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Optimization of Culture Conditions for Enhanced Antimicrobial Activity of *Rhodococcus erythropolis* VLK-12 Isolated from South Coast of Andhra Pradesh, India

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Authors' contributions

This work was carried out in collaboration between all authors. Author KN performed the statistical analysis, wrote the protocol, and the first draft of the manuscript. Author RKM managed the analyses of the study. Author UKM managed the literature searches, author VM designed the study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To Isolate and characterize the antimicrobial actinomycetes from the marine habitats of south coast of Andhra Pradesh, India.
Place and Duration of the Study: Marine habitats of south coast of Andhra Pradesh, India, between June 2011 and July 2012.
Methodology: The soil samples were collected, pre-treated and plated on yeast extract-malt extract dextrose agar medium. Identification of the strain was carried out by employing the polyphasic taxonomical studies including the 16S rRNA sequence based analysis. Phylogenetic tree was constructed using the Molecular Evolutionary Genetic Analysis (MEGA) version 5. The influence of culture conditions and the effect of environmental factors on the biomass and antimicrobial activy\ity of the strain was the focus of this study.
Results: A total of 20 actinobacteria were isolated from the marine habitats of south coast of Andhra Pradesh, India, and screened for antimicrobial activity against test bacteria and fungi. The potent bioactive metabolite producing strain was designated as VLK-12.

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Further polyphasic studies revealed that the Isolate VLK-12 belongs to the genera *Rhodococcus.* Phylogenetic analysis of 16S rRNA sequencing studies revealed that the strain is closely related to *Rhodococcus erythropolis.* The crude ethyl acetate extract obtained by culturing the strain on YMD inhibited Gram positive and Gram negative bacteria along with fungi.

Conclusion: *Rhodococcus erythropolis* isolated from the marine habitats of south coast of Andhra Pradesh exhibited antimicrobial activity against pathogens.

Keywords: Rhodococcus erythropolis; antimicrobial profile; bioactive metabolites; nutritional factors; culture conditions and environmental parameters.

1. INTRODUCTION

Actinobacteria are Gram positive, free living, saprophytic bacteria widely distributed in different habitats, frequently filamentous and sporulating with DNA rich in G+C (55-75%). They are the potential source for many bioactive secondary metabolites [1]. More than 9000 biologically active molecules have been isolated from actinobacteria yielding more than 60 pharmaceutical agents used in medical and agricultural fields [2]. Nearly 22,000 compounds have been reported from the living beings of marine origin. Majority of them have been reported from marine plants and animals whereas the microbiological component of this ecosystem remains relatively unexplored. As marine environmental conditions are extremely different from terrestrial ones, so that marine actinomycetes have different characteristics from those of terrestrial counterparts and, therefore, they might produce different types of bioactive compounds. Therefore, we have focused our attention towards marine actinomycetes.

Marine actinobacteria have been the main stay for antibiotic discovery efforts during the past few decades. They have the capability to produce a wide variety of biologically active secondary metabolites such as antibiotics, pesticides, herbicides and enzymes like cellulase and xylanase used in waste treatment [3]. The search for novel antimicrobial agents is very useful to prevent the infectious diseases as they are the main cause of death worldwide and antibiotic resistance has become a global concern [4]. They are also used in areas of medicine as a means to fight infection in the food industry, and in water sanitation to inhibit the growth of microorganisms in drinking water [5]. Fungal plant diseases are often controlled by fungicides, however, extended use of fungicides has not only created problem of fungicidal resistance and increased contamination of the environment, hence there has been an increasing interest in using eco-friendly methods such as antifungal compounds from microbes [6]. The emergence of drug resistant pathogens and the increase in diseases affecting the immune system have greatly intensified the need to investigate new bioactive metabolites for potential pharmaceutical and industrial applications.

As a part of the effort to enhance the potent bioactive compounds to act as biocontrol agents as well as to combat multiple drug resistant pathogens, we have selected marine habitats from south coast of Andhra Pradesh, India as a source for the isolation of potent actinobacteria with broad spectrum antimicrobial activity.

2. MATERIALS AND METHODS

Soil samples collected in sterile polythene bags from marine habitats of south coast of Andhra Pradesh, India, were air dried at room temperature (30 ± 2°C) for 2-4 days. The air - dried soil samples were pretreated with calcium carbonate (10:1w/w) and incubated at 30°C for four days. The samples were appropriately diluted with distilled water and plated on yeast extract- malt extract dextrose (YMD) agar with pH 7.0. The diluted soil sample (0.5mL) was spread on the YMD agar medium supplemented with nystatin (50 µg/mL), tetracycline (50 µg/mL) and incubated at 30±2ºC for 7-14 days. Colonies of actinobacteria were isolated, subcultured and preserved on YMD agar slants at 4°C.The strains were initially screened for antimicrobial activity against test bacteria such as *Bacillus megaterium, B. subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* and fungi like *Aspergillus niger, Botrytis cinerea, Candida albicans, Fusarium solani* and *F. oxysporum.* Preliminary screening for inhibitory ability of the isolate was tested by Cross streak method. The isolates were inoculated as a single streak in the centre of the Petridish and incubated at 30ºC for 3-4 days to permit growth and antibiotic production. Later the test bacteria and fungi were inoculated by streaking perpendicular to the growth of isolate. The plates were incubated for 24-48 hours at 30ºC in case of bacteria and 72 hours for fungi. After incubation, inhibition of test bacteria and fungi around the isolate was taken as positive for inhibitory activity.

Among the 20 isolates tested for biological activity, one isolate designated as VLK-12 was found to be potent as it exhibited high antimicrobial activity and was identified based on cultural, morphological, physiological and biochemical characters along with molecular approaches. The strain VLK-12 was grown on seven International Streptomyces Project (ISP) media and three non-ISP media to determine morphological, biochemical and physiological and cultural characteristics [7,8,9]. The morphological characteristics were assessed using scanning electron microscopy (SEM: Model- JOEL-JSM 5600, Japan) of 4-day old culture grown on ISP2 medium (YMD) at various magnifications.

2.1 Identification of the Culture Based on 16s rRNA Sequence

The strain was grown in YMD broth for 3 days and was centrifuged at 10,000 rpm for 20min and the pellet was used for the extraction of DNA [10]. PCR mixture consisted of 2.5 µl of 10× buffer, 3.5 µl of $MgCl_2$ (25 mM), 2 µl of dNTP (0.4 mM), 1 µl of 16S rDNA actino specific Primer - forward (10 pmol/µl), 1 µl of 16S rDNA actino specific Primer- reverse (10 pmol/µl), Taq polymerase (2 U/µl) and 2 µl template DNA. PCR amplification was carried out as follows: initial denaturation step at 94 ºC for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1min and extension at 72°C for 1 min, with a further 5min extension at 72°C. The PCR product was purified with Agarose Gel DNA Purification Kit (SoluteReady® Genomic DNA purification kit, PCR Master Mix, Agarose gel electrophoresis consumables and Primers are from HELINI Biomolecules, Chennai, India). The 750 bp 16S rDNA sequence was determined with 16S rDNA actino specific forward and reverse primers. The 16S rRNA gene fragment was amplified using actino specific forward primer -5'-GCCTAACACATGCAAGTCGA-3' and actino specific reverse primer-5'-CGTATTACCGCGGCTGCTGG-3' [11]. The deduced 16s rRNA sequence was compared with the sequences in Gene Bank (http://www.ncbi. nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) then aligned with the related reference sequences retrieved from NCBI Gene Bank databases using the Clustal *W* method. Phylogenetic and molecular

evolutionary analyses were conducted using Molecular Evolutionary Genetic analysis (*MEGA)* version 5.0 [12].

2.2 Growth Pattern of the Strain

To determine the growth pattern, strain VLK-12 was activated by growing in the seed medium for 48h and then inoculated into 250 ml flasks containing 100 ml YMD broth followed by incubation at 30 ±2°C on a rotary shaker at 180 rpm. At every 24 h interval, the flasks were harvested and the growth of the strain was measured by weighing the dry weight of biomass. The culture filtrate was extracted with ethyl acetate and used for testing antimicrobial activity.

2.3 Optimizing the Culture Conditions for Enhanced Antimicrobial Activity

Attempts were made to enhance antimicrobial activity by optimizing the culture conditions such as pH, temperature, culture media, minerals, carbon and nitrogen sources.

2.4 Influence of Culture Media on Biomass and Antimicrobial Activity

In order to determine ideal conditions for the maximum antimicrobial activity from the strain VLK-12, the strain was cultured on ten different media such as tryptone yeast extract broth (ISP-1), yeast extract-malt extract-dextrose broth(ISP-2), Oat meal broth (ISP-3), starch inorganic salts broth (ISP-4), glycerol-asparagine broth (ISP-5), starch casein broth(ISP-6), tyrosine broth (ISP-7), nutrient broth, Czapek-Dox broth and yeast extract- starch broth. Influence of nutritional conditions are important to enhance the antimicrobial activity [15].The biomass accumulation and bioactive metabolite production in each medium was evaluated. The medium in which the strain exhibits optimum level of antimicrobial activity was used for subsequent study.

2.5 Effect of pH and Temperature on Biomass and Antimicrobial Activity

To determine the influence of initial pH on growth and antimicrobial activity of the strain VLK-12 was cultured in the medium with different initial pH levels ranging from 4-10 and at different temperatures from 25 to 45°C. The biomass and bioactive metabolite production were estimated to determine optimal pH and temperature which were used for further study [16,17].

2.6 Impact of Carbon and Nitrogen Sources on Biomass and Antimicrobial Activity

To determine the impact of carbon sources on biomass and antimicrobial activity of the strain, different carbon sources like maltose, lactose, fructose, sucrose, dextrose, starch, mannitol, xylose, starch and cellulose (each at a concentration of 0.5%) were added separately to the optimized medium [18]. Influence of several nitrogen sources on bioactive metabolite production was evaluated by supplementing the medium with different nitrogen sources like yeast extract, sodium nitrate, proline, tryptophan, histidine, cystein, alanine, tyrosine, urea and asparagine (each at a concentration of 0.5%) to the optimized medium containing the superior carbon source obtained in this study.

2.7 Effect of Minerals on Biomass and Antimicrobial Activity

The impact of minerals on the production of biomass and antimicrobial activity was studied by supplementing different minerals like K_2HPO_4, $MgSO_4$, $FeSO_4$, K_2HPO_4 and $ZnSO_4$ each at a concentration of 0.05% (w/v) to the optimized medium [19].

2.8 Antimicrobial Activity against Test Organisms

The antimicrobial activity of the crude extract produced by strain VLK-12 was determined by agar well diffusion method [13]. Nutrient agar (NA) and Czapek-Dox (CD) agar media were used for culturing the test bacteria and fungi respectively. NA medium (100 ml) was sterilized at 15 lbs pressure (121ºC) for 15min, cooled and inoculated with 0.2 ml of test bacterial suspension. After solidification of agar medium, wells of about 5 mm diameter were punched into it with sterilized cork borer. In case of antifungal assay, spore suspension of test fungi was mixed with the cooled, molten CD agar medium and poured into Petri dishes. The crude extract dissolved in ethyl acetate at a concentration of 50ppm was added to the wells made in the medium. Adding only ethyl acetate to the wells served as control. The plates were incubated at 30°C for 24 h for bacteria and 24-72 h for fungi and the diameter of the inhibition zones was measured [14].

The antimicrobial activity of the crude extract produced by the strain under optimized conditions was tested against bacteria viz. *Streptococcus mutans* (MTCC 497), *Lactobacillus casei* (MTCC 1423), *Lactobacillus acidophilus* (MTCC 495), *Enterococcus faecalis* (MTCC 439), *Staphylococcus aureus* (MTCC3160), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus vulgaris*(MTCC 7299) and *Xanthomonas campestris* (MTCC 2286) and fungi such as *Candida albicans* (ATCC 10231), *Aspergillus niger* and *Fusarium oxysporum* (MTCC 3075) by agar-diffusion assay.

2.9 Statistical Analysis

Data obtained on the antimicrobial activity under different culture conditions are statistically analyzed and expressed as mean ± standard error with one-way analysis of variance (ANOVA).

3. Results and Discussion

During our search for the isolation of potent actinomycetes, 20 strains (VLK-1 to VLK-20) were isolated from Bheemunipatnam, south coast of Andhra Pradesh, India. Among them, one strain found potent was designated as VLK-12. Among the ten media tested, ISP-2 supported excellent growth of the culture followed by ISP-1, ISP-6, ISP-7, maltose- tryptone agar and Czapek- Dox agar. The aerial mycelium appeared to be pink and substrate mycelium was pale yellow. Pigmentation was not found on any medium (Table 1).

Table 1. Cultural characteristics of the strain VLK-12

S.No	Medium	Growth	Aerial Mycelium	Substrate Mycelium	Pigmentation
1	Tryptone yeast-extractagar(ISP-1)	Good	Pink	Pale yellow	Nil
2	Yeast extract malt extract dextrose agar (ISP-2)	Excellent	Pink	Pale yellow	Nil
3	Oat-meal agar (ISP-3)	Poor	Pink	Pale yellow	Nil
4	Inorganic salts starch agar (ISP-4)	Moderate	pink	Paleyellow	Nil
5	Glycerol asparagine agar (ISP-5)	Moderate	pink	Paleyellow	Nil
6	Starch-casein agar(ISP-6)	Good	Pink	Pale yellow	Nil
7	Tyrosine agar (ISP-7)	Good	Pink	Pale yellow	Nil
8	Czapek-Dox agar	Good	Pink	Pale yellow	Nil
9	Maltose tryptone agar	Good	Pink	Pale yellow	Nil
10	Nutrient agar	Good	Pink	Pale yellow	Nil

The Morphological characters (Plate 1), physiological and biochemical characteristics of the strain VLK-12 are summarized in the Table 2. The strain was Gram positive and showed negative result for indole, Voge's – Proskaeur, citrate utilization and H_2S production tests. Sodium chloride tolerance of the strain was also studied as the salt concentration has a profound effect on the production of antibiotic from microorganisms due to its effect on the osmotic pressure to the medium [20]. The strain could tolerate NaCl up to 14% and was able to grow at a wide pH range (4-10) with optimum being pH 7. The optimum temperature recorded for growth was 30^0C while the range was found to be 25-35^0C. The strain could produce several enzymes like amylase, caseinase, chitinase, urease, cellulase, lipase and asparaginase and negative for arginine hydrolase.

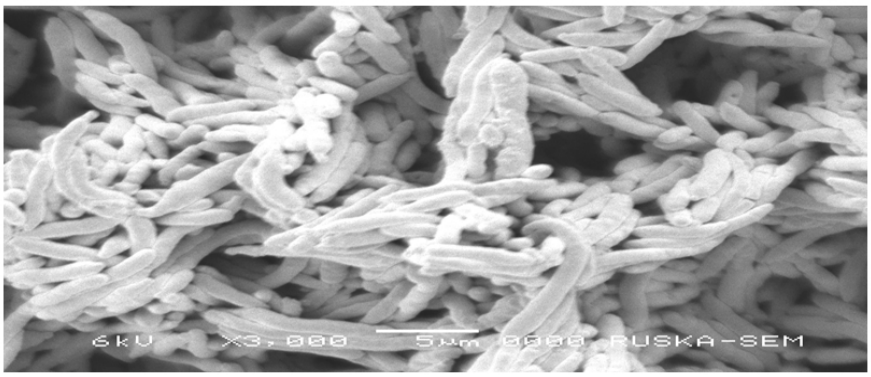

Plate 1. Scanning Electron Microscopic photograph of as *Rhodococcus erythropolis* VLK-12 (X 3000)

Table 2. Morphological characters, physiological characters and biochemical characters of the strain VLK-12

Character	Response
Cell shape	Fragmented mycelium, non sporulating
Color of aerial mycelium	Pink
Color of substrate mycelium	pale yellow
Physiological characters	
Gram's reaction	+
Production of melanin pigment	–
Range of temperature for growth	25-35°C
Optimum temperature for growth	30 °C
Range of pH for growth	4-10
Optimum pH for growth	7
NaCl (%) tolerance	14
Biochemical characters	
Catalase production	+
Hydrogen sulfide production	-
Nitrate reduction	-
Starch hydrolysis	+
Methyl red test	–
Voges proskauer test	–
Indole production	–
Citrate utilization	–
Enzymatic activity	
Amylase	+
Caseinase	+
Chitinase	+
Urease	+
Cellulase	+
Arginine hydrolase	–
L-Asparaginase	+
Lipase	+

Gene sequence of 16S rRNA of VLK-12 was blasted against nucleotide database of the NCBI and the sequences were aligned with the set of published sequences on the basis of the conserved primary sequence and also by nucleotide BLAST similarity search analysis. The strain showed a close relation with *Rhodococcus erythropolis* based on the 16S rRNA gene sequence (Fig. 1). The 16S rRNA sequence was deposited in the GenBank database of NCBI with the accession number KC106730. Basing on the morphological, physiological, biochemical characteristics and molecular characterization by 16s rRNA sequencing, the strain has been identified as *Rhodococcus erythropolis VLK-12.*

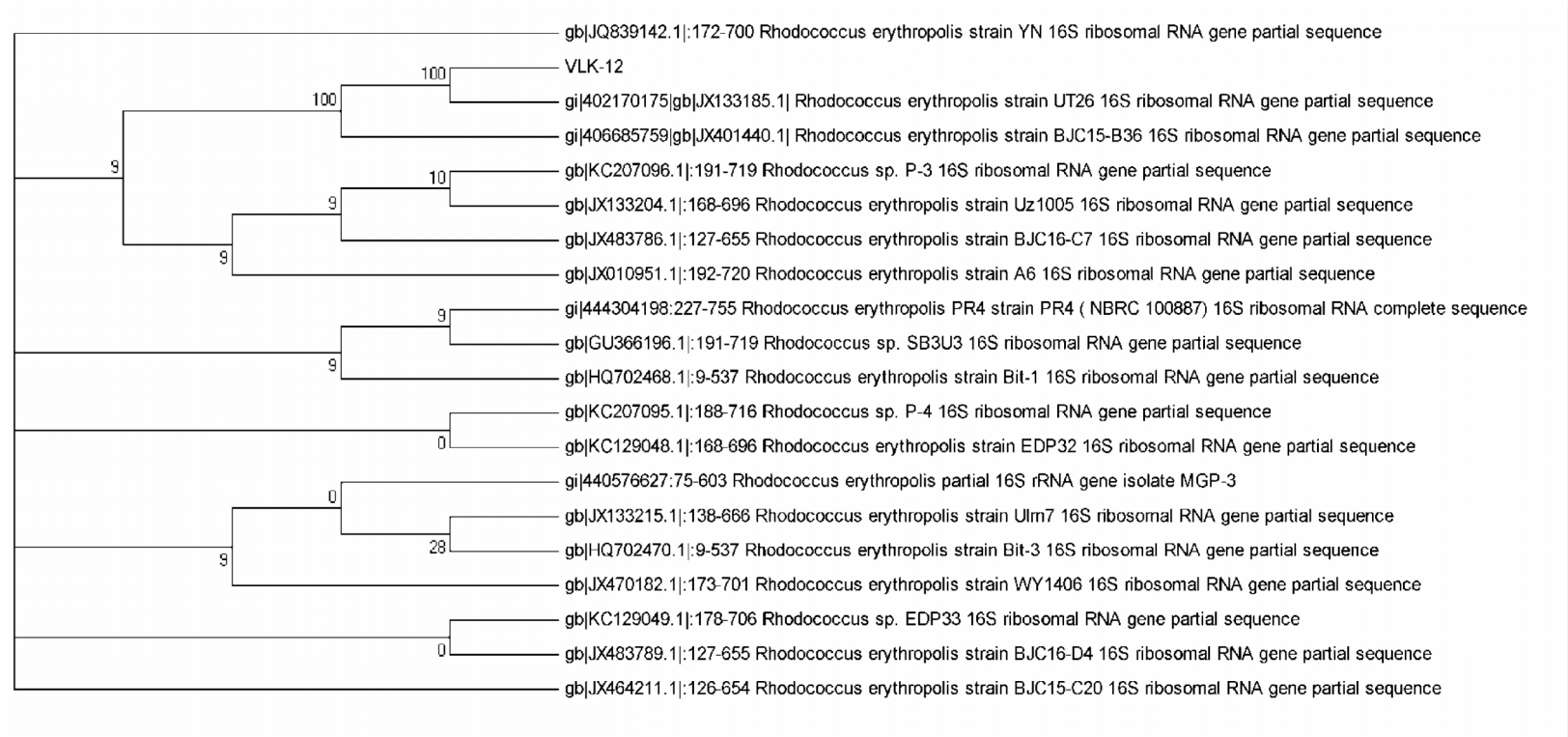

Fig. 1. Maximum Parsimony tree based on partial 16S rRNA gene sequence showing relationship between *Rhodococcus erythropolis* strain VLK-12 and related members of the genus *Rhodococcus erythropolis.*

3.1 Effect of Incubation Period on Biomass and Antimicrobial Activity

The growth pattern of the strain VLK-12 was studied on YMD broth. The stationary phase of the strain VLK-12 extended from 96 h to 120 h of incubation (Fig. 2). The crude extract obtained from five day old culture exhibited high antimicrobial activity against the test microorganisms.

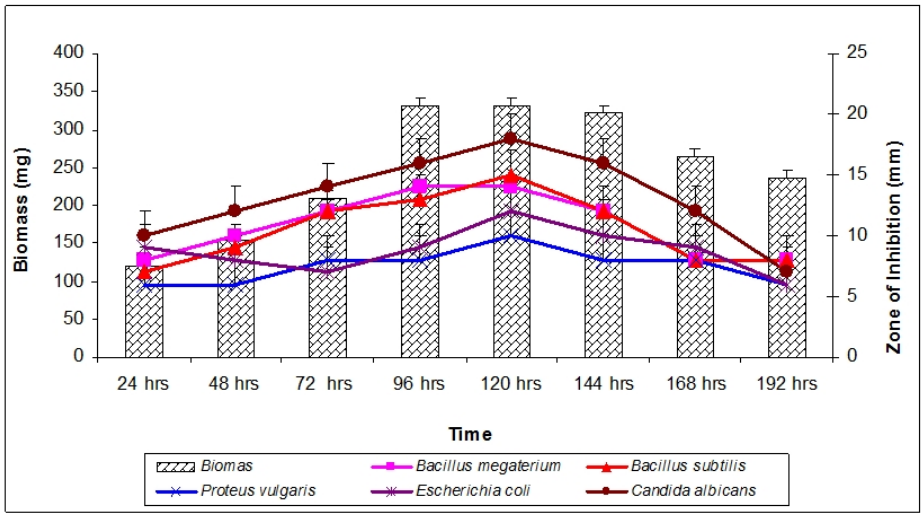

Fig. 2. Growth pattern of strain VLK-12. Data are statistically analyzed and found to be significant at 5%.

3.2 Influence of Culture Media on Biomass and Antimicrobial Activity

Biomass and antimicrobial activity of the strain were studied in different culture media (Fig. 3). Among the ten media tested, ISP-2 supported the production of compounds with high antimicrobial activity followed by yeast extract-starch broth, NAM and ISP-6.

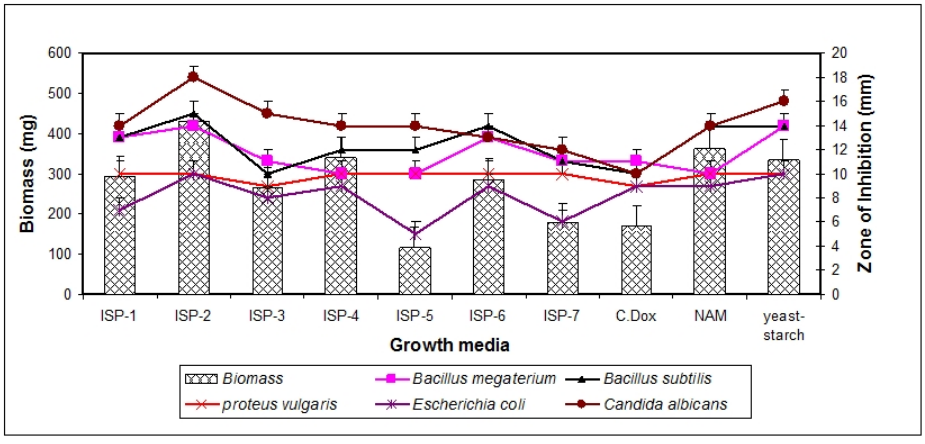

Fig. 3. Influence of different culture media on biomass and antimicrobial activity of the strain VLK-12. Data are statistically analyzed and found to be significant at 5%.

3.3 Impact of pH and Temperature on Biomass and Antimicrobial Activity

The strain was able to grow over a wide range of pH. Maximum growth and antimicrobial activity by the strain was found at pH 7 (Fig. 4). Bioactive metabolites obtained from the Isolate *Streptomyces* sp. VITSVK 9 grown at pH 7 exhibited good antimicrobial activity [17].

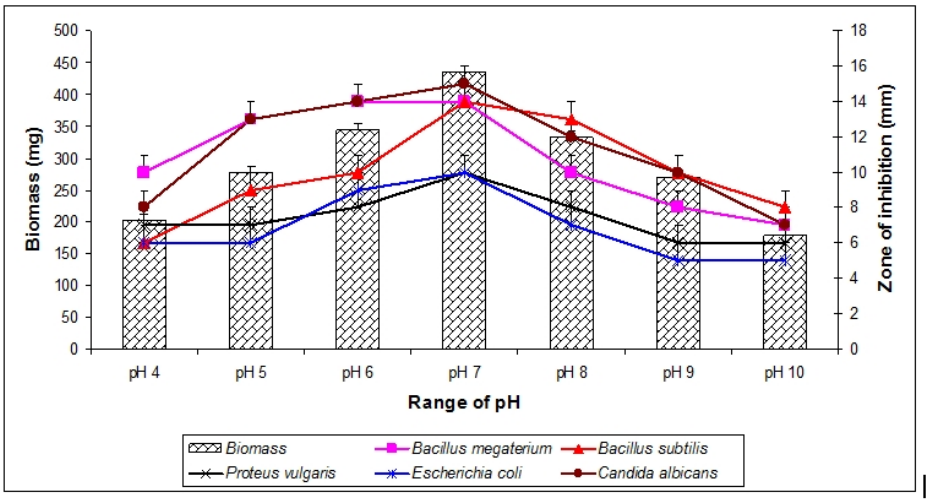

Fig. 4. Effect of pH on biomass and antimicrobial activity of the strain VLK-12. Data are statistically analyzed and found to be significant at 5%.

The growth as well as the antimicrobial activity of the strain was increased with rise in the incubation temperature from 25°C -30°C (Fig. 5). However, further increase in temperature (above 35°C) resulted in the decreased growth and antimicrobial activity.

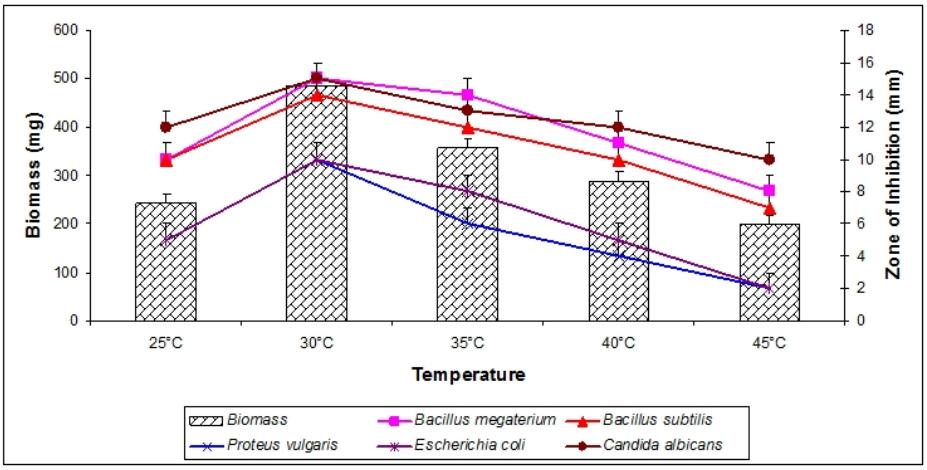

Fig. 5. Effect of temperature on biomass and antimicrobial activity of the strain VLK-12. Data are statistically analyzed and found to be significant at 5%.

3.4 Effect of Carbon and Nitrogen Sources on Biomass and Antimicrobial Activity

The effect of carbon sources on production of biomass and antimicrobial activity by the strain VLK-12 are presented in Fig. 6. Significant activity was obtained in lactose amended medium followed by fructose, galactose and dextrose. Similarly, the production of biomass was high with galactose, xylose and fructose followed by dextrose. As lactose emerged as the most preferred carbon source for high antimicrobial activity of the strain, varying concentrations of lactose (0.1-1%) were tested to determine the optimal concentration. Lactose at 0.5% showed optimal production of biomass and antimicrobial activity (Fig. 7).

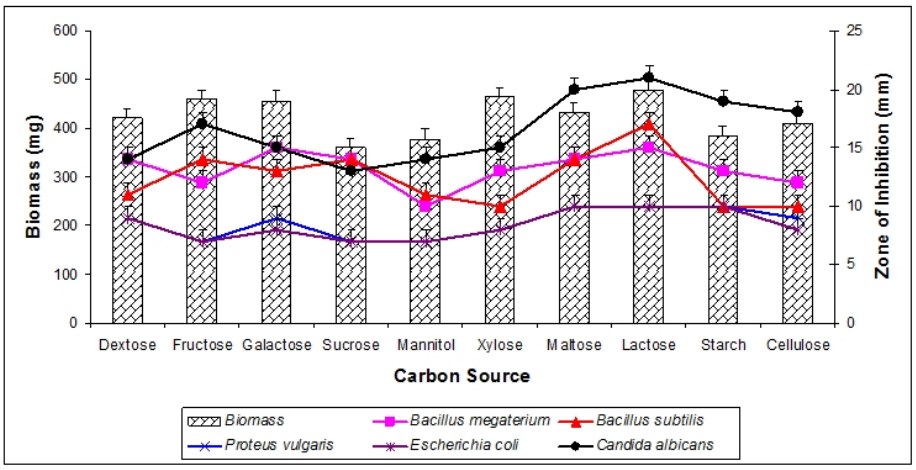

Fig. 6. Impact of different carbon sources supplemented in modified YMD broth on biomass and antimicrobial activity of the strain VLK-12. Data are statistically analyzed and found to be significant at 5%

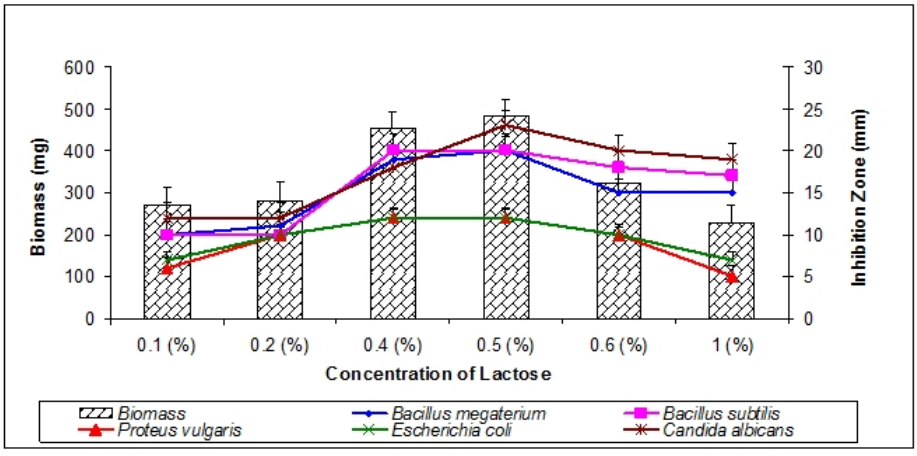

Fig. 7. Effect of different concentration of lactose on biomass and antimicrobial activity of the strain VLK-12. Data are statistically analyzed and found to be significant at 5%.

In order to make effective composition of growth medium, different nitrogen sources were evaluated for their influence on growth and antimicrobial activity of the strain. Of all the nitrogen sources tested, asparagine was found to be good for growth followed by proline and cystein, while asparagine and yeast extract were efficient for the antimicrobial activity (Fig. 8). As asparagine enhanced the biomass and antimicrobial activity of the strain VLK-12, effect of different concentrations of asparagine was tested and 0.5% was found to be best for improved antimicrobial activity (Fig. 9).

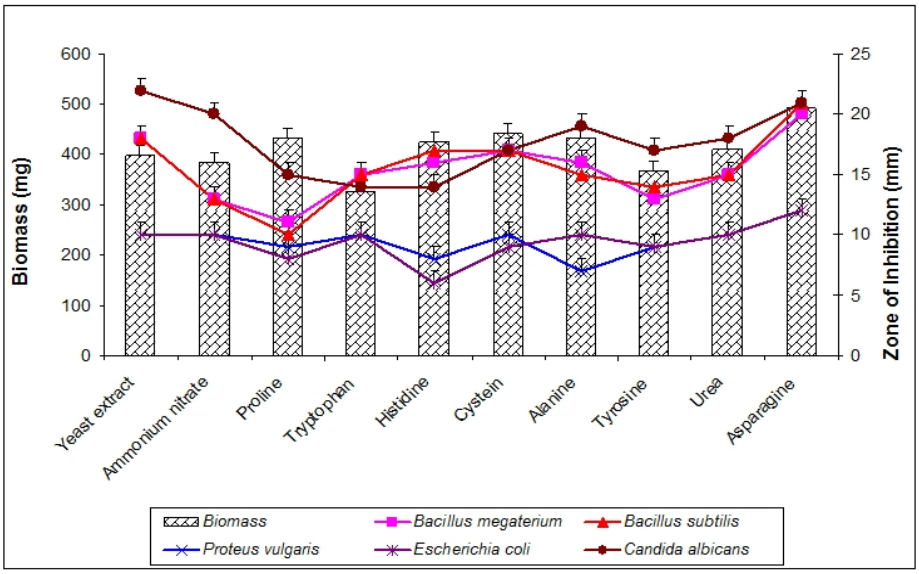

Fig. 8. Effect of different nitrogen sources supplemented in modified YMD broth on biomass and antimicrobial activity of the strain VLK-12. Data are statistically analyzed and found to be significant at 5%

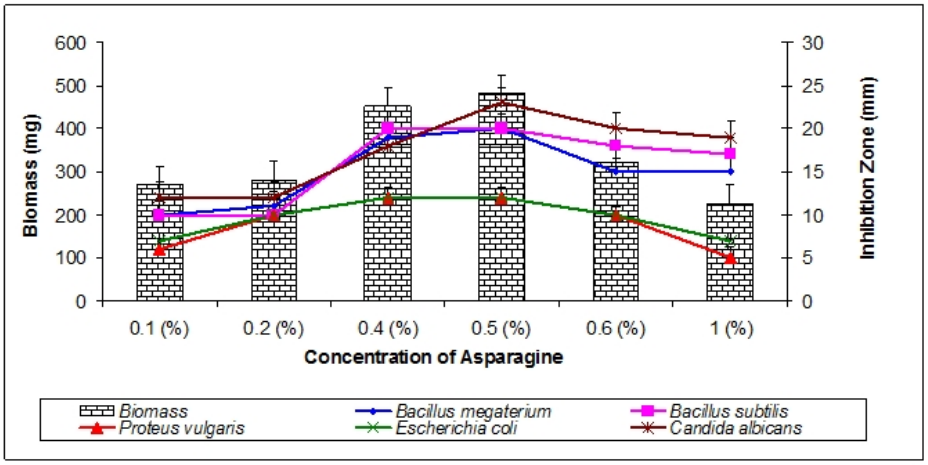

Fig. 9. Effect of different concentration of asparagine on biomass and antimicrobial activity of the strain VLK-12. Data are statistically analyzed and found to be significant at 5%.

3.5 Impact of Minerals on Biomass and Antimicrobial Activity

Effect of minerals on growth and secondary metabolite production by the strain VLK-12 is shown in Fig. 10. Among the minerals tested, K_2HPO_4 supported high rates of biomass and antimicrobial activity. Similar results were reported for *Streptomyces albidoflavus* [21].

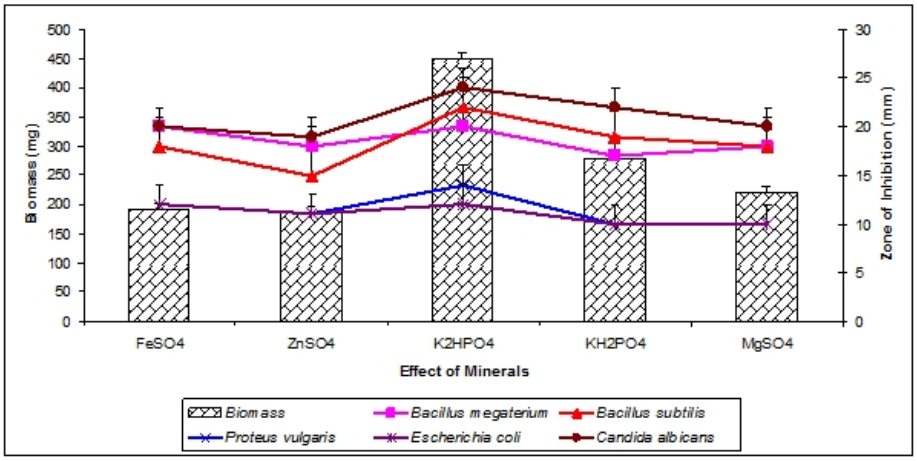

Fig. 10. Effect of different minerals on biomass and antimicrobial activity of the strain VLK- 12. Data are statistically analyzed and found to be significant at 5%.

4. CONCLUSION

In the present study, *Rhodococcus erythropolis VLK-12* exhibited high antimicrobial activity when cultured on ISP-2 broth amended with lactose (0.5%), asparagine (0.5%), NaCl (12%) and K_2HPO_4 (0.05%) with pH 7.0 and incubated at 30^{o}C for 120 h. Among the bacteria tested, *Bacillus subtilis* (Plate 2) and *B. megaterium* were highly sensitive to the crude extract followed by, *Enterococcus faecalis* and *Staphylococcus aureus* while *Candida albicans* (Plate 3) exhibited high sensitivity followed by *Fusarium oxysporum* and *Rhizoctonia* among fungi.(Fig. 11 and 12).This is the first report on the antimicrobial activity of *Rhodococcus erythropolis VLK-12.* Isolation, purification and chemical characterization of bioactive compounds produced by *Rhodococcus erythropolis VLK-12* is under progress.

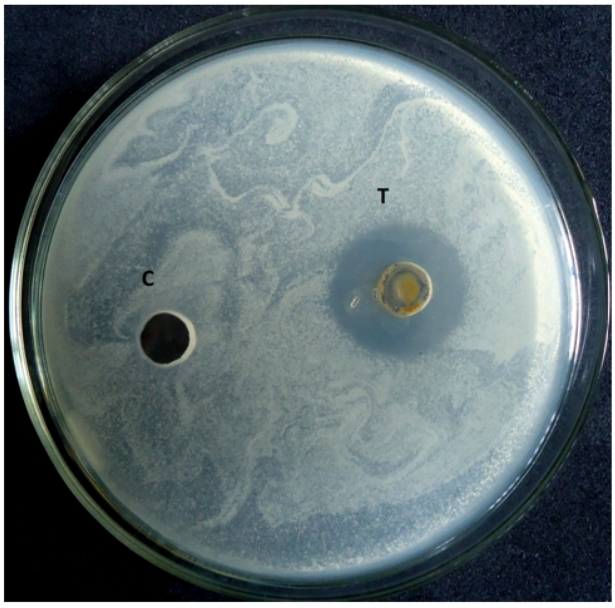

Plate 2. Antibacterial activity of the Strain VLK-12 against *Bacillus subtilis*
T= Ethyl acetate extract, C= Solvent (Ethyl acetate)

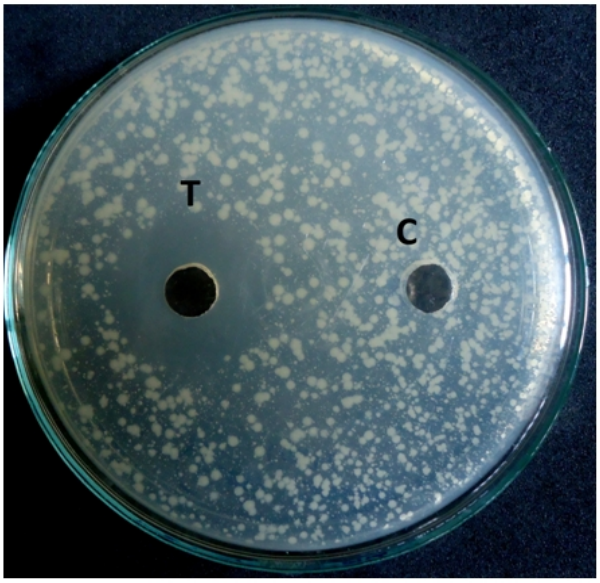

Plate 3. Antifungal activity of Strain the Strain VLK-12 against *Candida albicans*
T= Ethyl acetate extract, C= Solvent (Ethyl acetate)

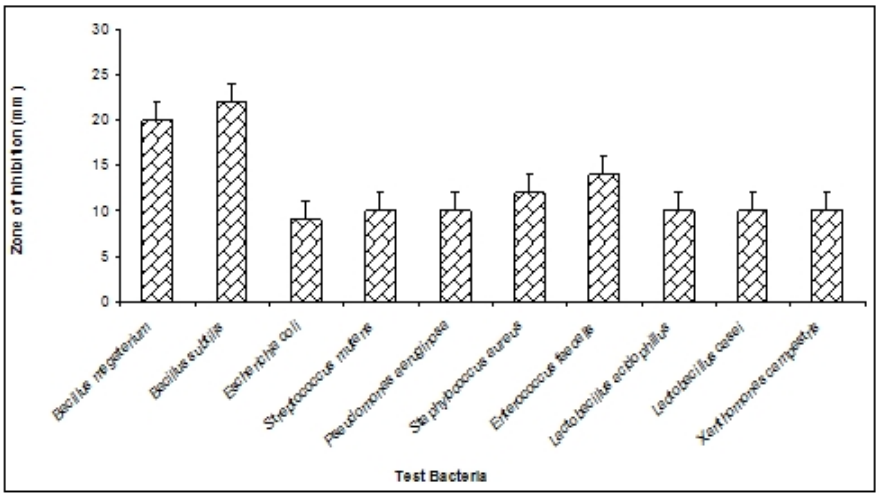

Fig. 11. Anti-bacterial activity of the strain VLK-12 (presented in terms of zone of inhibition) under optimized conditions tested against various bacteria. Data are statistically analyzed and found to be significant at 5%.

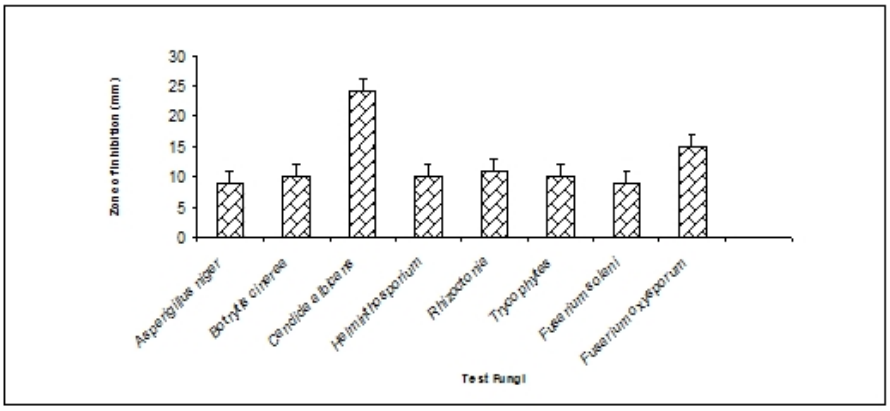

Fig.12. Anti-fungal activity of the strain VLK-12 (Presented in zone of inhibition) under optimized conditions tested against various fungi. Data are statistically analyzed and found to be significant at 5%

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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