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Optimization of Culture Conditions for Production of Bioactive Metabolites by *Streptomyces* spp. Isolated from Soil

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Abstract

The current work was carried out under a screening program targeted at isolation of bioactive *Streptomyces* species from soil samples. A total of 54 *Streptomyces* species were isolated from soil samples, out of which 4 isolates were found to be promising. These isolates were identified as *Streptomyces spectabilis*, *Streptomyces purpurascens*, *Streptomyces coeruleorubidus* and *Streptomyces lavendofoliae* and their sequences have been deposited in the GenBank. The influence of culture conditions including, incubation time, incubation temperature, initial pH and different carbon and nitrogen sources on growth and bioactive compound formation was investigated. Isolate R1, identified as *Streptomyces spectabilis*, showed maximum bioactive metabolite production with cellobiose and peptone as the carbon and nitrogen sources, on the 5th day at pH 5 at 30˚C. The optimum conditions for production by isolate R3, identified as *Streptomyces purpurascens*, were observed to be starch and casein as the carbon and nitrogen sources, pH 7, temperature 30˚C and an incubation period of eight days. For isolate R5, identified as *Streptomyces coeruleorubidus*, maximal production resulted on the sixth day at pH 6 and temperature of 35˚C with mannitol and JBM. Isolate Y8, identified as *Streptomyces lavendofoliae*, was found to produce high levels of bioactive metabolites in the medium supplemented with starch and peptone on the 10th day at pH 7 and at an incubation temperature of 30˚C. The four strains tested here behaved differently, each one requiring specific conditions for maximum growth as well as bioactive metabolite production.

Keywords

***Streptomyces*, Bioactive Metabolites, Culture Conditions**

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1. Introduction

Actinomycetes, especially *Streptomyces* species are a rich source of several useful bioactive natural products with potential applications [1]-[3]. They are prolific producers of secondary metabolites, many of which have commercial importance as antibiotics, antiparasitic and antifungal agents, anticancer and immunosuppressive agents [1] [4]-[7].

However, the ability of *Streptomyces* cultures to form these bioactive products is not a fixed property but can be greatly increased or completely lost under different conditions of nutrition and cultivation [8]. This is because antibiotic biosynthesis is a specific property of microorganisms which depends greatly on culture conditions. Improvement in the growth and antibiotic production can be carried out by manipulating the nutritional and physical parameters of the culturing conditions. Hence media composition plays a vital role in the efficiency and economics of the ultimate process. Therefore, designing an appropriate fermentation medium is of critical importance in the production of secondary metabolites [9]. Changes in the nature and type of carbon and nitrogen sources have been reported to affect antibiotic biosynthesis in *Streptomyces* [10]-[12]. Also several cultivation parameters like pH, incubation period and temperature play a major role in the production of bioactive metabolites [13].

In our search for bioactive secondary metabolites from indigenous *Streptomyces*, 54 *Streptomyces* strains were isolated from the soil samples collected from in and around Nagpur. Four isolates, isolate R1, isolate R3, isolate R5 and isolate Y8 were found to be potent and were selected for further studies. The microscopic, morphological, biochemical and physiological characterization strongly suggests that the isolate belongs to the genus *Streptomyces*. These strains were identified by 16S rRNA gene sequencing as *Streptomyces spectabilis*, *Streptomyces purpurascens*, *Streptomyces coeruleorubidus* and *Streptomyces lavendofoliae*. Their sequences have been deposited in the GenBank under the accession numbers KF468818, KF395224, KF527511 and KF681281respectively.

The present work describes the effect of different carbon and nitrogen sources and minerals on growth and bioactive compound production by the four *Streptomyces* strains. Further, the influence of pH, temperature and incubation time on growth and bioactive compound production was also studied.

2. Materials and Methods

2.1. Inoculum Preparation

The seed cultures to be used as the fermentation inoculum were prepared by transferring a colony from the agar plates to 100 ml basal medium which consisted of (g/1): glucose 10.0, soybean meal 10.0, NaCl 10.0, $CaCO_3$ 2.0.in a 250 ml Erlenmeyer flask. Seed cultures were grown in a shaker incubator, set at 28˚C, 130 rpm for 7 days. The culture broth was centrifuged at 5000 rpm for 10 min at 25˚C ± 2˚C. The pellet of the isolates was washed twice by suspension in sterile distilled water and resuspended in 10 ml of sterile distilled water. This suspension was used as inoculum (2% v/v) in the study [14].

2.2. Effect of Carbon and Nitrogen Sources on Biomass and Bioactive Metabolite Production

The effect of different carbon sources on growth and bioactive metabolite production was studied by replacing glucose in the basal medium with other carbon sources like galactose, maltose, lactose, cellobiose, starch etc. Four flasks were set up for each carbon source tested.

After the incubation period, to study the effect of carbon sources on biomass production, the mycelia was separated by centrifugation and dried at 70˚C until a constant weight was obtained which was expressed as mg/100 ml [15].

The triplicate flasks were used for ethyl acetate extraction of the filtrate, in order to determine the effect of carbon and nitrogen sources on the production of bioactive metabolites. The extracts were concentrated and used for testing bioactivity. The bioactivity was determined by agar well diffusion method against *Bacillus cereus*. 15 µl of the ethyl acetate extract (1 mg/ml) was used to determine antimetabolite production. The diameters of the zones of inhibition were noted [16]. Similarly, the effect of various nitrogen sources like casein, peptone, jack bean meal, ammonium sulphate and potassium nitrate was assessed by replacing soybean meal in the basal me-

dia. Data obtained was statistically analyzed. ANOVA on ranks was carried out and *P* value was determined [9].

2.3. Effect of Incubation Period

The isolates were inoculated into the basal medium and incubated up to 20 days in a rotary shaker at 130 rpm at 28˚C. For the determination of bioactive metabolite production, 1 ml aliquots were withdrawn after every 24 hours and subjected to centrifugation. The cell free supernatant thus obtained was concentrated five-fold in the vacuum concentrator and 50 µl was used to determine antimicrobial activity. The diameters of zones of inhibition were noted [11].

2.4. Effects of Initial pH on Biomass and Bioactive Metabolite Production

The initial pH levels of the basal media were adjusted from 4 to 11 and the isolates were grown for 10 days at 130 rpm and 28˚C. The biomass and bioactive metabolite production was estimated as described above. Similarly, the optimum temperature for growth and bioactive metabolite production was measured by incubating the isolates at temperatures ranging from 20˚C to 40˚C. The growth was measured as dried cell mass (mg/100 ml) and antimetabolite production was determined on the basis of diameters of zones of inhibition [17].

2.5. Effects of Minerals on Biomass and Bioactive Metabolite Production

To evaluate the effects of minerals on the growth and bioactive metabolite production, the optimized medium with superior carbon and nitrogen sources was supplemented individually with different minerals, such as $MgCl_2$, $CuSO_4$, $MnSO_4$, $FeCl_3$, $CoCl_2$, $(NH_4)_2MoO_4$ and $ZnCl_2$, each at a concentration of 0.05% (w/v) [13].

3. Results

3.1. Effect of Carbon Sources on Growth and Bioactive Metabolite Production

The effect of different carbon sources on growth and bioactive metabolite production by the isolates is presented in the **Figure 1** and **Figure 2**. Growth was estimated as dry cell mass/100 ml of the medium and bioactive metabolite production was estimated on the basis of diameter of zones of inhibition. All the carbon sources tested, supported the growth of all the four isolates. However, in general, it was found that monosaccharides were more effective carbon sources for growth of the isolates whereas cellobiose and starch were the best carbon sources for bioactive compound production.

3.2. Effect of Nitrogen Sources on Growth and Bioactive Metabolite Production

The different nitrogen sources were tested with the best five carbon sources obtained for each isolate. For all the four isolates, Jack bean meal (JBM) was found to be the best nitrogen source with all the tested carbon sources for maximum cell growth. Maximum bioactive metabolite production was observed in the medium with peptone (isolate R1), casein (isolate R3), JBM (isolate R5) and peptone (isolate Y8). Out of the twenty-five different Carbon + Nitrogen (C + N) combinations tested for each isolate, for isolate R1, Inositiol + JBM was the best for cell growth and starch + peptone for production. For isolate R3, cellobiose + JBM supported maximum growth and cellobiose + casein supported maximum production. For isolate R5, galactose + JBM was the best combination which supported both cell growth as well as bioactive metabolite production. For isolate Y8, Mannose +JBM supported growth whereas starch + peptone supported bioactive metabolite production.

3.3. Effect of Incubation Period on Bioactive Metabolite Production

The bioactive metabolite production by the four isolates was monitored over a period of 20 days. The bioactive metabolite production started only after 48 hours for all the four isolates. It reached a maximum (19 mm) for **isolate R1** on the 4th day, remained almost stable till the 10th day and decreased gradually to again increase on the 18th day. A similar pattern was observed for **isolate R5** where, maximum bioactive metabolite production was observed on the 6th day (19 mm) and then again on the 14th day. But for **isolate R3**, maximum production reached on 8th day (19 mm) which gradually went on decreasing from the 10th day (**Figure 3**). For **isolate Y8**, the production started on the 2nd day and went on increasing till it reached a maximum (28 mm) on the 10th day.

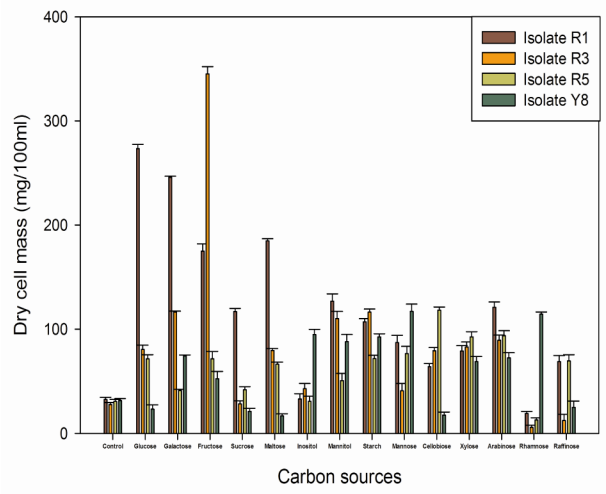

Figure 1. Effect of carbon sources on growth of the *Streptomyces* species.

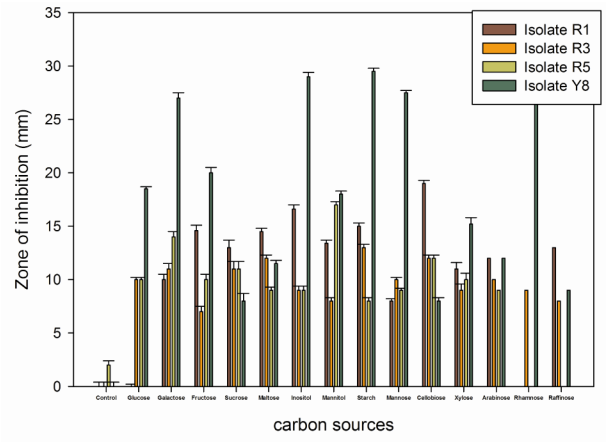

Figure 2. Effect of carbon sources on bioactive metabolite production by the *Streptomyces* species.

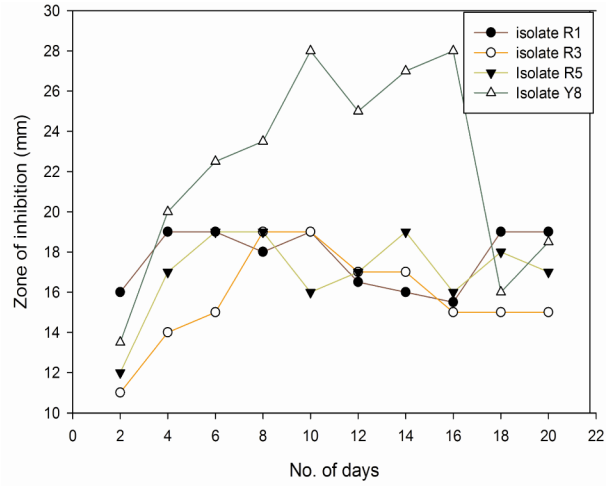

Figure 3. Effect of incubation period on bioactive metabolite production by the *Streptomyces* isolates.

The production remained more or less constant till the 16th day after which there was a sharp decrease (16 mm) on the 18th day.

3.4. Effect of pH on Growth and Bioactive Metabolite Production

The effect of initial pH of the medium on growth and bioactive metabolite production was studied for all the four isolates. The growth and production were maximum for isolate R1 and R3 at pH 5 and pH 7 respectively. However, for isolate **R5**, pH 5 was optimum for growth (97.6 mg) and pH 6 for bioactive metabolite production (19 mm). Also pH 10 showed maximum bioactive metabolite production (19 mm) but the cell mass was 25% less than that at pH 7. For **isolate Y8**, pH 7 proved to be the best for growth with 105.2 mg /100 ml cell mass and pH 10 proved to be the best for bioactive metabolite production (16 mm) closely followed by pH 7 (14 mm). None of isolates showed any bioactive metabolite production at pH 4 nor did it support much growth. The final pH of all the flasks for all the isolates was found to be in the range of 6.2 to 8.4 irrespective of the initial pH except for isolate Y8, where the final pH was recorded to be between 4 - 5.5. **Figure 4** and **Figure 5** represent the effect of initial pH on growth and bioactive metabolite production respectively by the isolates. ANOVA on ranks was carried out against pH 4 flask and the initial pH was found to have a significant effect on both growth as well as production at a P value of 0.05.

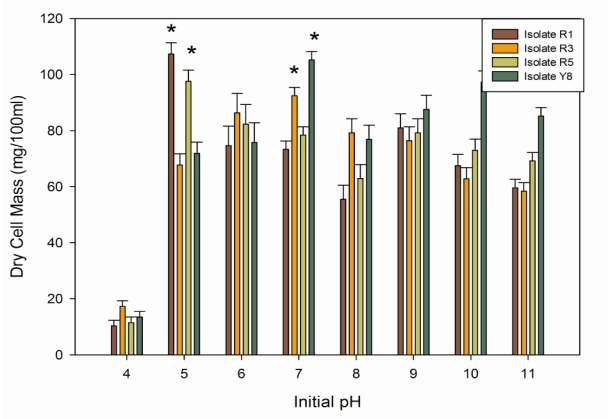

Figure 4. Effect of initial pH on growth of the *Streptomyces* isolates. Data expressed as mean ± SD (n = 3). $^{*}P \leq 0.05$ (vs pH 4).

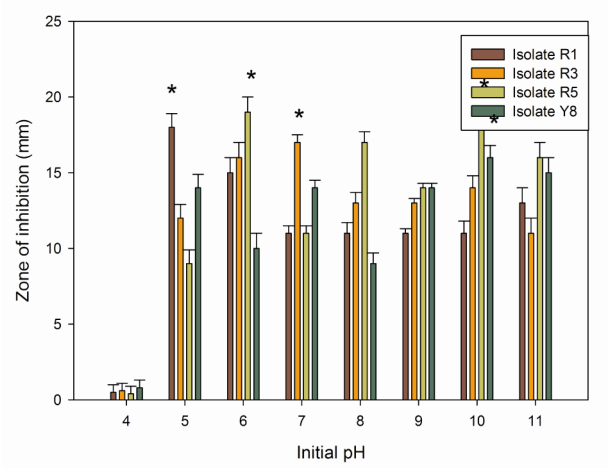

Figure 5. Effect of initial pH on bioactive metabolite production by the *Streptomyces* isolates. Data expressed as mean ± SD (n = 3). $^{*}P \leq 0.05$ (vs pH 4).

3.5. Effect of Temperature on Growth and Bioactive Metabolite Production

The effect of incubation temperature on growth and bioactive metabolite production was studied for the four selected isolates. 30˚C was observed to be optimum temperature for the growth of all the four isolates studied. Except **isolate R5**, which showed maximum bioactive metabolite production at 35˚C, all other isolates were found to have 30˚C as the optimum temperature for bioactive metabolite production. **Figure 6** and **Figure 7** depicts the effect of incubation temperature on growth and bioactive metabolite production by the isolates.

3.6. Effect of Minerals on Growth and Bioactive Metabolite Production

For the evaluation of the effect of minerals on the growth and bioactive metabolite production, the medium with optimized carbon and nitrogen sources was supplemented individually with different minerals, such as $CuSO_4$, $MnSO_4$, $MgCl_2$, $FeCl_3$, $CoCl_2$, KNO_3, $(NH_4)_2MoO_4$ and $ZnSO_4$, each at a concentration of 0.05%. The effect of minerals on growth is represented in **Figure 8**. As is evident, $CuSO_4$ and $ZnCl_2$ did not seem to have much effect on growth of isolates whereas $MgCl_2$ and $(NH_4)_2MoO_4$ significantly enhanced the growth. $CoCl_2$ and $FeCl_3$ were not found to have much effect on growth. As represented in **Figure 9**, $MgCl_2$ and KNO_3 significantly enhanced the production of bioactive metabolites in these isolates.

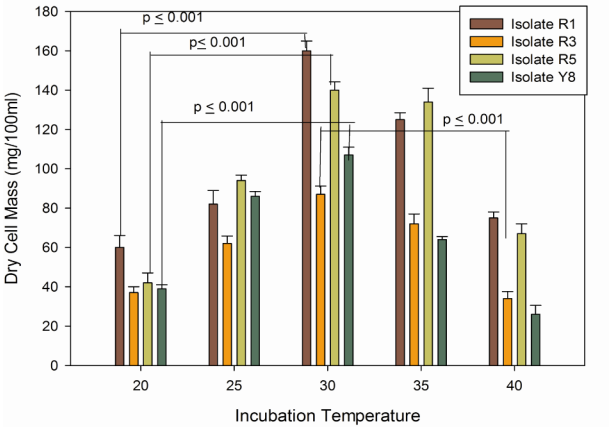

Figure 6. Effect of temperature on growth of the *Streptomyces* isolates. Data expressed as mean ± SD (n = 3). Significant differences with respective *P* values indicated above the bars.

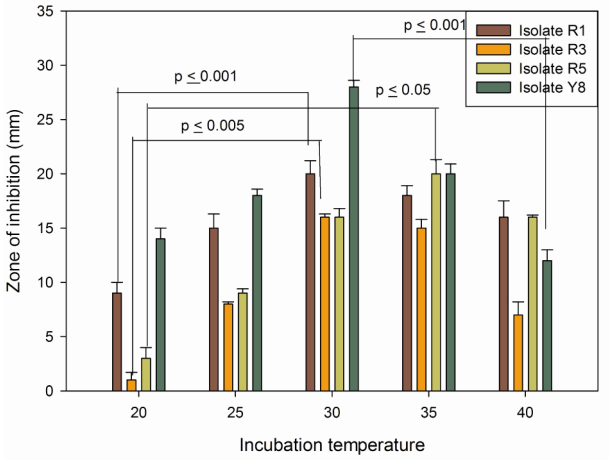

Figure 7. Effect of temperature on bioactive metabolite production by the *Streptomyces* isolates. Data expressed as mean ± SD (n = 3). Significant differences with respective *P* values indicated above the bars.

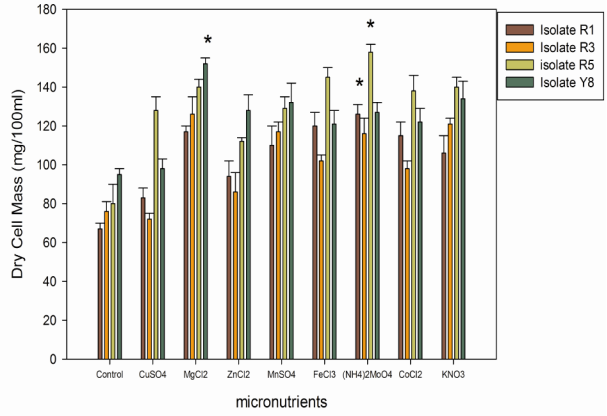

Figure 8. Effect of minerals on growth of the *Streptomyces* isolates. Data expressed as mean ± SD (n = 3). $^{*}P \leq 0.05$ (vs control).

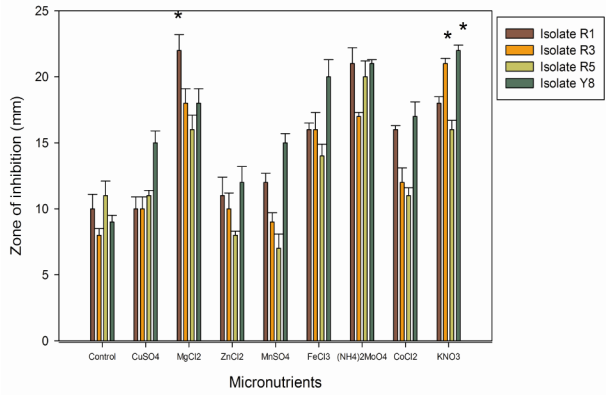

Figure 9. Effect of minerals on bioactive metabolite production by the *Streptomyces* isolates. Data expressed as mean ± SD (n = 3). $^{*}P \leq 0.001$ (vs control).

4. Discussion

Development of an efficient fermentation process for the production of secondary metabolites by *Streptomyces* species requires examination of a diverse array of species-specific features, including physical and chemical factors. Carbohydrates and nitrogen play key roles as structural and energy compounds in cell. Also several cultivation parameters like pH, incubation period and temperature play a major role in the production of bioactive metabolites [13]. Thus, to determine the optimal medium and culture conditions for antibiotic production by the four isolates, various carbon and nitrogen sources were tested.

We used a complex medium rather than a defined for optimization studies because antibiotic producing organisms usually produce limited quantities of antibiotics in defined media and growth is also lower [18].

4.1. Effect of Carbon Sources on Growth and Bioactive Metabolite Production

Isolate R1, isolate R3 and isolate Y8, showed maximum cell growth in medium amended with glucose, fructose and mannose but very poor bioactive metabolite production. On the other hand, maximum bioactive metabolite production was observed in medium amended with complex carbon sources like cellobiose and starch respectively. This may be because monosaccharides have been reported to be suitable carbon sources for growth but not for biosynthesis of antibiotics [19]. Actinomycete cell metabolism under conditions of nutritional excess is known to be directed towards the generation of cell mass rather than production of secondary metabolites. Also, glucose is known to cause catabolite repression, in which production of enzymes of secondary metabolite bio-

synthesis is inhibited [11] [20]. It has been observed that secondary metabolite production is often stimulated by slowly assimilated complex carbon sources like polysaccharides [21]. Optimal production has been achieved by cultivating organisms in media containing slowly utilized nutrient sources [22] [23]. Starch has been found to be the best carbon source for antibiotic production by several researchers [24] [25].

Isolate R5, preferred carbon sources like cellobiose, xylose and arabinose for maximum cell mass and mannitol for production. Dekleva (1985), in his studies on *Streptomyces peucetius* has reported that xylose and arabinose are not very good carbon sources for growth and antibiotic production [18]. Our results were in agreement with these for isolate R1, isolate R3 and isolate Y8, but for isolate R5, arabinose and xylose were the second and third best carbon sources for growth, and bioactive metabolite production was also moderate.

4.2. Effect of Nitrogen Sources on Growth and Bioactive Metabolite Production

In this study, it was clear from the results that the growth of the isolates was greatly influenced by the nature and type of nitrogen source supplemented in the medium. In comparison with the inorganic nitrogen sources, organic nitrogen sources induced relatively higher biomass yield as well as bioactive metabolite production. In this study, Jack bean meal proved to be the best nitrogen source tested for growth and the metabolite production was also good. Soybean meal has been found to be the best organic nitrogen source by Narayana and Vijaylakshmi on their studies on *S. albidoflavus* and also by Wu *et al*., on their studies on polyene antibiotics by *S. padamus* [26] [27].

This study also showed that peptone and casein resulted in maximum bioactive metabolite production in the isolates. Peptone followed by casein has been observed to be the best nitrogen sources by Gopi Reddy *et al*., on their studies on *S. rochei* [11]. Peptone has been found to favour antibiotic production by other authors too [28] [29].

4.3. Effect of Incubation Period on Bioactive Metabolite Production

Generally, it has been observed that *Streptomyces* show progressive increase of biomass during the first 4 - 7 days of incubation. Antibiotic production usually starts on the second or third day but maximum antibiotic activity is recorded on ninth or tenth day, that is, in the stationary phase. It has been reported that two phases are observed during the propagation of antibiotic producers. The first phase (trophophase) is characterized by rapid growth (biomass production) and the second phase (idiophase) is characterized by a slow growth and maximal productivity of antibiotics [30]. For isolate R3 and Y8, a similar result was recorded where maximum antibiotic activity could be seen on eighth and tenth day and then it was almost stable. In the case of isolate R1 and R5 maximum antibiotic activity was seen on the fourth and sixth day. Thus, though the stationary phase must have begun on the tenth day, maximum antibiotic production had started in the mid log and late log phase and continued in the stationary phase.

4.4. Effect of pH on Growth and Bioactive Metabolite Production

According to Guimaraes, the pH of the culture medium is one of the most important environmental factors, because it exerts a marked effect on the activity of several enzymes that catalyze metabolic reactions, as well as exerting significant influence on complex physiological phenomena such as membrane permeability and cell morphology [31]. Changes in the initial external pH affect many cellular processes such as regulation and biosynthesis of secondary metabolites [32] [33]. Hence, an attempt was made to determine the optimum external initial pH for each isolate.

Generally, in most published literature, optimum pH for antibiotic production in *Streptomyces* cultures has been reported to be near neutral [17] [34] [35]. For isolate R3, this was found to be true, where pH 7 was found to be optimum both for growth as well as bioactive metabolite production. But for isolate R1, pH 5 proved to be optimum for growth as well as production. In the case of isolate R5, pH 5 gave maximum cell mass but pH 6 was optimum for bioactive metabolite production. Similarly, for isolate Y8, pH 7 was optimum for growth but pH 10 was optimum for bioactive metabolite production. Similar results have been reported by Guimaraes in his work on retamycin production by *S. olindensis* where, the optimum pH for cell growth was slightly different from that for retamycin production [31]. In the present work, the final pH of pH flasks 5 to 11 after an incubation period of 12 days was found to be in the range of 6.2 - 8.4. This could be because of the buffering action of

the media constituents.

4.5. Effect of Temperature on Growth and Bioactive Metabolite Production

The incubation temperature also was found to have an effect on growth as well as bioactive metabolite production. 30˚C was observed to be the optimum temperature for the growth of all the four *Streptomyces* species in this study. Except isolate R5, for which the optimum temperature for bioactive metabolite was 35˚C, all the other isolates showed maximum bioactive metabolite production at 30˚C. This is in agreement with the report of Oskay on *Streptomyces* sp. KGG32, wherein maximum biomass and antimicrobial production was observed at 30˚C [17]. Hassan also has observed maximum antibiotic production by *Streptomyces violatus* at 30˚C [36]. However, El-Mehalawy had observed that maximum antifungal production by *Streptomyces lydicus* was at 24˚C whereas by *Streptomyces erumpens* and *Streptomyces antimycoticus* was at 28˚C [37].

4.6. Effect of Minerals on Growth and Bioactive Metabolite Production

Among the minerals tested, $MgCl_2$ and KNO_3 had a positive effect on both growth and bioactive metabolite production by the *Streptomyces* isolates. $ZnCl_2$ and $MnSO_4$ showed a negative effect on bioactive metabolite production by isolate R5 in particular, as compared to that of control. Narayana and Vijaylakshmi have reported $MgSO_4$ to have a positive effect and $MnCl_2$ and $ZnSO_4$ to have a negative effect on antimicrobial metabolite production by *Streptomyces albidoflavus* [26]. According to Hassan, iron and manganese could play an important role in antibiotic production. He observed a slight increase in antibiotic production for $CuSO_4$ whereas $ZnSO_4$ addition lowered the antibiotic production as compared to control [36]. Kiranmayi too observed decrease in antibiotic production with $ZnSO_4$ and $FeSO_4$ [13]. Similar results have been recorded by Ripa *et al*., and Majumdar [38] [39].

The study showed that pH, temperature, incubation time, carbon and nitrogen source directly influenced the production of these bioactive metabolites. The four strains tested here behaved differently, each one requiring specific conditions for maximum growth as well as bioactive metabolite production.

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