British Microbiology Research Journal
4(4): 428-442, 2014

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Optimization of Process Parameters for Improved Production of Bioactive Metabolites by *Streptomyces tritolerans* DAS 165^T

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Authors' contributions

This work was carried out in collaboration between all authors. Author UKM performed the experimental part. Authors VM and DA designed the study, author SP performed literature search and statistical analysis. All authors read and approved the final manuscript.

Original Research Article

Received 24th September 2013
Accepted 14th November 2013
Published 12th January 2014

ABSTRACT

Aims: To optimize the process parameters for enhanced production of bioactive metabolites by *Streptomyces tritolerans* DAS 165^T.
Place and Duration of Study: Department of Botany and Microbiology, April 2012 to August 2012.
Methodology: Agar well diffusion assay was employed to study the effect of environmental parameters such as incubation period, pH, temperature and salt concentration and influence of various nutrients such as carbon and nitrogen sources and minerals on the bioactive metabolite production by *Streptomyces tritolerans* DAS 165^T.
Results: The production of antimicrobial metabolite was high when the strain was cultured for six days at 35ºC in medium (pH 7.5) with sucrose at the concentration of 2% (carbon source), soya peptone at the concentration of 1% (nitrogen source) and sodium chloride at the concentration of 5%.
Conclusion: This is the first report on the optimization of bioactive metabolite production

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by *Streptomyces tritolerans* DAS 165[T]. As the strain exhibited potent antimicrobial activity, it may be explored for biotechnological purposes.

Keywords: Optimization studies; streptomyces tritolerans; bioactive metabolites; environmental parameters; nutritional factors; antimicrobial activity.

1. INTRODUCTION

The intensified search for novel antibiotics and bioactive compounds is due to the establishment of multiple drug resistance in pathogenic bacteria and lack of compelling therapies against infectious diseases [1]. Actinomycetes are well documented sources for the aspired natural structures providing the skeletal scaffold for the biosynthesis of significant molecules of pharmaceutical interest [2]. They are responsible for approximately 70% of the antibiotics that are of clinical use [3]. New trends in drug discovery from natural sources accentuate in probing the extreme ecosystems to uncover multitudinal knotty chemical molecules which become the new source for lead molecules for treatment of many diseases [4]. Actinomycetes are wide spread in nature and occur in extreme environments like acidophilic, alkaliphilic, psychrophilic, thermophilic etc [5]. They have the potential to produce array of molecules with exclusive structures due to their adaptation to highly physical and chemical rugged conditions prevail in extreme ecosystems [6]. Most of the studies related to extremophilics are confined to actinomycetes due to their molecular evolution of life and stability of macromolecules. [7].

Actinomycetes occur in a multiplicity of natural and manmade environments and illustrate divergent physiological and metabolic properties. The search for new antibiotics is generally based on the screening of naturally dwelling actinomycetes [8]. Boundless number of antimicrobial agents is unlocked from actinomycetes by screening extremophilic natural habitats such as alkaline soils and marine water bodies [9,10]. Most of the microbial compounds screened and structurally elucidated so far have been originated from the actinomycetes dwelling in extreme environments. *Streptomyces spp.* are Gram positive actinobacteria renowned for their ability to produce clinically important secondary metabolites. Several studies have been under taken for the isolation of novel *Streptomyces* species from different habitats [11]. Among the 23,000 bioactive secondary metabolites reported from microbes, over 10,000 are produced by actinomycetes representing 45% of all the bioactive compounds [12,13].

Accomplishment to rewards in natural product discovery is the manipulation of the nutritional and environmental factors nourishing the secondary metabolite biosynthesis. Production of secondary metabolites is the specific property of actinomycetes which depends on the growth conditions. The potential to impact the quality and diversity of the fermentation products results from the minor variation in the nutrition or the environment. Many of the substrates have an effect on the production of the secondary metabolites [14]. The efficiency and economics of the ultimate product depend on the media composition as it not only influences the growth and metabolism but also the product titer and the process economics. Generally adapted strategies for selecting the media include easy availability and low cost of the components [15]. The important aspects to be considered are the design of the culture media and the culture conditions.

This paper is worked upon the manipulation of nutritional and environmental factors of *Streptomyces tritolerans* DAS 165[T] which has the ability to tolerate high salinity, alkalinity and

temperature to mimic the pathways of secondary metabolite biosynthesis so as to synthesize chemical entities with complex skeletons and potent anti-microbial activity.

2. METHODOLOGY

Streptomyces tritolerans was isolated from an alkaline soil sample (pH 9-10) collected from Gulbarga, Northern Karnataka, Southern India by using a standard serial dilution plating technique on starch casein agar medium and further maintained on yeast extract malt extract dextrose (ISP-2) slants at 4ºC and as glycerol suspensions at -20ºC [16]. The 16s rRNA gene sequence of the strain has been deposited in Genbank under the accession number DQ345779. The test microorganisms used in the study were procured from ATCC, University Boulevard, Manassas, USA and MTCC, IMTECH, Chandigarh, India and preserved at 4ºC.

The production of bioactive metabolites by the strain was optimized by using different environmental and nutritional parameters such as pH, temperature, NaCl, carbon, nitrogen and minerals.

2.1 Influence of Incubation Period on Growth and Bioactive Metabolite Production by the Strain

The growth pattern and bioactive metabolite production of the strain was studied at regular intervals up to 10 days. Initially the strain was cultured for one week and then transferred into the starch casein salts broth (seed medium) and incubated for 48h. Exponential phase of seed culture at the rate of 10% was inoculated into the asparagine glucose broth (production medium).The fermentation process was carried out for 10 days under agitation (150 rpm) at 35ºC in order to estimate the growth and antimicrobial activity of the strain. The flasks were harvested at 24 h interval to separate the biomass and to extract the bioactive metabolite from the culture filtrate. Growth of the strain was expressed as dry weight of the biomass per 100ml of culture medium while the production of bioactive metabolites was measured in terms of diameter of inhibition zone against the test microorganisms. The crude bioactive compound produced in the fermentation medium by the isolate was extracted twice with equal volume of ethyl acetate (1:1) in a separating funnel. The solvent layer thus collected was evaporated in a rotary evaporator under vacuum to obtain the crude residue which was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1000µg/ml and employed for antimicrobial activity against test microorganisms like *Streptococcus mutans* (MTCC 497), *Staphylococcus aureus* (MTCC 3160), *Salmonella typhi* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231) by agar well diffusion method [17].

2.2 Effect of Temperature and pH on Growth and Bioactive Metabolite Production by the Strain

The optimum temperature for growth and antimicrobial metabolite production was measured by inoculating the culture in the production medium and incubated at different temperatures ranging from 20ºC to 50ºC. Similarly the initial pH values of production medium were adjusted from 5 to 10 to study the impact of pH. The biomass and bioactive metabolite production were estimated and the optimum pH and temperature achieved for maximum antimicrobial metabolite production were used for subsequent study [18,19].

2.3 Effect of NaCl on Growth and Bioactive Metabolite Production by the Strain

The impact of salinity on growth and bioactive metabolite production by *S. tritolerans* was carried out by growing the strain in fermentation medium with NaCl at concentrations ranging from 0.1 to 9% at 35ºC for six days [7].

2.4 Influence of Supplementary Carbon and Nitrogen Sources on Growth and Bioactive Metabolite Production by the Strain

To determine the influence of carbon sources on growth and bioactive metabolite production by the strain, the production medium was amended with different carbon sources such as maltose, sucrose, fructose, lactose, glucose, starch, mannitol, inositol, raffinose and rhamnose (each at a concentration of 1%). The effect of varying concentrations (0.5-5%) of the best carbon source selected on the growth and antimicrobial metabolite production was also investigated. Similarly several nitrogen sources such as soya peptone, arginine, asparagine, meat extract, yeast extract, tryptone, soya flour, casein, beef extract and glycine (each at a concentration of 0.5%) were individually supplemented into the fermentation medium containing an optimum amount of the superior carbon source as described above. The impact of different concentrations of optimized nitrogen source (0.1-2%) was also studied to enhance active metabolite production [20].

2.5 Impact of Minerals on Growth and Bioactive Metabolite Production by the Strain

The impact of minerals on growth and bioactive metabolite production by *Streptomyces tritolerans* was carried out by culturing the strain in different concentrations of K_2HPO_4 and $MgSO_4$ ranging from 0.01 to 0.1%, supplemented into the fermentation medium.

2.6 Evaluation of Antimicrobial Activity

The antimicrobial activity was evaluated by agar well diffusion assay [17]. The test microorganisms used to evaluate the production of bioactive metabolites were *Staphylococcus aureus* (MTCC 3160)*, Streptococcus mutans* (MTCC 497)*, Bacillus subtilis* (ATCC 6633)*, Lactobacillus casei* (MTCC 1423)*, Lactobacillus acidophilus* (MTCC 495)*, Xanthomonas campestris* (MTCC 2286)*, Bacillus megaterium, Escherichia coli* (ATCC 35218)*, Enterococcus faecalis* (MTCC 439)*, Pseudomonas aeruginosa* (ATCC 9027), *Salmonella typhi* (ATCC 14028), *Proteus vulgaris* (MTCC 7299)*, Candida albicans* (ATCC 10231)*, Aspergillus niger, Aspergillus flavus, Fusarium oxysporum* (MTCC 3075) *and Penicillium citrinum.*

2.7 Statistical Analysis

Results on growth and the production of bioactive metabolites by *S. tritolerans* exposed to different cultural conditions are statistically analyzed with one way and two way analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

3.1 Influence of Incubation Period on Growth and Bioactive Metabolite Production by the Strain

The growth pattern of *S. tritolerans* was studied on asparagine-glucose broth. The stationary phase of the strain extended from 96 h to 144 h of incubation. The secondary metabolites obtained from six day old culture exhibited high anti microbial activity against the test microorganisms (Fig. 1). The incubation time for the production of bioactive metabolites seems to be different among *Streptomyces* strains. Thakur [21], reported that metabolites elaborated from 6 day old culture of *Streptomyces* sp. 201 showed good antimicrobial activity, whereas metabolites collected from 7-day old culture of *Streptomyces tanashiensis* strain A2D also exhibited good antimicrobial activity [7]. Bioactive metabolites collected from 4 day old culture of *Streptomyces psammoticus* elaborated good antimicrobial activity against methicillin resistant *Staphylococcus aureus* [22]. Antimicrobial metabolite production by the new *Streptomyces* strain RUPA-08PR isolated from Bangladesh soil was reported to start after 10 days of incubation [23]. The secondary metabolites obtained from 9-day old culture of *Streptomyces* VITSVK-9 sp. exhibited good antimicrobial activity [19] against the test microorganisms.

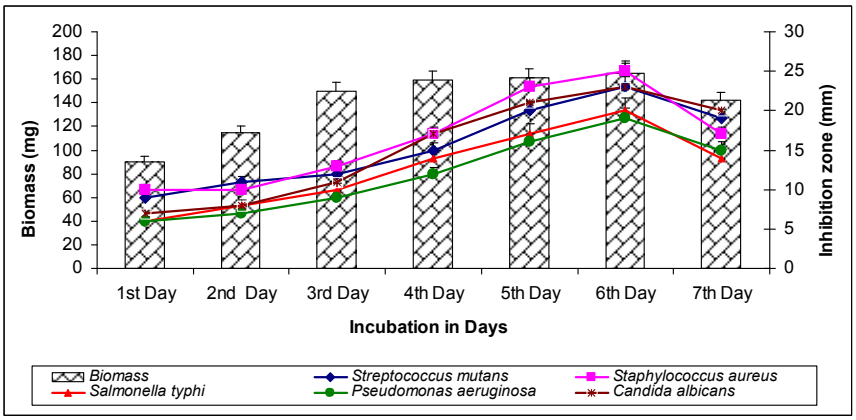

Fig. 1. Growth Pattern of *Streptomyces tritolerans* and antimicrobial metabolite production. Data are statistically analyzed by two way ANOVA and found to be significant at 1%. ($P< 0.01$)

3.2 Effect of Temperature and pH on Growth and Bioactive Metabolite Production by the Strain

The impact of environmental factors such as pH and temperature on growth and bioactive metabolite production was studied. There was an increase in the growth as well as bioactive metabolite production with the increase of incubation temperature from 20ºC to 35ºC. However further increase in temperature (above 35ºC) resulted in the decline of growth and bioactive metabolite production. Maximum growth as well as antimicrobial metabolite production of the strain was obtained at 35ºC (Fig. 2). These results are in complete accordance with those reported by Saha et al. [24] and Narayana and Vijayalakshmi [25].

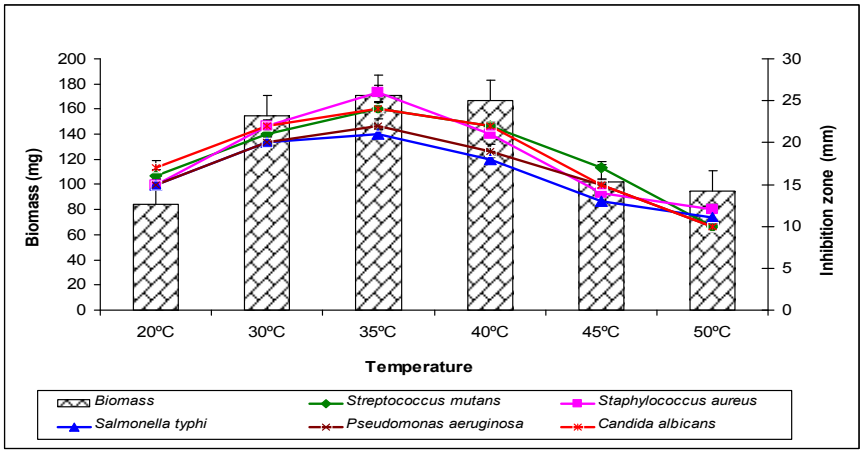

Fig. 2. Effect of temperature on growth and bioactive metabolite production by *Streptomyces tritolerans.* Data are statistically analyzed by two way ANOVA and found to be significant at 1%. (*P*< 0.01)

The antimicrobial metabolite production was found to be influenced by pH of the medium. The maximum growth as well as bioactive metabolite production of the strain was obtained at pH 7.5 (Fig. 3), suggesting its inclusion in the neutrophilc actinomycetes group. Medium maintained at pH 7.5 was reported to support enhanced antimicrobial metabolite production by *Streptomyces* sp. 201 [21] and *Streptomyces* sp. KGG32 [26]. It has been reported that environmental factors like pH, temperature and incubation period have profound influence on antibiotic production [22]. Changes in external pH affect many cellular processes such as regulation and biosynthesis of secondary metabolites [27,28,29].

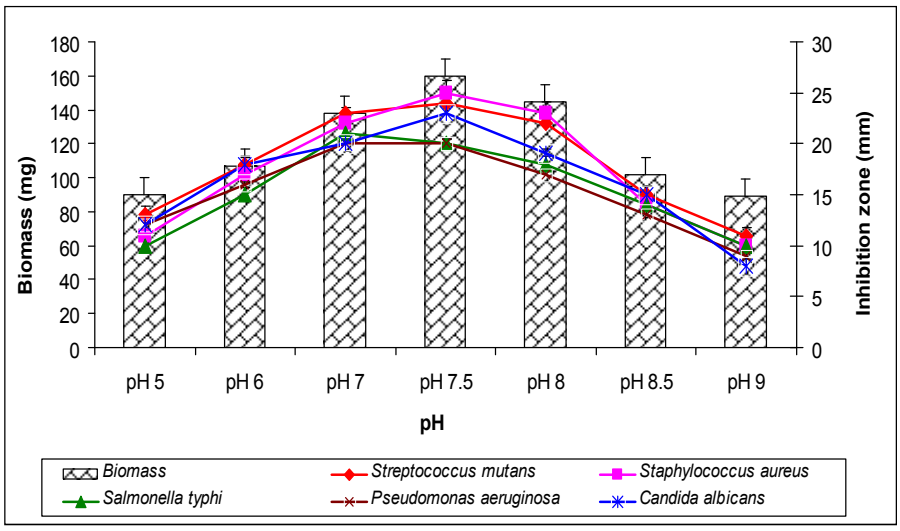

Fig. 3. Effect of pH on Growth and Bioactive metabolite production by *Streptomyces tritolerans*. Data are statistically analyzed by two way ANOVA and found to be significant at 1%. (*P*< 0.01)

3.3 Effect of NaCl on Growth and Bioactive Metabolite Production by the Strain

Optimum salt requirement for bioactive metabolite production was examined in the production medium supplemented with different salt concentrations ranging from 0.1- 9%. NaCl at 5% concentration was found to be optimum for maximum growth as well as antimicrobial metabolite production (Fig. 4). Further increase in salt concentration resulted in the decreased production of bioactive metabolites. The requirement of NaCl for the production of bioactive metabolites seems to be different among actinomycete strains. Saha et al. [24], reported that high antimicrobial potential of actinomycete isolate grown in the production medium with NaCl @ 5%. Singh et al. [7], showed that antibiotic production by *Streptomyces tanashiensis* A2D was maximum at 2% NaCl in the medium. Culture medium with NaCl @1% was reported to support high antimicrobial metabolite production by *Nocardioides luteus* [30].

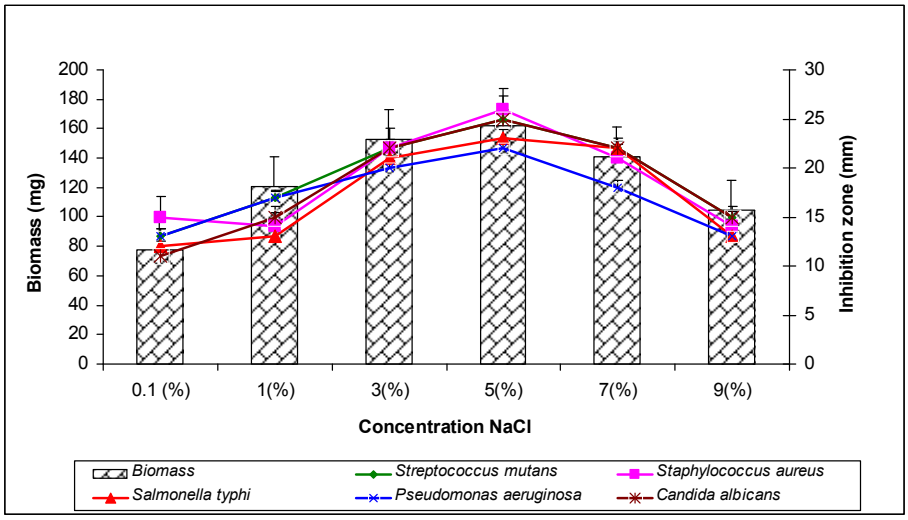

Fig. 4. Effect of NaCl on growth and bioactive metabolite production by *Streptomyces tritolerans*. Data are statistically analyzed by two way ANOVA and found to be significant at 1%. (*P*< 0.01)

3.4 Influence of Supplementary Carbon and Nitrogen Sources on Growth and Bioactive Metabolite Production by the Strain

In order to develop effective medium for bioactive metabolite production, the impact of carbon and nitrogen sources need to be evaluated. Among the various carbon sources tested, significant production of antimicrobial metabolites was obtained in sucrose amended medium followed by glucose and mannitol while the production of biomass was high with mannitol followed by sucrose and starch (Fig. 5). The results showed that bioactive metabolite production was high in medium amended with sucrose as carbon source. The result is in conformity with that reported for *Streptomyces sp.,* KGG32 [26] and *Streptomyces rochei* G-164 [31]. The carbon source needed for maximal yield of antibiotic production seems to be different for actinobacterial strains. Glycerol was found to be the best carbon source for antibiotic production by *Streptomyces hygroscopicus* 1.5. [32]. Thakur et al. [21], reported that mannitol followed by sucrose and glycerol favored high rates of antibiotic production by *Streptomyces* sp. 201 isolated from tea plantations of Assam. El-refai et al.

[30], stated that actinomycin production from *Nocardioides luteus* was high in medium with starch as carbon source while biomass was high with maltose. Abdelhalem et al. [33], also reported that optimal actinomycin production by *Streptomyces* sp. AH 11.4 was found with starch as the carbon source in the culture medium.

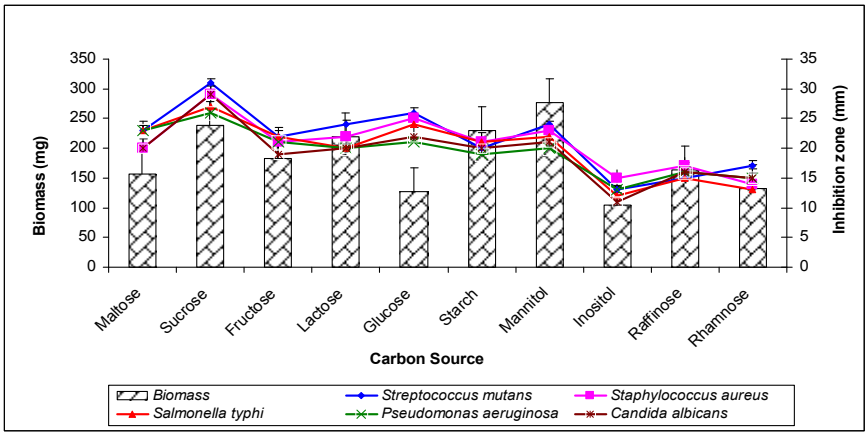

Fig. 5. Effect of different carbon sources on growth and bioactive metabolite production by *Streptomyces tritolerans*. Data are statistically analyzed by two way ANOVA and found to be significant at 1%. (*P*< 0.01)

As sucrose emerged as the most preferred carbon source for antimicrobial metabolite production by the strain, different concentrations of sucrose (0.5-5%) were tested to determine its optimal concentration. As shown in Fig. 6, maximum growth and antimicrobial activity was obtained in culture medium supplemented with 2% sucrose. Similar results were reported for *Nocardia levis* MK_VL 113 [34]. A few reports suggested that medium containing 2% glucose supported maximum levels of Natamycin production by *Streptomyces natalensis* [35] as well as by *Thermomonospora* sp. [36].

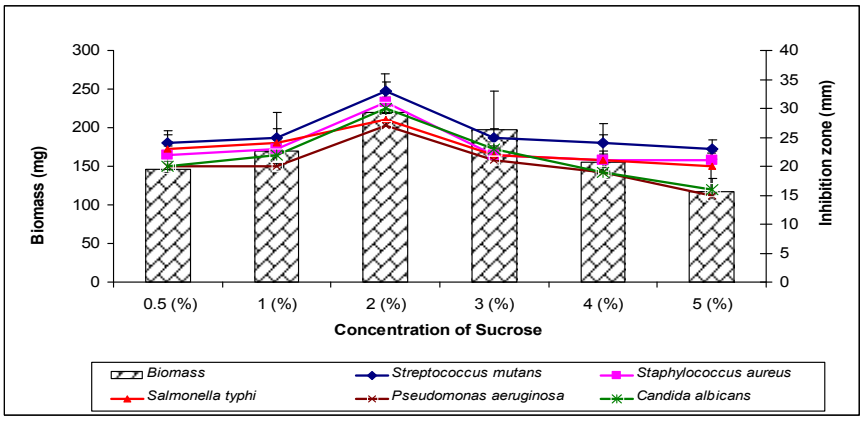

Fig. 6. Effect of different concentrations of sucrose on growth and production of bioactive metabolite by *Streptomyces tritolerans* data are statistically analyzed by two way ANOVA and found to be significant at 1%. (*P*< 0.01)

It was reported that complex nitrogen sources could increase the production of antibiotics by *Streptomyces* spp. These sources could strengthen high antibiotic titer which can be linked to the slow release of nitrogenous components during the course of fermentation. Different nitrogen sources were found to have significant effect on growth and secondary metabolite production by *Streptomyces tritolerans.* Among the nitrogen sources tested, soya peptone was found to be the best for growth as well as bioactive metabolite production followed by yeast extract and meat extract (Fig. 7). The utilization of nitrogen sources for the production of bioactive metabolites is reported to vary for actinomycete strains. Singh et al [7], recorded that soya bean meal increased antibiotic production by *Streptomyces tanashiensis* strain A2D and similar results were recorded by Narayana and Vijayalakshmi [25], for *Streptomyces albidoflavus.* Peptone was identified as the best nitrogen source for the production of antimicrobial metabolites by *Streptomyces sp.,* KGG32 [26] and *Streptomyces cheonanensis* VUK-A [37].

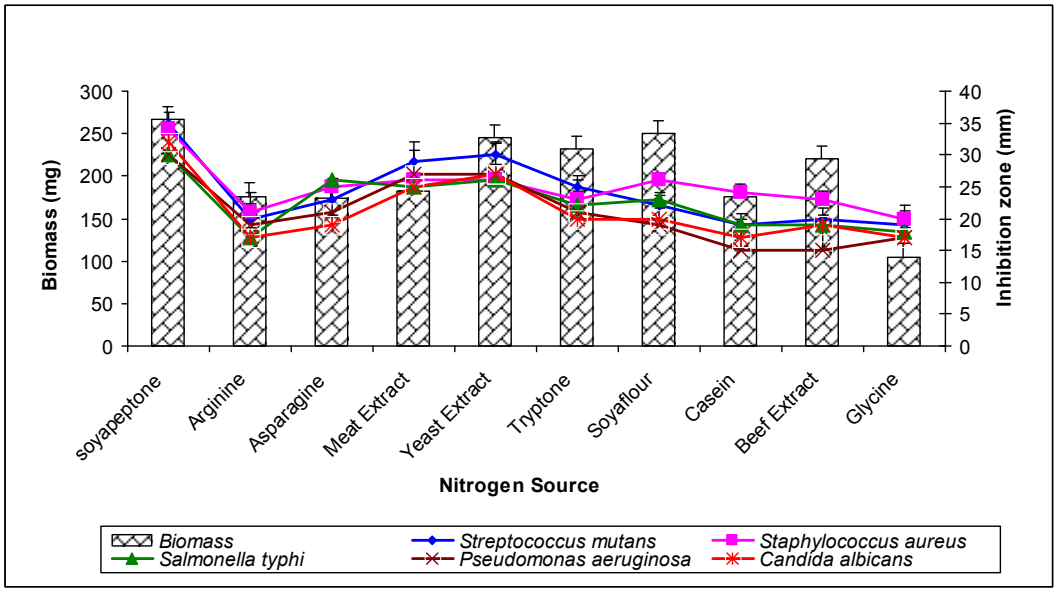

Fig. 7. Effect of different nitrogen sources on growth and bioactive metabolite production by *Streptomyces tritolerans,* Data are statistically analyzed by two way ANOVA and found to be significant at 1%. (*P*< 0.01)

Influence of different concentrations of soya peptone on the production of bioactive metabolites is represented in Fig. 8. Soya peptone at the concentration of 1% and 0.5% supported optimal production of biomass and bioactive metabolite respectively. Singh et al [7], reported that optimal antibiotic activity was obtained with 1% of soya bean meal in the culture medium by *Streptomyces tanashiensis* strain A2D. Viana et al. [38], recorded that soya bean flour at the concentration of 2% enhanced the clavulanic acid production by *Streptomyces* DAUFPE 3060, while soyabean meal at the concentration of 1.12% increased the fungichromin production by *Streptomyces padanus* PMS-702, as reported by Wu et al [39].

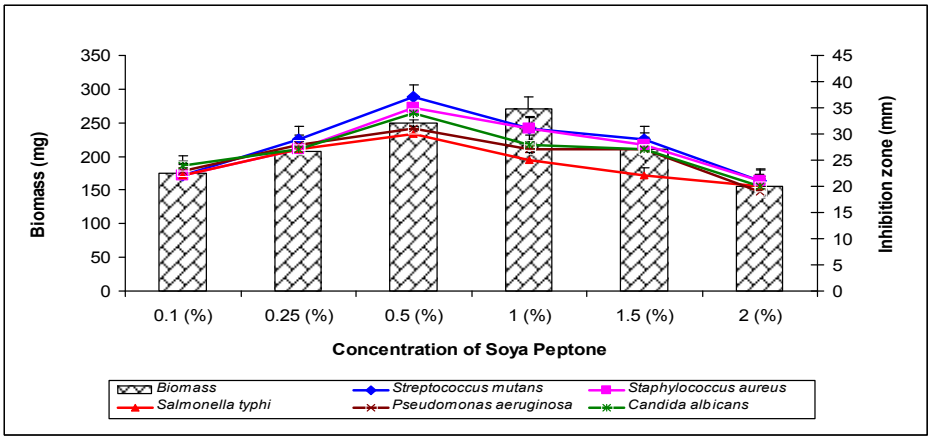

Fig. 8. Effect of concentration of Soya peptone on growth and bioactive metabolite production by *Streptomyces tritolerans*, Data are statistically analyzed by two way ANOVA and found to be significant at 1%. (*P*< 0.01)

3.5. Impact of Minerals on Growth and Bioactive Metabolite Production by the Strain

Among the minerals tested, slight enhancement in growth and antimicrobial activity was obtained in culture medium supplemented with K_2HPO_4 (0.05%) and $MgSO_4$ (0.1%) (Fig. 9 and 10). Majumdar and Majumdar [40], also observed the positive effect of K_2HPO_4 on neomycin production by *Streptomyces fradiae.* Ripa et al. [23], reported that K_2HPO_4 and $MgSO_4$ exerted positive effect on antibiotic production by a *Streptomyces* spp. RUPA-08PR.

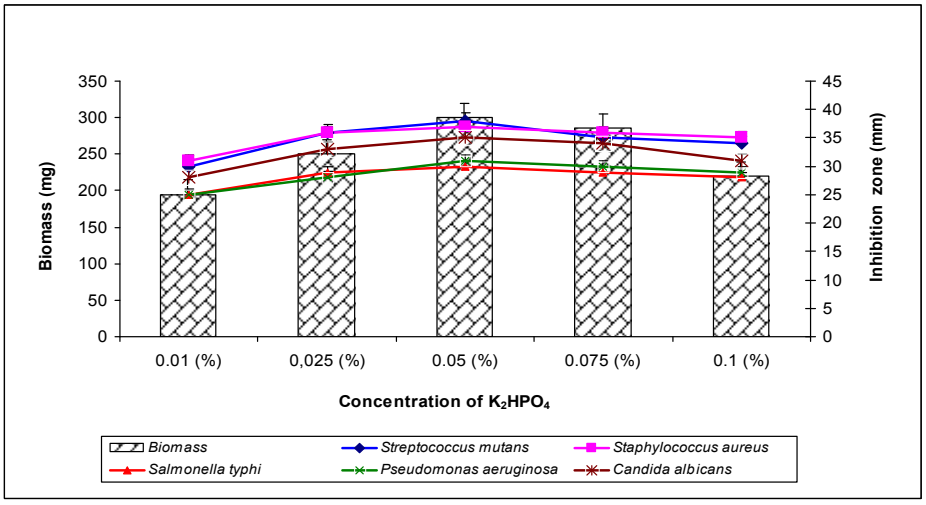

Fig. 9. Impact of K_2HPO_4 on growth and bioactive metabolite production of strain *Streptomyces tritolerans.* Data are statistically analyzed by two way ANOVA and found to be significant at 1%. (*P*< 0.01)

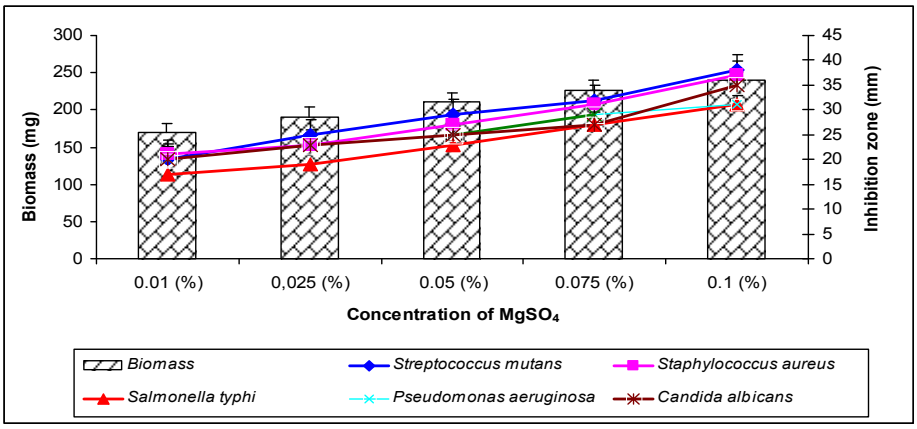

Fig. 10. Impact of $MgSO_4$ on growth and bioactive metabolite production of strain *Streptomyces tritolerans.* · Data are statistically analyzed by two way ANOVA and found to be significant at 1%. (*P*< 0.01)

The production of antimicrobial metabolites was high when the strain was grown in optimized culture medium containing sucrose at the concentration of 20g/L as carbon source, soya peptone at the concentration of 10g/L as nitrogen source, K_2HPO_4 at the concentration of 0.5g/L, MgSO4 at the concentration of 1g/L as mineral sources and 5%NaCl with an initial pH 7.5 for six days at 35ºC. The antimicrobial metabolite produced by the strain under optimized conditions was tested against different test bacteria and fungi and the results are presented in (Fig. 11,12 and 13). Among the bacteria tested *Streptococcus mutans* and *Staphylococcus aureus* were found to be highly sensitive to the metabolites produced by *S. tritolerans* followed by *Xanthomonas campestris* and *Bacillus megaterium,* while *Candida albicans* appeared to be more sensitive among the fungi.

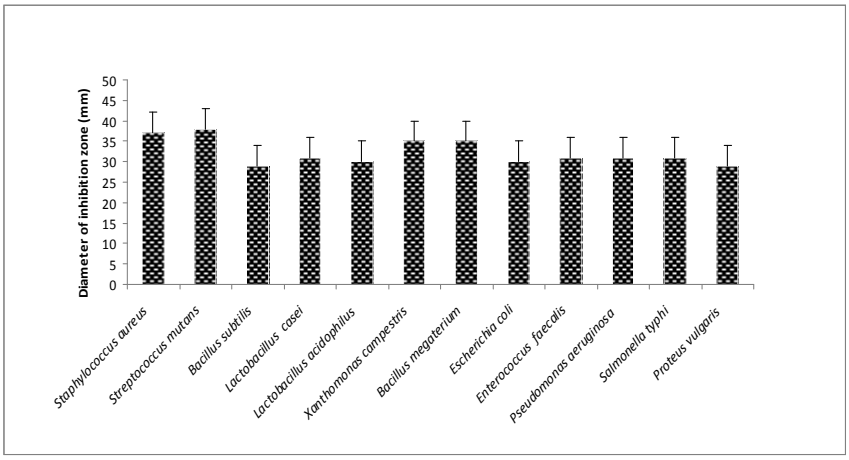

Fig. 11. Antibacterial activity of bioactive metabolites produced by *Streptomyces tritolerans* under optimized conditions. Data are statistically analyzed by One way ANOVA and found to be significant at 1%. (*P*< 0.01)

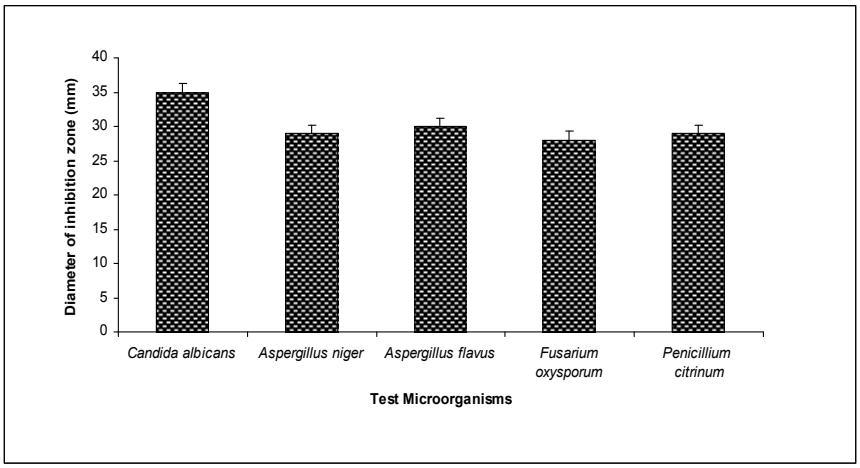

Fig. 12. Antifungal activity of bioactive metabolites produced by *Streptomyces tritolerans* under optimized conditions. Data are statistically analyzed by one way ANOVA and found to be significant at 1%. (P< 0.01)

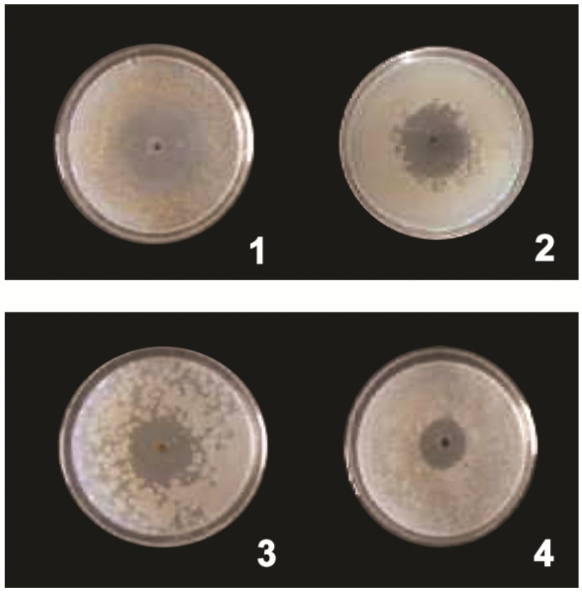

Fig. 13. Antimicrobial activity of *Streptomyces tritolerans* against 1. *Xanthomonas campestris* (MTCC 2286) 2. *Escherichia coli* (ATCC 35218) 3. *Candida albicans* (ATCC 10231) 4. *Proteus vulgaris* (MTCC 7299).

4. CONCLUSION

The process parameters for enhanced production of bioactive metabolites by *Streptomyces tritolerans* DAS 165^T were optimized. The strain grown in optimized culture medium exhibited potent antimicrobial activity against the bacteria and fungi tested.

ACKNOWLEDGEMENTS

Financial assistance from CSIR, New Delhi is gratefully acknowledged.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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