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OPTIMIZATION OF SOME CULTURE CONDITIONS FOR IMPROVED BIOMASS AND ANTIBIOTIC PRODUCTION BY *STREPTOMYCES SPECTABILIS* ISOLATED FROM SOIL

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ABSTRACT: A *Streptomyces* strain was isolated from soil and was identified as *Streptomyces spectabilis* on the basis of 16 S rRNA gene sequencing. On screening for its ability to produce antimicrobial compounds, it was found to be active only against Gram positive bacteria. Attempts were made to optimize the culture conditions for the production of antimicrobial compounds. Basal medium supplemented with glucose as a carbon source was found to be the best for growth but not for antibiotic production. Antibiotic production proved to be highest in the medium amended with cellobiose. Jack bean meal (JBM) and peptone were the best nitrogen sources for growth and antibiotic production respectively. Antibiotic production appeared to start after 48 hours and reached a maximum on the fourth day. Maximum growth and antibiotic production occurred when the initial pH was adjusted to 5.

INTRODUCTION: Microbial natural products are the origin of most of the antibiotics on the market today. There is an alarming scarcity of new antibiotics currently under development in the pharmaceutical industry. Still, microbial natural products remain the most promising source of novel antibiotics, although new approaches are required to improve the efficiency of the discovery process. Actinomycetes have provided important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances [1]. These searches have been remarkably successful and approximately two-thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes [2-3].

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About 61% of all the bioactive microbial metabolites are isolated from actinomycetes especially from Streptomycetes. Hence, the search for novel antibiotics often includes the isolation of *Streptomyces* species from soil [4].

The ability of *Streptomyces* cultures to form bioactive products is not a fixed property but can be greatly increased or completely lost under different conditions of nutrition and cultivation [5]. 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.

Changes in the nature and type of carbon and nitrogen sources have been reported to affect antibiotic biosynthesis in *Streptomyces* [6-7]. Also several cultivation parameters like pH, incubation period and temperature play a major role in the production of bioactive metabolites [8].

This study involves the identification of a *Streptomyces* strain isolated from soil and optimization of the culture conditions and process parameters to facilitate improved growth and production of the biologically active compound.

MATERIALS AND METHODS:

The organism: The Streptomycete was isolated from the soil collected from Nagpur, Maharashtra, India. The soil (10 g) was air dried for three days and added to a conical flask containing 100 ml of sterile water and few drops of Tween 80. The flask was shaken for 30 min in an orbital shaker incubator. This soil suspension was serially diluted (1:100, 1:1,000, and 1:10,000) and plated on Potato dextrose agar and Yeast extract malt extract agar (ISP2) media. After an incubation of 7 days at 28°C, a typical red colored colony was picked up and re-streaked to ensure the purity of the colony. The culture was maintained at 4°C on ISP 2 media slants.

Characterization of the organism: The *Streptomycete* was characterized morphologically and physiologically following the methods given in the International *Streptomyces* project (ISP)[9]. The isolate was identified as species belonging to the genus *Streptomyces* by analyzing its morphological characteristics. The morphology of aerial hyphae and substrate mycelia and spore chains was determined by direct light-microscopy examination of cultures after 10 days of growth at 28°C[10]. The color determinations of the aerial mass, substrate mycelium (reverse color), and pigment production were examined in ISP2, inorganic salt-starch agar (ISP4), and glycerolasparagine agar (ISP5) respectively [9]. Melanin production was observed on Peptone yeast extract iron agar (ISP 6).

Molecular characterization of the isolate: A confirmatory taxonomic identification was done by the nucleotide sequencing of the 16S rRNA gene. Genomic DNA extraction, amplification and sequencing of the 16S rRNA gene were performed as described earlier[11]. The 16S rRNA gene was amplified with primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500r (5'AGAAAGGAGGTGATCCAGCCA-3'). The amplified DNA fragment was separated on 1 % agarose gel, eluted from the gel and purified using QIAquick gel extraction kit (Qiagen).

The purified PCR product was sequenced with 27f, 519r (5-GWATTACCGCGGCKGCTG-3), 1087r (5-CTCGTTGCGGGACTTAACCC-3), 530f (5-TTCGTGCCAGCAGCCGCGG-3), 945f (5-GGGC CCGCACAAGCGGTGG-3) and 1492r respectively (*Escherichia coli* numbering system). The rDNA sequence was determined by the dideoxy chain-termination method using the Big-Dye terminator kit using ABI 310 Genetic Analyzer (Applied Biosystems, USA). Almost complete sequence (1158 bp) of 16S rRNA was determined and was compared with those of other closely related taxa retrieved from the GenBank database.

A phylogenetic tree was constructed by Neighbour-Joining plot [12]. A sequence similarity search was done using GenBank BLASTN [13]. Sequences of closely related taxa were retrieved; aligned using Clustal X programme [14] and the alignment was manually corrected. For the Neighbour-joining analysis, the distances between the sequences were calculated using Kimura's two-parameter model [15]. Bootstrap analysis was performed to assess the confidence limits of the branching [16].

The antimicrobial potential of the Streptomycete: Antimicrobial activity of the *Streptomyces* isolate was detected using agar cross streak method [17]. The isolate was streaked as a dense straight line on nutrient agar plate and incubated at 28°C for 6 days (144 h). After the 6th day, test bacterial strains were streaked at right angle, but not touching each other, and then incubated at 37°C for 24 h.

The antimicrobial activity was observed by determining the distance of inhibition between the test organisms and the *Streptomyces* isolate colony margin. The test organisms used were *Micrococcus luteus* MTCC 106, *Bacillus subtilis* MTCC 441, *Escherichia coli* MTCC 443 and *Pseudomonas aeruginosa* MTCC 741, *Candida albicans* MTCC 227, and *Aspergillus niger* MTCC 282.

Antibiotic production & extraction: The spore suspension (5%, v/v) of the isolate was inoculated into 100 ml Potato dextrose broth and the flask was kept for incubation in a shaker incubator at 28°C at 120 rpm for 10 days. After incubation the mycelium was separated from the broth by filtration.

Ethyl acetate (50 ml) was added to the mycelium and filtrate (1:1 v/v) and shaken vigorously for 20 min. The organic layers were collected and the organic solvent was evaporated to dryness in a vacuum concentrator to obtain the crude cell extract and crude broth extract. The dried crude extracts were resuspended in methanol and used for antimicrobial testing using agar well diffusion method [18-19] against *Bacillus subtilis*. 15 µl of 1 mg/ml dried extract was used for this purpose and the zones of inhibition obtained were measured.

Effect of Carbon and Nitrogen sources on biomass and antibiotic production: A basal medium containing glucose (10g/l), soyabean (10g/l), NaCl (10g/l) was used for optimization studies. The pH of the medium was adjusted to 7. The effect of varying carbon sources on growth and antibiotic production was studied by replacing glucose in the basal medium with 1% of the carbon sources like galactose, maltose, lactose, cellobiose, starch etc. Triplicate flasks, set up for each carbon source tested, were then incubated on a rotary shaker incubator (REMI CIS-24 BL) at 120 rpm at 28±2°C for 10days. After 10 days, the mycelia was separated by centrifugation and dried at 70°C until a constant weight was obtained and expressed as mg/100 ml [20-21].

The final pH of the filtrate was recorded and the filtrate was concentrated five-fold in a vacuum concentrator and preserved for analysis of antibiotic production. The duplicate flasks were used for ethyl acetate extraction of mycelium as well as filtrate. The extracts were concentrated and used for testing bioactivity. The bioactivity was determined by agar well diffusion method against *B.subtilis*. 50µl of concentrated supernatant and 15µl of the ethyl acetate cell extract and broth extract (1mg/ml) of each carbon and nitrogen flask were used to determine bioactive metabolite production. The zones of inhibition were noted. Similarly, the effect of various nitrogen sources like casein, peptone, jack bean meal, ammonium sulphate and potassium nitrate was assessed by adding them in place of soybean meal in the basal media at a concentration of 1 %.

Effect of Incubation period on biomass and antibiotic production: The isolate was inoculated into the basal medium and incubated up to 15 days in a rotary shaker at 120 rpm at 28°C. 1 ml sample was withdrawn every day and the cells were separated by centrifugation at 5000 rpm. The cell free broth was concentrated five-fold in a vacuum concentrator and 50µl was used to determine the antimicrobial activity by agar well method in the nutrient agar plate already seeded with *Bacillus subtilis*. The plate was incubated at 37°C for 24 h and the zone of inhibition measured. The growth of the isolate was measured as dry weight per ml of the sample withdrawn.

Effects of initial pH on biomass and antibiotic production: The initial pH levels of the basal media were adjusted from 4 to 11 and incubated for 10 days after inoculation. The biomass and antibiotic production was estimated as described above.

RESULTS AND DISCUSSION:

Morphological and cultural characterization of the organism: One of the more efficient ways of discovering novel metabolites from microorganisms is through the isolation of new microbial species. With this perspective, a Streptomycete was isolated from the local soil sample. **Figure 1** shows it to be Gram-positive filamentous, indicating it to belong to *Streptomyces* genus. Morphological observation of the 7-15 day old culture grown on yeast-malt extract agar (ISP2) [9] revealed that both aerial and vegetative hyphae were abundant. The isolate developed well on several media including ISP1, ISP2, ISP3, ISP4, ISP5, and PDA. The cultural characteristics of strain are evident from **figure 2**. Aerial mycelium was abundant, well-developed and varied from orange to pink red on all media tested. The substrate hyphae varied from yellow to orangish red. Diffusible pigments were not produced on any tested media. Melanin production was observed on ISP6.

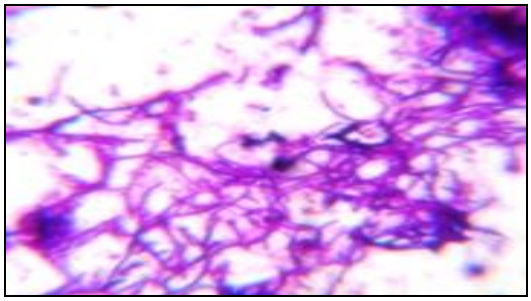

FIGURE 1: GRAM POSITIVE FILAMENTS OF *STREPTOMYCES SPECTABILIS* AS SEEN BY LIGHT MICROSCOPY (1000X)

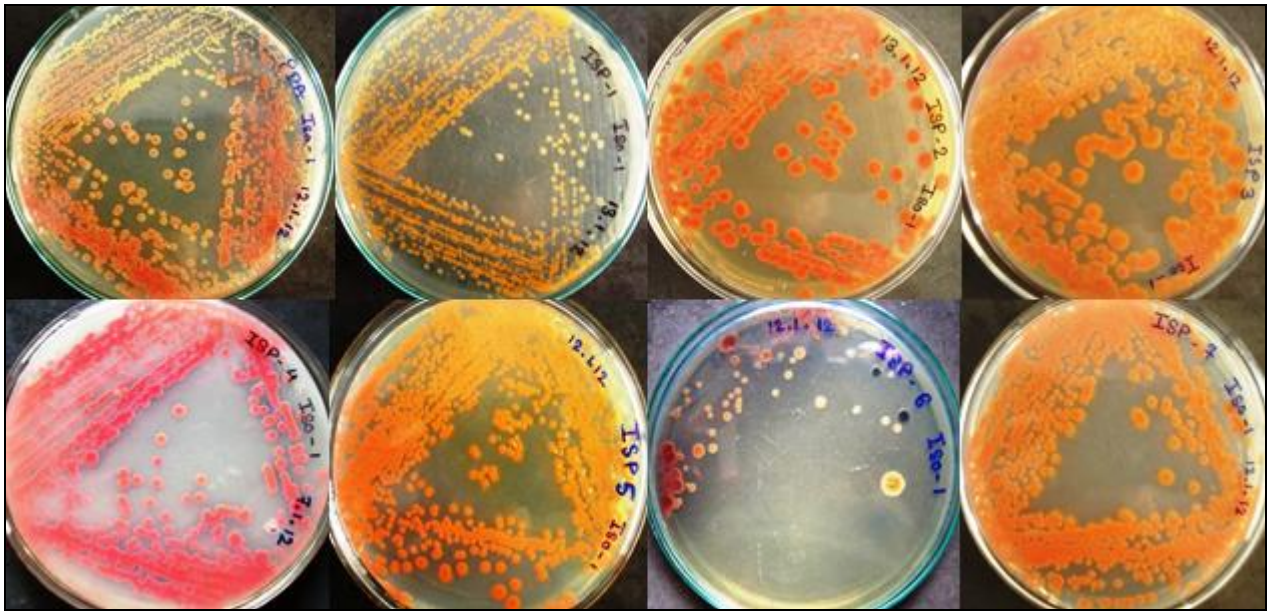

FIGURE 2: COLONY CHARACTERISTICS OF *STREPTOMYCES SPECTABILIS* ON DIFFERENT ISP MEDIA AFTER 7 DAYS OF INCUBATION AT 28°C

Molecular identification of the isolate: The comparison of the partial sequences (1158 bp) of 16S rRNA of the isolate with those found in databases was done by BLASTN analysis. Result showed that query sequence of the isolate was best pair-wise aligned with 16S rRNA partial gene sequence of *Streptomyces spectabilis* strain BJHS7 with similar sequence homology and identity of 100%.

Figure 3 shows the N-J phylogenetic tree of the 16S rDNA sequence data confirming that the isolate was most closely related to *Streptomyces spectabilis* strain BJHS7. Out of 1158 16S rRNA nucleotides of the isolate all were identical with those of *Streptomyces spectabilis* strain BJHS7, indicating that the sequence similarity of the 16S rRNA of the two bacteria was 100%. The culture has been deposited at National Centre for Cell Sciences, Pune, India.

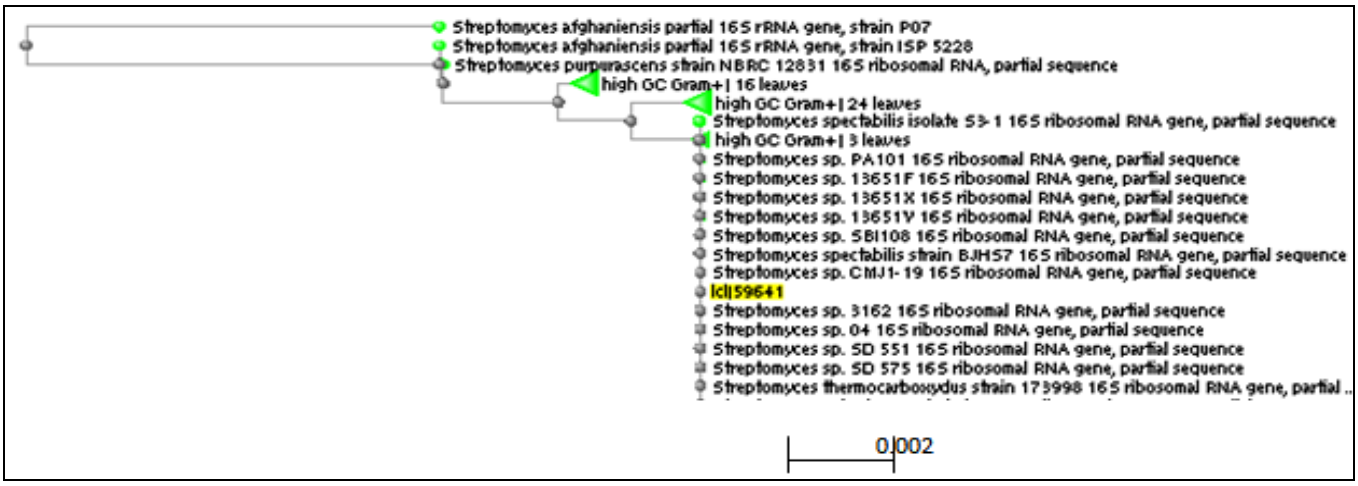

FIGURE 3: NEIGHBOUR-JOINING TREE BASED ON 16S RRNA (1158 BASES) SEQUENCES, SHOWING THE PHYLOGENETIC RELATIONSHIP BETWEEN THE *STREPTOMYCES* STRAIN AND OTHER PHYLOGENETIC NEIGHBOURS.

The antimicrobial potential of *Streptomyces spectabilis*: The antimicrobial activity of *Streptomyces spectabilis* was screened against various Gram-positive and Gram-negative organisms. It showed a narrow spectrum antimicrobial activity against Gram-positive bacteria such as *Micrococcus luteus* and *Bacillus subtilis*. However, it did not affect the growth of Gram-negative organisms. It was also found to have an antifungal activity against *Aspergillus niger* and *Candida albicans* (**Fig. 4**).

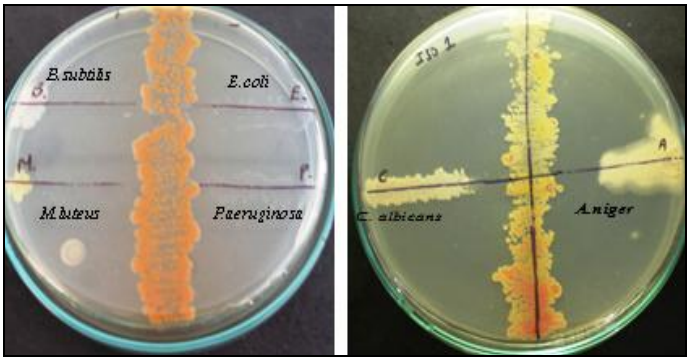

FIGURE 4: SCREENING FOR ANTIMICROBIAL ACTIVITY OF *STREPTOMYCES SPECTABILIS* BY CROSS STREAK PLATE METHOD. INHIBITION OF GRAM POSITIVE BACTERIA, *C. ALBICANS AND A. NIGER* CAN BE SEEN.

Antibiotic production & extraction: As depicted in **figure 5** both ethyl acetate cell extract and broth extract were found to have immense antibacterial activity against *B. subtilis*. However, the extracts failed to show any appreciable activity against *Candida albicans*.

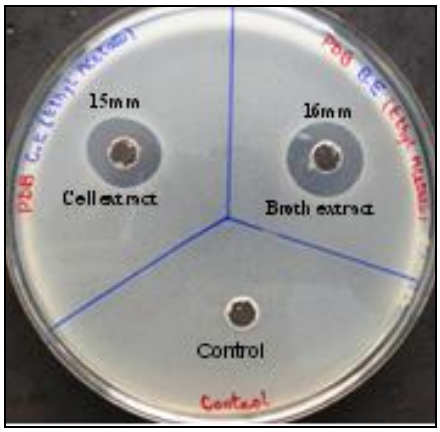

FIGURE 5: ANTIBACTERIAL ACTIVITY OF ETHYL ACETATE CELL EXTRACT AND BROTH EXTRACT AGAINST *B.SUBTILIS* BY AGAR WELL DIFFUSION METHOD. 15UL OF 1MG/ML EXTRACTS USED.

Effect of Carbon and Nitrogen sources on biomass and antibiotic production: 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 sources play key roles as structural and energy compounds in cell.

Thus, to determine the optimal medium for antibiotic production by the isolate, various carbon and nitrogen sources were tested.

The effect of different carbon sources on growth and antibiotic production *Streptomyces spectabilis* is presented in the **figure 6**. All the Carbon sources tested, supported the growth of *Streptomyces spectabilis*. Glucose proved to be the best carbon source for cell growth (273.3mg) followed by galactose (245.8mg) and maltose (184.8) whereas, cellobiose was the best carbon source for antibiotic production (19mm) followed by inositol (16.6mm) and starch (15mm).

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 [22]. The isolate showed maximum cell growth in medium amended with glucose but no antibiotic production. On the other hand, maximum antibiotic production was observed in medium amended with complex carbon sources like cellobiose.

Monosaccharides have been reported to be suitable carbon sources for growth but not for biosynthesis of antibiotics. Secondary metabolite production is often stimulated by slowly assimilated complex carbon sources like polysaccharides. A possible explanation for this phenomenon is that glucose causes catabolite repression, in which production of enzymes of secondary metabolite biosynthesis is inhibited [7, 23-24]. Starch has been found to be the best carbon source for antibiotic production by Gao *et al* [25]. Optimal production has been achieved by cultivating organisms in media containing slowly utilized nutrient sources [23, 26].

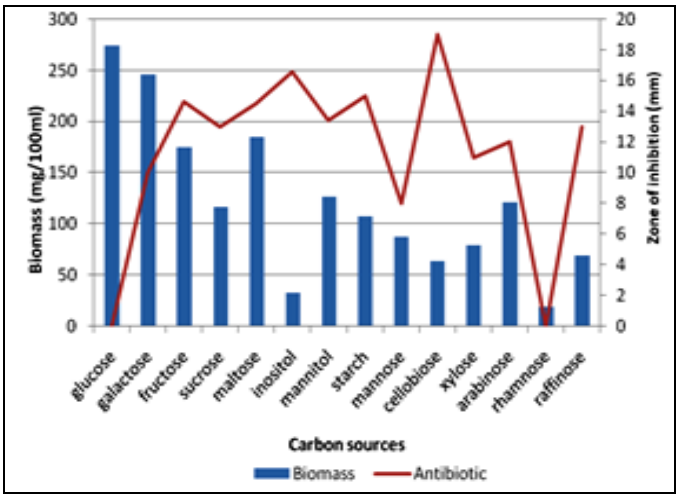

FIGURE 6: EFFECT OF DIFFERENT CARBON SOURCES ON GROWTH AND ANTIBIOTIC PRODUCTION BY *STREPTOMYCES SPECTABILIS*

The different nitrogen sources were tested with the best five carbon sources obtained. As is evident from **figure 7a**, Inositol + JBM supported maximum growth and Starch + peptone supported maximum antibiotic production (**fig. 7b**). It was clear from the results that the growth of the isolate 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 antimetabolite production. This is in accordance with Yu *et al*, [27] and Vahidi *et al*, [28] who reported that organic nitrogen sources are superior for antibiotic production.

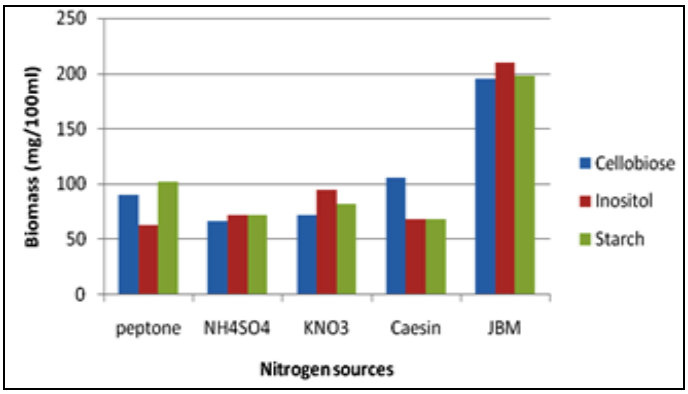

FIGURE 7A: EFFECT OF DIFFERENT NITROGEN SOURCES AND SELECTED CARBON SOURCES ON GROWTH OF *STREPTOMYCES SPECATBILIS*

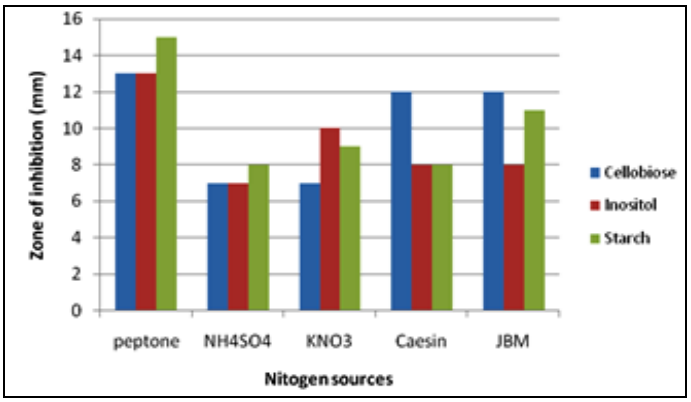

FIGURE 7B: EFFECT OF DIFFERENT NITROGEN SOURCES AND SELECTED CARBON SOURCES ON ANTIBIOTIC PRODUCTION BY STREPTOMYCES *SPECATBILIS*

Effect of Incubation period on biomass and antibiotic 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 [29].

The growth and antibiotic production by *Streptomyces spectabilis* was monitored over a period of 20 days. As is evident from the **figure 8**, the growth reached a maximum on 9th day (19mg) and from there on remained almost constant indicative of stationary phase. The antibiotic production started only after 48 hours. It reached a maximum (19mm) on the 4th day, remained almost stable till the 10th day and decreased gradually. It can be concluded that maximum biomass production was seen on tenth day but maximum antibiotic activity was seen on the fourth day. Thus, though the stationary phase began on the tenth day, maximum antibiotic production had started in the mid log and late log phase and continued in the stationary phase.

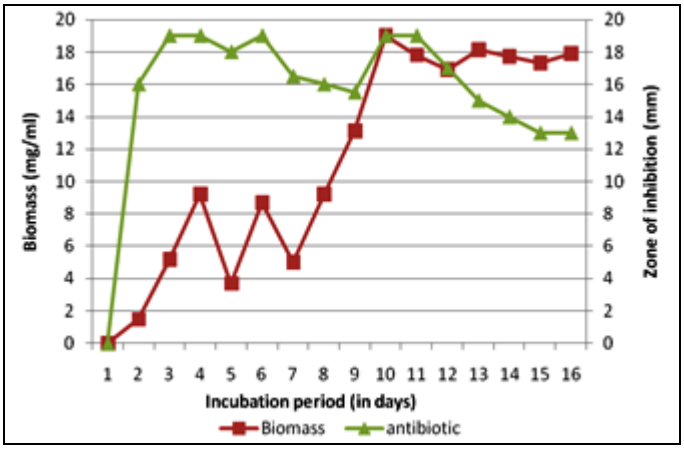

FIGURE 8: EFFECT OF INCUBATION PERIOD ON GROWTH AND ANTIBIOTIC PRODUCTION BY *STREPTOMYCES SPECTABILIS.*

Effects of initial pH on biomass and antibiotic production: Changes in the initial external pH affect many cellular processes such as regulation and biosynthesis of secondary metabolites [30]. The effect of pH on growth and antibiotic production for *Streptomyces spectabilis* is presented in the figure 9. pH 5 was found to be the optimum for growth (107.3 mg) as well as antibiotic production for isolate.

pH 4 did not support antibiotic production and the growth was also not very appreciable **(**10.3 mg). Thakur [31] and Singh [32] reported that pH 7.5 and 8 were optimum in their respective studies on *Streptomyces* species. Generally, in most published literature, optimum pH for antibiotic production in *Streptomyces* cultures has been reported to be near neutral.

Our studies however, showed pH 5 to be optimum for both growth and antibiotic production, indicating it to be an acidophile.

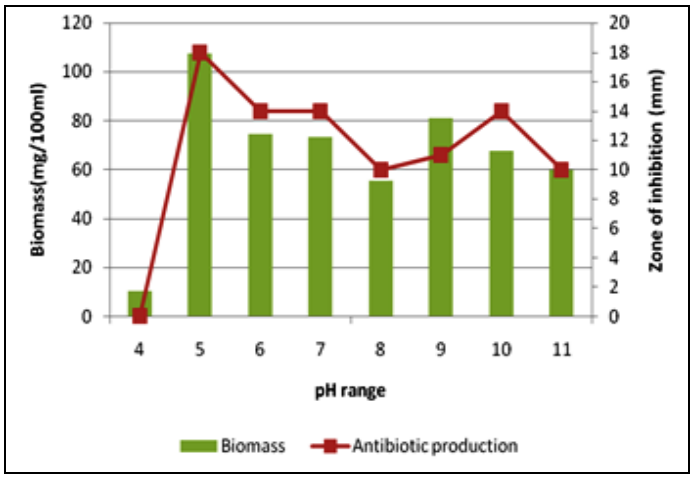

FIGURE 9: EFFECT OF INITIAL pH OF MEDIUM ON GROWTH AND ANTIBIOTIC PRODUCTION BY *STREPTOMYCES SPECTABILIS*

CONCLUSION: The results of this study showed that *Streptomyces spectabilis* produces anti-metabolites which are particularly active against Gram positive bacteria. The study showed that pH, incubation time, carbon and nitrogen source directly influenced the production of these antimetabolites.

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