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EFFECT OF CORBON AND NITROGEN SOURCE FOR THE PRODUCTION OF TETRACYCLINE ANALYSIS BY USING HPLC

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ABSTRACT

Tetracyclines are broad spectrum antibiotic, belong to the family of polyketide antibiotic ,produced by *Streptomyces aureofaciens* of actinobacteria. The organism *Streptomyces aureofaciens 325* was obtained MTCC and cultured on the starch casein media. Screening the organism for the production was confirmed by antibiogram test. The production media was optimized by using carbon sources, and nitrogen sources , Maximum production of antibiotic was shown in soluble starch with 3.0gm concentration tryptophan with the concentration of 0.2gm, and potassium nitrate with 50 mg concentration. The production was increased from 4^{th} day to 7^{th} day and gradually decreased. After the production of tetracycline is quantified by using HPLC.

Keywords: Tetracycline, Antibiogram, Actinobacteria, Polyketide and HPLC

[I] INTRODUCTION

Tetracycline is a broad spectrum antibiotic. These refer to those antibiotics that act against both gram positive and gram negative bacteria. They belong to the family of polyketide antibiotics produced by *Streptomyces aureofacians* of actinobacteria [1]. Tetracyclines bind to the 30S subunit of microbial ribosomes. They inhibit protein synthesis by blocking the attachment of charged aminoacyl-tRNA to the A site on the ribosome. Thus, they prevent introduction of new amino acids to the nascent peptide chain.[2]

The reports on the clinical use of tetracyclines have been confined to respiratory tract infections, sinuses, middle ear infections, urinary tract infections, intestinal infections, and also gonorrhea [3], [4]. Tetracyclines are active against malaria, and this has unexpectedly become important for prophlylaxis following the rapid increase of mefloquine-resistant *P. falciparum* strains [5]. Some applications of tetracycline are in pharmokinetic behavior, human therapy, veterinary medicines, and animal growth promoters [6]. Tetracyclines are used as marker of bone growth in human biopsies [7], tetracycline are also used in transcriptional activation [8].

The effect of medium ingredients such as carbon, inorganic and organic nitrogen sources by the various strains of *Streptomyces* [*S.aureofaciens*], *S.rimosus* and *S.viridifacians* in solid state fermentations was observed [9]. In my present

study, Tetracycline has been produced by submerged fermentation, study evaluating the effect of carbon, nitrogen, lipid and cofactors for increase in the production of antibiotic.

[II] MATERIALS AND METHODS

2.1Culture condition:

The *Streptomyces aureofaciens* 325 was obtained from MTCC . The culture of *Streptomyces* was maintained on the starch casein agar media at 28 °C. The composition of basal media(starch casein agar): Agar- 1.5gm, soluble starch-1gm, potassium phosphate dibasic -0.2gm, potassium nitrate- 0.2gm, sodium chloride -0.2gm, casein- 0.03gm, Magnesium sulphate – 0.005gm, calcium carbonate -0.002gm, Ferrous sulphate -0.001gm, distilled water -100ml.

2.1Screening of Organism:

Antibiogram test was performed, freshly prepared starch casein agar media poured on the whatman filter paper 2cm width and allow it for solidification, *Streptomyces* was used to lawn on the media and kept for 72hr incubation. Another LB agar plate was prepared, where DH5α was used to lawn on the media and whatman filter paper from the starch casein agar media was taken and placed reversely on the DH5α lawn culture plate, kept for 24 hr incubation. Zone was observed surrounding the filter paper.

2.3 Effect of carbon sources:

The different carbon sources (Soluble starch, fructose, maltose, dextrose, sucrose) were added in the basal broth media : Soluble starch – 1gm,potassium phosphate dibasic – 0.2gm, potassium nitrate – 0.2gm, sodium chloride - 0.2gm,casein -0.3gm,magnesium sulphate - 0.005gm, calcium carbonate -0.002gm, Ferrous sulphate – 0.001gm, distilled water-100ml, kept for production for 3-7days, and production was confirmed by well diffusion method by using Muler-Hilton agar plates against against gram negative bacteria - Escherichia coli, by inoculating bacterial broth of 0.5 McFarland standard turbidity in Muller-Hinton agar [10]

2.4 Effect of amino acids :

The different amino acids (phenylalanine, Tryptophan, asparaginine, serine), were added in the basal broth media: Citric acid -1gm, potassium phosphate dibasic -0.2gm, potassium nitrate- 0.2gm, sodium chloride -0.2gm, casein- 0.3gm, magnesium sulphate – 0.005gm, calcium carbonate – 0.002gm, Ferrous sulphate – 0.001gm, distilled water – 100ml, kept for production for 3-7 days, and production was confirmed by well diffusion method by using Muler – Hilton agar plates.

2.5 Effect of cofactors:

The different cofactors (Potassium nitrate, ammonium nitrate, ammonium sulphate) were added in the basal broth media, and kept for 3-7days, and production was confirmed by well diffusion method by using Muler- Hilton agar plates.

2.6 Purification of antibiotic:

The optimized sample was then purified by charcoal method where 2% of charcoal was added to the filtrate and extracted with 1.2% acidified methanol and the eluent obtained was left it for air dry. The air dry sample was mixed with the phosphate buffer (0.5ml), and then the prepared sample was quantified.

2.7 HPLC Analysis:

Tetracycline was estimated using Waters 510 HPLC System, column was varion 250*46, C18, 5 micron and mobile phase was Acetonitrile : water ::50:50 , pH was adjusted to 3.5 by adding orthophosphoric acid, Flow rate 1ml/min, UV Detector 215nm.

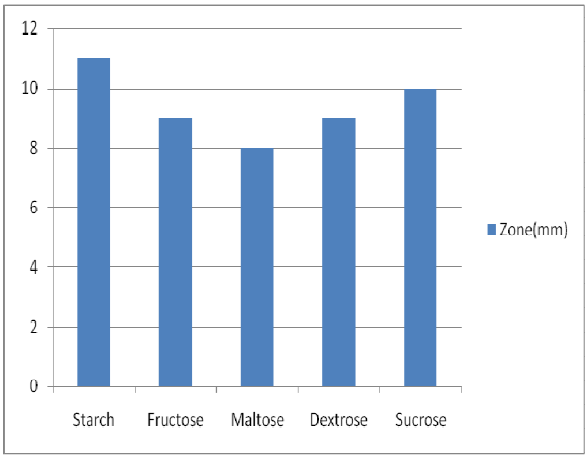
[III] RESULTS AND DISSCUSSION

The *Streptomyces aureofaciens* was obtained from MTCC and screened for the production of tetracycline **(fig 1)** and kept for the production. Tetracycline production in *Streptomyces viridifaciens* with sweet potato residue by solid state fermentations [10]. Tetracycline production by different *Streptomyces* strains in solid state fermentation [11] different *Streptomyces* strain [*S. aureofaciens* NCIM (2417, 2614, and 2615), *S.rimosus* NCIM 2213 and *S. viridifaciens* NCIM

2506]. The production of antibiotic by using *Streptomyces hygroscopicus* CH – 7(Slavica *et al*.)[12].

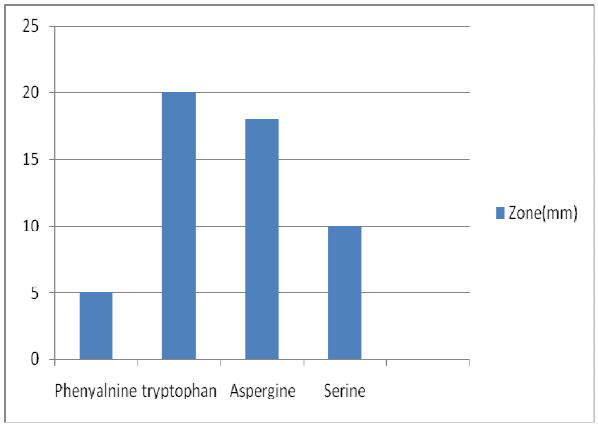
3.2 Effect of different sources:

Different carbon sources supplemented in the production media (Soluble starch, fructose, maltose, dextrose, sucrose [**Graph 1**], for different concentration of Soluble starch ,where we observe maximum zone of inhibition with 3.0gm **[fig 2]** achieved a good yield. Maltose and soluble starch stimulated 2.24-2.69 [10], sucrose stimulated 11.6% while glucose and galactose inhibited 60.2-68.2%.Supplement with a small amount of Soluble starch or other fermentable polysaccharide might be good for secondary metabolite production. D- Galactose showed the best yield where as dextrin, soluble starch, potato starch showed the moderate yield [13]. It has found that 80-90% chlorotetracycline is formed from starch and furthermore, that the metabolic pathway for the biosynthesis of chlorotetracycline does not involve fructose, pentose, krebs cycle, shikimic acid and fixation of carbon dioxide.



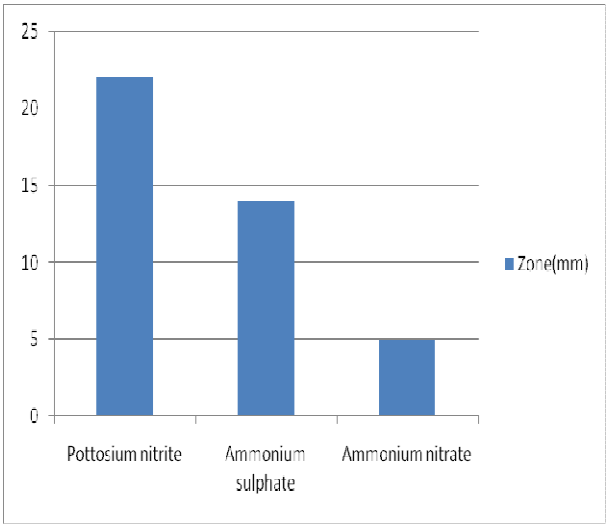
Graph1 Corbon Sources .

Different amino acids supplemented in the production media (phenylalanine, tryptophan, asparaginine, and serine), [Graph2] for different concentration of tryptophan, we observe maximum zone of inhibition to be with 200mg [Table 1] and the increase in production of Tetracycline as amino acids with 4.76% [fig 3], and other nitrogen sources moderately enhanced the production. On comparison ,the nitrogen sources supplement with 0.5% ammonium sulphate yielded the maximum tetracycline potency[10] , when the concentration of ammonium sulphate was more than 1%, the cell growth was stimulated and the production was suppressed, when ammonium nitrate was used as a sole nitrogen sources, the tetracycline and chlorotetracycline were 59.4% and 28.1%, while peanut meal and ammonium sulphate were used as the combined nitrogen sources, the tetracycline and chlorotetracycline were 18.3% and 38.6% .The addition of 1% calcium carbonate resulted in the maximal tetracycline secretion with *Streptomyces* strain, the presence of Sodium Chloride and magnesium sulphate moderately enhanced the tetracycline production. The biosynthesis of compounds on the enzyme level suggest that the formation of chlorotetracycline in high production strains of *S. aureofaciens* cannot be accounted simply by an intensive carboxylation of Acetyl Co-A . Under the fermentation conditions, the medium contains the proteins or other precursors or metabolic pathway that might lead to the formation of malonyl – CoA. Incorporation of 2- C-Glycine and CH_3-methionine into the group of ring A of the chlorotetracycline molecule. In my present study, the production of tetracycline, casein as the precursors have been used in all the production media, as metabolic pathway leads to the formation of malonyl –CoA.



Graph 2 Amino acids

Where as in the cofactors, different cofactors were supplemented in the production media(potassium nitrate, ammonium sulphate, and ammonium nitrate),(Graph3)for different concentration of Potassium nitrate and Ammonium sulphate ,we observe the maximum zone of inhibition to be with 50mg and 100mg[Table 1] and the increase in tetracycline as cofactors with 4.3% and 30%[fig 4]. The supplementation of various nitrogen sources at 10% concentration on tetracycline production was studied[11] all the supplemented organic nitrogen sources were founded to enhance the tetracycline secretion. Among all organic nitrogen sources peanut meal enhances maximum antibiotic secretion .Then, 6 non-amino acid nitrogen compounds were examined as nitrogen sources for growth of *Streptomyces* and its biosysnthesis of antibiotic. Of the nitrogen sources, ammonium sulphate was the best with respect to formation of rapamycin, and supported cell growth comparable to the organic nitrogen sources used in the control chemically defined medium ie. aspartate, arginine, histidine [12] . We can conclude that for the cofactors, effect on the production is the best showing ammonium sulphate and potassium nitrate of 50mg and 100mg in the production media of 100ml.



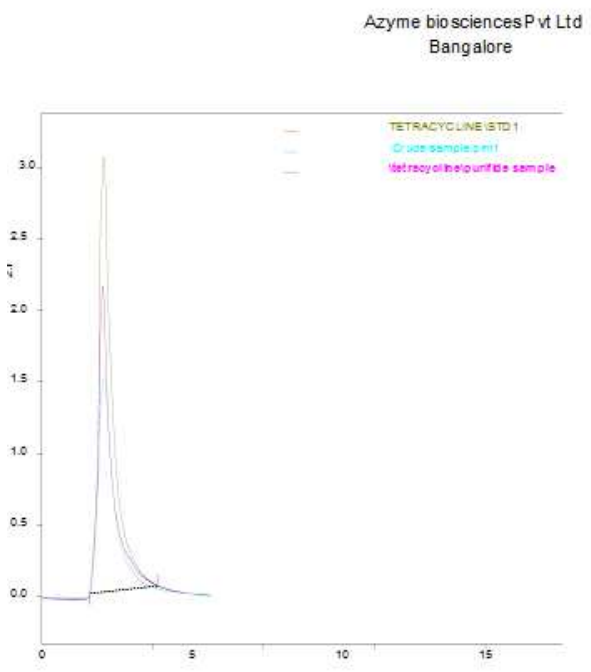
Graph 3

Sl No	Conc (mg)	Zone of Inhibition in mm		
		Tryptophan	Potassium Nitrite	Ammonium Sulphate
1	50	10	23	10
2	100	12	15	20
3	150	14	13	15
4	200	21	12	14
5	250	15	12	14

Table 1

The suitable media selected as a result of the present study consisted of Starch 3.0gm, Tryptophan 0.2gm Potassium nitrate 50mg and ammonium sulphate 100mg per 100ml.

The purification procedure used a biological assay to determine which fractions contained biological activity and effectively separated compounds based on polarity. In some cases, however, purification procedures can be detrimental due to the separation of components thatwork in synergy or provide an additive effect[13]. This work shows that the A. americanum-derived methanolic extract can be partially purified while maintaining activity; in fact, the active compound(s) became concentrated and more effective during purification. After purification qualitative and quantitative by using HPLC [**Graph 4**] according to the HPLC result fold purification is 1.28



Graph4

Samples	Rt	Area
Tetracycline standard	2.79	131.778
Crude Sample	2.81	71.343
Purified Sample	2.74	91.139

Table 2

[IV]**REFERENCES**

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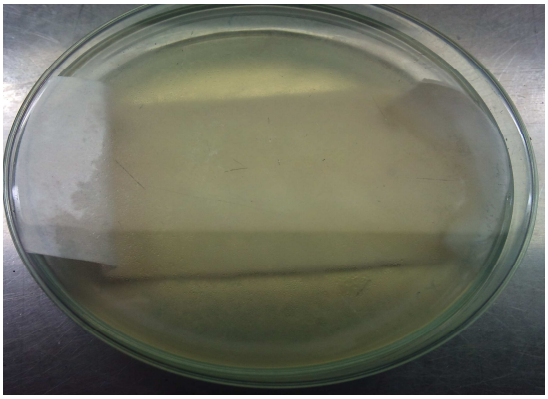

Fig 1: Antibiogram test

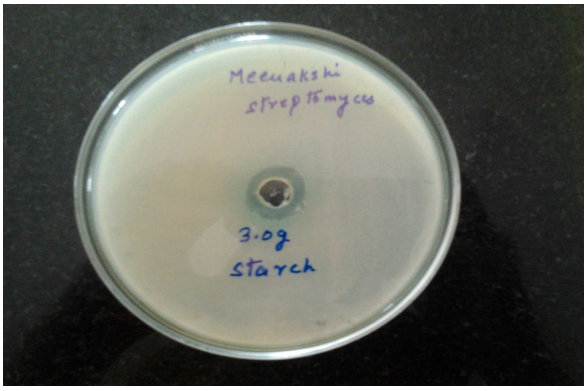

Fig 2: Carbon source with concentration 3.0gm

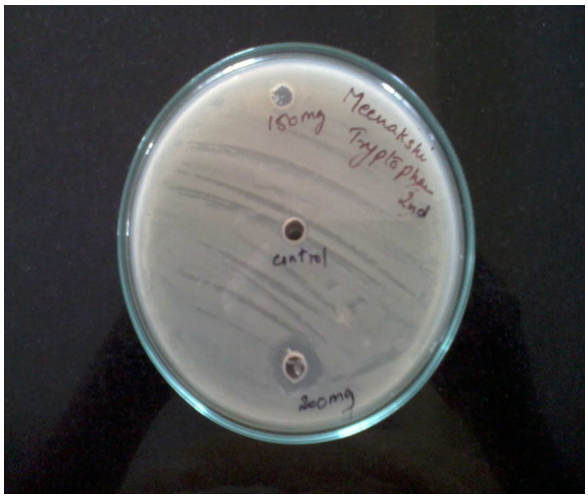

Fig 3: Nitrogen source with 0.2gm concentration

Fig 4: Cofactors with 50 mg and 100mg concentration