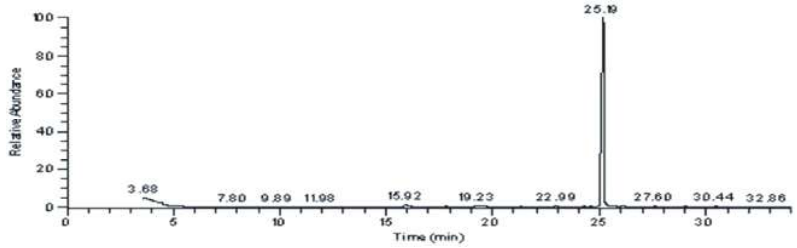


Fig. 5a: GLC chromatogram of di-(2-ethylhexyl) phthalate (DEHP).

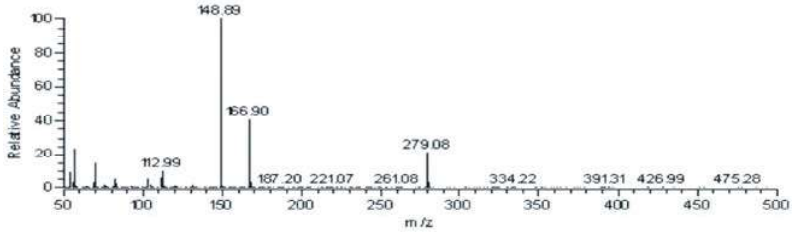


Fig. 5b: MS spectrum of di-(2-ethylhexyl) phthalate (DEHP).

multiplet at δ 4.21, for protons bonded to a C-O linkage. Additionally, the spectrum showed multiplets around δ 0.89-1.66 for a number of methylenes and methyl groups

From the aforementioned spectroscopic data, the molecular structure of the isolated compound was determined to be di-(2-ethylhexyl) phthalate (DEHP) (Fig.7). Phthalate compounds are petrochemicals used as plasticizers or solvents in a variety of industrial products. Nevertheless, many phthalate derivatives have been isolated from terrestrial and marine organisms including plants [32], fungal and bacterial culture broths, especially those belonging to the genus *Streptomyces* [33]. Di-(2-ethylhexyl) phthalate (DEHP) has been already described from *Streptomyces bangladeshiensis* [34]. Other phthalate derivatives have been isolated from *Streptomyces* species, such as the dibutyl phthalate [35, 36].

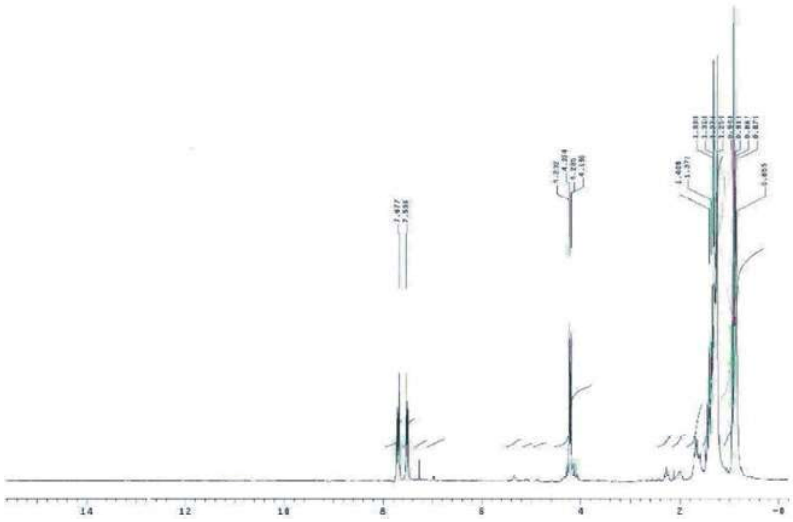


Fig. 6: ^{1}HNMR-Spectrum of di-(2-ethylhexyl) phthalate (DEHP) in $CDCl_3$.

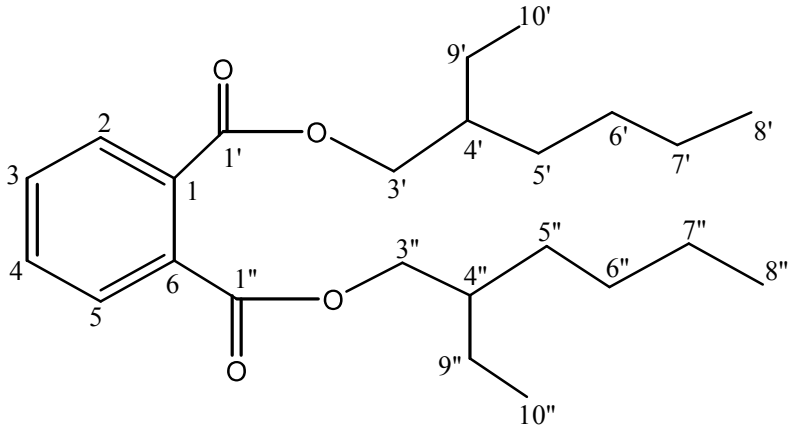


Fig. 7: The molecular structure of di-(2-ethylhexyl) phthalate (DEHP) produced by *Streptomyces mirabilis*, NSQu-25.

Table 3: Antimicrobial activities (MICs µg/ml) of di-(2-ethylhexyl) phthalate (DEHP) produced by *Streptomyces mirabilis*, NSQu-25.

Test organism		MIC (µg/ml)
1. Bacteria	*Bacillus subtilis* ATCC 6633	3.5
	Staphylococcus aureus ATCC 6538	1.47
	Streptococcus equosemens ATCC 12388	2.37
	Escherichia coli ATCC 7839	5.4
	Pseudomonas aeruginosa ATCC 9027	6.2
	Closteridium perfringens ATCC 3626	>50
2. Yeasts	*Candida albicans* ATCC 10231	1.2
3. Fungi	*Aspergillus niger ATCC 16404*	>100
	Aspergillus flavus ATCC16883	>100
	Aspergillus fumigatus	>100

Biological Activity of the Isolated Compound (DEHP)

Antimicrobial Spectrum: The minimum inhibitory concentrations (MICs) of the purified compound (Table 3) proved active against a number of Gram-positive bacteria; *Bacillus subtilis* ATCC 6633 with MIC 3.5 µg/ml, *Staphylococcus aureus* ATCC 6538 with MIC 1.47 µg/ml and *Streptococcus equosemens* ATCC 12388 with MIC 2.37 µg/ml but the inhibition of Gram negative bacteria was lower; *Escherichia coli* ATCC 7839 with MIC 5.4 µg/ml, *Pseudomonas aeruginosa* ATCC 9027 with MIC 6.2 µg/ml and *Closteridium perfringens* ATCC 3626 with MIC >50 µg/ml. On the other hand, the compound has strong effect against *C. albicans* with MIC 1.2 µg/ml whereas *A. niger* and *A. flavus* were even less susceptible to the compound. Antimicrobial activity of DEHP isolated from *Streptomyces avidinii* strain SB9 against various

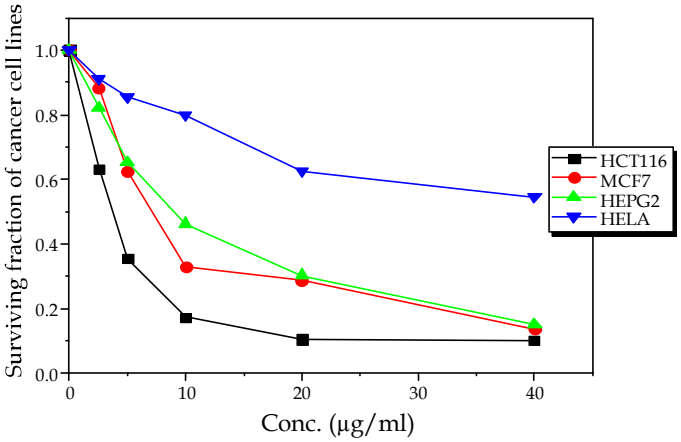


Fig. 8: Cytotoxicity profile of di-(2-ethylhexyl) phthalate (DEHP) against different human cancer cell lines.

microorganisms is in accordance with previous reports proving that DEHP is a biologically active compound [37, 38]. A potent antimicrobial activity was also found for DEHP isolated from the microorganism *Streptomyces bangladeshiensis* [34].

Cytotoxic Activities of (DEHP) Compound: The isolated compound was evaluated for its cytotoxic activity against number of human cancer cell lines (Fig. 8), DEHP exhibited strong cytotoxicity against human colon carcinoma cells (HCT 116) with an IC_{50} of 3.681 µg/ml and human breast carcinoma cells (MCF7) with an IC_{50} of 6.941 µg/ml. Its cytotoxicity is moderate against human liver carcinoma cells (HEPG2) with an IC_{50} of 9.028 µg/ml. On the other hand, it remained inactive against human cervix carcinoma cells (HELA). The DEHP is considered as pro inflammatory agent in other studies [39, 40]. The same compound was isolated from the plant *Aloe vera* and was found to have antileukemic and antimutagenic effects [32]. The 16S rRNA gene sequence of *Streptomyces mirabilis* NSQu-25 was compared to other *Streptomyces* species. It has been shown that the highest binary similarity value of 99% with the different *Streptomyces* species (Fig. 3) which was differed for them in the morphological characters and carbon utilization where the different detection limits of the methods make presence/absence comparisons difficult [13]. On the other hand, the binary similarity between this isolate and the known producers of DEHP from *Streptomyces* strains [33-36] was low. But this compound has not been reported to be produced by any of the *S. mirabilis* known strains. Therefore, *Streptomyces mirabilis* NSQu-25 is suggested to be new *Streptomyces* species.

CONCLUSION

In conclusion, the data obtained in the present work report, the isolation of di-(2-ethylhexyl) phthalate (DEHP) as a major bioactive compound produced in the culture filtrate of the newly isolated soil *Streptomyces* (*Streptomyces mirabilis* strain NSQu-25). Finding an organism with a potential to produce only one major bioactive compound provides a biotechnological advantage in the industrial downstream processing and lead to improvements in bioprocess control for existing products in large-scale fermentations.

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