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OPTIMIZATION STUDIES FOR ENHANCED BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES VIOLACEORUBER* VLK-4 ISOLATED FROM THE SOUTH COAST OF ANDHRA PRADESH, INDIA

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Keywords:

Streptomyces violaceoruber, Antimicrobial profile, Nutritional factors and Environmental parameters

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ABSTRACT: The aim of the present study was to design the suitable culture medium and also optimize the culture conditions for enhanced production of antimicrobial metabolites by *Streptomyces violaceoruber* VLK-4 which exhibited a broad spectrum of activity against bacteria and fungi. Production of bioactive metabolites by the strain was high in modified yeast extract-malt extract dextrose (ISP-2) broth as compared to other media tested. Mannitol (0.4%) and asparagine (1%) were found to be the most suitable carbon and nitrogen sources for the optimum production of bioactive metabolites as well as growth. Maximum production of bioactive metabolites was found in the culture medium with an initial pH 7.0 incubated for five days at 30 ºC under shaking conditions. This is the first report on the optimization of bioactive metabolites by *Streptomyces violaceoruber* VLK-4.

INTRODUCTION: Natural products are chemical compounds derived from living organisms including plants, animals and microorganisms [1]. Special interest was focused on the microbes that have been proved as the natural dumps for the bioactive metabolites [2]. Nearly 22,000 compounds have been reported from the organisms of marine origin. Majority of them have been reported from marine plants and animals, whereas the microbiological component of the marine ecosystem remains relatively unexplored. The most promising source of the future antibiotics that the society expects is the natural microbial products [3].

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Many microbes that thrive in extreme environments have the potentiality to produce atypical bioactive metabolites [4]. Production of the secondary metabolites by the microbes differs in quality and quantity based on the type of strains used [5]. Actinomycetes capable of producing many types of secondary metabolites represent a group of prokaryotic organisms which are Gram-positive, free-living, saprophytic bacteria widely distributed in different habitats, frequently filamentous and sporulating with DNA rich in G+C.

They symbolized one of the most studied and exploited classes of bacteria for their capability to make a wide range of biologically active metabolites. They play an important role among the marine bacterial communities due to their diversity and competency to produce novel chemical compounds of high commercial value [6]. As a part of the effort to enhance the potent bioactive compounds from *Streptomyces violaceoruber*

VLK-4, an attempt has been made in the present study to optimize the cultural parameters.

MATERIALS AND METHODS:

Isolation: Soil samples collected from marine habitats of the 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 diluted with distilled water were plated on starch-casein agar medium (ISP-6) supplemented with secnidazole (25µg/mL) and tetracycline (25µg/mL) and incubated at 30 ± 2 °C for 7 -14 days. Colonies of actinomycetes were isolated, subcultured and preserved on ISP-6 agar slants at 4 °C [7].

Among the 20 isolates tested for biological activity, one isolate designated as VLK-4 was found to be potent against microorganisms. Based on cultural, morphological, physiological and biochemical characters along with molecular approaches, it was identified as *Streptomyces violaceoruber* VLK-4. The 16s rRNA sequence of the strain was submitted to the Genbank with accession number KF908011 [8].

Optimization of Culture Conditions for the Enhanced Production of Bioactive Metabolites: Attempts were focused to enhance the production of bioactive metabolites by altering the parameters such as pH, temperature, culture media, minerals, carbon, and nitrogen sources.

Impact of Incubation Period on Biomass and Bioactive Metabolite Production by *Streptomyces violaceoruber* VLK-4: The growth pattern and bioactive metabolite production of the strain were studied at regular intervals up to 8 days. The actively growing culture was inoculated into ISP-6 broth and incubated at 30 ± 2 ºC on a rotary shaker at 120 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 extracted with ethyl acetate was concentrated and used as crude extract for testing antimicrobial activity employing agar well–diffusion method against test microorganisms [9].

Effect of Culture Media on Biomass and Production of Bioactive Metabolites by *Streptomyces violaceoruber* VLK-4: To determine the ideal conditions for the maximum production of antimicrobial agents, the strain was cultured on ten different media including tryptone yeast extract broth (ISP-1), yeast extract-malt extract-dextrose broth (ISP-2), oatmeal 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 is important to enhance the production of bioactive metabolites [10].

The biomass and bioactive metabolite production in each medium are evaluated. The medium in which the strain exhibits optimum levels of bioactive metabolite production was used for subsequent study.

Influence of pH and Temperature on Biomass and Bioactive Metabolite Production by *Streptomyces violaceoruber* VLK-4: To find out the influence of initial pH on growth and bioactive metabolite production, the strain was cultured in the medium with different initial pH levels ranging from [4-10]. The biomass and bioactive metabolite production were estimated and the optimum pH achieved for maximum bioactive metabolite production was used for further study [11]. Similarly, the optimum temperature for biomass and bioactive metabolite production was determined by incubating the culture at different temperatures ranging from 25 to 45 °C, while maintaining all other conditions at optimum levels [12, 13].

Effect of Carbon and Nitrogen Sources on Biomass and Bioactive Metabolite Production by *Streptomyces violaceoruber* VLK-4: To determine the impact of carbon sources on biomass and bioactive metabolite production by the strain, different carbon sources like maltose, lactose, fructose, sucrose, dextrose, starch, mannitol, xylose and cellulose, each at a concentration of 0.5% were added separately to the medium [14]. Effect of several nitrogen sources on bioactive metabolite production was evaluated by supplementing with different nitrogen sources like yeast extract, ammonium nitrate, proline, tryptophan, histidine, cysteine, alanine, tryptone, urea and asparagine, each at a concentration of 0.5% were incorporated into the fermentation medium. Further, the impact of different levels of optimized carbon and nitrogen

sources was studied to enhance antimicrobial metabolite production [15].

Influence of Minerals on Biomass and Bioactive Metabolite Production by *Streptomyces violaceoruber* VLK-4: To evaluate the effects of minerals on the production of biomass and bioactive metabolites, the strain was cultured in the optimized medium by supplementing different minerals like K_2HPO_4, $MgSO_4$, $FeSO_4$, KH_2PO_4 and $ZnSO_4$ each at a concentration of 0.05% (w/v) [16].

Antimicrobial Activity of *Streptomyces violaceoruber* VLK-4 Against Test Organisms: The antimicrobial metabolites produced by the strain cultured under optimized conditions were tested by the agar well diffusion assay against test bacteria including *Streptococcus mutans* (MTCC 497), *Lactobacillus casei* (MTCC 1423), *Lactobacillus acidophilus* (MTCC 495), *Enterococcus faecalis* (MTCC 439), *Staphylococcus aureus* (MTCC3160), *Bacillus subtilis* (ATCC 6633), *B. megaterium*, *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella typhi*, *Proteus vulgaris* (MTCC 7299) and *Xanthomonas campestris* (MTCC 2286) and fungi such as *Candida albicans* (ATCC 10231), *Aspergillus niger*, *A. flavus*, *Fusarium oxysporum* (MTCC 3075) and *Penicillium citrinum*.

Statistical Analysis: Data obtained on the bioactive metabolite production under different culture conditions are statistically analyzed and expressed as mean ± standard error with one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION:

Effect of Incubation Period on Biomass and Bioactive Metabolite Production by *Streptomyces violaceoruber* VLK-4: The growth pattern of the strain was studied on starch-casein broth. The stationary phase of the strain VLK-4 extended from 96 h to 120 h of incubation **Fig. 1**. The crude extract obtained from 120 h old culture exhibited high antimicrobial activity against the test microorganisms. Similarly, metabolites collected from 5-day-old culture of *Rhodococcus erythropolis* VLK-12 exhibited good antimicrobial activity against the test bacteria and fungi [17]. Extracts of four-day-old cultures of *Nocardia Levis* MK_VL113 [18], *Streptomyces tendae* TK-VL_333 [19], *S. cheonanensis* VUK-A [20] and *Pseudonocardia* sp. VUK-10 [21] exhibited high antimicrobial activity against the test microorganisms.

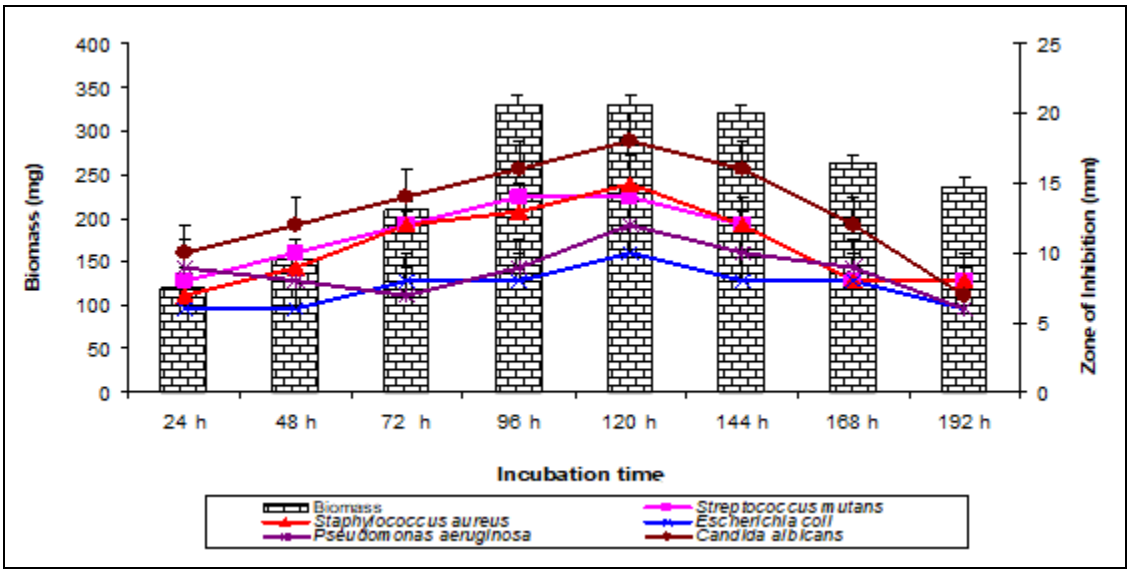

FIG. 1: GROWTH PATTERN AND ANTIMICROBIAL METABOLITE PRODUCTION (EXPRESSED IN TERMS OF ZONE OF INHIBITION) OF *STREPTOMYCES VIOLACEORUBER* VLK-4. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%

Influence of Culture Media on Biomass and Bioactive Metabolite Production by *Streptomyces violaceoruber* VLK-4: Biomass and bioactive metabolite production by the strain were studied in different culture media **Fig. 2**. Among the ten media tested, ISP-2 supported high levels of bioactive metabolites followed by yeast extract-starch broth, nutrient broth, and ISP-6. The

antimicrobial activity of actinomycete isolates could be increased or decreased remarkably under different cultural conditions [22]. Modified YMD broth supported the production of bioactive metabolites by *Pseudonocardia* sp. VUK-10 [21]. Czapek- Dox broth favored high rates of antibiotic production by *Streptomyces* sp. MNK-7 [23].

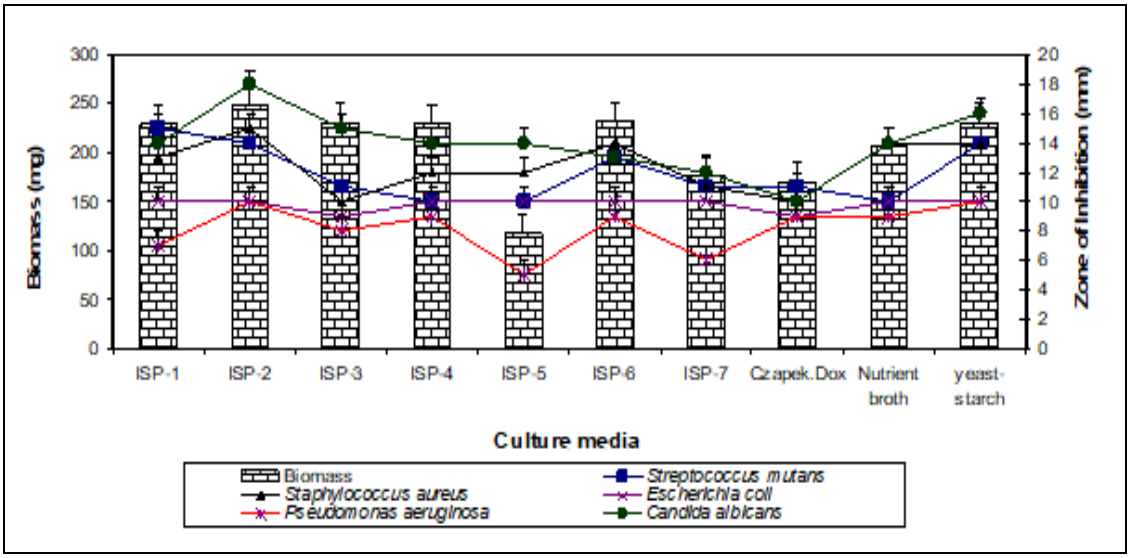

FIG. 2: IMPACT OF DIFFERENT CULTURE MEDIA ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES VIOLACEORUBER* VLK-4. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%

Impact of pH and Temperature on Biomass and Bioactive Metabolite Production by *Streptomyces violaceoruber* VLK-4: The strain was able to grow over a wide range of pH. Maximum growth and antimicrobial production by the strain was found at pH 7 **Fig. 3**. Bioactive metabolites obtained from *Streptomyces* sp. VITSVK 9 (24), *Streptomyces albidoflavus* [16] and *Streptomyces cheonanensis* VUK-A (20) at pH 7 exhibited good antimicrobial activity. The biomass, as well as the production of bioactive metabolites, was increased with the rise in the incubation temperature from 25°C-30°C **Fig. 4**. However, further increase in temperature (above 30 °C) resulted in the decline in growth and production of bioactive metabolites. These results are in complete accordance with the earlier reports [25, 26]. In terms of its optimum temperature for growth and production of bioactive metabolite, the strain VLK-4 appeared to be mesophilic.

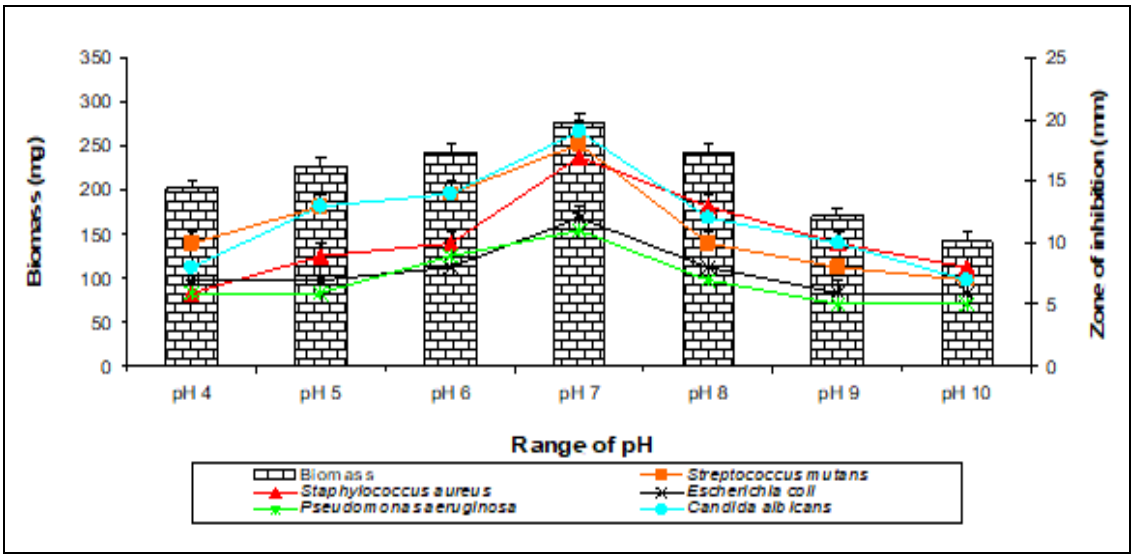

FIG. 3: INFLUENCE OF pH ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES VIOLACEORUBER* VLK-4. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%.

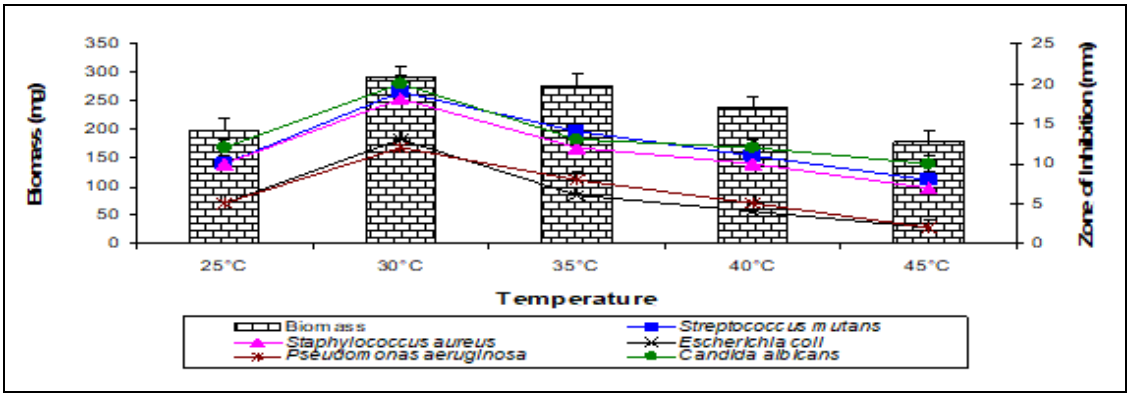

FIG. 4: EFFECT OF TEMPERATURE ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES VIOLACEORUBER* VLK-4. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%

Effect of Carbon and Nitrogen Sources on Biomass and Bioactive Metabolite Production by *Streptomyces violaceoruber* VLK-4: The effect of carbon sources on production of biomass and bioactive metabolites by the strain is presented in **Fig. 5**. Significant production of bioactive metabolite was obtained in mannitol amended medium followed by fructose, galactose, and dextrose, while the production of biomass was high with dextrose followed by sucrose, starch, and fructose. As mannitol emerged as the most preferred carbon source for bioactive metabolite production by the strain, varying concentrations of mannitol (0.1-1%) were tested to establish the optimal concentration. Mannitol at 0.4% showed optimal yields of bioactive metabolites **Fig. 6**.

The growth and bioactive metabolite production by *Streptomyces* sp. was good with glucose [27, 28] while *Streptomyces hygroscopicus* CH-7 utilized lactose as a carbon source for antibiotic production [29].

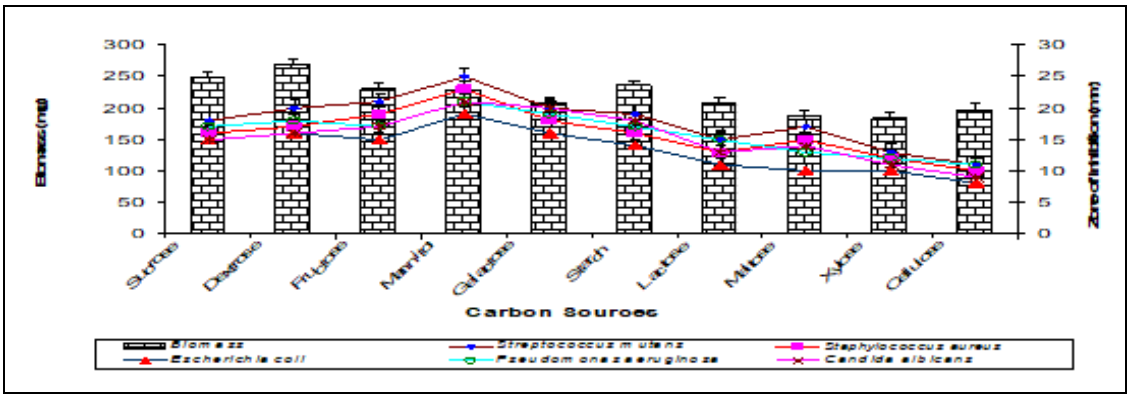

FIG. 5: EFFECT OF DIFFERENT CARBON SOURCES SUPPLEMENTED IN MODIFIED YMD BROTH ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES VIOLACEORUBER* VLK-4. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%

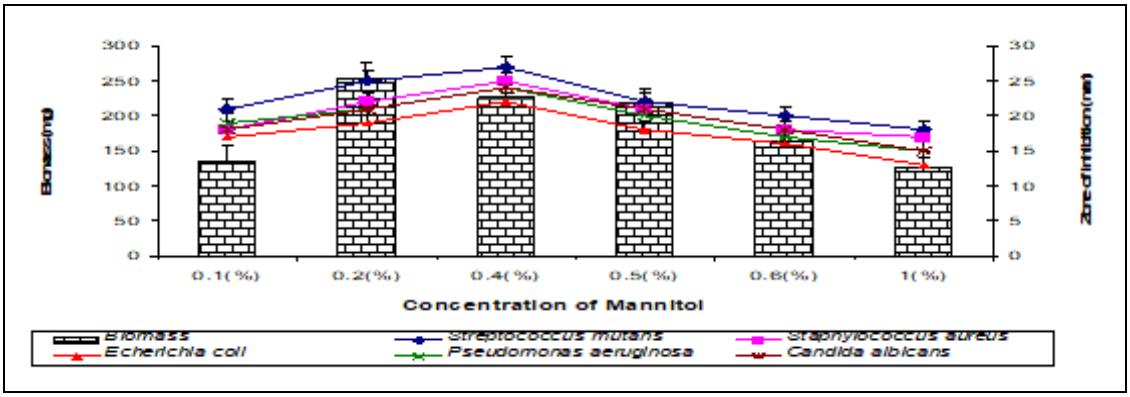

FIG. 6: IMPACT OF DIFFERENT CONCENTRATIONS OF MANNITOL ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES VIOLACEORUBER* VLK-4. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%

In order to make an effective composition of growth medium, the different nitrogen sources were evaluated for their influence on biomass and antimicrobial metabolite production by the strain VLK-4. Of all the nitrogen sources tested, asparagine followed by tryptophan and yeast extract was found to be the best for growth, while asparagine and yeast extract were efficient for the production of bioactive metabolites **Fig. 7**. As asparagine enhanced the biomass and bioactive metabolite production by the strain, effect of different concentrations of asparagine was tested, and 1.0% was found to be good for the production of bioactive metabolites **Fig. 8**. Peptone was reported to enhance the biomass and bioactive metabolite production by *Streptomyces* VITSVK 9 sp. [25] and *Streptomyces cheonanensis* VUK-A [20].

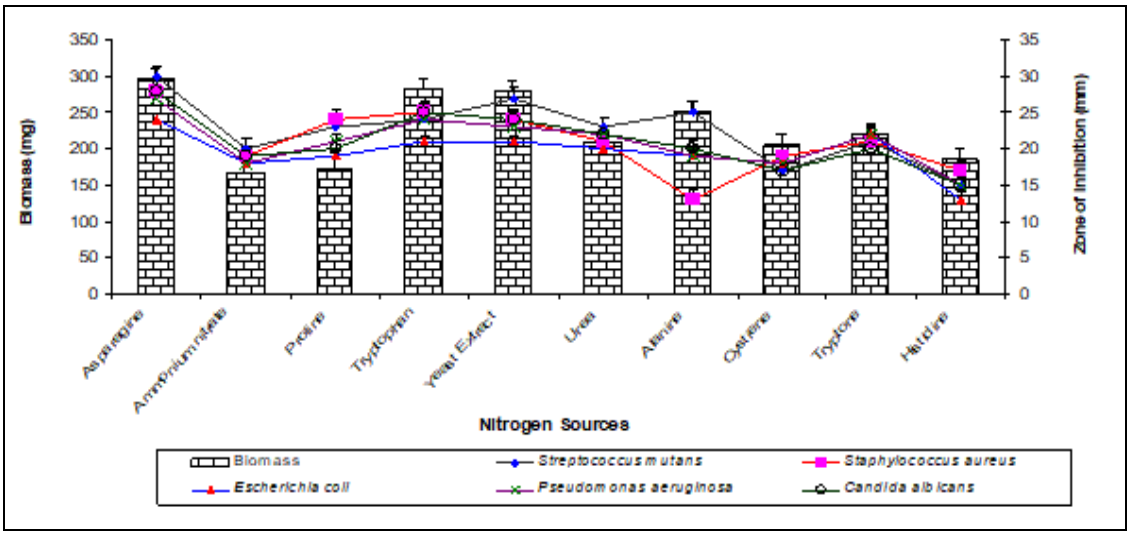

FIG. 7: IMPACT OF DIFFERENT NITROGEN SOURCES SUPPLEMENTED IN MODIFIED YMD BROTH ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES VIOLACEORUBER* VLK-4. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%

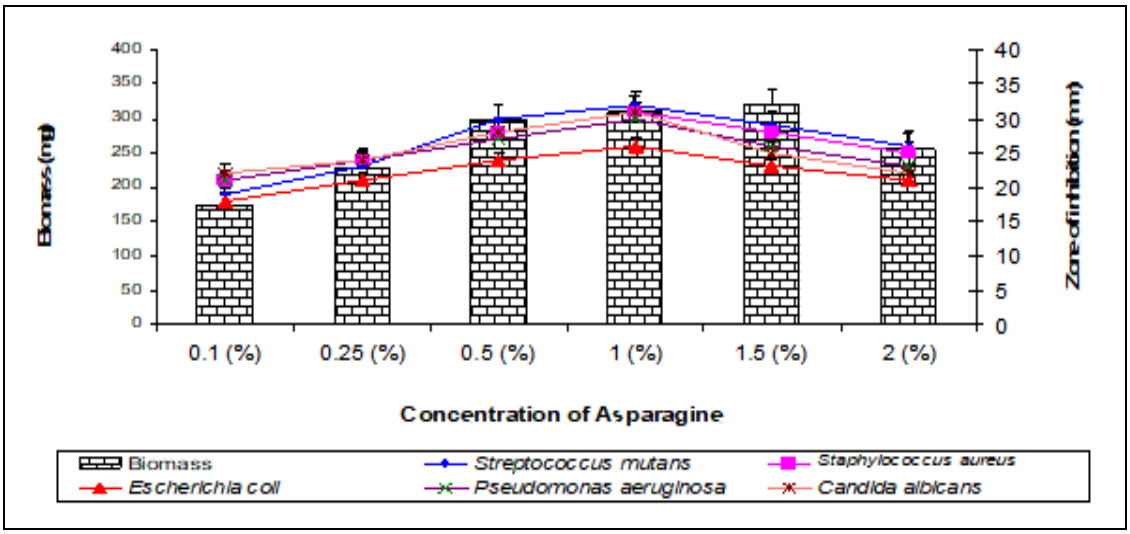

FIG. 8: INFLUENCE OF DIFFERENT CONCENTRATIONS OF ASPARAGINE ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES VIOLACEORUBER* VLK-4. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%

Impact of Minerals on Biomass and Bioactive Metabolite Production by *Streptomyces violaceoruber* VLK-4: Effect of minerals on growth and secondary metabolite production by the strain is shown in **Fig. 9**. Among the minerals tested, K_2HPO_4 supported high biomass and bioactive metabolite production. Similar results were reported for *Streptomyces albidoflavus* [16], *Streptomyces* sp. RUPA-08PR [30], *Streptomyces cheonanensis* VUK-A (20) and *Pseudonocardia* sp. VUK-10 [21].

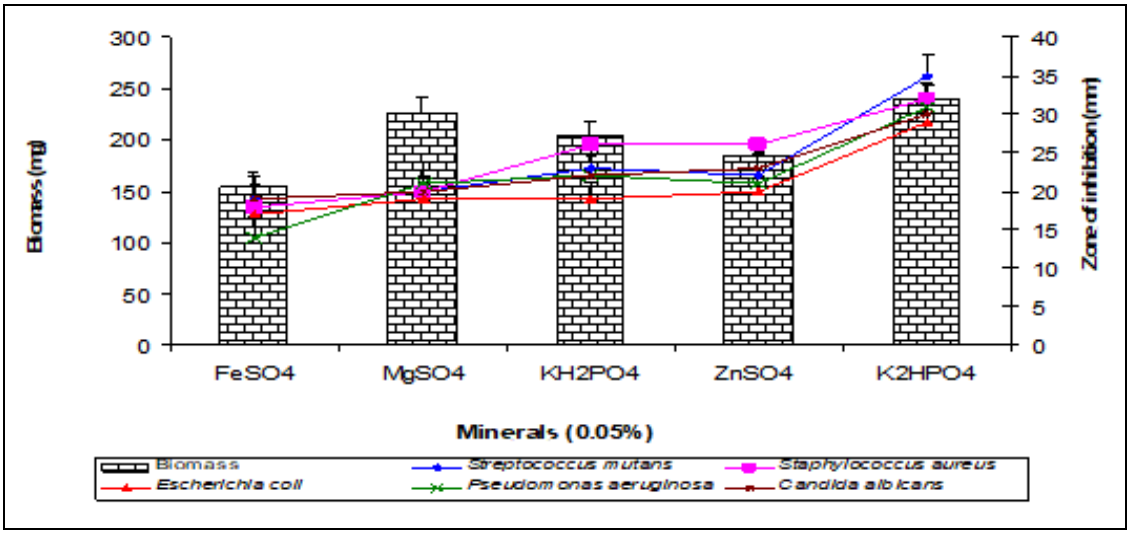

FIG. 9: INFLUENCE OF DIFFERENT MINERALS ON BIOMASS AND BIOACTIVE METABOLITE PRODUCTION BY *STREPTOMYCES VIOLACEORUBER* VLK-4. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%

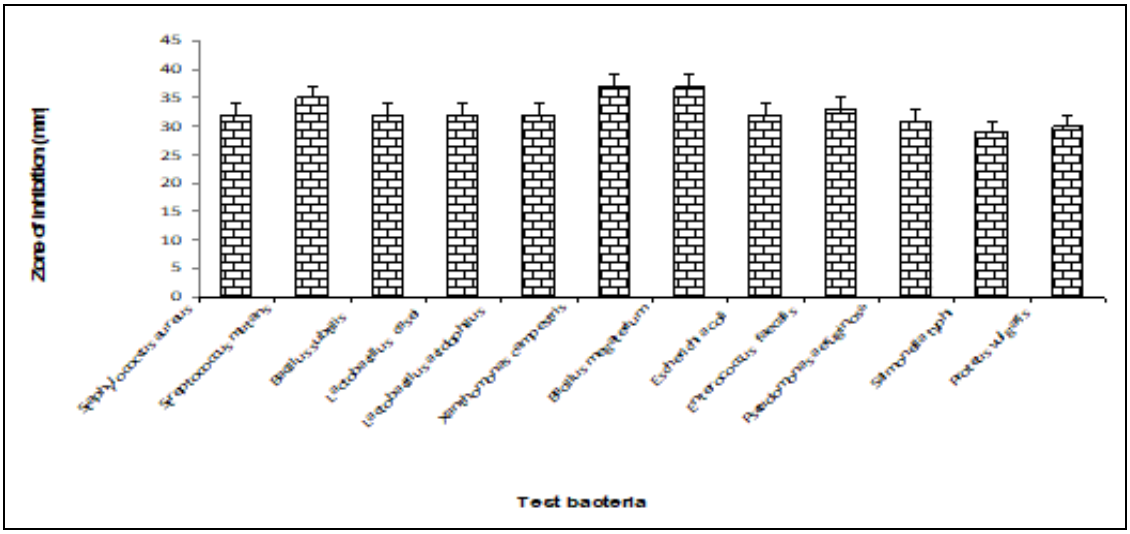

FIG. 10: ANTI-BACTERIAL ACTIVITY OF *STREPTOMYCES VIOLACEORUBER* VLK-4 GROWN UNDER OPTIMIZED CONDITIONS. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%

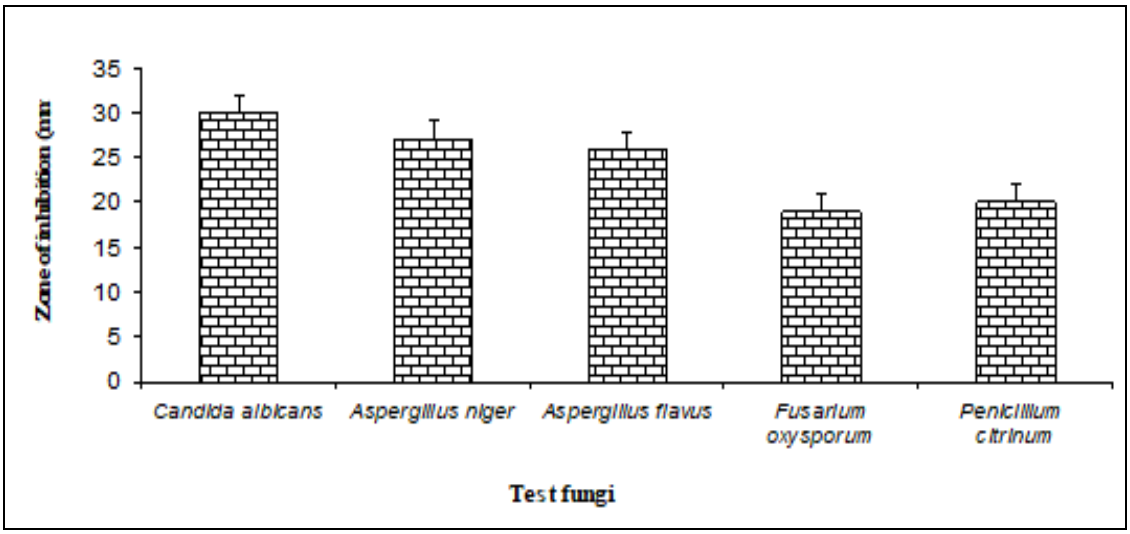

FIG. 11: ANTI-FUNGAL ACTIVITY OF *STREPTOMYCES VIOLACEORUBER* VLK-4 (PRESENTED IN TERMS OF ZONE OF INHIBITION) CULTURED UNDER OPTIMIZED CONDITIONS. DATA ARE STATISTICALLY ANALYZED AND FOUND TO BE SIGNIFICANT AT 5%

CONCLUSION: In the present study, *Streptomyces violaceoruber* VLK-4 exhibited high antimicrobial activity when cultured on modified ISP-2 broth amended with mannitol (0.4%), asparagine (1%), NaCl (3%) and 0.05% K_2HPO_4 with pH 7.0 incubated at 30 ºC for 120 h. Among the bacteria tested, S*treptococcus mutans, Xanthomonas campestris* **Plate 1** and *Bacillus megaterium* were highly sensitive to the metabolites followed by *Enterococcus faecalis* and *Staphylococcus aureus*, while *Candida albicans* **Plate 2** exhibited high sensitivity followed by *Aspergillus niger* and *A. flavus* among fungi **Fig 10, 11**. Consequently, further studies on purification, characterization, and identification of bioactive metabolites of *Streptomyce violaceoruber VLK*-4 are in progress. It is the first report on the culture conditions for enhanced production of bioactive metabolites by *S. violaceoruber* VLK-4.

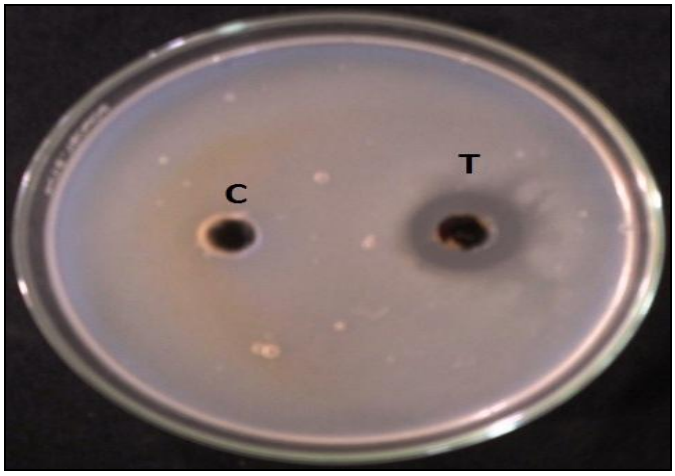

PLATE 1: ANTIBACTERIAL ACTIVITY OF *STREPTOMYCES VIOLACEORUBER* VLK-4 AGAINST *XANTHOMONAS CAMPESTRIS*. T= Ethyl acetate extract; C= Ethyl acetate (Control)

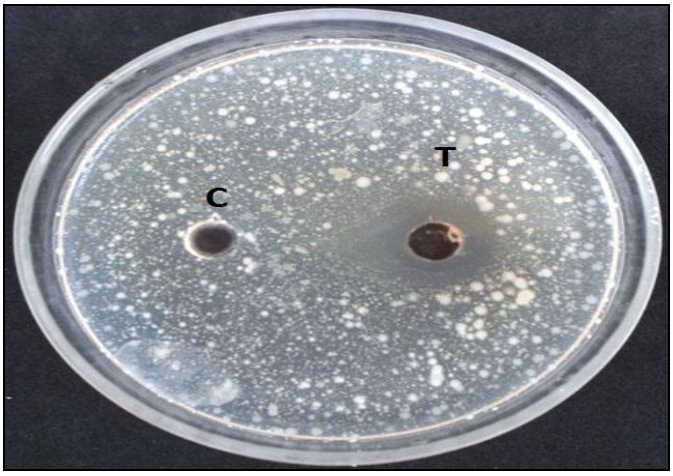

PLATE 2: ANTIFUNGAL ACTIVITY OF *STREPTOMYCES VIOLACEORUBER* VLK-4 AGAINST *CANDIDA ALBICANS*. T= Ethyl acetate extract; C= Ethyl acetate (Control)

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CONFLICT OF INTEREST: Nil

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