Optimization of the Cultural parameters for Improved Production of Antimicrobial Metabolites by *Streptomyces gulbargensis* DAS 131^T

ABSTRACT

Aims: To investigate the influence of appropriate culture medium by optimizing the cultural and environmental conditions affecting the growth and bioactive metabolite production by *Streptomyces gulbargensis* DAS 131^T under submerged culture conditions in order to reduce the cost of fermentation process and improve the formation of antimicrobial compounds.

Place and Duration of Study: Department of Botany and Microbiology, January 2012 to May 2012.

Methodology: The impact of environmental parameters such as incubation period, pH, temperature and salt concentration and effect of various nutrients such as carbon and nitrogen sources and minerals on the antimicrobial metabolite production by *Streptomyces gulbargensis* DAS 131^T was evaluated by employing agar well diffusion assay. Growth was measured in the form of dry mycelial weight.

Results: The optimum pH and temperature for bioactive metabolite production were 7 and 35°C respectively. Highest anti microbial metabolite production was found when the strain was inoculated into the medium amended with glucose @ 2%, soya peptone @ 1% and NaCl @ 5% and incubated for six days under shaking conditions. The metabolites showed good antimicrobial activity against Gram positive and Gram negative bacteria, as well as unicellular and multicellular fungi.

Conclusion: This is the first report on the optimization studies of bioactive metabolite production by *S.gulbargensis* DAS 131^T. It was found that the antimicrobial metabolite production by *S. gulbargensis* was positively influenced by carbohydrates, nitrogen sources and minerals. As the strain exhibited potent antimicrobial activity further studies regarding the characterization of bioactive compounds by *S. gulbargensis* are in progress.

Keywords: Optimization, Bioactive metabolites, Nutritional factors, Antimicrobial activity, Streptomyces gulbargensis DAS 131^T.

1. INTRODUCTION

The microbes are the source for many important drugs including antibiotics, antitumor compounds, Immunosuppressants, antiviral and antiparasitic agents. Over 10,000 of bioactive compounds have been produced by Actinomycetes which contribute to 45% of all the bioactive secondary metabolites discovered [1]. Microbes dwelling in extreme habitats have been focused as an important source for novel compounds in recent years. The majority of studies with microbes from extreme environments were confined to bacteria and the actinomycetes from these habitats have been relatively less explored [2]. As highlighted in many reviews [3], natural products are the origin for most of the antibiotics in the market today. These products are an important source for both the existing and new drugs. Among these, actinomycetes are a biotechnologically priceless group of prokaryotes. Actinobacteria form a distinct line in the 16S rDNA tree and produce metabolites that have medical contribution from antibiotics to enzyme inhibitors. They are ubiquitously distributed in terrestrial, fresh water and extremophilic environments such as marine ecosystems and alkali soils [4]. They are considered to be the important group of microbes due to their ability to produce novel chemical compounds that are complex and commercially important (5). The solution to combat multidrug resistance of pathogens is to search for novel antimicrobial compounds so as to find a solution to overcome the global resistance to pathogenic bacteria.

It is widely accepted that alkaliphilic actinomycetes are a valuable source for medicinal and industrial products [6]. Extensive exploration of actinomycetes having unique therapeutic properties continues to be an important area of research. *Streptomyces* species belonging to actinomycetes have been known as prolific producers of useful bioactive metabolites. These species are also recognized as industrially important organisms for their ability to synthesize different kinds of novel secondary metabolites, accounting for 70- 80% of all natural compounds produced by actinomycetes. *Streptomyces* are well documented as source for novel drug metabolites [7]. Some of the important compounds obtained from the alkaliphilic *Streptomyces* species include Pyrocoll [8], Chinikomycin and Lajollamycin, Mediomycins A and B, Clethramycin [9], Bleomycin [10] and Caboxamycin [11] with anti-tumor, anti-parasitic and anti-microbial properties. Several studies were aimed at isolation of *Streptomyces* and screening them for new antibiotics. Novel actinomycetes documented and the products derived from poorly explored habitats stress the need to probe into new habitats [2].

Media supplemented with carbon, nitrogen sources [12], sodium chloride [13] and mineral salts [14] and physico-chemical parameters like temperature, pH and incubation period also play a major role on growth and production of anti-microbial metabolites. The type, addition, deletion and concentration of carbon, nitrogen, and phosphate together with trace elements are reported to influence the antibiotic biosynthesis by *Streptomyces* [15]. In order to achieve the highest level of metabolite production, the optimization of process parameters is very critical [16, 17]. Hence an effort was made to understand the impact of different carbon and nitrogen sources, temperature, pH and incubation period on growth and bioactive metabolite production by *Streptomyces gulbargensis* DAS 131^{T}

2. MATERIALS AND METHODS

2.1. Isolation

During the course of screening for industrially important microorganisms, an alkali-tolerant and thermo-tolerant actinomycete isolate identified as *Streptomyces gulbargensis* DAS 131^{T} was isolated from semi-arid soils of Gulbarga, Northern Karnataka province, India, by standard serial dilution technique using starch casein agar medium [18] and further

maintained on Yeast extract malt extract dextrose (ISP-2) agar medium at 4°C [19]. The strain has been deposited in the NCBI genbank with the accession number DQ 317411 [20].

2.2. Effect of Incubation period

The growth pattern and bioactive metabolite production by the strain was studied at regular intervals up to 10 days. One week old culture was cultivated in seed medium (ISP-2 broth) at 37°C for 48 h. Seed culture at a rate of 10% was inoculated into the production medium (starch casein broth). The fermentation process was carried out for 10 days under agitation at 150 rpm. At every 24 h interval, the flasks were harvested and the biomass was separated from the culture filtrate. Biomass was determined in terms of dry weight and antimicrobial metabolite production was determined in terms of their antimicrobial spectrum [21]. 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 at periodic intervals. The solvent layer was collected and evaporated in a rotary evaporator under vacuum. The crude residue thus obtained was dissolved in DMSO (Dimethylsulfoxide) 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 [22].

2.3. Selection of culture conditions for the optimum production of bioactive metabolites

Antimicrobial metabolite production by the strain was optimized by using different parameters such as pH, temperature, NaCl, carbon, nitrogen sources and minerals.

2.4. Effect of pH and temperature

To determine the influence of initial pH on growth and bioactive metabolite production, the strain was cultivated in the medium with different initial pH values ranging from 5 to 10. The strain was inoculated into production medium and grown at temperatures ranging from 20 to 50°C to study the impact of temperature. The biomass and bioactive metabolite production were estimated and optimal pH and temperature achieved in this step was used for subsequent study.

2.5. Effect of NaCl concentration

The impact of salinity on growth and bioactive metabolite production by *S. gulbargensis* DAS 131^T was recorded by cultivating the strain in the fermentation medium amended with different concentrations of NaCl (1-10%).

2.6. Effect of carbon and nitrogen sources

To determine the effect of carbon sources on biomass and bioactive metabolite production, different carbon sources like galactose, lactose, fructose, sucrose, glucose, starch, mannitol, arabinose, raffinose and rhamnose each at a concentration of 1% were added separately into the production medium. The effect of varying concentrations of the best carbon source (0.5 - 5%) on bioactive metabolite production was examined. Similarly, the influence of various nitrogen sources on antimicrobial metabolite production was evaluated by amending different nitrogen sources like 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 production medium containing an optimum amount of the superior carbon source. Further, the impact of varying concentrations of optimized

nitrogen source (0.1-2%) was studied to standardize the maximum antimicrobial metabolite production.

2.7. Impact of K_2HPO_4

To study the impact of K_2HPO_4 on growth and bioactive metabolite production, the strain was grown in the fermentation medium amended with different concentrations of K_2HPO_4 (0.01 to 0.1%).

2.8. Statistical analysis

Results on cell growth and the production of bioactive metabolites by *S. gulbargensis* under different cultural conditions tested are statistically analyzed with Two way analysis of variance (ANOVA).

The metabolites produced by the strain under optimized conditions were tested against bacteria and fungi by agar-well diffusion assay (22). 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* (NCIM 2187), *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.*

3. RESULTS AND DISCUSSION

3.1. Effect of incubation period

The growth pattern of *S.gulbargensis* DAS 131^T was studied on starch casein broth. The stationary phase extended from 120 h to 144 h of incubation (Fig.1). The secondary metabolites obtained from six day old culture exhibited high antimicrobial activity against the test microorganisms which was in complete accordance with the earlier reports [23,7]. The incubation period for the production of bioactive metabolites seems to vary among *Streptomyces* strains. Metabolites elaborated from 5 day old culture of *Streptomyces* sp. KGG32 [24] and *S.ramulosus*-AZ-SH-29 [25] showed good anti microbial activity. Metabolites collected from 10-day old culture of Hygromycin-B producing *S.crystallinus* AZ-A151 exhibited good anti microbial activity [26].

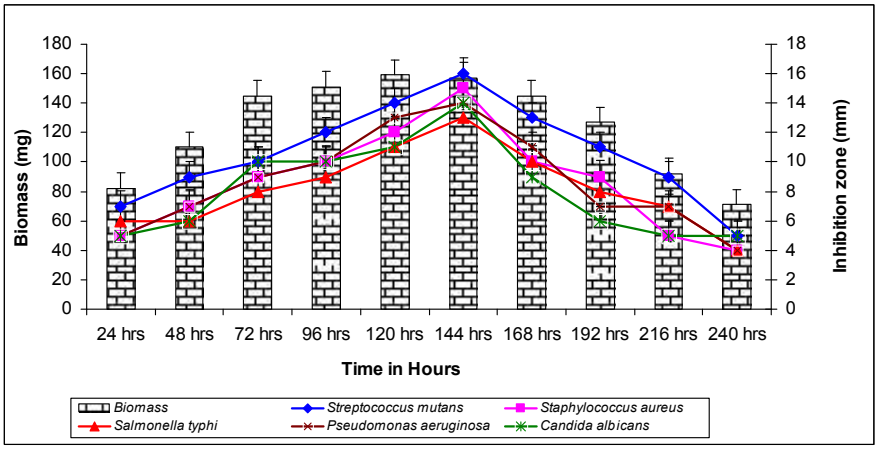

Fig.1. Growth pattern and anti-microbial activity of *S. gulbargensis* DAS 131^T.

**Data on cell growth and bioactive metabolite yield were statistically analyzed by Two-way ANOVA and found to be significant at 1%.*

3.2. Effect of initial pH and incubation temperature

The environmental requirements and cultural conditions for growth and bioactive metabolite production by *S. gulbargensis* DAS 131^T were studied. The antimicrobial metabolite production was found to be influenced by pH of the medium. The maximum biomass and bioactive metabolite production by the strain was obtained at pH 7 suggesting its inclusion in the neutrophilic actinomycetes group (Fig. 2). Medium maintained at pH 7.0 was reported to support enhanced anti microbial metabolite production by *Streptomyces rochei* G 164 [27], *Streptomyces marinensis* [28], *Streptomyces albidoflavus* [21], *Streptomyces torulosus* KH-4 [29] and *Streptomyces cheonanensis* VUK-A [31].

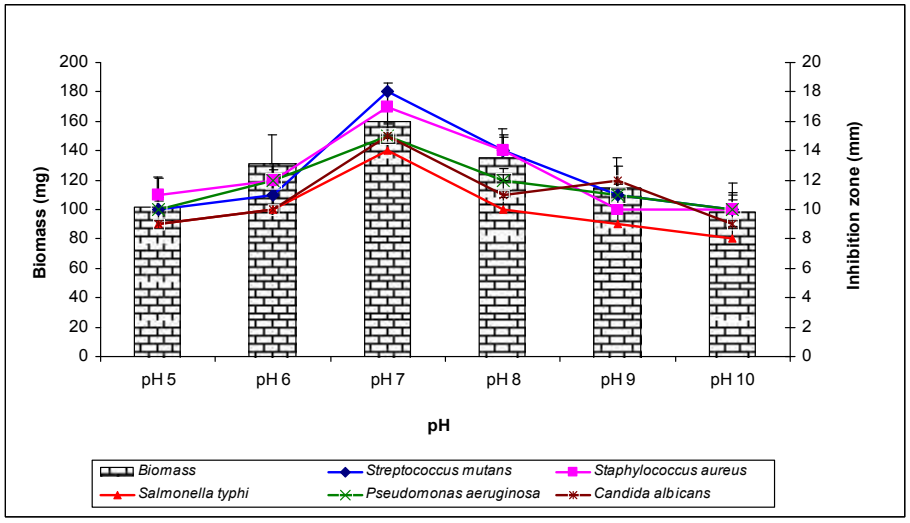

Fig. 2. Effect of pH on growth and bioactive metabolite yield of *S. gulbargensis* DAS 131^T

**Data on cell growth and bioactive metabolite yield were statistically analyzed by Two-way ANOVA and found to be significant at 1%.*

The increase of incubation temperature from 20°- 35°C enhanced the biomass and the production of bioactive metabolites (Fig.3). In terms of its optimum temperature for growth, the organism appeared to be mesophilic in nature. Atta *et al.* [26] reported that *Streptomyces crystallinus*, AZ-A151 produced high levels of Hygromycin-B production at 35ºC. Ushakiranmayi *et al.* [33] stated that the optimum temperature capable of promoting antimicrobial metabolite produced by *Pseudonocardia* sp.VUK-10 isolated from Nizampatnam mangrove ecosystem was 35ºC. Similar results have been reported earlier for several *Streptomyces* spp. [34, 21, 35, 30].

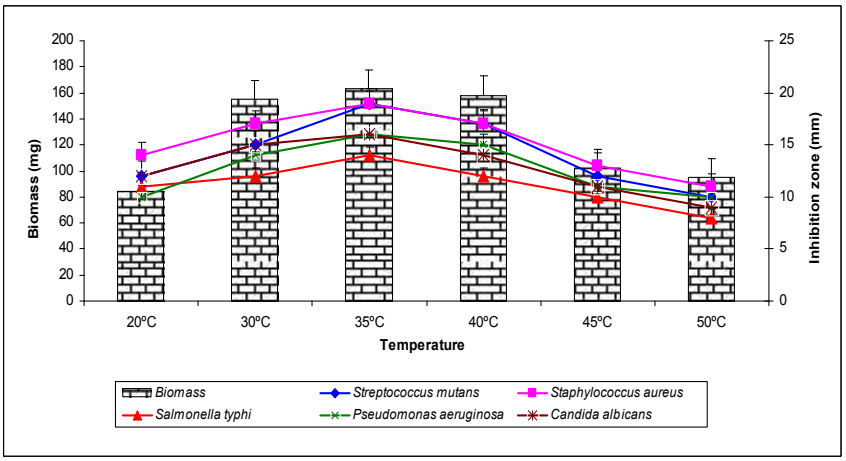

Fig.3. Effect of temperature on growth and bioactive metabolite yield of *S.gulbargensis* DAS 131^{T}.

**Data on cell growth and bioactive metabolite yield were statistically analyzed by Two-way ANOVA and found to be significant at 1%.*

3.3. Effect of NaCl

Optimum salt requirement for bioactive metabolite production was examined in the production medium supplemented with different salt concentrations ranging from 1-10%. NaCl @ 5% was found to be optimum for maximum growth as well as antimicrobial compound production by *S. gulbargensis* DAS 131^{T} (Fig. 4). Further increase in salt concentration reduced the antimicrobial agent biosynthesis. The requirement of NaCl for the production of bioactive metabolites seems to be different among actinomycete strains. Optimum NaCl concentration for maximum growth as well as antimicrobial metabolite production was reported to be 2% for *Streptomyces tanashiensis* A2D [2], 1% for *Streptomyces felleus* YJ1 [36] and 5% for *Streptomyces* VITSVK9 [30].

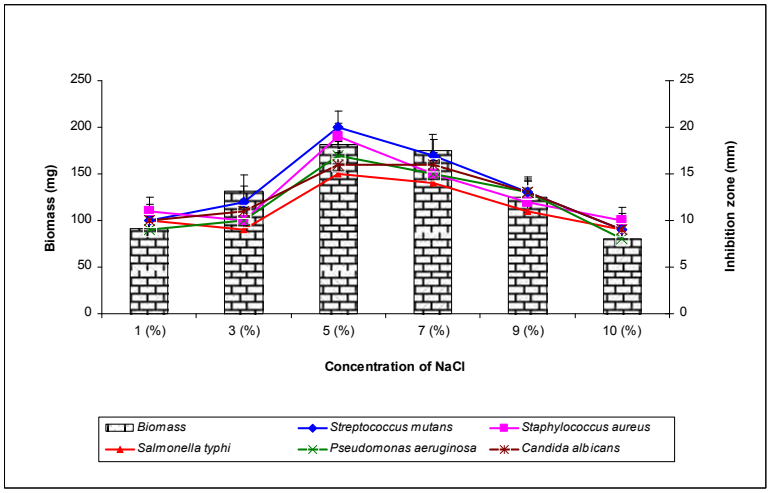

Fig. 4. Effect of NaCl on growth and bioactive metabolite yield of *S. gulbargensis* DAS 131^{T}.

Data on cell growth and bioactive metabolite yield were statistically analyzed by Two-way ANOVA and found to be significant at 1%.

3.4. Effect of carbon and nitrogen sources

Carbon and nitrogen sources play key role as structural and energy compounds in cells to enhance antibiotic production. Information related to various carbon and nitrogen sources that support good microbial growth, maximize the product yield, minimize the synthesis of compounds closely related to the product and enhance product recovery is required [37, 38].

The effect of carbon sources on biomass and bioactive metabolite production by *S. gulbargensis* DAS 131^{T} was evaluated. The antimicrobial activity production seems to be no way correlated to the biomass yield (Fig. 5). The production of biomass was high with lactose followed by sucrose and starch, while significant bioactive metabolite production was obtained by the strain in glucose amended media followed by galactose and fructose. El-Enshasy *et al.* [39] reported that glucose and sucrose in pure or in polymer forms were the best C-sources for erythromycin production. Antibiotic production from alkaliphilic *S. tanashiensis* strain A2D was high in medium containing glucose as carbon source [2]. Similarly glucose was found to be the best carbon source for antibiotic production by *Streptomyces torulosus* KH-4 [29], *S. griseocarneus* [40] and *S. kanamyceticus* M27 [41].

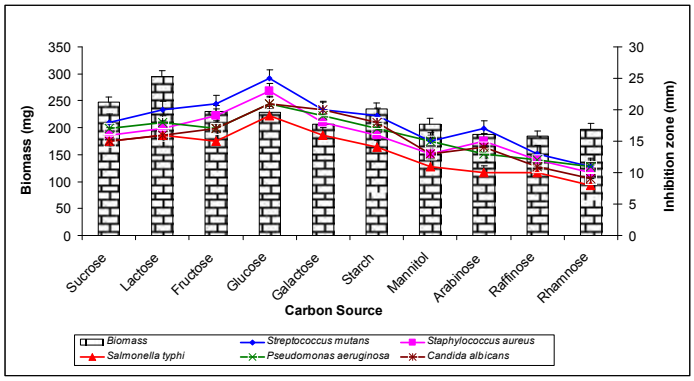

Fig. 5. Effect of different carbon sources on growth and bioactive metabolite yield of *S. gulbargensis* DAS 131^{T}.

Data on cell growth and bioactive metabolite yield were statistically analyzed by Two-way ANOVA and found to be significant at 1%.

As glucose emerged as the most preferred carbon source for bioactive metabolite production by the strain, varying concentrations of glucose (0.5-5%) was tested to determine its optimal concentration. It is noted that glucose at 3% and 2% concentrations showed optimal yields of biomass and bioactive metabolites respectively (Fig.6). Medium containing 2% glucose supported maximum levels of Natamycin production by *Streptomyces natalensis* and *Thermomonospora* spp. [38, 42] while Atta *et al.* [25] reported that medium containing 2.5% glucose supported antibiotic production by *Streptomyces ramulosus* AZ-SH-29.

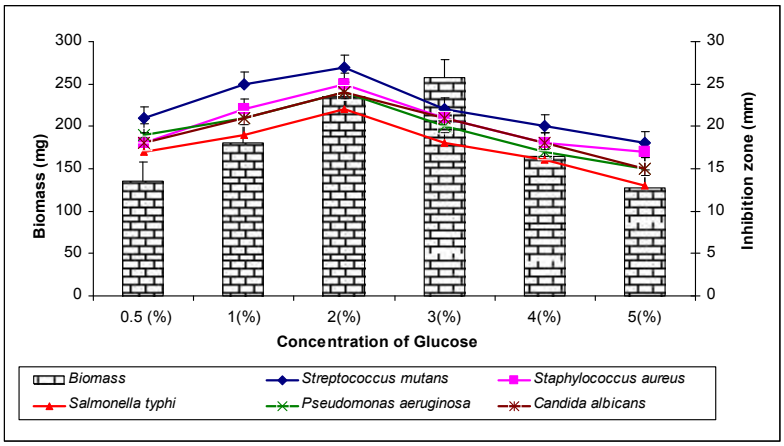

Fig.6. Effect of different concentrations of glucose on growth and production of bioactive metabolite by *S.gulbargensis* DAS 131^{T}.

**Data on cell growth and bioactive metabolite yield were statistically analyzed by Two-way ANOVA and found to be significant at 1%.*

Nitrogen sources are important for the production of bioactive metabolites by micro organisms. Changes in the nature and concentration of nitrogen source seem to affect antibiotic biosynthesis in different organisms. Different nitrogen sources were found to have significant effect on growth and secondary metabolite production by *S. gulbargensis* DAS 131^{T}. Among the nitrogen sources tested amendment of soya peptone in the culture medium enhanced the biomass and bioactive metabolite production by the strain (Fig. 7). Viana *et al.* [43] recorded that soya bean flour increased the clavulanic acid production by *Streptomyces* DAUFPE 3060. In contrast Thakur *et al*. [7] found that basal medium amended with asparagine as nitrogen source was proved to be the best for 2-methylheptyl isonicotinate production by *Streptomyces sp.*201.

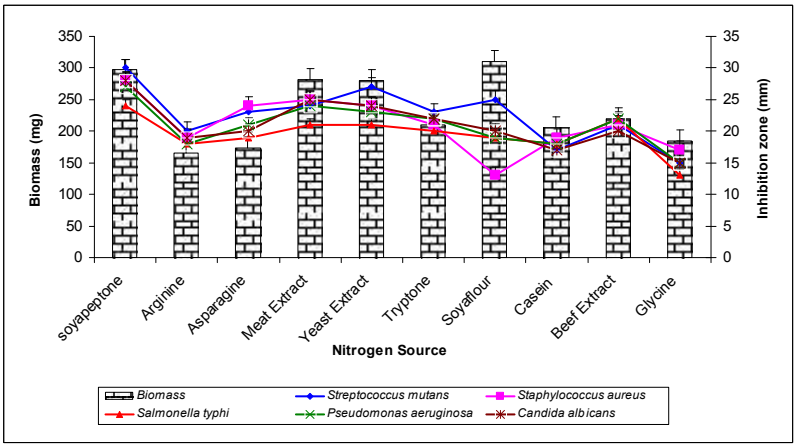

Fig.7. Effect of different nitrogen sources on growth and bioactive metabolite production by *S. gulbargensis* DAS 131^{T}.

**Data on cell growth and bioactive metabolite yield were statistically analyzed by Two-way ANOVA and found to be significant at 1%.*

Influence of different concentrations of soya peptone on the production of bioactive metabolites is represented in Fig.8. It is noted that soya peptone at a concentration of 1.5%

and 1% exhibited optimal production of biomass and bioactive metabolites respectively. Himabindu and Jetty [44] reported that soya bean meal at a concentration of 1% and 0.5% enhanced growth and gentamicin production by *Micromonospora echinospora.* Whereas Qin Song *et al.* [36] stated that soya bean meal at a concentration of 2% increased the bioactive metabolite production by *Streptomyces felleus* YJ1.

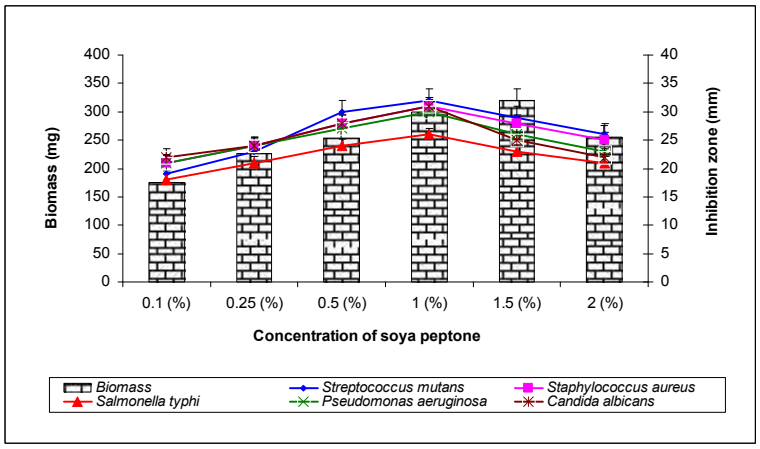

Fig.8. Effect of different concentrations of soya peptone on growth and production of bioactive metabolite by *S. gulbargensis* DAS 131^{T}.
**Data on cell growth and bioactive metabolite yield were statistically analyzed by Two-way ANOVA and found to be significant at 1%.*

3.5. Effect of K_2HPO_4

Effect of K_2HPO_4 on biomass and bioactive metabolite production by the strain (Fig. 9) was studied. A slight enhancement in growth and antimicrobial activity was obtained in medium supplemented with 0.05% of K_2HPO_4. Ripa *et al*. [32] reported that among different minerals tested, K_2HPO_4 showed positive influence on antibiotic production by *Streptomyces* RUPA-08PR. Narayana and Vijayalakshmi [21] also recorded that K_2HPO_4 slightly enhanced the production of biomass and bioactive metabolites of *Streptomyces albidoflavus.* Production of gentamicin by *M.purpurea* and antibiotic tylosin by a *Streptomyces* sp. was inhibited by high phosphate concentrations [45, 46].

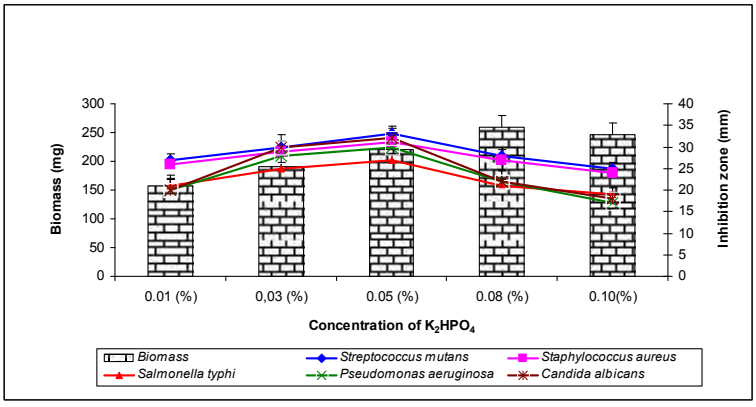

Fig.9. Impact of K_2HPO_4 on growth and bioactive metabolite production of *S. gulbargensis* DAS 131^T.

**Data on cell growth and bioactive metabolite yield were statistically analyzed by Two-way ANOVA and found to be significant at 1%.*

The antimicrobial metabolite produced by the strain under optimized conditions was tested against various test bacteria and fungi (Table.1 & Fig.10). Among the bacteria tested, *Xanthomonas campestris* and *Bacillus megaterium* are highly sensitive to the metabolites produced by *S. gulbargensis* DAS 131^T followed by *Streptococcus mutans* and *Enterococcus faecalis.* Among the fungi tested, *Candida albicans* was highly sensitive to the metabolites produced by the strain followed by *Aspergillus niger* and *Aspergillus flavus.* A significant antimicrobial activity was reported on the opportunistic and pathogenic bacteria and fungi tested.

Table1. Antimicrobial activity of *S.gulbargensis* against opportunistic and pathogenic bacteria and fungi under optimized conditions.

Antimicrobial Activity of Bioactive Metabolite Produced by Strain - DAS-131 (Streptomyces gulbargensis) under Optimized Conditions	
Bacteria	
Test Microorganisms	***Zone of Inhibition*** *(mm)*
Staphylococcus aureus	*31*
Streptococcus mutans	*33*
Bacillus subtilis	*30*
Lactobacillus casei	*32*
Lactobacillus acidophilus	*31*
Xanthomonas campestris	*34*
Bacillus megaterium	*33*
E.coli	*31*
Enterococcus faecalis	*33*
Pseudomonas aeruginosa	*30*
Salmonella typhi	*27*
Proteus vulgaris	*28*
Fungi	
Candida albicans	*32*
Aspergillus niger	*27*
Aspergillus flavus	*26*

Fusarium oxysporum	*19*
Pencillium citrinum	*20*

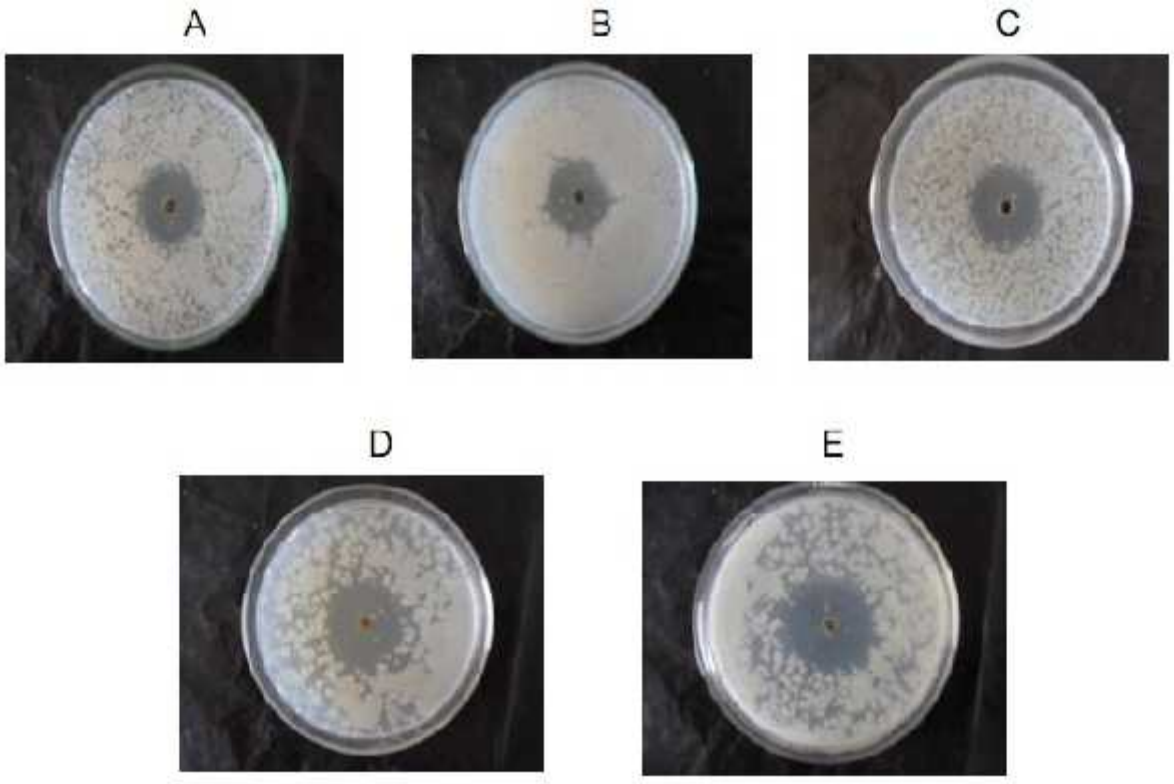

FIG. 10: Antimicrobial activity of *Streptomyces gulbargensis* DAS 131^T against A. *Pseudomonas aeruginosa* B. *Escherichia coli* C. *Salmonella typhi* D. *Bacillus subtilis* E. *Candida albicans.*

4. CONCLUSION

The results obtained in the present investigation revealed that the growth and bioactive metabolite production of *S. gulbargensis* DAS 131^T is influenced by environmental and cultural conditions. Metabolite production was high when the culture was grown in medium containing glucose @ 20 g/L as carbon source, soya peptone @ 10 g/L as nitrogen source, K_2HPO_4 @ 0.5g/L as mineral source and 5% NaCl with an initial pH 7 for six days at 35°C.

In the present study, the metabolites produced by *S. gulbargensis* DAS 131^T grown under optimized conditions exhibited significant antimicrobial activity against Gram positive, Gram negative bacteria and fungi. Hence, further studies regarding the purification, characterization and identification of bioactive compounds produced by the strain are in progress. This is the first report on the optimization of bioactive metabolites produced by novel *S. gulbargensis* DAS 131^T.

ACKNOWLEDGEMENTS:

Financial assistance from CSIR, New Delhi is greatfully acknowledged.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

UKM performed the experimental part, MVL& DA designed the study and SP performed literature search and statistical analysis. All authors read and approved the final manuscript.

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