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Isolation and Structural Confirmation of Bioactive Compounds Produced By the Strain *Streptomyces Albus* CN-4

B.Chinna Narasaiah[1],*V.Leelavathi[2], G.Sudhakar,[1] P.Mariyadasu,[3] G.Swapna[2], Anil Kumar. Manne[2].

1*Department of Botany & Zoology, S.V Arts & Science College, Giddaluru, A.P, India*
2Department of Biotechnology, Acharya Nagarjuna University, Guntur, A.P. India
3Department of Zoology, Acharya Nagarjuna University, Guntur, A.P. India

Abstract: *Actinomycetes have the potential to produce most promising secondary metabolites like antibiotics, anticancer drugs, immunosuppressors and enzyme inhibitors. Out of 10 actinomycetes strains isolated from two different media, one predominant strain designated as CN-4 was chosen for bioactive metabolite production. The fermentation was carried out in 1L Roux bottles for 120 h at 30 °C. The culture filtrate (30L) was extracted twice with an equal volume of ethyl acetate, pooled and the organic layer was concentrated in a Rotavac. The deep brown semi solid compound was extracted for structural elucidation. GC-MSD Analysis revealed that strain CN-4 was produce 14 fractions. The structure of all these fractions was analyzed on the basis of GC-MSD analysis.*

Keywords: *GC-MSD Analysis, Bioactive metabolites, Actinomycetes, Anticancer drugs*.

I. Introduction

Actinomycetes have been useful to the pharmaceutical industry for their unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities. The practical importance of antibiotics and other secondary metabolites is tremendous. They are broadly used in the human therapy, veterinary, agriculture, scientific research and in countless other areas. Remarkably, the vast majority of these compounds are derived from the single actinomycete genus Streptomyces, raising the intriguing possibility that additional chemically prolific taxa await their discovery. Discoveries of important, novel bioactive compounds depend upon the development of objective strategies for the isolation and characterization of novel and rare micro-organisms for existing and new screens [1]. Although soils have been screened by the pharmaceutical industry for about 50 years, only a miniscule fraction of the surface of the globe has been sampled, and only a small fraction of actinomycete taxa have been discovered [2,3]. A very frightening consequence of indiscriminate use, over prescription of antibiotics is the development of antibiotic resistant bacteria. According to the World Health Organization, the emergence rate of drug resistant strains is quicker than the rate of discovery of new drugs and antibiotics. The multiple drug resistance problem demands that a renewed effort be made to seek antimicrobial agents effective against these MDROs. Polyenes like amphoterecin B, nystatin and natamycin are widely used for the treatment of candidiasis, coccidiodal meningitis, cutaneous dermatophytes and histoplasmosis. Wide spread use of the limited numbers of antifungal agents for control of mycotic diseases also led to the development of drug resistance. The life saving drugs used for treating the bacterial, fungal and candidal infections at present mostly are the products of terrestrial actinomycetes. By this it is apparent that continuous research on terrestrial actinomycetes may result in the isolation of novel actinobacterial species as well as bioactive metabolites active against MDROs. Proving their excellence in the field of medicine, actinomycetes extend their contribution towards agriculture. Fungal diseases in agriculture crops pose a great challenge. Fungicides are extensively used to ensure crop quality and production. The currently used fungicides have lethal effects that are not restricted to their target species and their application may have negative impact on organisms that benefit the wider agro-ecosystem. Hence, it is necessary to develop environmentally safer and potent fungicides of natural origin. Microbial metabolites have been expected to minimize the deleterious side effects caused by synthetic fungicides. These metabolites are degraded within a month or even in a few days when exposed to natural environmental conditions, thus leading to low residual levels. The biological control of fungal pathogens causing plan diseases using extracellular metabolites of Streptomyces was previously reported [3,4,5,6,7]. Numerous environmental factors including nutrients (nitrogen, phosphorous and carbon sources), growth rate, enzyme inactivation and other physical conditions (oxygen supply, temperature and pH) are require to biosynthesis of the secondary metabolites [8].

II. Materials And Methods

Isolation and screening of actinomycetes strains from laterite Soil sample Laterite soil sample collected for the isolation of potent actinomycetes strains was initially analysed for physico-chemical properties such as moisture content (%), pH, organic carbon (%) and total nitrogen content (%).

2.1 Isolation of actinomycetes strains

Laterite soil sample collected from Kandukuru, Prakasam (Dist) a depth of 5 – 8 cm was pretreated with calcium carbonate (1:1 w/w) and dried at 45oC for 1 h in order to reduce the abundance of bacteria and fungi [10]. Yeast extract malt extract dextrose agar (YMD) and starch casein salts agar media were prepared, sterilized at 15 lbs pressure (120°C) for 15 min and poured into Petri plates under aseptic conditions. Both streptomycin (50 µg/ml) and nystatin (50 µg/ml) were added to the media just before pouring into Petri plate. Soil dilution plate technique was employed for isolation of actinomycete strains [11]. The pretreated soil (1 g) sample was suspended in 100 ml of sterile distilled water. Serial dilutions were prepared and 0.1ml of 10-3 and 10-4 dilutions were plated on media with the help of a spreader. The inoculated plates were incubated at 30°C for 7-14 days. After incubation, actinomycete colonies were isolated from soil. Streak plate method was used to purify the cultures of actinomycete strains. The colonies were picked with the loop according to the condition. The picked up specks of the colonies were streaked over YMD agar medium followed by incubation at 30oC for 7 days. Further, pure cultures were maintained on YMD agar slants and stored at 4oC for further study[11].Screening of predominant actinomycete strain for bioactive metabolites Out of 10 actinomycete strains isolated from two different media, one predominant strain designated as CN-4 was chosen for bioactive metabolite production. The secondary metabolites produced by actinomycete strain were extracted by the method of [12]. The pure culture of the strain was transferred aseptically into the seed medium (ISP-2 broth). After 24 h of incubation, the seed culture at a rate of 10% was inoculated into the production medium of ISP-2 broth. Fermentation was carried out at 30°C for 1 week under agitation at 180 rpm. At every 24 h interval, the flasks were harvested and the biomass was separated from the broth. The dry weight of the biomass was recorded and expressed as g/100ml. The culture filtrate was extracted twice with ethyl acetate and the pooled solvent extracts were concentrated under vacuum to yield a crude extract. The residue dissolved in methanol was used for extraction of bioactive compounds.

2.2 Extraction and structural confirmation of bioactive compounds produced by the strain CN-4

For the large scale production of bioactive compounds from the strain, 10 % of seed broth was inoculated into the optimized production medium (dextrose @ 5g, yeast extract @ 5g, malt extract @ 10g, sodium chloride @ 10g dissolved in one liter distilled water and adjusted to pH 7.0) for the enhanced secondary metabolite production. The fermentation was carried out in 1L Roux bottles for 120 h at 30 °C.

2.3 Isolation and identification of bioactive compounds

The bioactive compounds from the fermented broth was harvested by filtration of biomass through Whatman filter paper no. 42 (Merck, Mumbai, India). The culture filtrate (30L) was extracted twice with an equal volume of ethyl acetate, pooled and the organic layer was concentrated in a Rotavac. The deep brown semi solid compound was extracted for structural elucidation.

III. Results And Discussion

Extraction, structural elucidation and biological evaluation of bioactive metabolites produced by Streptomyces sp. CN-4 For the production of bioactive compounds, seed broth (10%) was inoculated into the optimized production medium. The fermentation was carried out in 1L Roux bottles for 120 h at 30°C. The harvested broth (30L) extracted with ethyl acetate was concentrated in a Rotavac. The deep brown concentrated semi solid compound (4g) obtained served as the crude antimicrobial compound.

3.1 Isolation of bioactive compounds from crude extract

The most important task of the present study is to extract, isolate and elucidate the structures of the bioactive compounds produced by the strain Streptomyces albus CN-4. Seed broth (10%) was inoculated into the optimized production medium for the production of bioactive compounds. The fermentation was carried out in 1L Roux bottles incubated at 30°C and terminated after 120 h. The harvested broth (30L) extracted with ethyl acetate was concentrated in a Rotavac. The deep brown concentrated semi solid compound (4 g) obtained served as the crude antimicrobial compound. Structural elucidation was done in the LUCID laboratories Pvt. Ltd. Balanagar, Hyderabad. GC-MSD Analysis revealed that strain CN-4 was produce 14 fractions. The structure of all these fractions was analyzed on the basis of GC-MSD analysis. compounds such as -3,5,6-trychloropyridine, Dichloro acetic acid, 4-hexadecylester. Phenol,4(1,1,3,3, tetramethylbutyl), Phenol,4(1,1,3,3,tetramethylbutyl), 1- Nonadecene,Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2- methylpropyl), Pyrrolo[1,2-a]pyrazine-1,4-

dione,hexahydro-3-(2-methylpropyl),2,5-Piperazinedione,3,6-bis(2-methylpropyl), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl), 1,2-Benzenedicarboxylic acid, diisooctyl ester.

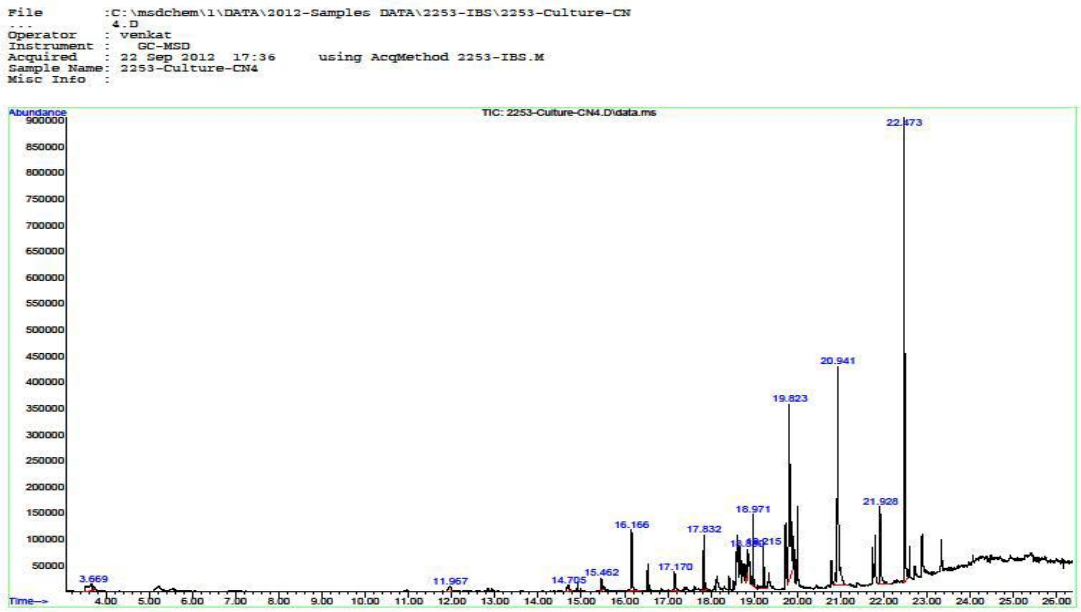


Fig: 1 GC-MSD Spectrum of Streptomyces albus CN-4

3.2 Physico- chemical properties and of bioactive compounds

3.2.1 Butyric acid

Butyric acid, also known under the systematic name butanoic acid, is a carboxylic acid with the structural formula CH3CH2CH2- COOH. Salts and esters of butyric acid are known as butyrates. Formula: C4H8O2, Boiling point: 163.5 °C Density: 959.50 kg/m³,Molar mass: 88.11 g/mol, Melting point: -7.9 °C, CAS: 107-92-6; Butyric acid is used in the preparation of various butyrate esters. As a consequence, they find use as food and perfume additives. It is also used as an animal feed supplement, due to the ability to reduce pathogenic bacterial colonization [13]. The use of butyrate changes differs between normal and cancerous cells Which is called as the "butyrate paradox. A review suggested that the chemo- preventive benefits of butyrate depend in part on amount, time of exposure with respect to the tumorigenic process, and the type of fat in the diet. The production of volatile fatty acids such as butyrate from fermentable fibers may contribute to the role of dietary fiber in colon cancer[14].

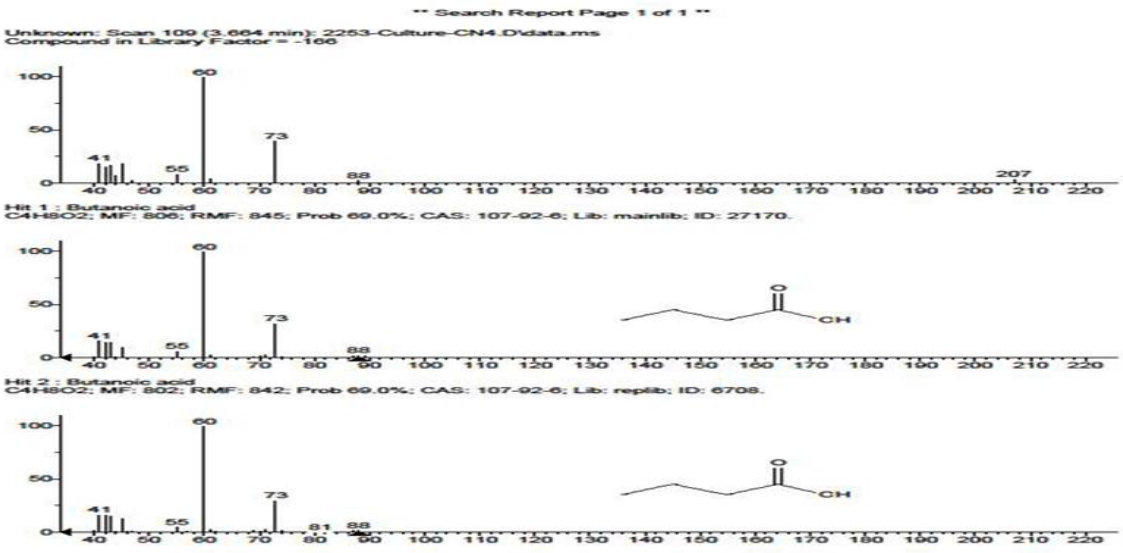


Fig: 2 Mass spectrum and chemical structure of Butanoic acid by Streptomyces albus CN-4

3.2.2 Hexanoic acid

Hexanoic acid also known as caproic acid, general formula C5H11COOH and CAS: 142-62-1. It is a colorless oily liquid with an odor that is fatty, cheesy, waxy, and like that of goats or other barnyard animals (Merck, 1989). It is a fatty acid found naturally in various animal fats and oils, and is one of the chemicals that give the decomposing fleshy seed coat of the ginkgo its characteristic unpleasant odor. The primary use of hexanoic acid is in the manufacture of its esters for artificial flavors, and in the manufacture of hexyl derivatives, such as hexylphenols. (Merck, 1989). The salts and esters of this acid are known as hexanoates or caproates. Caproic, caprylic, and capric acids (capric is a crystal- or wax-like substance, whereas the other two are mobile liquids) are not only used for the formation of esters, but also commonly used "neat" in: butter, milk, cream, strawberry, bread, beer, nut, and other flavors.

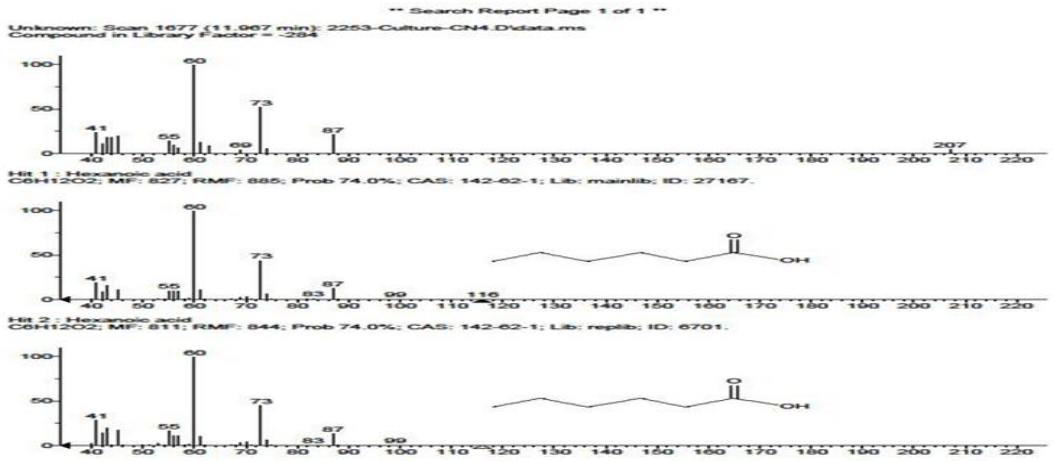
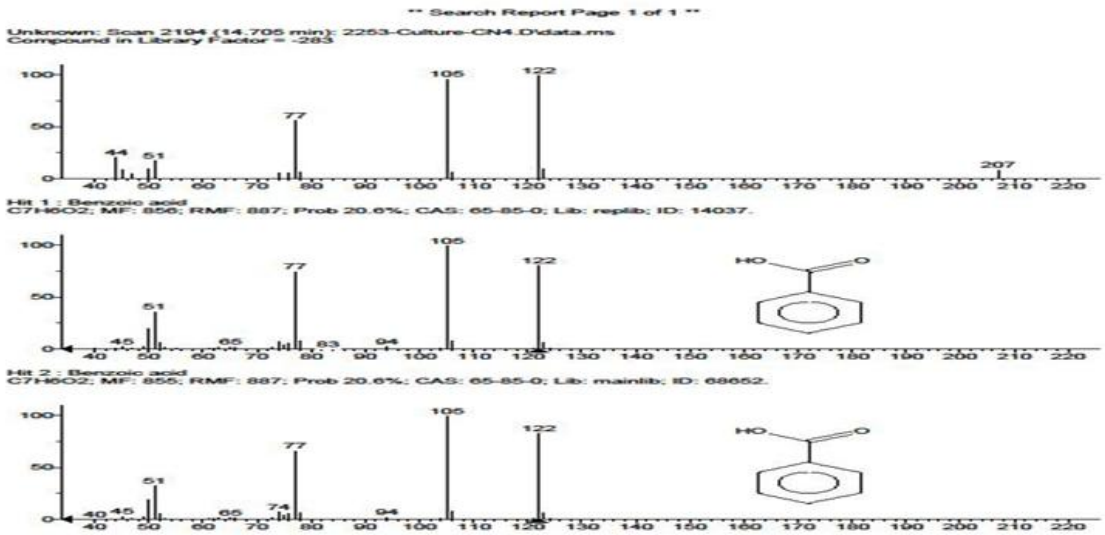


Fig: 3 Mass spectrum and chemical structure of Hexanoic acid by Streptomyces albusCN-4

3.2.3Benzoic acid

Benzoic acid was discovered in the sixteenth century. The dry distillation of gum benzoin was first described by Nostradamus (1556), and then by Alexius Pedemontanus (1560) and Blaise de Vigenère (1596). Formula: C7H6O2, CAS: 65-85-0. Benzoic acid and its salts are used as a food preservatives. It inhibits the growth of mold and yeast and some bacteria. It is either added directly or created from reactions with its sodium, .potassium, or calcium salt. The efficacy of benzoic acid and benzoate is thus dependent on the pH of the food. Acidic food and beverage like fruit juice (citric acid), sparkling drinks (carbon dioxide), soft drinks (phosphoric acid), pickles (vinegar) or other acidified food are preserved with benzoic acid and benzoates. Benzoic acid is a constituent of Whitfield's ointment which is used for the treatment of fungal skin diseases such as tinea, ringworm, and athlete's foot. As the principal component of benzoin resin, benzoic acid is also a major ingredient in both tincture of benzoin and Friar's balsam.



Fig; 4 Mass spectrum and chemical structure of Benzoic acid by Streptomyces albus CN-4.

3.2.4 Benzeneacetic acid

Also known Phenylacetic acid (abr. PAA and synonyms are: α- toluic acid, benzeneacetic acid, alpha tolylic acid, 2-phenylacetic acid) is an organic compound containing a phenyl functional group and a carboxylic acid functional group. It is a white solid with a disagreeable odour. Because it is used in the illicit production of phenylacetone. Molecular formula C8H8O2 , CAS: 103-82-2. Phenylacetic acid is used in some perfumes, possessing a honey-like odour in low concentrations, and is also used in penicillin G production. It is also employed to treat type II hyperammonemia to help reduce the amounts of ammonia in a patient's bloodstream by forming phenylacetyl-CoA, which then reacts with nitrogen-rich glutamine to form phenylacetylglutamine.

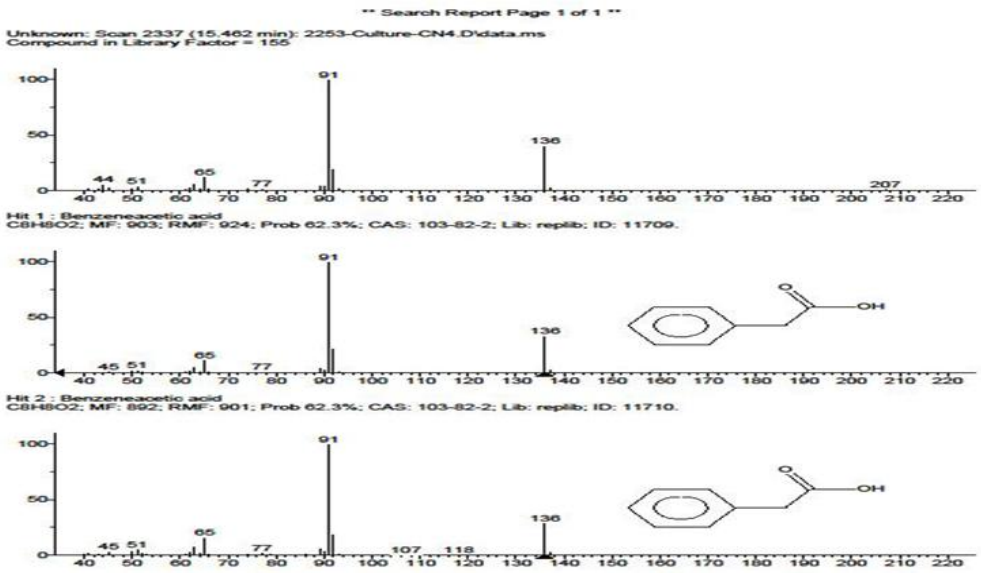


Fig: 5 Mass spectrum and chemical structure of Benzeneacetic acid by Streptomyces albus CN-4

3.2.5 Benzenepropanoic acid

Molecular formula C9H10O2; CAS: 501-52-0, Molecular Weight: 150.1745. Also known as hydrocinnamic acid, 3-Phenylpropanoic acid, Benzylacetic acid, Dihydrocinnamic acid, Benzenepropanoic acid, Benzenepropionic acid. Benzenepropanoic acid is widely used for flavoring, food additives, spices, fragrance, and medicines as it acts as a fixative agent, or a preservative. And used in the food industry to preserve and maintain the original aroma quality of frozen foods. It can also be used to add or restore original color to food. Shelved foods are protected from microorganism by adding Benzenepropanoic acid to prevent deterioration to the food by microorganisms as well as acting as an antioxidant to prolong shelf life foods. Benzenepropanoic acid is also added to food for technological purposes in a wide variety including manufacturing, processing, preparation, treatment, packaging, transportation or storage, and food additives.

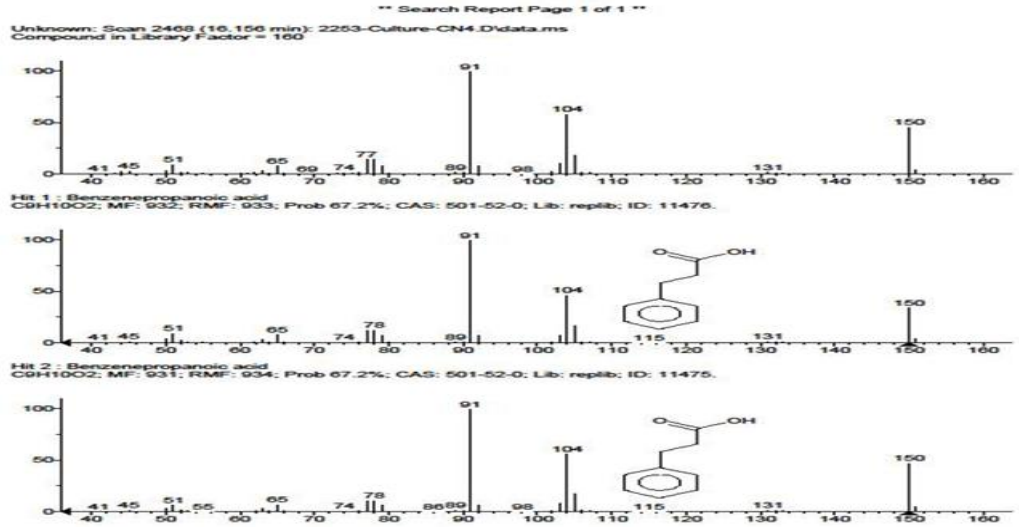


Fig: 6 Mass spectrum and chemical structure of Benzenepropanoic acid (Hit-1 and 2) by Streptomyces albus CN-4.

3.2.6 1, 2-Benzenedicarboxylic acid, diisooctyl ester

Molecular formula is C24H38O4, CAS: 27554-26-3, 1,2- Benzenedicarboxylic acid, diisooctyl ester also called as Bis(6- methylheptyl) benzene-1,2-dicarboxylate . Molecular Weight 390.62, appearance Oily colorless liquid with a slight ester odor.Insoluble in water, Melting Point -4, Boiling Point 370, Used as Plasticizer for poly vinyl chloride jackets for building wire.

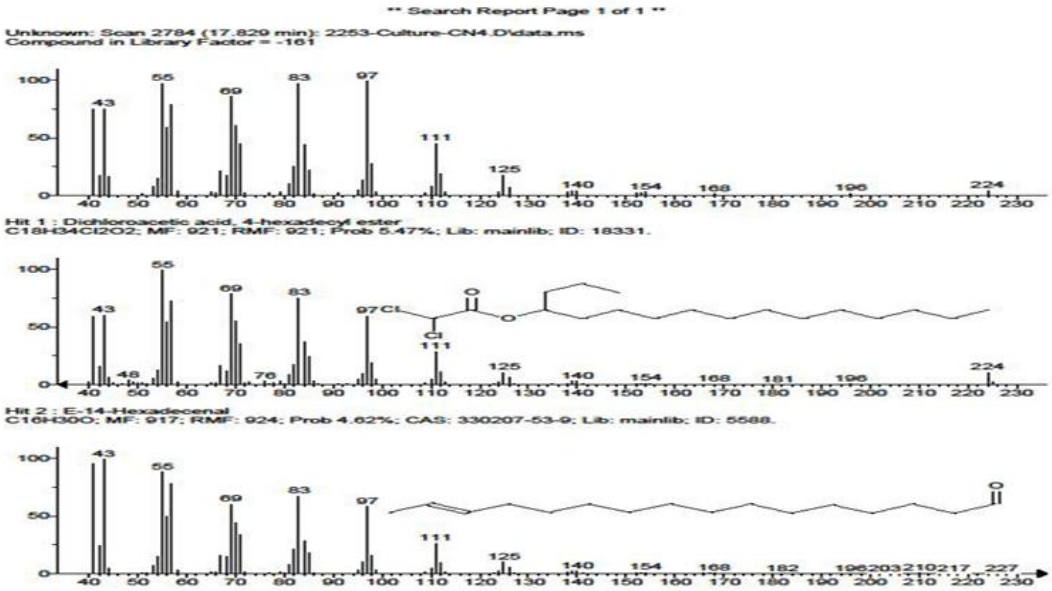


Fig: 7 Mass spectrum and chemical structure of Dichloroacetic acid,4-hexadecy ester(Hit-1) and E-14 Hexadecenal(Hit-2) by Streptomyces albus CN-4

IV. Conclusion

In the present work demonstrated the production of bioactive compounds of a predominant actinomycete strain CN-4 isolated from laterite soil. The strain produced different bioactive metabolites structurally confirmed on the basis of mass spectroscopy techniques, proving their excellence for the production of natural products with wide pharmaceutical magnitude.

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