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Rare actinobacteria *Nocardiopsis lucentensis* VLK-104: Optimization of cultural conditions and GC-MS analysis of bioactive metabolites

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Abstract:

An attempt was made to enhance the bioactive metabolites by *Nocardiopsis lucentensis* VLK-104 under optimized conditions and evaluation of its bioactive metabolites by GC-MS analysis. Soil dilution plate technique was employed for the isolation of the strain on Starch-Casein agar (ISP-6) medium. *Nocardiopsis lucentensis* VLK-104 isolated from South Coast of Andhra Pradesh, India. The strain exhibited good antimicrobial activity against Gram-positive, Gram-negative and fungi. Five-day old culture exhibited maximum antimicrobial activity when cultured at pH 7.0 and temperature 30°C. Production of bioactive compounds by the strain varied significantly with different carbon and nitrogen sources. The productivity of the strain VLK-104 was enhanced by amending with carbon (Mannitol) and nitrogen (Peptone) sources @0.4% and 0.5% and K_2HPO_4(0.05%). As the strain exhibited good antimicrobial activity, it may be explored for biotechnological purposes. The crude ethyl acetate extract was analyzed by Gas Chromatography-Mass Spectroscopy. It reveals the metabolites produced by the strain and evidenced the presence of 41 diversified compounds according to the available library data, NIST MS Search (ver. 2.0). Hence it could be a possible source for investigate novel bioactive compounds by employing modern biotechnological aspects.

Keywords: Mangrove ecosystem, *Nocardiopsis lucentensis*, Optimization of Culture conditions, Bioactive compounds, GC-MS analysis

1. INTRODUCTION:

Microorganisms in extreme environmental conditions are prolific producers of numerous bioactive compounds due to adaptation of different conditions. Actinobacteria are well known for their capacity to produce bioactive metabolites with complex diversity and various biological activities [1]. The mangrove ecosystem exists between terrestrial and marine environments that supports rich and diverse group of microorganisms. There may be no other group of plants with such highly developed morphological, biological, ecological and physiological adaptations to extreme conditions [2]. Microorganisms

existed under extreme conditions, eventually adapted for their survival and evolved to produce novel bioactive secondary metabolites[3].

Actinobacteria are saprophytic, free living, Gram positive bacteria widely distributed in different habitats, frequently filamentous and sporulating with DNA rich in G+C (55-75%). Actinobacteria are classified into two groups, namely *Streptomyces* or non-*Streptomyces* or rare actinobacteria [4]. Rare actinobacteria have been considered as actinobacteria with lesser exploration for bioactive metabolites [5]. Now a days pathogens were developing resistance to traditional antibiotics [6&7]. There is an urgent need to find safer and more effective compounds with broader spectrum of bio-activity [8]. There are evidences that bioactive compounds with unique structures have been isolated from mangrove actinobacteria in recent years [9]. Cultural conditions and media constituents are the significant factors for influencing the high yield of bioactive compounds. Hence the present work was designed to study the optimization of cultural conditions for the production of bioactive metabolites can be attained by different parameters are influenced the high yield of secondary metabolites by the strain *Nocardiopsis lucentensis* VLK-104.

2. MATERIALS AND METHODS:

2.1 Isolation of the strain:

The strain *Nocardiopsis lucentensis* VLK-104 was isolated from sediment soil samples of Mangrove ecosystem of Krishna District, Andra Pradesh, India, by using the soil dilution plate technique on Starch-Casein agar (ISP-6) medium with pH 7.0 [10]. The pure culture was maintained on ISP-2 agar medium at 4^{o}C. The strain has been deposited in the NCBI GenBank with the accession No. KF317772 [11].

2.2. Test microorganisms

The strain VLK-24 was tested against one day old test bacteria Bacillus *subtilis* (ATCC 6633), *B. megaterium* (NCIM2187), *Staphylococcus aureus* (MTCC 3160*), Xanthomonas campestris* (MTCC 2286), *Proteus vulgaris (*MTCC 7299*), Streptococcus mutans (*MTCC 497*).* Gram negative bacteria *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae (*ATCC10031*), Vibrio parahaemolyticus* (ATCC 43996) and fungi like *Aspergillus niger, A. flavus, Fusarium oxysporium (*MTCC 3075) and *Candida albicans* (ATCC 10231) by agar well diffusion assay[12&13].

2.2 Effect of incubation period on growth and bioactive metabolite production

The incubation period and bioactive metabolite production of the strain VLK-104 was studied at regular intervals up to eight days. two-day old culture of the strain was used as seed medium (ISP-2 broth) and then inoculated into 500 ml flasks containing 250 ml YMD broth. The fermentation process was carried out for one week under agitation at 180 rpm. At every 24h interval, the flasks were harvested and bioactive compounds production was determined in terms of their antimicrobial activity [13]. The culture filtrate was extracted with ethyl acetate. The extracts were concentrated and 50µl of crude extract was used for antimicrobial activity.

2.3 Optimization of cultural parameters

To enhance the compounds by the strain *Nocardiopsis lucentensis* VLK-104 was grown under different conditions such as pH, temperature, different culture media, carbon sources, nitrogen sources and minerals. The effect of initial pH on the bioactive metabolite production was determined by altering pH of the production medium from 4 to 10. The optimal pH achieved at this step was fixed for further study [14]. Similarly, the optimum temperature for bioactive metabolite production was determined by incubating the strain at different temperatures ranging from 20 to 40°C, while maintaining all other conditions at optimum levels [15]. The influence of culture media on the production of bioactive metabolites was studied by culturing the strain separately in ten different media such as ISP-1, ISP-2, ISP-3, ISP-4, ISP-5, ISP-6, ISP-7, Czepak-dox, nutrient broth and yeast-starch broths.

The impact of carbon sources on bioactive metabolite production was determined by supplementing the production medium (YMD) with different carbon sources such as fructose, glucose, lactose, starch, maltose, mannitol, sucrose, xylose, cellulose and galactose each at a concentration of 0.4% (w/v) to the optimized media by replacing their carbon source and keeping the other ingredients constant [16]. Influence of varying concentrations of the best carbon source (0.2–1.5% w/v) on bioactive metabolite production was also studied. Likewise, the effect of various nitrogen sources such as yeast extract, sodium nitrate, proline, tryptophan, peptone, cysteine, alanine, tyrosine, urea and histidine were studied by adding (0.4%) to the medium with an optimized carbon source. Further, the optimal levels of the suitable nitrogen source (0.2–1.5% w/v) for high yields of bioactive metabolites were also recorded [17]. To assess the effect of mineral salts, the optimized medium containing the carbon and nitrogen source was added separately with mineral complements such as $ZnSO_4$, $MgSO_4$, $FeSO_4$, K_2HPO_4 and KH_2PO_4.

2.4 Antimicrobial activity under optimized conditions

The strain *Nocardiopsis lucentensis* VLK-104 was grown under optimized conditions for 120 h. The culture broth separated by sterile Whatman filter (No.1) paper was extracted with ethyl acetate and dried in a rotary evaporator at 40°C. The antimicrobial activity of the compounds produced by the strain VLK-104 was done by agar well diffusion method [18]. Nutrient agar and Czapek-Dox agar media were used for culturing the test bacteria and fungi respectively. Ethyl acetate extract (50µl) was added to each well and only ethyl acetate served as control. The plates were incubated at 30°C and the diameter of the inhibition zone was measured after 24 h of incubation for bacteria and 24-72h for yeast.

2.5 Extraction and characterization of bioactive metabolites

For extraction of bioactive compounds, actively growing pure culture of the strain VLK-104 was inoculated into the optimized production medium (ISP-2). Composed of mannitol (0.4%), peptone (0.5%), malt extract (1%), calcium carbonate (0.2%) (pH 7.0) and K_2HPO_4(0.05%). The flasks were incubated on a rotary shaker (180 rpm) at 30^{o}C for 5 days. After five days, the fermentation broth attained was extracted with ethyl acetate and concentrated in roto-evaporator to yield a crude extract of the strain (2 g). The secondary metabolites of the ethyl acetate extract produced by the strain *Nocardiopsis lucentensis* VLK-104 was analyzed on Agilent GC–MS system (GC: 5890 series II; MSD 5972). The fused-silica HP-5 capillary column (30 m×0.25 mm, ID, film thickness of 0.25 µm) was directly coupled to the MS. The carrier gas was helium with a flow rate of 1.2 mL/min. Oven temperature was programmed (50°C/min, then 50–280°C @ rate of 5°C/min) and subsequently, held isothermally for 20 min. The temperature of the injector port was maintained at 250°C and that of detector at 280°C. The peaks of components in gas chromatography were subjected to mass spectral analysis. The spectra were analyzed from the available library data, NIST MS Search (ver. 2.0) (included with NIST'02 mass spectral library, Agilent p/n G1033A).

2.6 Statistical analysis:

The results of *Nocardiopsis lucentensis* VLK-104 under different cultural conditions were statistically analyzed with one -way analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION:

3.1 Growth pattern and bioactive compounds production by the strain VLK-104

The growth pattern of *Nocardiopsis lucentensis* VLK-104 was studied on ISP-2 broth. The strain entered logphase after 24 h and showed exponential growth upto 96 h followed by stationary phase extended up to 120 h. The bioactive compounds obtained from 5-day-old culture exhibited good antimicrobial activity against the test microorganisms (Fig. 1).

The bioactive compounds of 5-day-old culture produced by *Nocardiopsis* sp. [19], *Nocardiopsis halotolerans* VJPR-2 [20], *Rhodococcus erythropolis* VL-RK_5 [21], *Streptomyces cellulosae* VJDS-7 [22], *Rhodococcus erythropolis* VLK-12 [23], were exhibited high antimicrobial activity against Gram-positive as well as Gram-negative bacteria and fungi. Whereas the metabolites reported from 4-day-old cultures of *Nocardia metallicus* VJSY-14 [24], *Arthrobacter kerguelensis* VL-RK_09 (Rajesh kumar *et al*., 2015)[25], and *Nocardia levis* MK-VL_113 [26] were active against test bacteria and fungi. The secondary metabolites obtained from 8 day-old culture of *Nocardiopsis flavescens* VJMS-18 [27], , *Nocardiopsis*sp.VJRM-8 and *Nocardiopsis dassonvilla* VJRM-7 [28] showed high antimicrobial activity against the test microbes.

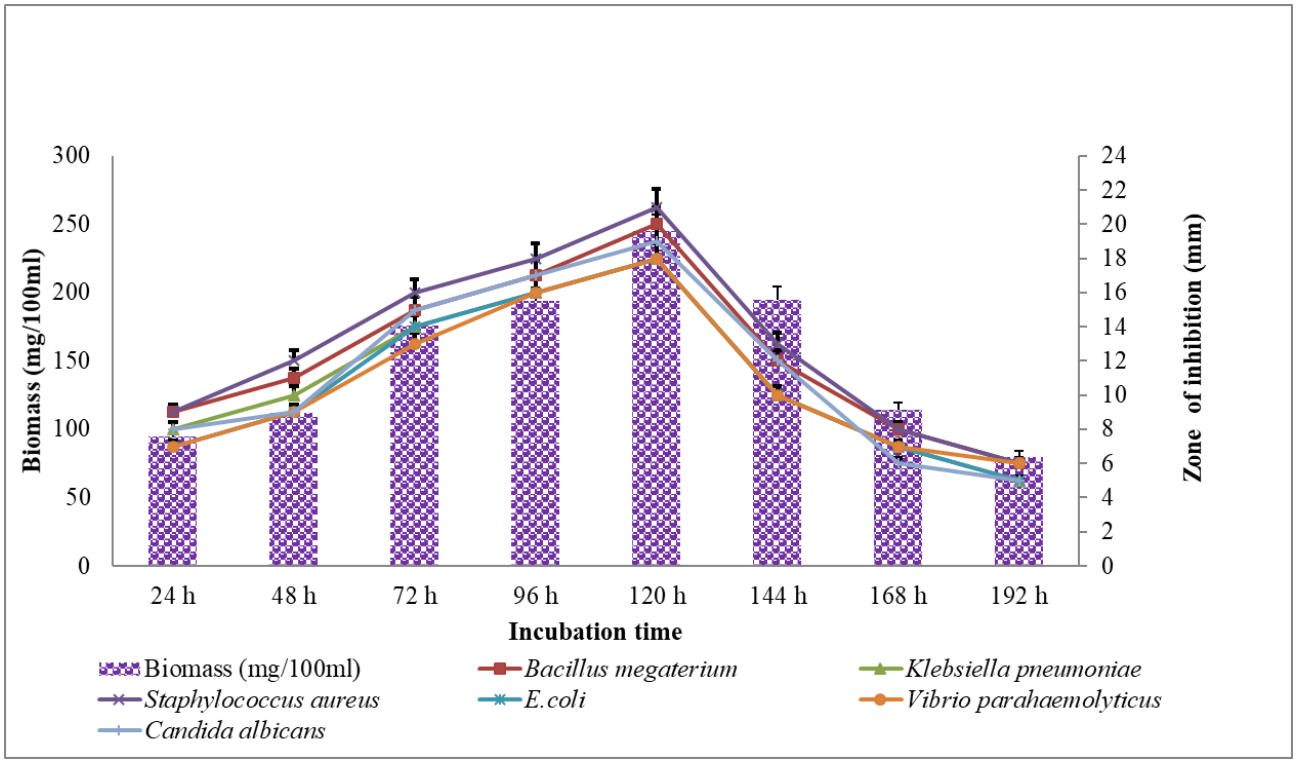

Fig. 1 Growth pattern and antimicrobial activity of strain VLK-104. Data are statistically analyzed and found to be significant at 5%.

3.2 Effect of pH and temperature on biomass and antimicrobial compounds

The impact of initial pH on growth and bioactive compounds of the strain VLK-104 was studied by altering the pH of fermentation broth from 4 to10. Maximum growth and secondary metabolites production by the strain-VLK-104 were found at pH 7.0 (Fig. 2). Similarly, the optimum pH for the production of antimicrobial compounds by several actinobacteria such as *Rhodococcus erythropolis* VLK-12 [23], *R. erythropolis* VL-RK_5 [21] *Nocardiopsis flavescens* VJMS-18 [28] *Nocardiopsis* sp. VJRM-8 and *Nocardiopsis dassonvilla* VJRM-7 [28] was reported as 7.0.

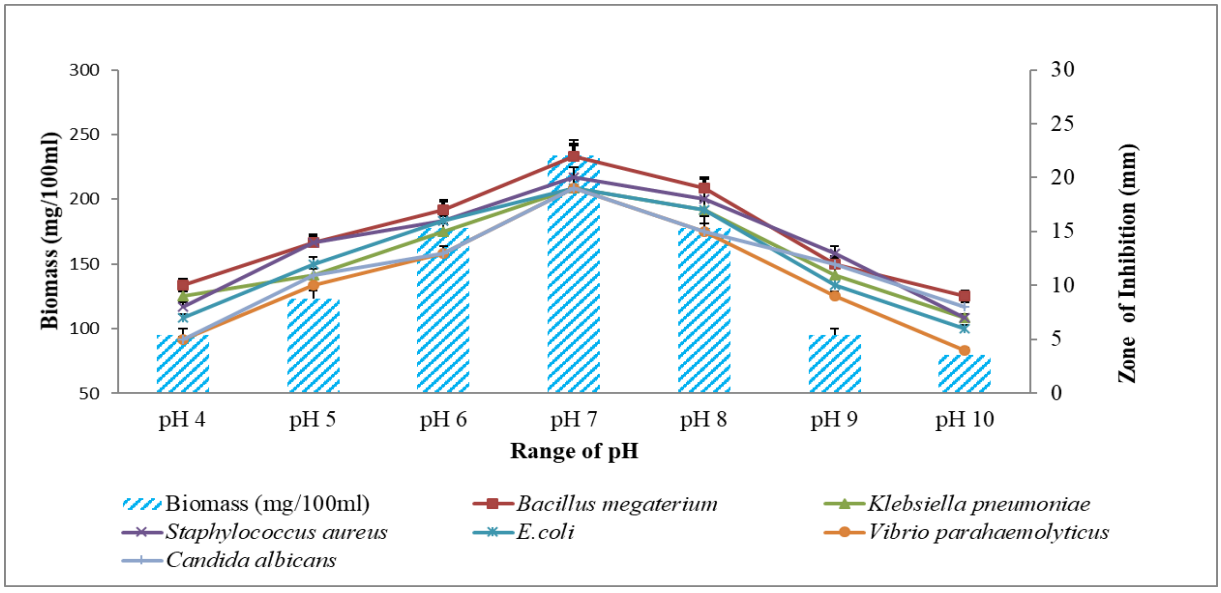

Fig. 2 Effect of pH on biomass and antimicrobial activity of the strain VLK-104. Dataarestatistically analyzed and found to be significant at 5%.

As well as the production of bioactive metabolites of the strain VLK-104 was also recorded when cultured at various temperatures range from 20-40°C and the optimum was noted at 30°C (Fig. 3).The rise of temperature from 25 to 30°C, there was an increase in bioactive metabolite production. Further increase in temperature (above 30°C) resulted in the declined production of bioactive compounds. The strain VLK-104 appeared to be mesophilic in nature. This is agreement with previous reports of several actinomycete species [29-33& 23,14]. However, Mogili and Muvva [26] and Ragireddypallem *et al*. [28] reported the optimum temperature was 35°C for the production of bioactive compounds *by Nocardiopsis flavescens* VJMS-18 and *Nocardiopsis dassonvilla* VJRM-7 .

3.3 Impact of the culture media on biomass and bioactive compounds

The influence of different culture media on the production of bioactive compounds of the strain was studied in different culture media (Fig. 4). Among the media tested, ISP-2 broth supported good growth as well as bioactive metabolites followed by Starch-Casein broth (ISP -6) and yeast-starch broth.

Modified ISP-2 medium enhanced the production of bioactive metabolites of the strain *Rhodococcus erythropolis* VL-RK_05 [21] *R. erythropolis* VLK-12 [23] and *Pseudonocardia* sp.VUK-10 [10].

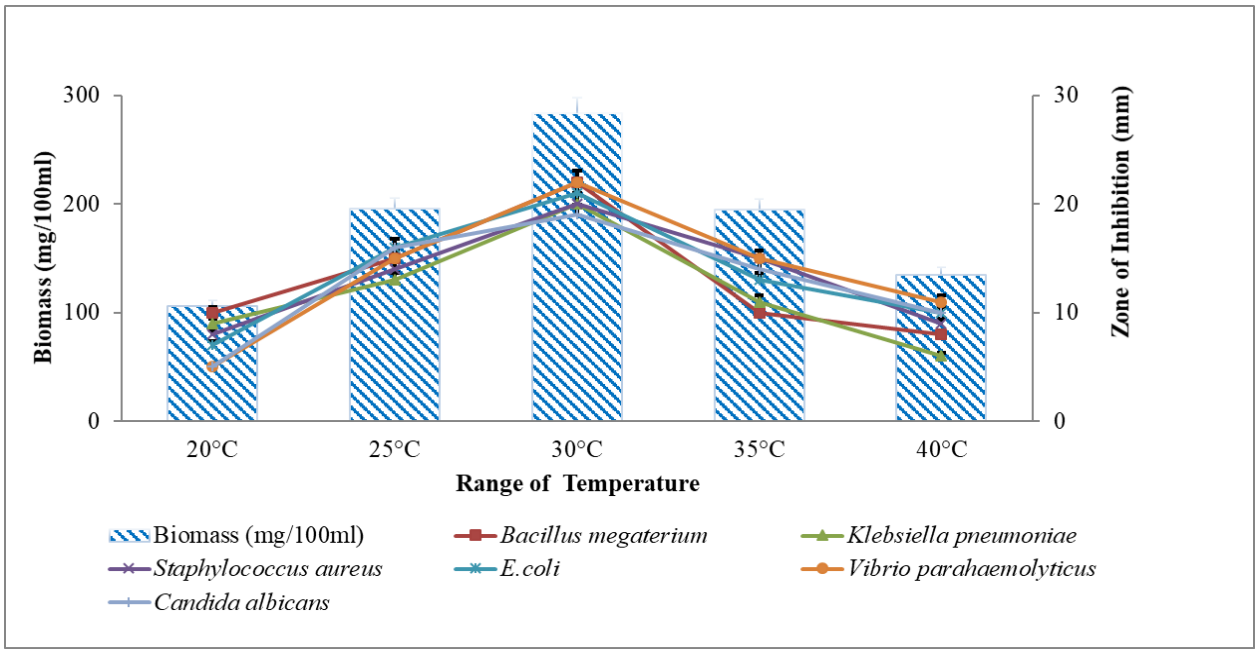

Fig. 3. Influence of temperature on biomass and antimicrobial activity of the strainVLK-104. Data are statistically analyzed and found to be significant at 5%.

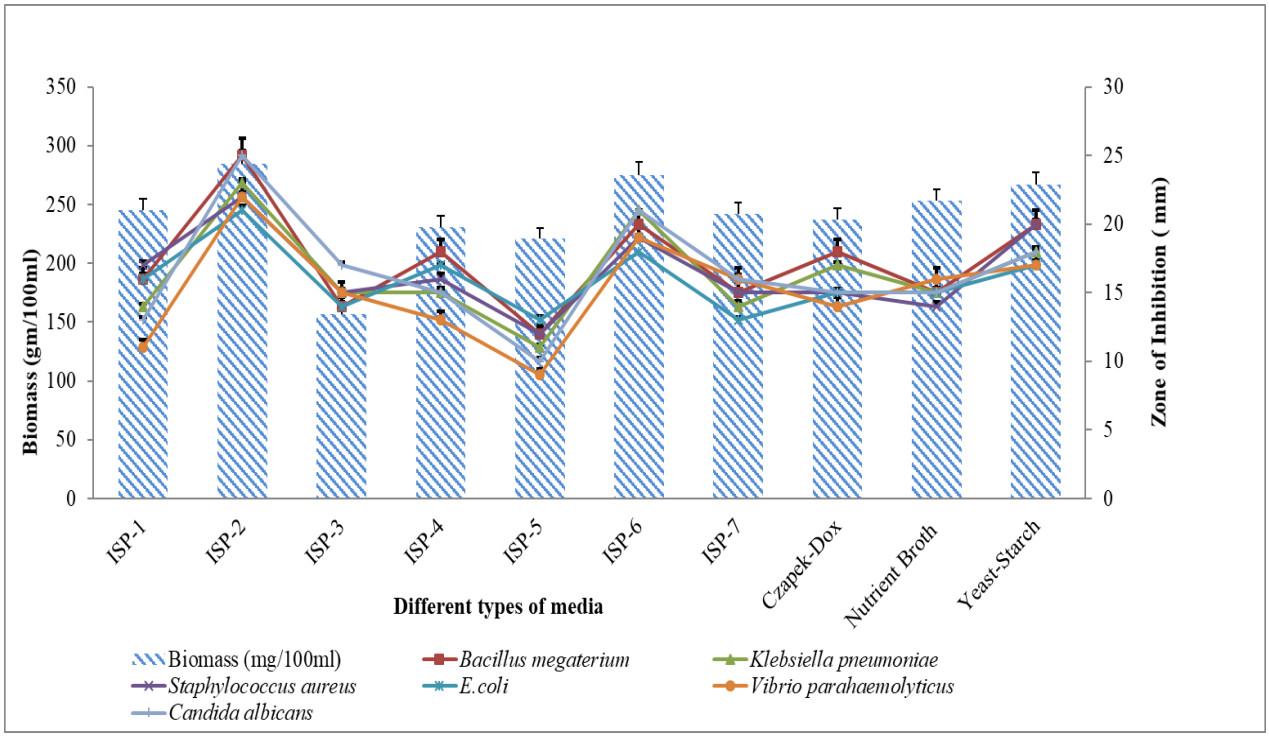

Fig. 6. Effect of different types of media on biomass and antimicrobial activity of the strain VLK-104. Data are statistically analyzed and found to be significant at 5%

3.3 Impact of carbon and nitrogen sources on biomass and bioactive compounds

The production of secondary metabolites varied with different carbon sources. The influence of various carbon sources on bioactive metabolite production was tested by adding to the ISP-2 broth at a concentration of 0.4% and other ingredients of the media same and incubated for 120 h at 30°C. The impact of various carbon sources on the growth and production of bioactive metabolites by the strain VLK-104 are presented in Fig. 4. Of all the carbon sources utilized, high yield of bioactive compounds was achieved with Mannitol followed by lactose and sucrose. Since mannitol enriched culture medium supported a high yield of bioactive metabolites, different concentrations of mannitol (0.2–1.5%) were tested to determine the optimal concentration. Mannitol @ 0.4 % concentration supported the optimal yields of bioactive compounds (Fig. 5).

The effect of various nitrogen sources on growth and bioactive metabolite production was tested by supplementing the medium with nitrogen sources at a concentration of 0.4%. Peptone was found to be good as compared to other nitrogen sources used by the strain VLK-

104 presented in Fig.6. Since peptone enhanced the antimicrobial metabolite production by the strain, the influence of different concentrations (0.2% -1.5%) of peptone was tested (Fig. 7). An enhanced level of bioactive metabolite production was found with peptone at a concentration of 0.5%. Similarly, Rajesh kumar *et al*. [14] reported that peptone @ 0.5% supported the highest antimicrobial compounds production in *Arthrobacter kerguelensis* VL-RK-09. Tryptone was reported as the suitable nitrogen source for optimum production of bioactive metabolites by *Nocardiopsis* sp.[19].

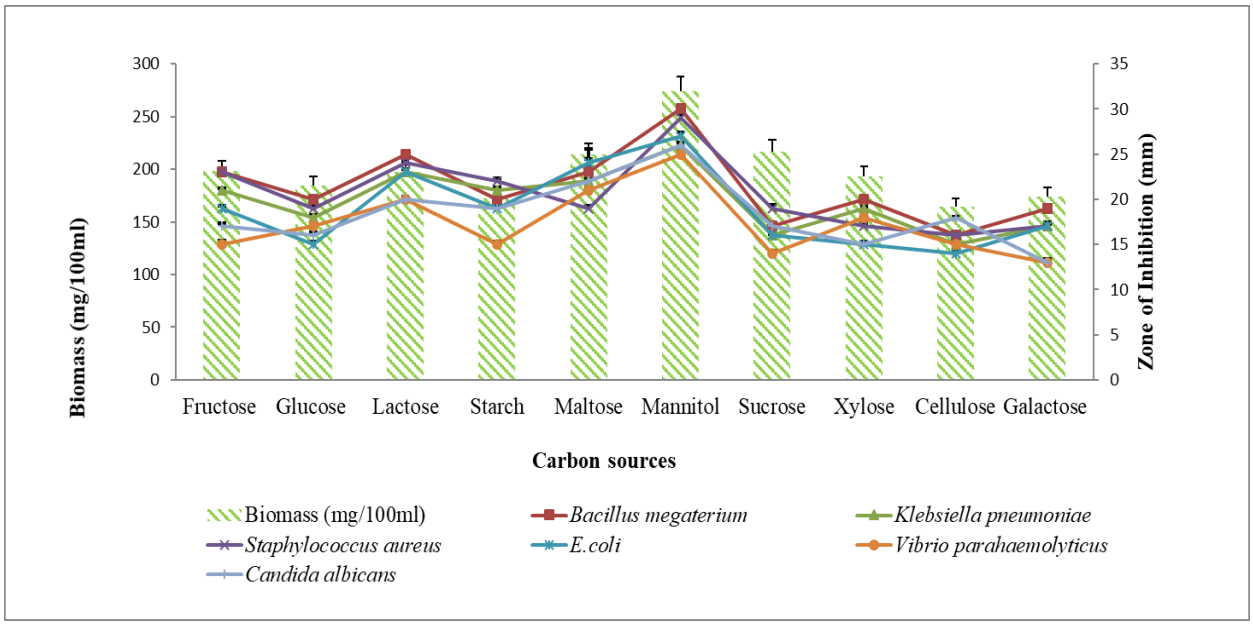

Fig. 4. Impact of different carbon sources supplemented YMD broth on biomass and antimicrobial activity of the strain VLK-104. Data are statistically analyzed and found to be significant at 5%.

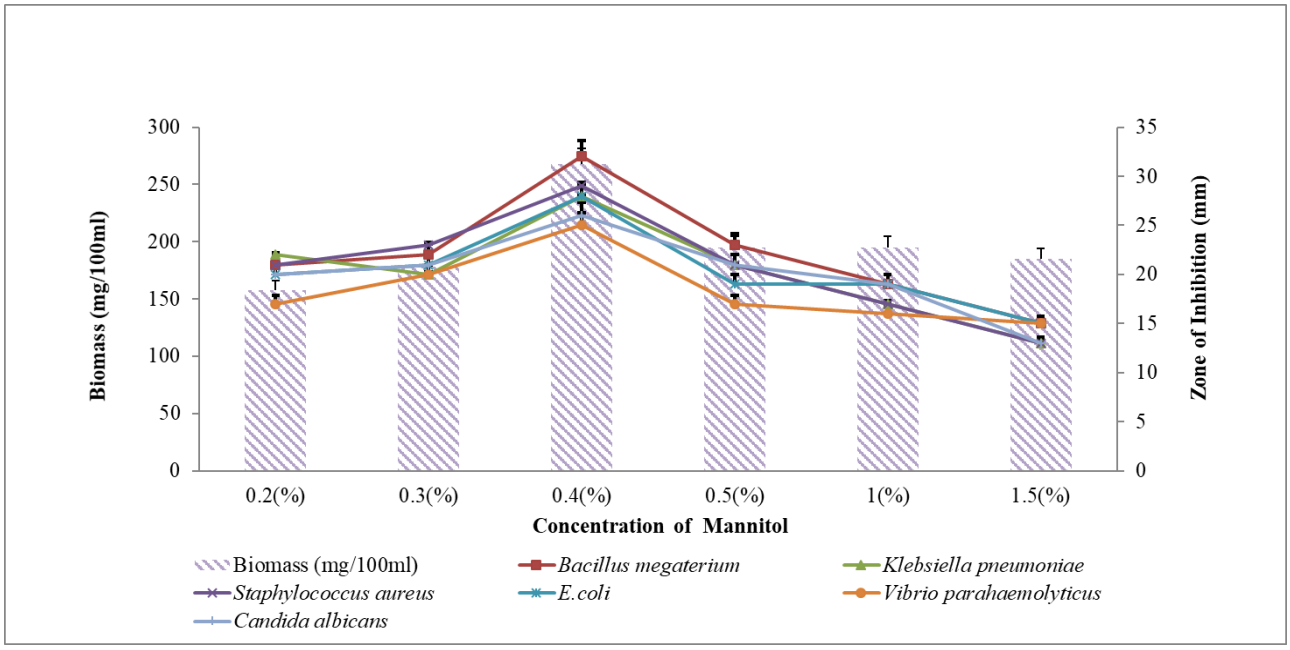

Fig. 5. Effect of different concentration of mannitol on biomass and antimicrobial activity of the strain VLK-104. Data are statistically analyzed and found to be significant at 5%.

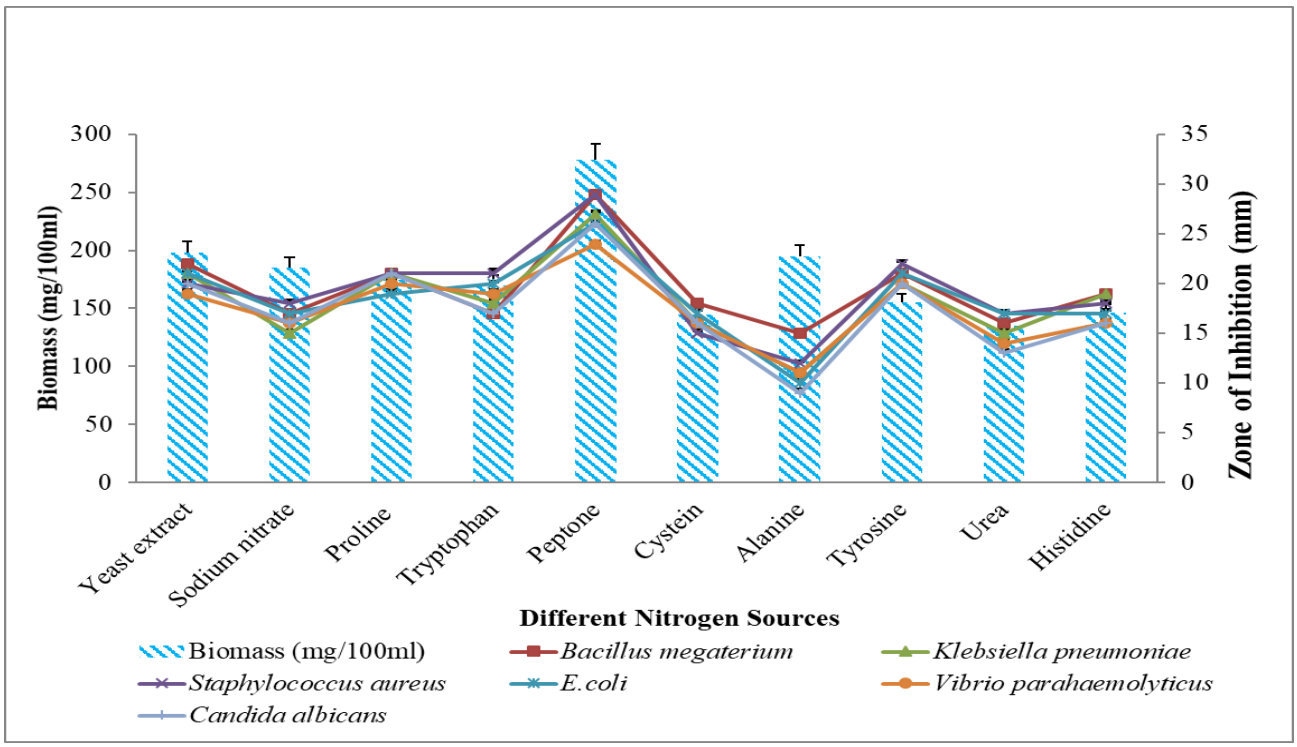

Fig. 6. Impact of different nitrogen sources supplemented in YMD broth on biomass and antimicrobial activity of the strain VLK-104. Data are statistically analyzed and found to be significant at 5%.

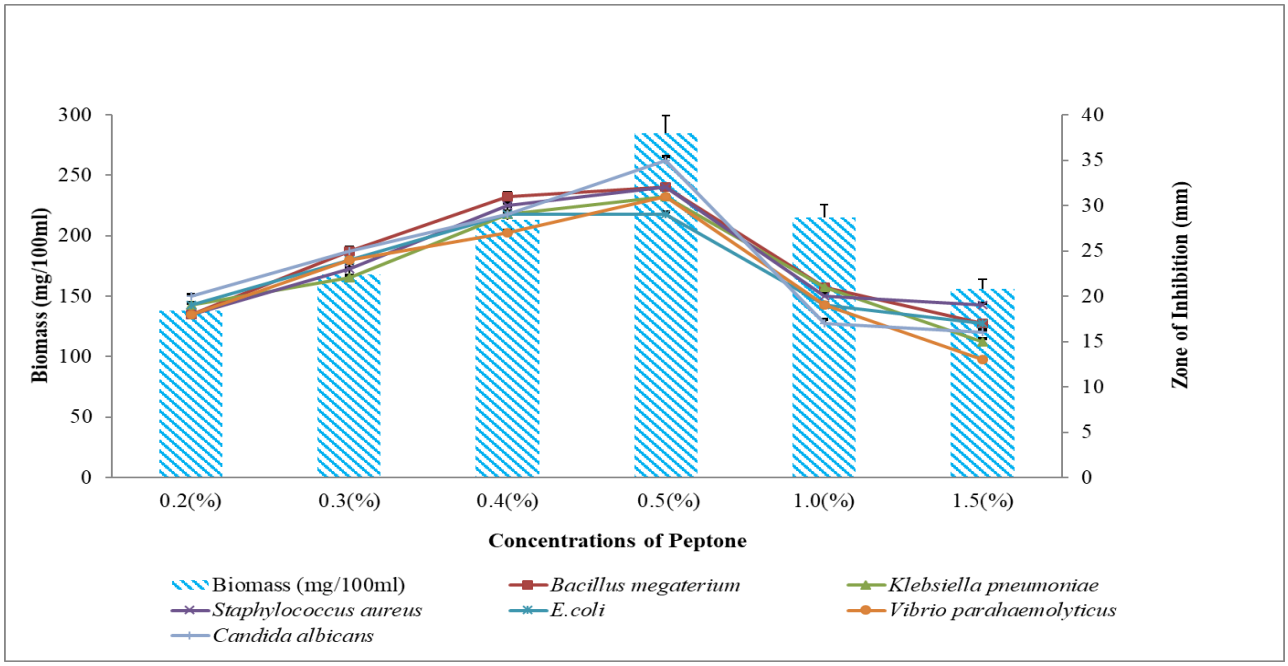

Fig. 7 Effect of different concentration of peptone on biomass and antimicrobial activity of the strain VLK-104. Data are statistically analyzed and found to be significant at 5%.

3.4 The influence of mineral on antimicrobial activity by the strain VLK-104

The impact of minerals on secondary metabolite production by the strain VLK-104 is shown in Fig. 8. Among the mineral tested, K_2HPO_4 supported the highest antimicrobial activity. Similar results were reported for *Arthrobacter kerguelensis* VL-RK-09 [14] and *Pseudonocardia* sp. VUK-10 [10].

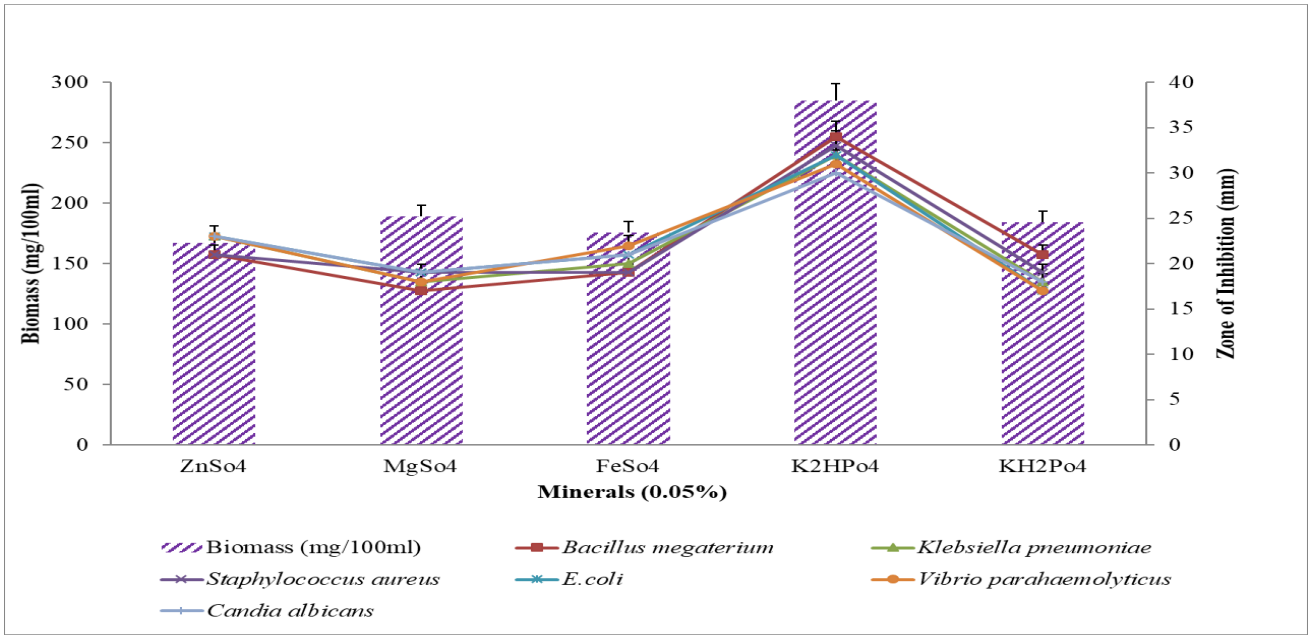

Fig. 8 Impact of different minerals on biomass and antimicrobial activity of the strain VLK- 104. Data are statistically analyzed and found to be significant at5%.

3.5 Antimicrobial activity of bioactive compounds under optimized conditions

The pure culture of the strain VLK-104 was inoculated into the optimized medium (ISP-2) composed of mannitol (0.4%), peptone (0.5%), malt extract (1%), calcium carbonate (0.2%) (pH 7.0) and K_2HPO_4(0.05%) and NaCl (3%) incubated at 30°C for five days. Among the bacteria tested *Bacillus megaterium, Staphylococcus aureus* and *Vibrio parahaemo lyticus* were highly sensitive to the compounds followed by *Streptococcus mutans, B. subtilis* and *Klebsiella pneumonia* (Fig.9).While *Candida albicans* exhibited high sensitivity followed by *Aspergillus niger* in case of fungi (fig.10).

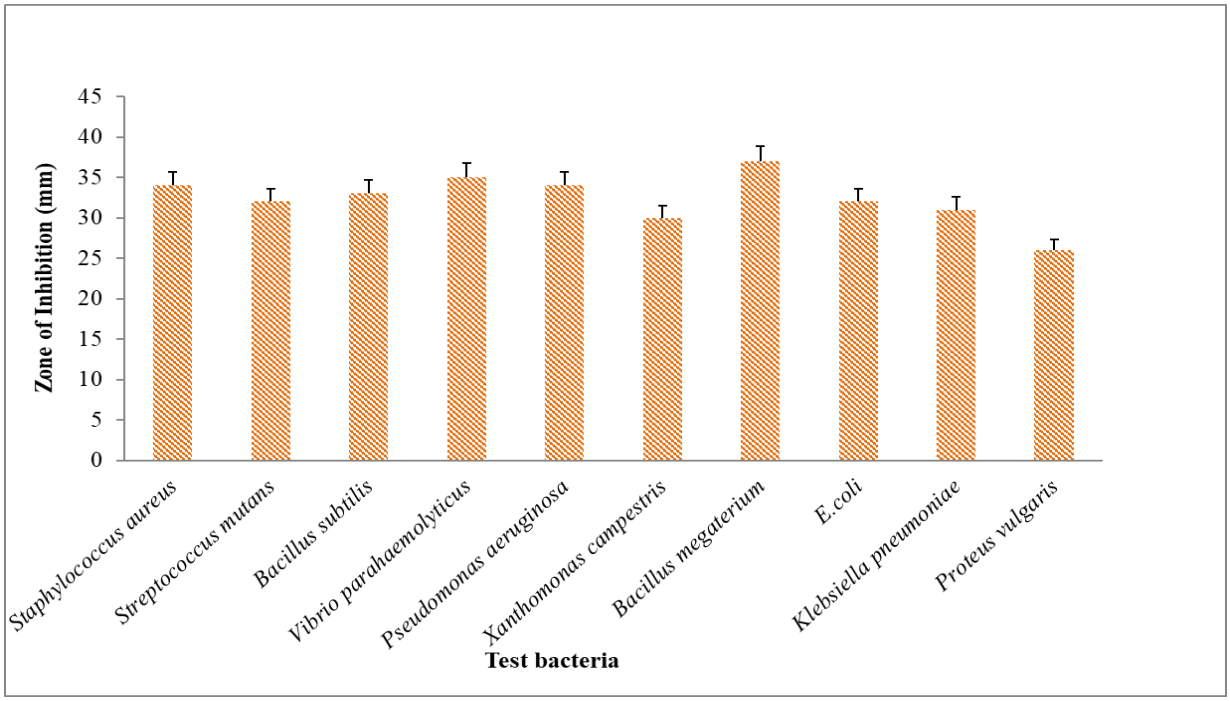

Fig. 9. Anti-bacterial activity of the strain VLK-104 under optimized conditions tested against various bacteria. Data are statistically analyzed and found to be significant at 5%.

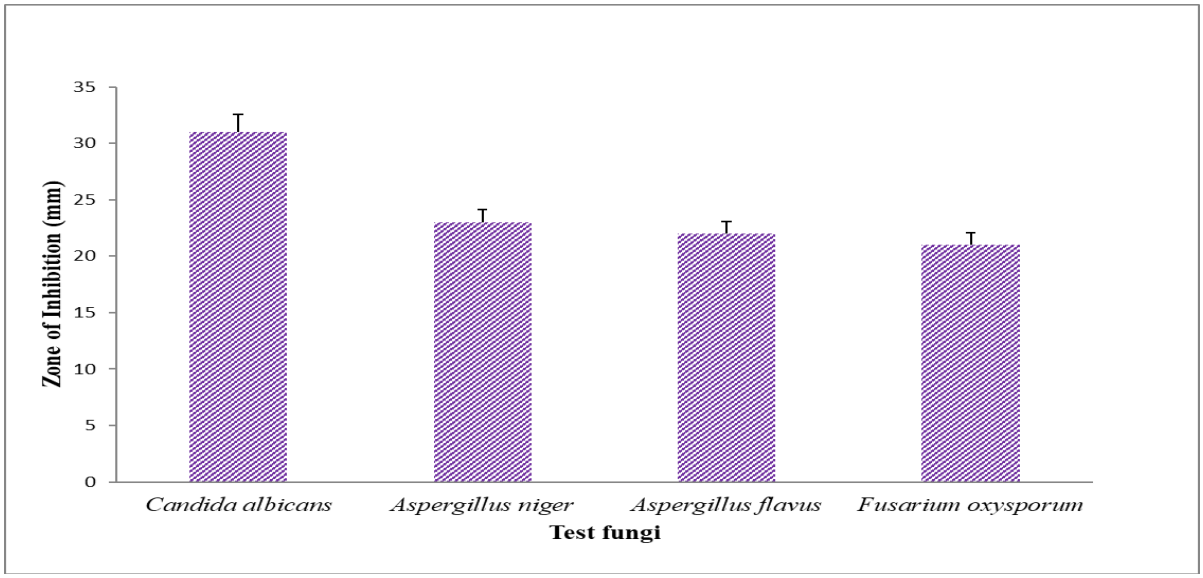

Fig.10. Anti-fungal activity of the strain VLK-104 under optimized conditions tested against test fungi. Data are statistically analyzed and found to be significant at 5%

3.2 Extraction and characterization of bioactive compounds by GC-MS Analysis

The culture broth collected after five days of fermentation were extracted twice with ethyl acetate and concentrated to yield a dark brown semi- solid compound. The components of ethyl acetate extract produced by the strain VLK-104 were revealed the presence of 41 peaks at different retention times by GC-MSD spectrum (Figure 3). According to the available library data, NIST MS Search (ver. 2.0) (included with NIST '02 mass spectral library, Agilent p/n G1033 A), compounds viz., 1 to 41 presented ethyl acetate extract of the strain VLK-104 and compounds which were found to have 70% or more similarity to the hits in database were selected. The retention time, molecular weight and the bioactivity of the compounds corresponding to the 15 peaks are represented (Table 1).

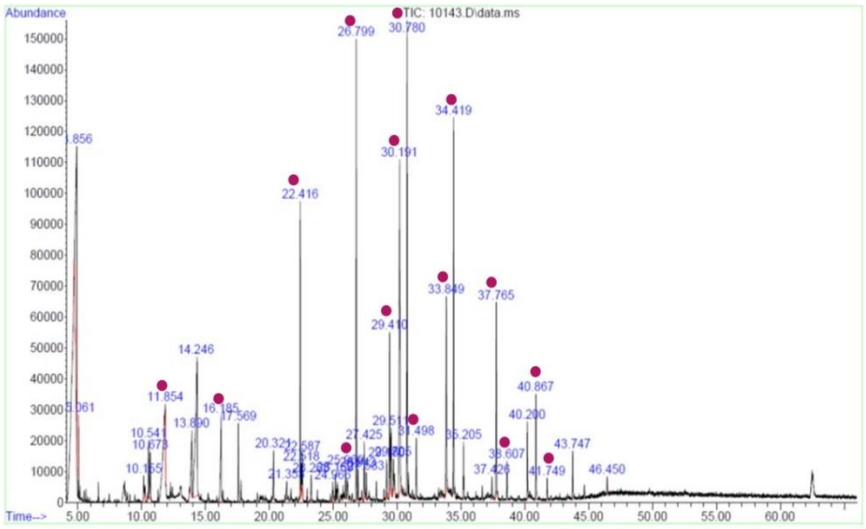

Fig.11 Total ion chromatogram of ethyl acetate extract of *Nocardiopsis lucentensis* VLK-104 showing the presence of bioactive compounds at various retention time.

Table1. GC-MS analysis of compounds presents in the ethyl acetate extract of *Nocardiopsis lucentensis* VLK-104.

S.NO	**Relative Time**	**Quality of similarity (%)**	**Relative abundance (%)**	**Compound name**	**Molecular formula**	**Molecular weight (g/mol)**
1	11.85	91	0.38	Benzoic acid	$C_7H_6O_2$	122
2	16.18	87	2.66	Benzenepropanoic acid	$C_9H_{10}O_2$	150
3	22.41	86	4.61	1-Nonadecene	$C_{19}H_{38}$	266
4	26.79	90	7.66	E-15-Heptadecenal	$C_{17}H_{32}O$	252
5	26.94	72	0.58	Oxalic acid	$C_{15}H_{28}O_4$	272
6	29.40	78	2.61	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276
7	30.19	96	10.98	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
8	30.77	95	8.54	3-Eicosene, (E)-	$C_{20}H_{40}$	280
9	31.49	72	1.20	4-Tridecene (Z)-	$C_{13}H_{26}$	182
10	33.84	99	4.23	Octadecanoic acid	$C_{18}H_{36}O_2$	284
11	34.42	97	6.38	1-Docosene	$C_{22}H_{44}$	308
12	37.76	81	3.83	10-Heneicosene (c.t)	$C_{21}H_{42}$	294
13	38.60	83	0.76	Oxalicacid, cyclo butyl hepta decyl ester	$C_{23}H_{42}O_4$	382
14	40.86	91	2.11	1-Deconol,2-hexyl-	$C_{16}H_{34}O$	242
15	41.74	80	0.50	1-Dodecanol,3,7,11-trimethyl-	$C_{15}H_{32}O$	228

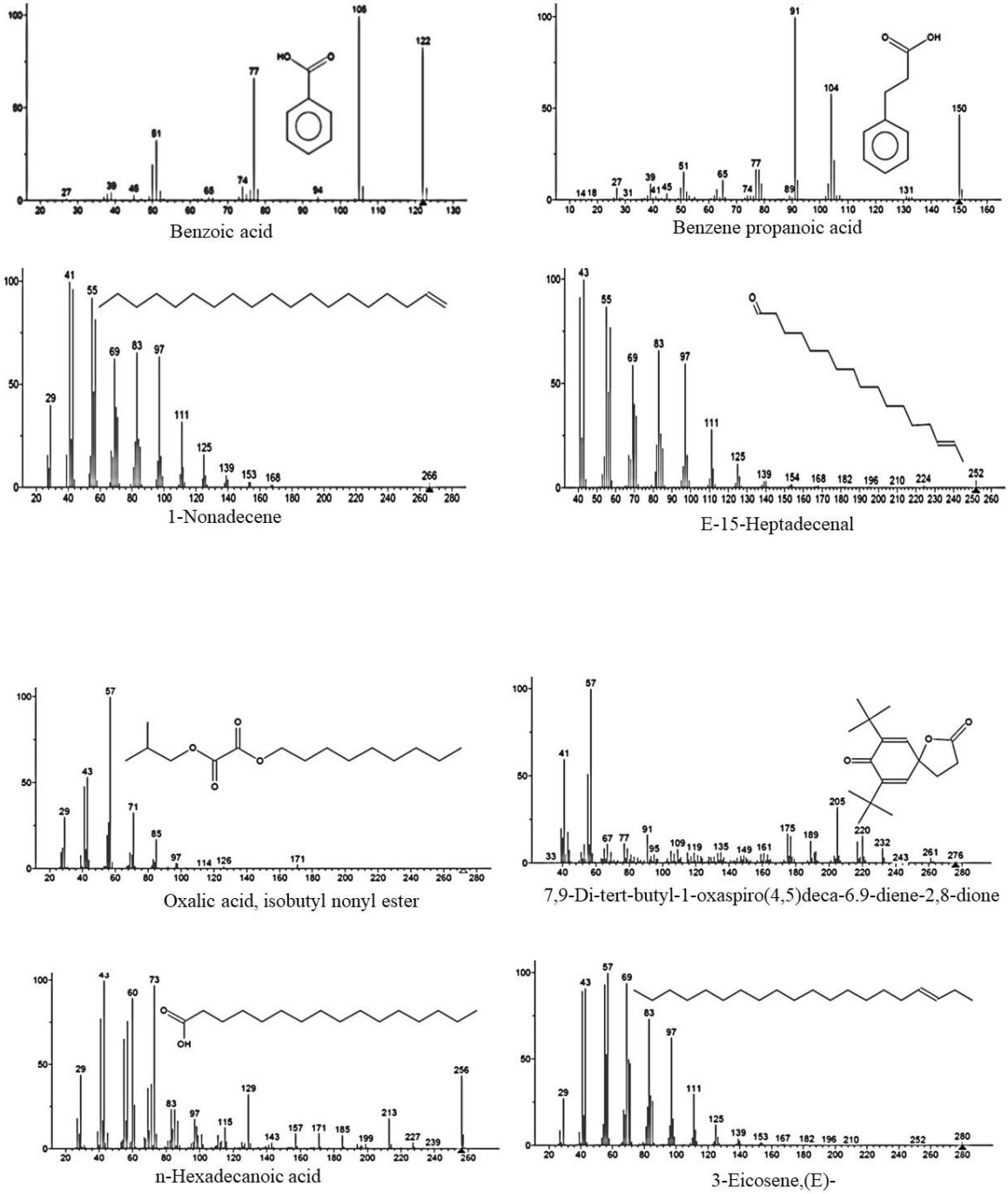

Fig. 12 Mass Spectrums of compounds from NIST library with Retention Time(RT) 11.85 (Benzoic acid), 16.18 (Benzene propanoic acid), 22.41(1-Nonadecene), 26.79(E-15-Heptadecenal), 26.94 (Oxalic acid, isobutyl nonyl ester), 29.40 (7,9-Di-tert-butyl-1-

oxaspiro(4,5)deca-6.9-diene-2,8-dione), 30.19 (n-Hexadecanoic acid) and 30.77(3-Eicosene,(E)-

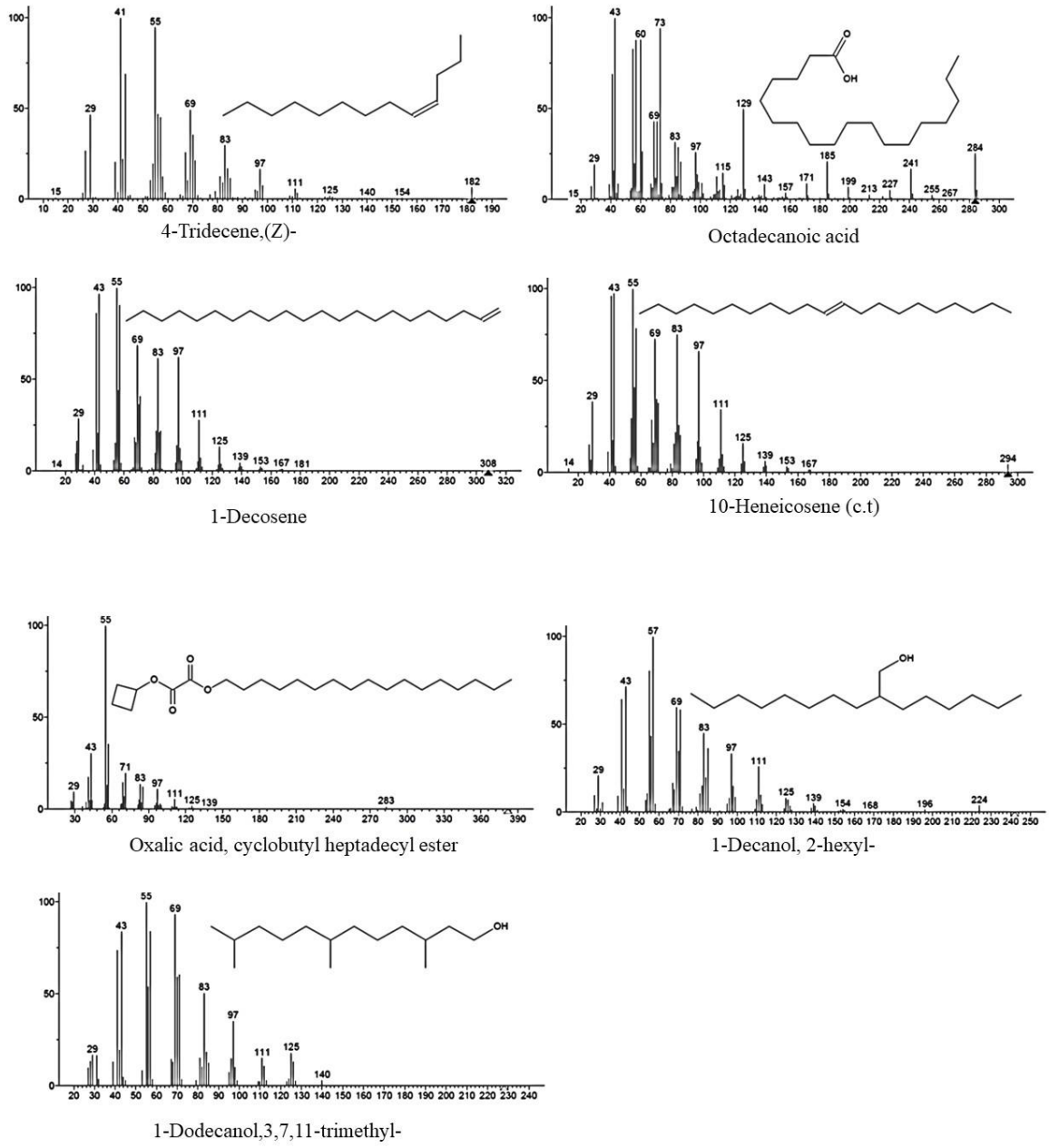

Fig. 13 Mass Spectrums of compounds from NIST library with Retention Time(RT) 31.49 (4-Tridecene,(Z)-,33.84(Octadecanoic acid), 34.42(1.Decosene), 37.76(10-Heneicosene (c.t), 38.60(Oxalic acid, cyclobuytlheptadecyl ester), 40.86(1-Decanol, 2-hexyl- and 41.74(1-Dodecanol,3,7,11-trimethyl-).

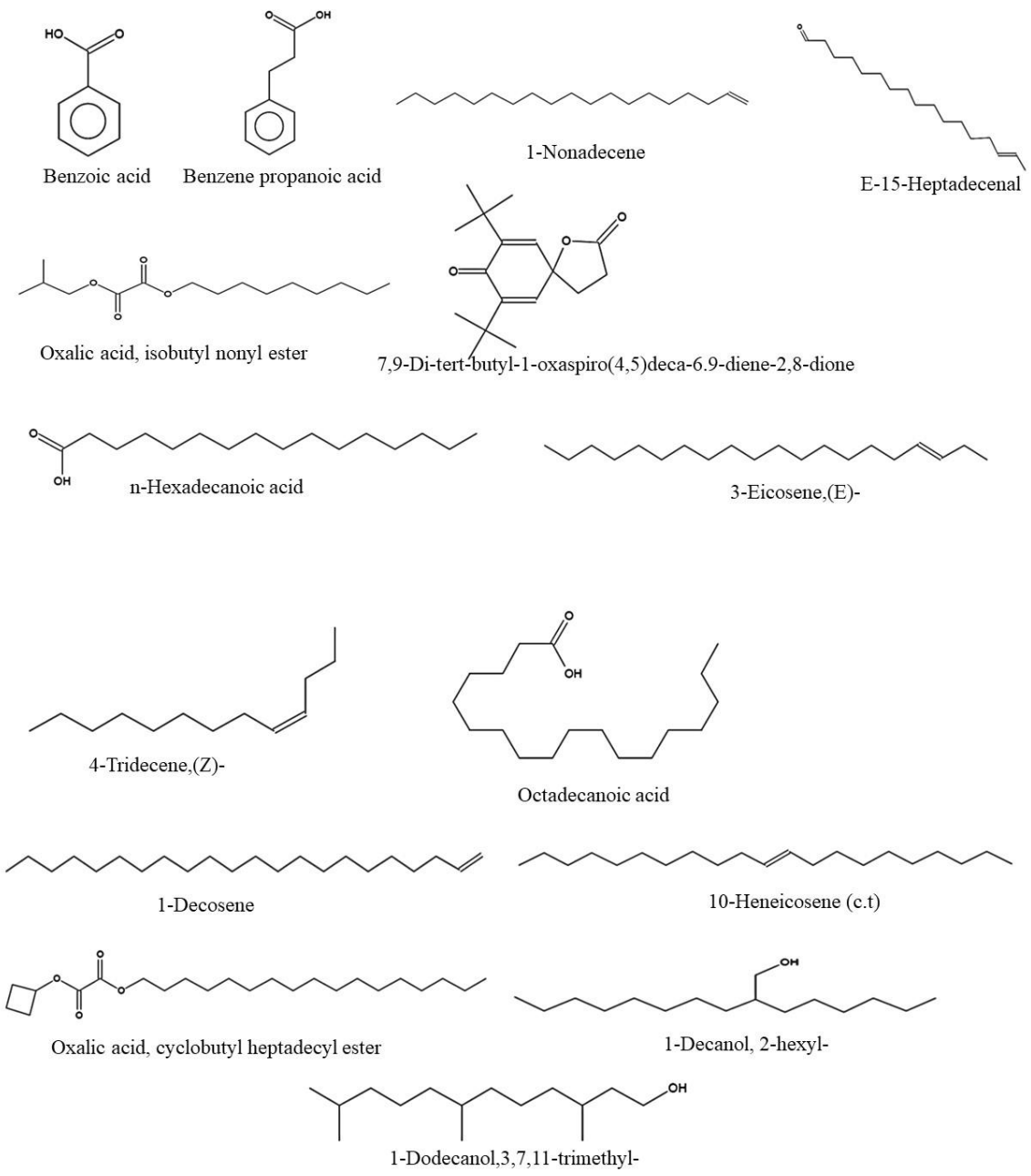

Fig.14. Structures of the compounds produced by the strain VLK-104

CONCLUSION:

The current study discovered that the *Nocardiopsis lucentensis* VLK-104 can produce antimicrobial compounds with board spectrum against Gram-positive as well as Gram-negative bacteria and fungi. Among the bacteria tested, *Bacillus megaterium* was highly sensitive to the metabolites followed by *Staphylococcus aureus*, *Bacillius subtilis* while

Candida albican exhibited high sensitivity in case of fungi and GC-MS analysis revealed different active compounds in crude extract of ethyl acetate.

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