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OPTIMIZATION AND EVALUATION OF ANTIMICROBIAL COMPOUNDS PRODUCED BY *STREPTOMYCES RUBRUS* VLK-24

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Keywords:

Streptomyces rubrus, Mangrove ecosystem, Antimicrobial compounds, Optimization, GC-MS analysis

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ABSTRACT: Optimizing the cultural conditions to enhance bioactive metabolite production by *Streptomyces rubrus* VLK-24 isolated from mangrove sediments as well as characterizing these compounds, has been taken up in this study. The culture broth inoculated in Yeast extract, Malt extract, and Dextrose broth and extracted with ethyl acetate exhibited high antimicrobial activity against test microorganisms, including Gram-negative and Gram-positive bacteria as well as fungi. The crude ethyl acetate extract exhibiting high antimicrobial activity was analyzed by Gas Chromatography-Mass Spectroscopy, which evidenced the presence of 39 compounds according to the available library data, NIST MS Search (ver. 2.0). The results of the study revealed the production of diversified metabolites by the strain; hence it could be a possible source for novel antimicrobial compounds.

INTRODUCTION: Mangrove ecosystems are salt-resistant forest ecosystems found in tropical and sub-tropical intertidal regions worldwide [1]. The soils of mangroves provide unique conditions for the growth of diverse microorganisms involved in recycling nutrients and producing secondary metabolites of pharmaceutical importance. Frequent variations in environmental factors in mangrove ecosystems lead to adaptations in organisms' metabolic pathways, resulting in the biosynthesis of unique metabolites [2].

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Less than 1% of the microbial diversity of mangrove ecosystems has been explored and only 5% of the microbes isolated have been studied for bioactive metabolite production [3]. Natural bioactive molecules are the best source for new drug development in the present scenario. The enhanced drug resistance in microbes is the chief cause of the discovery of new drugs. Microbes play a highly significant role in the drug discovery process.

Advanced techniques for identifying new chemical entities aided in enhancing the incidence of acquiring novel compounds [4]. Hence, we focused on isolating and identifying actinobacterial strains from the mangrove ecosystem of the south coast of Andhra Pradesh and their bioactive metabolites. The present study attempted to evaluate the antimicrobial compounds from *Streptomyces rubrus* VLK-24 by GC-MS analysis.

MATERIALS AND METHODS:

Microorganism: The strain VLK-24 isolated from the mangrove ecosystem of Krishna district of Andhra Pradesh, India, was identified as *Streptomyces rubrus* VLK-24 using polyphasic taxonomy and molecular (16S rRNA) analysis. The gene sequence of the strain was deposited in the GenBank database of NCBI with the accession number MG309760 [5].

Test Microorganisms: Antimicrobial activity of the strain VLK-24 was tested against bacteria such as *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (MTCC 3160*)*, *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae (*ATCC10031*)* and *Vibrio parahaemolyticus* (ATCC 43996) as well as fungi like *Candida albicans* (ATCC 10231) by agar well diffusion assay.

Optimization of Culture Conditions for Improved Bioactive Compounds: Attempts were made to optimize the culture conditions to enhance the antimicrobial activity of *Streptomyces rubrus* VLK-24, such as pH, temperature, carbon sources, nitrogen sources, and minerals. The bioactive metabolite production of the strain in terms of its antimicrobial activity was tested after 4 days of incubation. Fermentation was carried out in 500mL Erlenmeyer flasks with constant shaking at 120 rpm. The influence of initial pH on the production of secondary metabolites was determined by adjusting pH of the production medium from 4 to 10. Correspondingly, the optimal temperature for bioactive compound production was determined by inoculating the strain at temperatures ranging from 25 to 45°C, while maintaining all other conditions at optimum levels [6].

The strain was initially cultured on ten different growth media amended with 3% sodium chloride to determine the best medium for bioactive metabolite production. The impact of carbon sources on antimicrobial metabolite production was determined by adding different carbon sources to the production medium, such as maltose, sucrose, glucose, mannitol, lactose, starch, cellulose, fructose and xylose each at a concentration of 0.4% (w/v) [7]. The effect of different concentrations of the best carbon source (0.2–1% w/v) on bioactive compound production was also studied. Likewise, the impact of various nitrogen sources such as sodium nitrate, peptone, tryptophan, L-proline, tyrosine, urea, yeast extract, tryptophan, cysteine and alanine were studied by supplementing nitrogen source (0.4%) to the medium with an optimized carbon source. Further, the best nitrogen source (0.2-1% w/v) for improved production of bioactive metabolites was also recorded [8]. The medium with optimized carbon and nitrogen sources was amended with mineral salts such as KH_2PO_4, K_2HPO_4, $FeSO_4$, $ZnSO_4$ and $MgSO_4$, to assess the influence of minerals on the production of bioactive metabolites.

Biological Assay: The antimicrobial activity of bioactive compounds produced by the strain VLK-24 was done by agar well diffusion method [9]. Nutrient agar and Czapek-Dox agar media were used for culturing the test bacteria and fungi, respectively. Ethyl acetate extract (50 ppm) was added to each well of the seeded plate, with the solvent serving 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 24-72 h for fungi.

Fermentation and Extraction of Antimicrobial Metabolites: The antimicrobial activity of the strain *Streptomyces rubrus*VLK-24 was evaluated by extracting the fermentation broth with ethyl acetate. For extraction of secondary metabolites, an actively growing 2-day-old pure culture of the strain was inoculated into the optimized production medium (10 L) composed of glucose (0.5%), peptone (0.5%), malt extract (1%), calcium carbonate (0.2%) and K_2HPO_4 (0.05%) with pH 7.0. The flasks were incubated on a rotary shaker (120rpm) at 30°C for 4 days. The culture broth obtained after filtration was extracted twice with ethyl acetate and concentrated with a roto-evaporator for GC-MS analysis.

Characterization of Antimicrobial Metabolites by Gas Chromatography-Mass:

Spectroscopy Analysis: The secondary metabolite of the crude ethyl acetate extract of the strain 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 1.2 mL/min flow rate.

The oven temperature was programmed (50°C/min, then 50–280°C @ rate of 5°C/min) and held isothermally for 20 min. The temperature of the injector port was maintained at 250°C and that of the 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).

Statistical Analysis: Data obtained on the antimicrobial activity under different culture conditions are statistically analyzed with one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION:

Optimization of Cultural Conditions:

Influence of Incubation Period on Biomass and Antimicrobial Activity: The strain's growth pattern and antimicrobial compounds production were studied at regular intervals up to seven days. The stationary phase of strain continued from 72 h to 120 h of incubation **Fig. 1**. The bioactive compounds attained from the four-day-old culture exhibited good antimicrobial activity against the test microorganisms. The results conform with the earlier reports, which stated high antimicrobial activity with four-day-old cultures of *Streptomyces lavendulocolor* VHB-9 [10], *S. cheonanensis* VUK-A [11], *S. tendae* TK-VL_333 [12], *Nocardia Levis* MK_VL113 [13] and *Pseudonocardia* sp. VUK-10 [14].

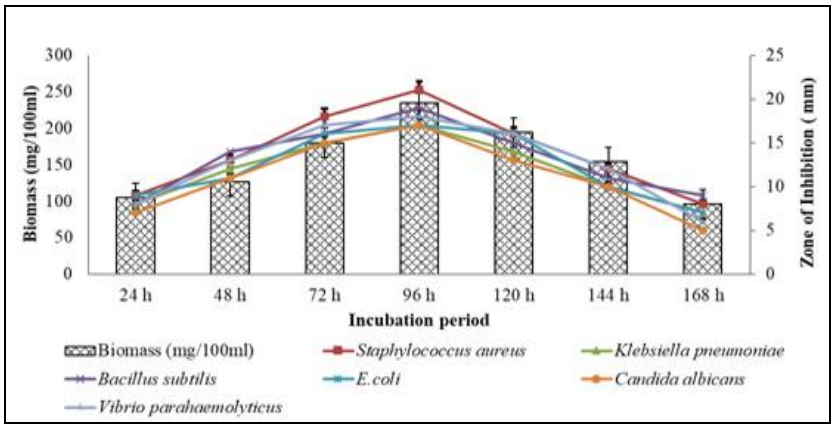

FIG. 1: GROWTH PATTERN AND ANTIMICROBIAL ACTIVITY BY *STREPTOMYCES RUBRUS* VLK-24. Data are statistically analyzed and found to be significant at 5%.

Effect of Culture Media on Biomass and Antimicrobial Metabolite Production: Biomass and antimicrobial compound production by the strain VLK-24 were studied in different culture media **Fig. 2**.

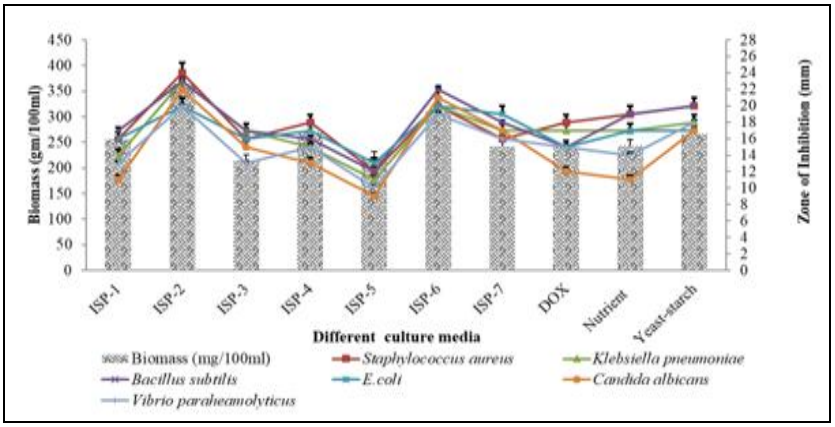

FIG. 2: EFFECT OF DIFFERENT CULTURE MEDIA ON BIOMASS AND BIOACTIVE COMPOUNDS PRODUCTION BY *STREPTOMYCES RUBRUS* VLK-24. Data are statistically analyzed and found to be significant at 5%.

Among the ten media tested, Yeast extract, malt extract, and dextrose (YMD) medium supported high levels of bioactive metabolites, followed by starch-casein and nutrient broth. YMD broth was

reported to support the production of antimicrobial compounds for *Streptomyces violaceoruber* VLK-4 [15] and *Pseudonocardia* sp. VUK-10 [14] while Czapek- Dox broth for *Streptomyces* sp. MNK-7 [16].

Effect of pH and Temperature on Biomass and Antimicrobial Activity of the Strain: Maximum growth and high antimicrobial metabolite production by the strain was found at pH 7.0 **Fig. 3**. Bioactive metabolites obtained from *Streptomyces albidoflavus* [17], *S. cheonanensis* VUK-A [11], *S. violaceoruber* VLK-4 [15] and S. *lavendulocolor* VHB-9 [10] also exhibited good antimicrobial activity when grown at pH 7.0.

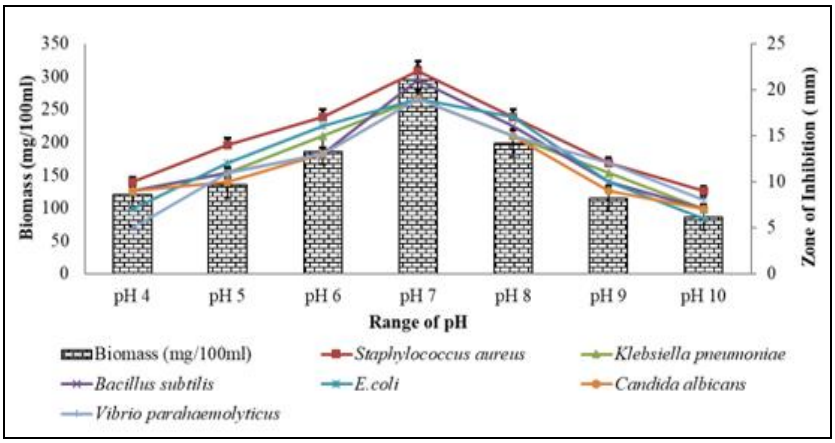

FIG. 3: INFLUENCE OF PH ON BIOMASS AND SECONDARY METABOLITE PRODUCTION BY *STREPTOMYCES RUBRUS* VLK-24. Data are statistically analyzed and found to be significant at 5%.

The biomass and bioactive compound production by the strainVLK-24 increased with the rise in the incubation temperature from 25 to 30°C **Fig. 4**. Further increase in temperature (above 30°C) resulted in a decline in growth and production of bioactive metabolites. A similar result was reported for *S. kanamyceticus* MTCC 324 [18] *S. albidoflavus*-143 [17], *S. cheonanensis* VUK-A [11], *S. violaceoruber* VLK-4 [9] and *S. lavendulocolor* VHB-9 [10].

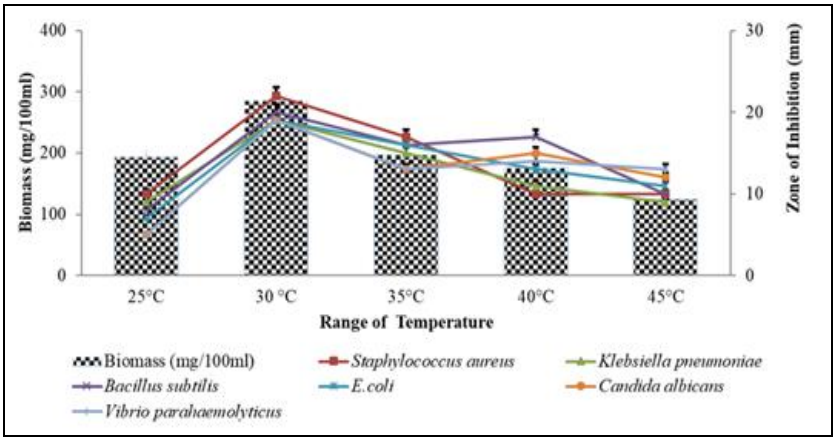

FIG. 4: EFFECT OF TEMPERATURE ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES RUBRUS* VLK-24. Data are statistically analyzed and found to be significant at 5%.

Impact of Carbon and Nitrogen Sources on Biomass and Antimicrobial Activity: The effect of carbon sources on the production of secondary metabolites as well as biomass by the strain VLK-24 is shown in **Fig. 5.** Significant production of bioactive compounds were found in a medium amended with glucose followed by lactose. In contrast, the growth of biomass was high with glucose followed by starch. As glucose appeared as the most favored carbon source for secondary metabolite production by the strain VLK-24, different glucose concentrations (0.2-1%) were added to find the optimal concentration. Glucose @ 0.5 % exhibited high yields of bioactive compounds **Fig. 6**. Glucose was reported as a best carbon source to enhance the growth and bioactive

metabolite production by *Streptomyces padanus* PMS-702 [19] and *Streptomyces tanashiensis* strain A2D [20], while mannitol for *Streptomyces violaceoruber* VLK-4 [15] and lactose for *Streptomyces lavendulocolor* VHB-9 [10] and *Streptomyces cheonanensis* VUK-A [11] served as good carbon sources for secondary metabolites production. Different nitrogen sources were tested for their impact on biomass and bioactive metabolite production by the strain VLK-24. Among the nitrogen sources used, medium with peptone supported high antimicrobial metabolite production. While peptone and histidine were effective in enhancing the growth of the strain **Fig. 7**. As peptone improved the biomass and bioactive compound production by the strain VLK-24, the influence of various concentrations of peptone was evaluated, and 0.5% was found to be good for producing bioactive metabolites **Fig. 9**. These results are comparable with *Streptomyces rochei* G164 [21] *S. cheonanensis* VUK-A [11] and *S. lavendulocolor* VHB-9 [10].

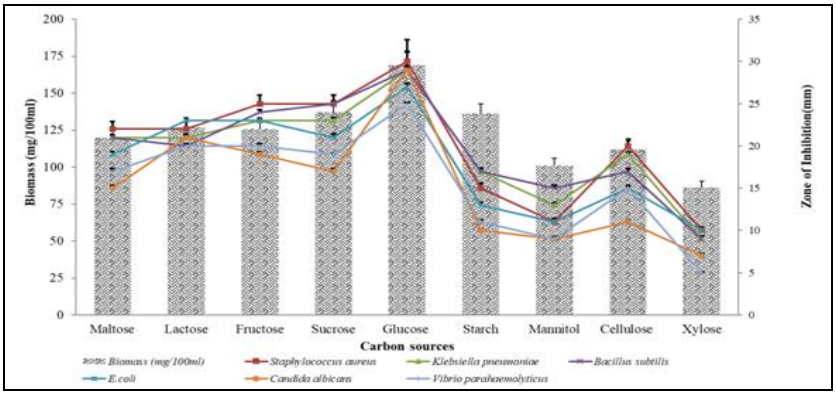

FIG. 5: INFLUENCE OF DIFFERENT CARBON SOURCES ON BIOMASS AND BIOACTIVE METABOLITES PRODUCTION BY *STREPTOMYCES RUBRUS* VLK-24. Data are statistically analyzed and found to be significant at 5%.

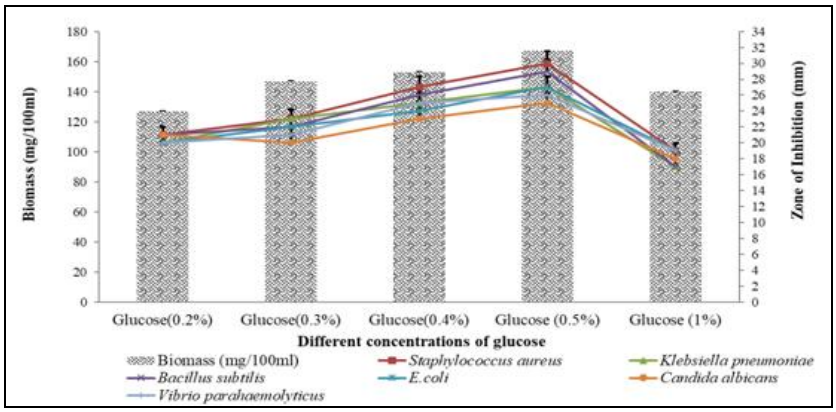

FIG. 6: IMPACT OF DIFFERENT CONCENTRATIONS OF GLUCOSE ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES RUBRUS* VLK-24. Data are statistically analyzed and found to be significant at 5%.

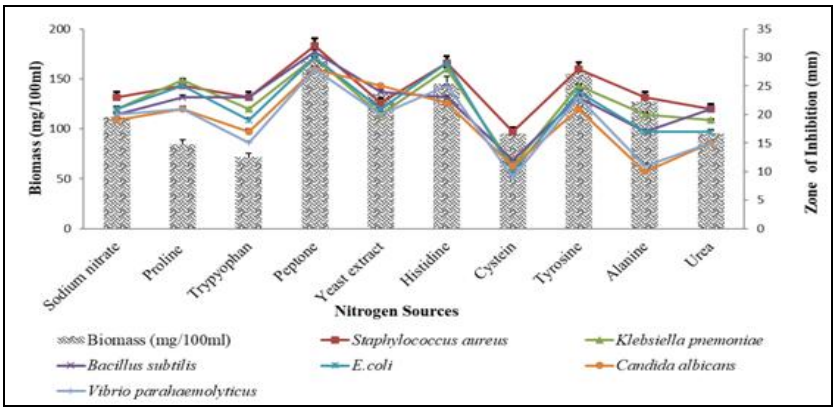

FIG. 7: EFFECT OF NITROGEN SOURCES ON BIOMASS AND SECONDARY METABOLITE PRODUCTION BY *STREPTOMYCES RUBRUS* VLK-24. Data are statistically analyzed and found to be significant at 5%.

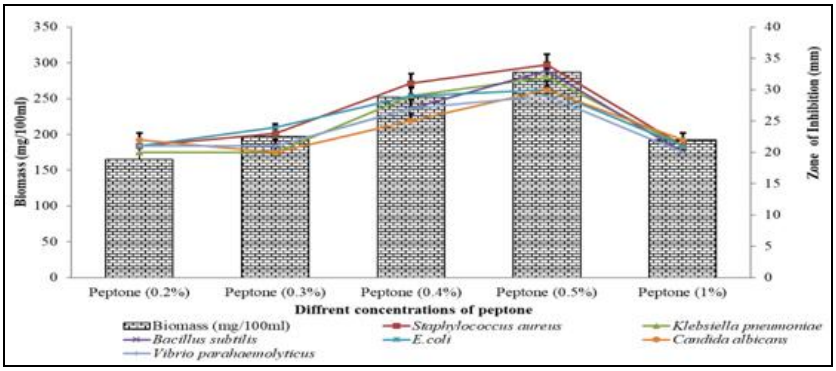

FIG. 8: INFLUENCE OF DIFFERENT CONCENTRATIONS OF PEPTONE ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES RUBRUS*VLK-24. Data are statistically analyzed and found to be significant at 5%.

Influence of Minerals on Biomass and Production of Antimicrobial Compounds: Effect of minerals on biomass and secondary metabolite production by the strain VLK-24 is shown in **Fig. 9**. Among the minerals tested, K_2HPO_4 followed by $FeSO_4$ and $ZnSO_4$ supported high yield of biomass and bioactive compound production. Similar results were reported for *Streptomyces lavendulocolor* VHB-9 [10], *S. violaceoruber* VLK-4 [15], *S. cheonanensis* VUK-A [11] and *S. albidoflavus*-143 [17].

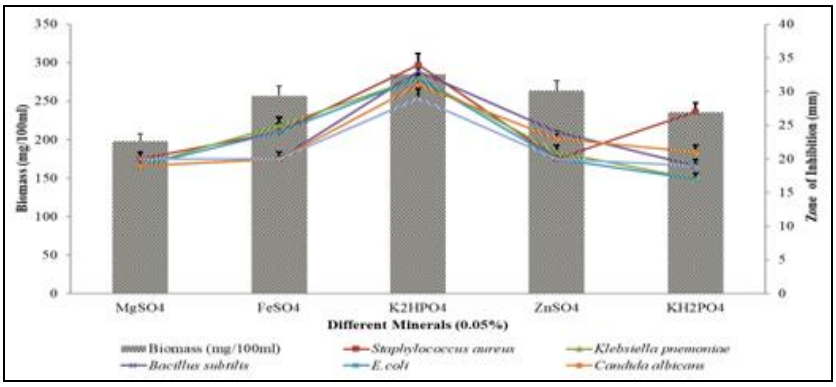

FIG. 9: IMPACT OF DIFFERENT MINERALS ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES RUBRUS*VLK-24. Data are statistically analyzed and found to be significant at 5%.

The optimized culture medium (YMD replaced with glucose @ 0.5%, peptone @ 0.5%, K_2HPO_4 0.05%with pH.7.0 and temperature 30^{o}C) was inoculated with the strain and incubated for four days. The culture broth was extracted with ethyl acetate and tested for antimicrobial activity. The solvent extract exhibited high antimicrobial activity against test bacteria and fungi **Fig. 10 & 11**. Among the bacteria tested, *Bacillus megaterium* was highly sensitive to the compounds produced by the strain, followed by *Vibrio parahaemolyticus* and *Staphylococcus aureus*. Among the fungi tested, *Candida albicans* showed high sensitivity.

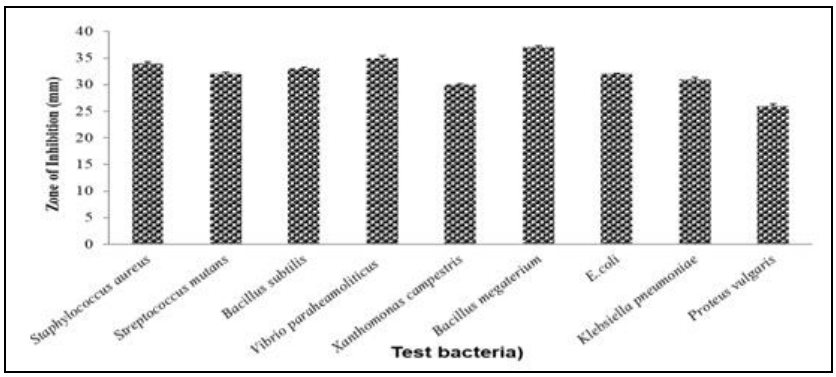

FIG. 10: ANTIBACTERIAL ACTIVITY OF *STREPTOMYCES RUBRUS* VLK-24 GROWN UNDER OPTIMIZED CONDITIONS. Data are statistically analyzed and found to be significant at 5%.

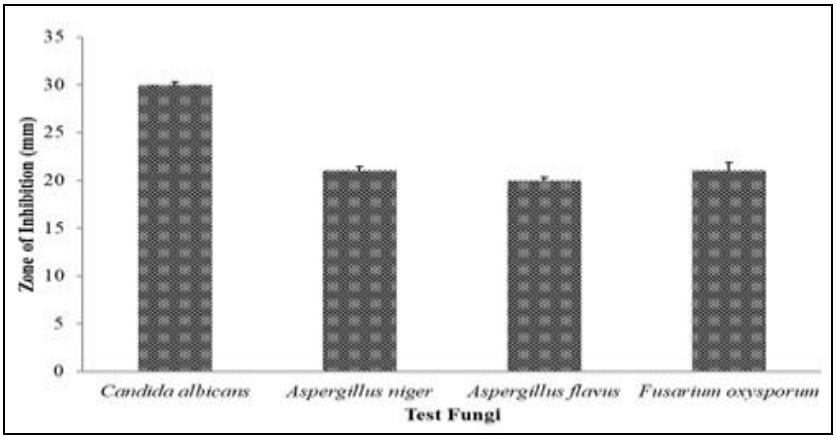

FIG. 11: ANTIFUNGAL ACTIVITY OF *STREPTOMYCES RUBRUS* VLK-24 GROWN UNDER OPTIMIZED CONDITIONS. Data are statistically analyzed and found to be significant at 5%.

Characterization of Bioactive Compounds by GC-MS Analysis: The culture broth pooled after4 days of fermentation was extracted twice with ethyl acetate and concentrated to yield a dark brown semi-solid compound. GC-MS analysis of the extract revealed the presence of 39 peaks at different retention times **Fig. 12.** According to the available library data, NIST MS Search (ver. 2.0) (included with NIST '02 mass spectral library, Agilent p/n G1033 A), the compounds in the ethyl acetate extract were identified. The details of the compounds are presented in **Table 1.**

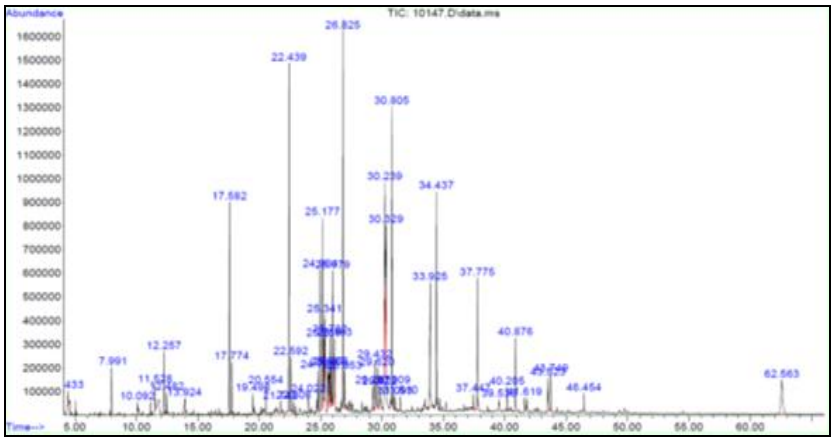

FIG. 12: GC MS SPECTRUM OF ETHYL ACETATE EXTRACT OF THE STRAIN VLK-24

TABLE 1: BIOACTIVE COMPOUNDS IDENTIFIED IN ETHYL ACETATE EXTRACT BY GC-MS ANALYSIS

Peak no.	Compound Name	Retention Time	Area%
1	Benzyl Alcohol	7.99	0.93
2	Benzyl carbamate	11.52	0.59
3	4-Decene	12.25	1.30
4	Benzene acetic acid	13.92	1.03
5	1-Hexadecene	17.58	5.00
6	Tetradecane	17.77	1.14
7	Phenol, 2,4-bis(1,1-dimethylethyl)-	20.55	0.68
8	Benzoic acid, 4-hydroxy-3-methoxy-	21.74	0.50
9	1-Hexadecene	22.43	8.85
10	Hexadecane	22.59	1.32
11	Phenol, 4-(1,1-dimethylpropyl)-	24.02	0.69
12	2-Acetamidotropone	24.79	0.99
13	Phenol, m-tert-butyl-	24.98	4.19
14	Phenol, m-tert-butyl-	25.17	4.49
15	Acetamide, N-(3-methylphenyl)-	25.26	1.30
16	Cyclohexene, 2-ethenyl-1,3,3-trimethyl-	25.33	1.04
17	Phenol, 4-(1,1-dimethylpropyl)-	25.58	0.79

18	Acetamide, N-(3-methylphenyl)-	25.77	1.59
19	Carbamic acid, N-[1,1-bis(trifluoromethyl)ethyl]-, 4-(1,1,3,3-tetramethylbutyl)phenyl ester	25.97	3.28
20	Phenol, 2-(1,1-dimethylethyl)-	26.16	0.81
21	1-Octadecene	26.82	10.10
22	Octadecane	26.95	0.79
23	1,2-Cyclopentanedione, 3,3,5,5-tetramethyl-	29.26	1.02
24	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	29.43	1.51
25	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	29.62	1.42
26	2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enone	29.82	0.83
27	Dibutyl phthalate	30.23	3.53
28	Tridecanoic acid	30.32	3.15
29	1-Nonadecene	30.80	8.19
30	Eicosane	30.90	0.50
31	Tetra decanoic acid	33.92	5.74
32	1-Docosene	34.43	5.37
33	1-Docosene	37.77	3.36
34	Phthalic acid, 2-ethylhexyl isohexyl ester	40.20	0.64
35	Docosyl trifluoroacetate	40.87	2.20
36	13-Docosenamide, (Z)-	43.52	0.94
37	Tri acontyl acetate	43.74	1.11
38	Cyclotriacontane	46.45	0.51
39	2-(2-Phenoxythiinyl) imidazolo[1,2-a]pyridine	62.56	3.63

The bioactive molecule dibutyl phthalate produced by *Streptomyces nasri*-H35, *S. melanofaciens* [22] and *S. albidoflavus* 321.2 [23] is reported to possess antimicrobial activity. Benzoic acid from *Streptomyces cheonanensis* VUK-A isolated from Coring mangroves showed potent antimicrobial activity [9] 1-nonadecene from *Streptomyces* sp. TN 256 [24], 1-Hexadecene and 1- nonadecene produced by *Streptomyces* sp. TN 272 [25] exhibited antimicrobial activity.

CONCLUSIONS: *Streptomyces rubrus* VLK-24 isolated from the mangrove ecosystem of the south coast of Andhra Pradesh, India, was tested for its antagonistic activity against several test bacteria and fungi. Attempts were made to optimize culture conditions, including pH, temperature, carbon and nitrogen sources, and minerals to enhance bioactive metabolite production.

The components of the optimized culture medium include glucose @ 0.5% (carbon source), peptone @ 0.5% (nitrogen source) 0.05% K_2HPO_4 and 3% sodium chloride with pH.7.0. The strain was cultured at 30ºC for four days in the optimized medium exhibited high antimicrobial activity. GC-MS analysis of ethyl acetate extract of the strain VLK-24 revealed the presence of significant bioactive compounds and hence the strain could be used as a source of novel antimicrobial compounds.

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CONFLICTS OF INTEREST: The authors revealed no potential conflicts of interest, financial or otherwise.

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