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RESEARCH ARTICLE

CULTURAL PARAMETERS INFLUENCING THE PRODUCTION OF ANTIMICROBIAL METABOLITES BY *RHODOCOCCUS ERYTHROPOLIS* VL-RK_05

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ABSTRACT

A study has been undertaken to optimize the production of antimicrobial metabolites by *Rhodococcus erythropolis* VL-RK_05 isolated from Mango orchards of Krishna District region by using basal medium as yeast extract malt extract dextrose medium. Five-day old culture showed maximum antimicrobial metabolite production when grown at pH 7.0 and temperature 30°C. The productivity of the strain was enhanced by amending the medium with sucrose and tryptophan each at a concentration of 1% (w/v) and 0.05% K_2HPO_4. The antimicrobial metabolites produced under optimized conditions exhibited broad spectrum of antimicrobial activity against different Gram positive and Gram negative bacteria as well as fungi.

INTRODUCTION

Microorganisms are capable of producing a broad spectrum of secondary metabolites, and the prokaryotic actinobacteria have been the most fruitful source of antibiotics, yielding 65-70% of all discovered antibiotics for the past five decades (Berdy, 2005). Therefore, screening of microorganisms for the production of new antibiotics continues to be an important approach in modern drug discovery programs. The class Actinobacteria accounts for a high proportion of soil microbial biomass and contains the most economically significant prokaryotes, producing more than half of the bioactive compounds (Lazzarini *et al*., 2000). Actinobacteria belonging to the genus *Streptomyces*, in particular, are excellent producers. Emergence of drug resistance in many bacterial pathogens and the current increase in the number of fungal infections has caused a resurgence of interest in finding new reserves of biologically active compounds (Samain *et al*., 1982). As the search for novel natural products continues, it becomes apparent that the rate of discovery of new compounds from soil *Streptomycetes* has decreased, whereas the rate of re isolation of known compounds is at increasing trend (Fenical *et al*., 1999). Recently, evidence has accumulated that rare actinomycete species, which are often very difficult to isolate and cultivate, represent a unique source of novel biologically active compounds (Baltz, 2006).

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On the other hand, new microbial habitats need to be examined in the search for novel bioactive compounds. As part of our ongoing research on bioactive metabolites of novel actinobacteria, one promising strain with good antimicrobial potential was identified as *Rhodococcus erythropolis* VL-RK_05 isolated from Mango orchards. The strain has been deposited in NCBI Genbank with an accession number JX885669. As the components of the culture medium and the conditions in which the organisms are cultured often influence the production of bioactive metabolites (Basak and Majumdar, 1973; Rabah *et al*., 2007; Rizk, 2007; Singh *et al*., 2008; Banga *et al*., 2008; Laidi *et al*., 2008), an attempt was made in the present study to optimize the cultural conditions for enhancing the production of bioactive metabolites by the strain.

MATERIALS AND METHODS

The strain *Rhodococcus erythropolis* VL-RK_05 was isolated from soil samples of Mango orchards of Vissannapet, Krishna Dist., Andra Pradesh, India, by using the soil dilution plate technique on yeast extract-malt extract-dextrose agar (ISP-2) with pH 7.0 (Waksman, 1961). The pure culture was maintained on YMD agar medium at 4°C (plate-1). The strain has been deposited in the NCBI GenBank with the accession No. JX885669. The test organisms used in the present study were procured from ATCC, University Boulevard, Manassas, USA and MTCC, IMTECH, Chandigarh, India and preserved at 4°C.

Plate-1. *Rhodococcus erythropolis* VL-RK_05 grown on ISP-2 (YMD) agar medium

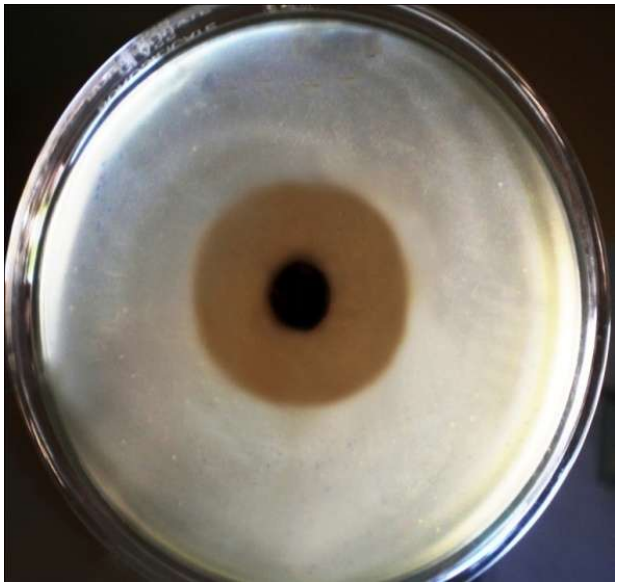

Plate-2. Antibacterial activity of the strain VL-RK_05 against *Pseudomonas aeruginosa*

Growth pattern and antimicrobial profile of R. erythropolis VL-RK_05

The growth pattern and antimicrobial profile of *R. erythropolis* VL-RK_05 was studied at regular intervals for up to 8 days. The strain was inoculated into 250 ml flasks containing 100 ml YMD broth and incubated at 30 ±2 °C for optimum yields on a rotary shaker at 120 rpm. At every 24 h interval, dry weight of the biomass of the strain and antimicrobial metabolites production in terms of their antimicrobial spectrum was determined. The culture filtrates were extracted with ethyl acetate and antimicrobial activity of crude extract was determined by agar well diffusion method. The production of bioactive metabolites was assessed by measuring the diameter of the inhibition zone against Gram positive bacteria - *Staphylococcus aureus* (MTCC 3160) and *Bacillus megaterium* (NCIM 2187) as well as Gram negative bacteria - *Shigella flexneri* (MTCC 1457), *Xanthomonas campestris* (MTCC 2286), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (ATCC 9027) and fungi including *Candida albicans* (MTCC 183).

Optimization of Fermentation process

Effects of initial pH and incubation temperature on biomass and bioactive metabolite production

Influence of initial pH on growth and bioactive metabolite production of the strain was determined by adjusting the pH of production medium ranging from 4-10. The optimal pH achieved at this step was used for further study (Oskay, 2011). Similarly, the optimum temperature for growth and bioactive metabolite yield was measured by incubating the production medium at temperatures ranging from 20-40ºC, while maintaining all other conditions at optimum levels (Ripa *et al*., 2009).

Selection of the culture media for influenced bioactive metabolite production

To select the suitable growth medium, the strain was grown in 10 different culture media, such as tryptone-yeast extract broth (ISP-1), YMD broth (ISP-2), Oat-meal broth (ISP-3), Inorganic salts starch broth (ISP-4), glycerol-asparagine broth (ISP-5), tyrosine broth (ISP-7), maltose tryptone broth, starch yeast extract broth, modified Czapek-Dox broth and starch-casein salts broth (Naragani *et al*., 2014). The biomass accumulation and bioactive metabolite production on different media were determined after 5th day of incubation. The medium in which the strain exhibited maximum bioactive metabolite production expressed in terms of zone of inhibition was fixed for further studies.

Effects of supplementary carbon and nitrogen sources on biomass and bioactive metabolite production

Carbon sources such as maltose, lactose, fructose, galactose, sucrose, glucose, starch, cellulose, mannitol and sorbitol at 1% concentration were supplemented separately into the fermentation medium. The effect of different concentrations of the best carbon source (0.5-4%) on the growth and bioactive metabolite production was also investigated. Similarly, various nitrogen sources, such as peptone, methionine, tryptophan, sodium nitrate, L-proline, leucine, tyrosine, valine, aspartic acid, urea, yeast extract and arginine @ 0.5% were individually supplemented into the fermentation medium (Majumdar and Majumdar, 1967). Further, the impact of different levels of optimized nitrogen source (0.1-1.5) was studied to enhance antimicrobial metabolite production (Kathiresan *et al*., 2005).

Effect of minerals on biomass and bioactive metabolite production

To evaluate the effects of minerals on growth and bioactive metabolite production, the optimized medium with superior carbon and nitrogen sources was amended separately with

different minerals, such as K_2HPO_4, KH_2PO_4, KCl, $MgSO_4$, $FeSO_4$, $MnCl_2$ and NaCl each at a concentration of 0.05% (w/v) (Ripa *et al*., 2009).

Test organisms

The antimicrobial metabolites produced by the strain under optimized conditions were tested against bacteria such as *Staphylococcus aureus* (MTCC 3160), *Bacillus megaterium* (NCIM 2187), *Bacillus subtilis, Lactobacillus casei, Lactobacillus acidophilus, Shigella flexneri* (MTCC 1457), *Xanthomonas campestris* (MTCC 2286), *Proteus vulgaris* (MTCC 7299), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 35218) and *Streptococcus mutans* (MTCC 497). *Candida albicans* (ATCC 10231), *Aspergillus niger, A. flavus, Fusarium solani, F. oxysporum* (MTCC 3075), *Penicillium citrinum* and *Alternaria sp.* were used for testing antifungal activity using agar plate diffusion assay (Cappuccino and Sherman, 2004).

Statistical analysis

Statistical data was recorded on cell growth of the strain and antimicrobial metabolite productiony by using One-way Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

Growth pattern and antimicrobial profile of the strain

The stationary phase of *Rhodococcus erythropolis* VL-RK_05 extended from 96 h to 144 h of incubation. The secondary metabolites obtained from five-day old culture showed high antimicrobial activity against the test microbes (Fig.1). Naragani *et al*., (2014) reported that metabolites obtained from five day old culture of *Rhodococcus erythropolis* VLK-12, a marine isolate showed maximum antimicrobial activity. Narayana *et al*., (2004) showed that *Streptomyces* sp isolated from virgin soil elaborated maximum antimicrobial metabolites production after 120 h. Narayana *et al*., (2008) stated that *Streptomyces albidoflavus* elaborated maximum antimicrobial metabolite production after 120 h. The secondary metabolites obtained from four-day old culture of *Nocardia levis* MK-VL_113 isolated from laterite soils of Guntur showed high antimicrobial activity against the test microbes (Kavitha and Vijayalakshmi, 2009).

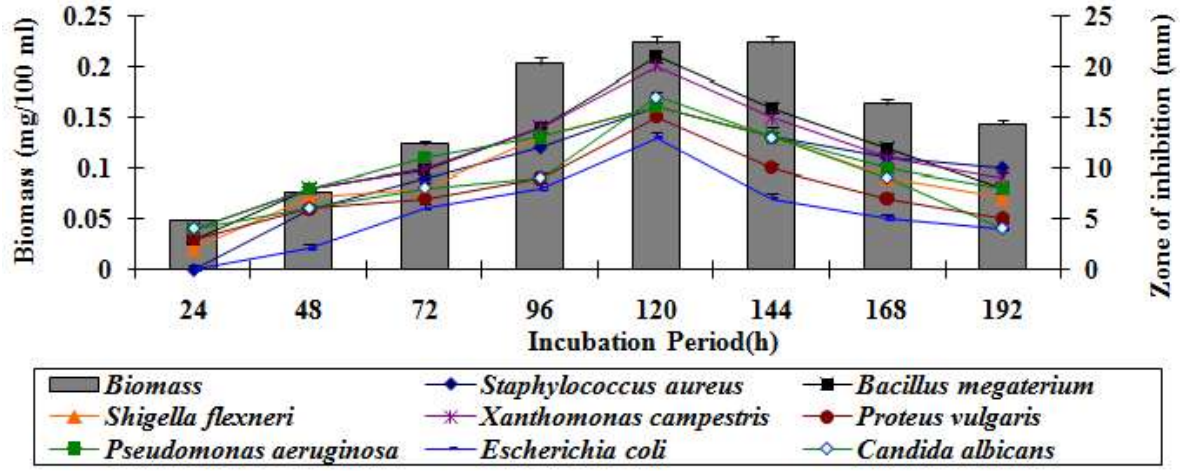

Fig.1. Effect of incubation period on biomass and bioactive metabolite production by *Rhodococcus erythropolis* VL-RK_05

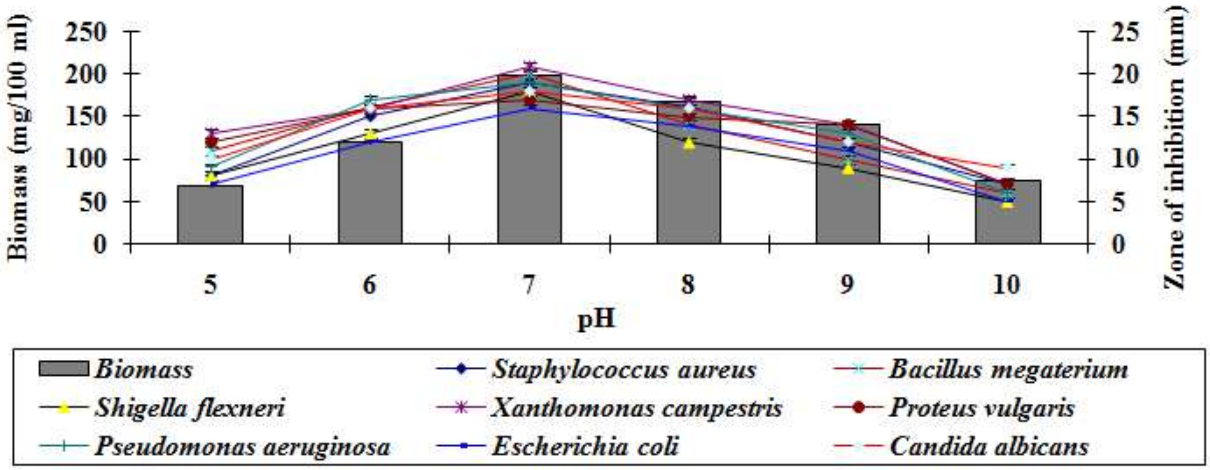

Fig.2. Effect of pH on biomass and bioactive metabolite production by *Rhodococcus erythropolis* VL-RK_05

Effect of initial pH and incubation temperature on biomass and bioactive metabolite production

The environmental requirements and cultural conditions for growth and bioactive metabolite production have been studied. Maximum growth and antimicrobial metabolite production was obtained at pH 7 (Fig.2). Similar results were reported for several *Streptomyces* sp. The influence of temperature on the biomass and bioactive metabolite production of the strain is presented in fig.3. Good growth as well as anti-microbial compound production was obtained at 30°C. In terms of its optimum temperature for growth, the organism appeared to be mesophilic. Actinomycetes such as *S.galbus* (Paul and Banerjee, 1983), *S.rochei* G164 (Chattopadhyay and Sen, 1997), *S.hygroscopicus* (Bhattacharyya *et al*., 1998), *S.marinensis* (Ellaiah *et al*., 2004), *S.fradiae*, *S.lavendualae*, *S.fulvissimus* (Rizk *et al*., 2007) and *Thermomonospora* sp. (Gupte and Kulkarni, 2003) showed optimum levels of antibiotic production at 30^{o}C.

Selection of culture media suitable for biomass and bioactive metabolite production

The influence of different media on the production of biomass and bioactive metabolites was recorded (Fig.4). Among the media tested, modified YMD broth supported good growth as well as bioactive metabolites, followed by maltose-tryptone broth and Inorganic salts starch broth. Modified YMD medium enhanced biomass and bioactive metabolite production of a marine isolate *Rhodococcus erythropolis* VLK-12 (Naragani *et al*., 2014).

Effect of supplementary carbon and nitrogen sources on biomass and bioactive metabolite production

Effects of different carbon and nitrogen sources were evaluated for their impact on growth and antimicrobial metabolite production (Figs.5 and 6). Among the various carbon sources tested, sucrose was the best one for bioactive metabolite production. Sucrose as the best carbon source for antibiotic production by *S. rochei* G164 was reported by Chattopadhyay and Sen (1997). Kavitha *et al.,* (2009) reported that *Nocardia levis* MK-VL_113 isolated from laterite soils of Acharaya Nagarjuna University utilized sucrose as the sole carbon source for antibiotic production. As sucrose was the most preferred carbon source for biomass and bioactive metabolite production by the strain, different levels of sucrose (0.5-4%) were tested to determine optimal concentration for bioactive metabolite production (Fig.7). One percent sucrose supplemented in the medium promoted the bioactive metabolite production. Different nitrogen sources were found to have significant effect on growth and secondary metabolite production by *Rhodococcus erythropolis* VL-RK_05.

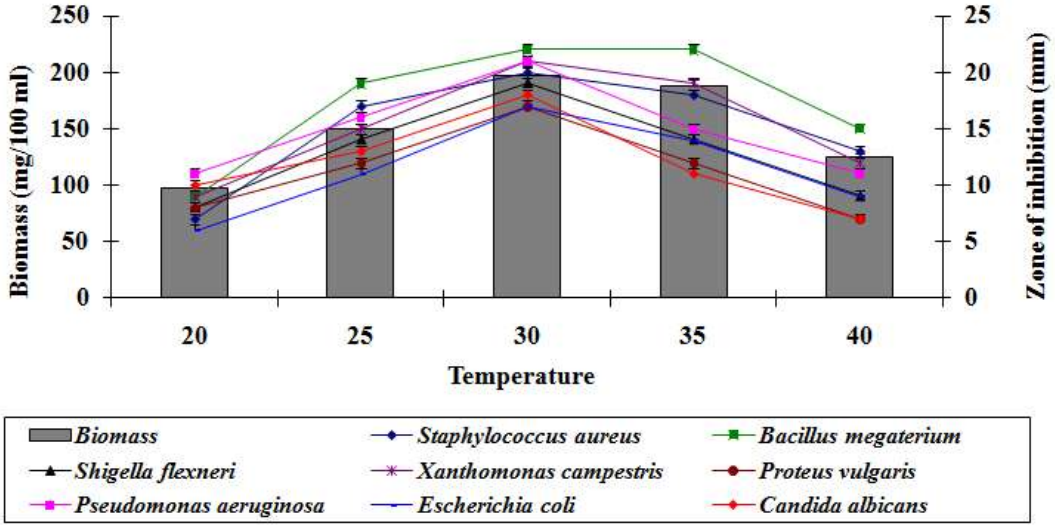

Fig.3. Effect of Temperature on biomass and bioactive metabolite production by *Rhodococcus erythropolis* VL-RK_05

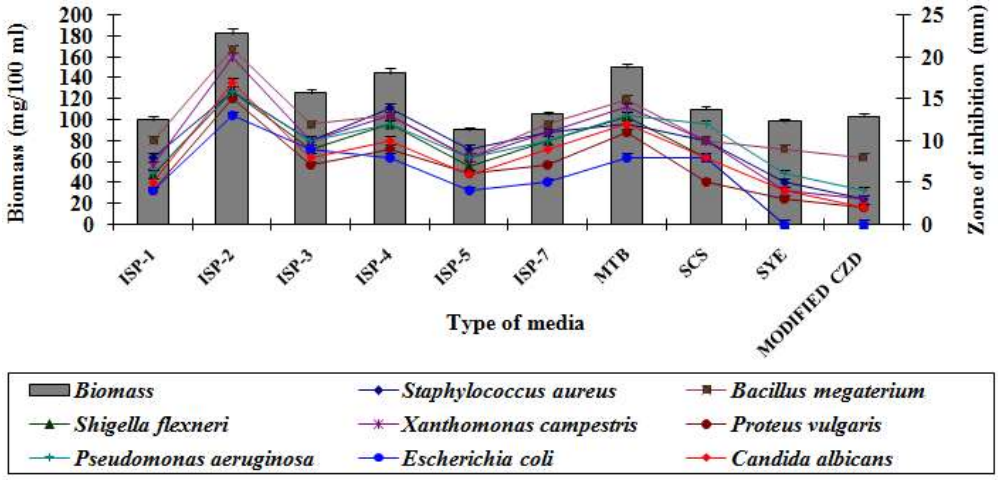

Fig. 4. Effect of different growth media on cell growth and bioactive metabolite production by *Rhodococcus erythropolis* VL-RK_05

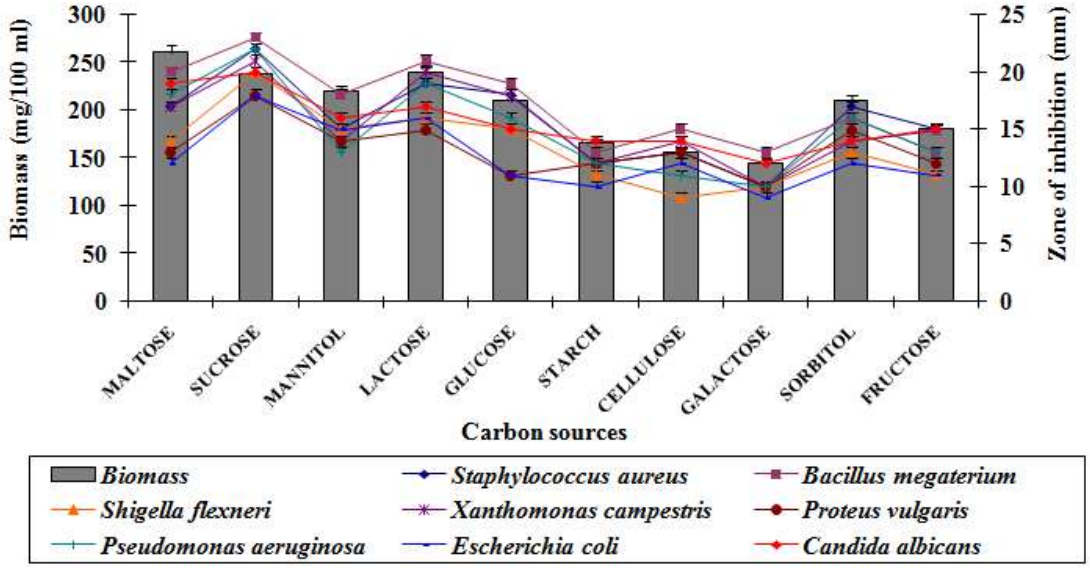

Fig. 5. Effect of different carbon sources on growth and bioactive metabolite production by *Rhodococcus erythropolis* VL-RK_05

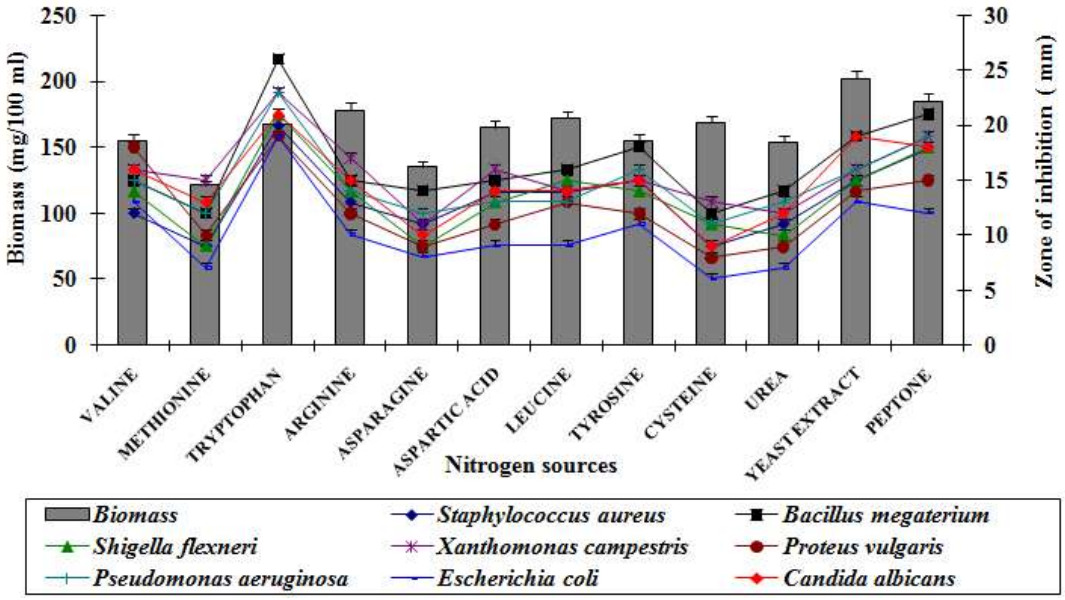

Fig. 6. Effect of different Nitrogen sources on growth and bioactive metabolite production by *Rhodococcus erythropolis* VL-RK_05

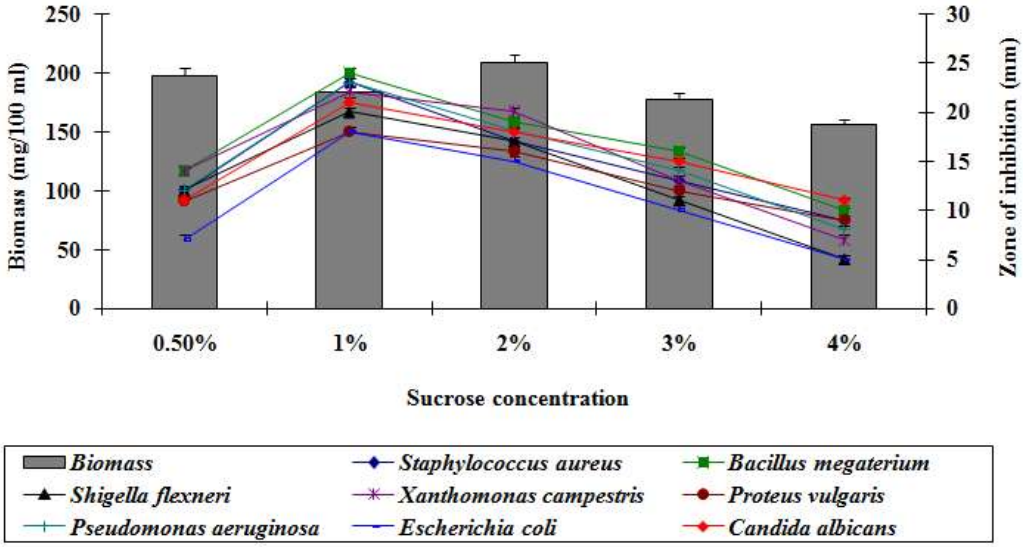

Fig. 7. Effect of Sucrose concentration on growth and bioactive metabolite production by *Rhodococcus erythropolis* VL-RK_05

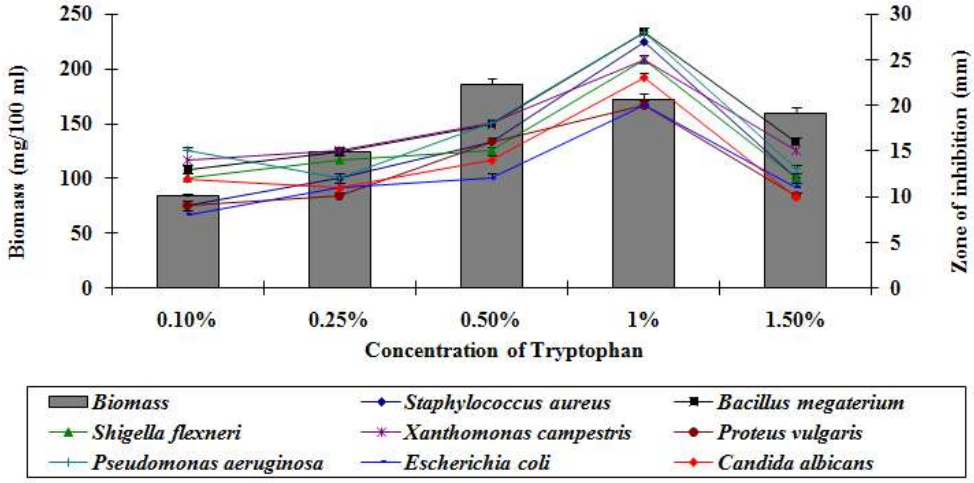

Fig. 8. Effect of different concentrations of Tryptophan on growth and bioactive metabolite production by *Rhodococcus erythropolis* VL-RK_05

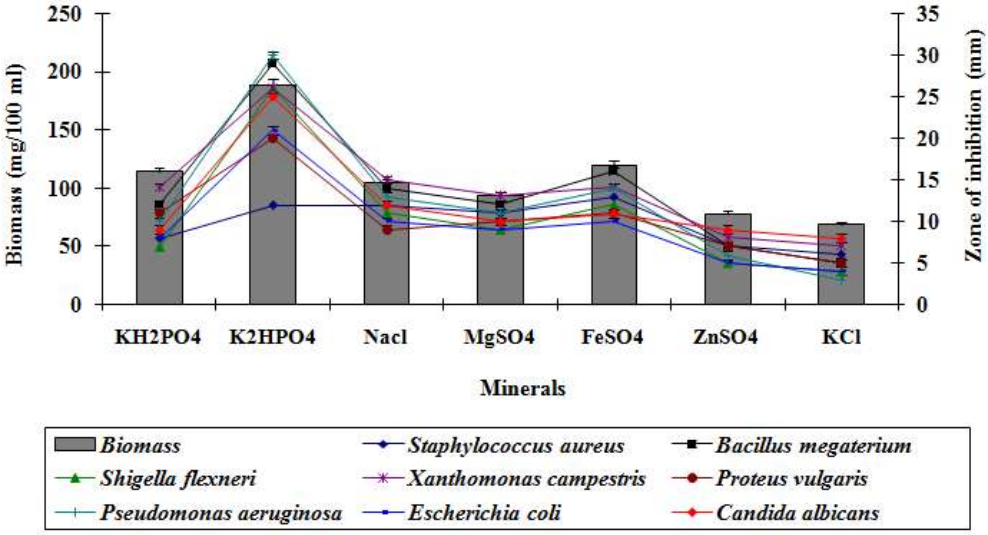

Fig.9. Effect of different minerals on biomass and bioactive metabolite production by *Rhodococcus erythropolis* VL-RK_05

Table 1. Antimicrobial activity of bioactive metabolites produced by *Rhodococcus erythropolis* VL-RK_05 under optimized conditions against bacteria and fungi

Test organisms	Zone of inhibition (mm)
Bacteria	
Staphylococcus aureus	28
Streptococcus mutans	24
Bacillus subtilis	28
Lactobacillus casei	32
Lactobacillus acidophilus	30
Xanthomonas campestris	29
Bacillus megaterium	30
Escherichia coli	23
Enterococcus faecalis	28
Pseudomonas aeruginosa	31
Shegella flexneri	27
Proteus vulgaris	22
Fungi	
Candida albicans	25
Aspergillus niger	16
A. flavus	15
Fusarium solani	18
F. oxysporum	20
Penicillium citrinum	19
Alternaria sp.	17

Maximum antimicrobial activity was obtained in culture filtrates supplemented with tryptophan followed by peptone and yeast extract, where as biomass production was found to be increased with yeast extract followed by peptone and arginine (Fig.6). Tryptophan (1%) supported high metabolite production (Fig.8). Growth and antibiotic production were found to be governed by nitrogen sources (Francois and Stephane, 2001) and the utilization of nitrogen sources for the production of bioactive metabolites seems to be different among actinomycete strains.

Effect of minerals on biomass and bioactive metabolite production

The influence of minerals on biomass and bioactive metabolite production by the strain is represented in Fig.9. K_2HPO_4 enhanced the production of biomass and antimicrobial metabolites. In contrast, the production of bioactive metabolites was very low with KCl followed by ZnSO4. Majumdar and Majumdar (1967) reported maximum yield of

neomycin by *Streptomyces fradiae* with K_2HPO_4 and least with $ZnSO_4$. Similar results have been recorded by Ripa *et al*. (2009).

Conclusion

In the present study, *Rhodococcus erythropolis* VL-RK_05 isolated from Mango orchards exhibited high antimicrobial activity when cultured in ISP-2 broth amended with Sucrose (1%), Tryptophan (1%) and K_2HPO_4 (0.05%) with pH 7.0 and incubated at 30^oC for 120 h. Among the bacteria tested, *Lactobacillus casei, Pseudomonas aeruginosa* (Plate-2) *Bacillus megaterium* and *Lactobacillus acidophilus* were highly sensitive to the crude extract followed by *Xanthomonas campestris* and *Bacillus subtilis* while *Candida albicans* exhibited high sensitivity followed by *Fusarium oxysporum* in case of fungi.

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