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NUTRITIONAL REQUIREMENTS OF STREPTOMYCES ROSEOCHROMOGENUS FOR THE PRODUCTION OF CYCLOSERINE

Part II. Effect of Phosphorus, Magnesium and Trace Metals

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The nutritional requirements of *Streptomyces roseochromogenus* NRRL-B2036 for cycloserine synthesis in shake flasks were studied. Single variable and factorial experiments were conducted to determine the effects of nitrogen, potassium, magnesium, iron, manganese and zin con the production of cycloserine. The medium which gave best result was (g/l): starch 50 g, urea 4.8, $MgSO_4.7H_2O$ 5.0, K_2HPO4 5.0, $FeSO_4$. $7H_2O$ 0.02, $ZnSO_4$ 0.02, and $MnSO_4$ 0.01. The yield of cycloserine was improved by increasing zinc or K_2HPO_4. Interactions were found between urea and $MgSO_4.7H_2O$, K_2HPO_4 and $MgSO_4.7H_2O$, urea and zinc, manganese and zinc and iron and manganese.

In continuation of the work reported earlier[1] on the effect of carbon and nitrogen sources on the synthesis of cycloserine, a study was made to determine the optimum concentration of phosphorus, magnesium and trace metals such as iron, zinc and manganese. Literature dealing with mineral metabolism of Actinomycetes was reviewed by Thornberry[2] and Thornberry and Anderson[3]. Temple,[4] Chaloupka,[5] Saunders and Sylnester,[6] and Johnstone and Wakesman[7] studied the effect of potassium, magnesium, iron, zinc and manganese on antibiotic production other than cycloserine.

In the present work, the influence of the various constituents of media were investigated on the production of cycloserine with single variable and with a factorial design with multiple variables.

Materials and Methods

Organism.—The strain of *Streptomyces roseochromogenus* NRRL B-2036 was used in the present study. The culture was maintained on the agar medium consisting of (g/l): glucose 10, agar 20, beef extract 1.0, yeast extract 1.0, casein hydrolysate 2.0. The cultures were grown at 30°C for 10 days.

Inoculum Preparation.—Vegetative inoculum was used in the present investigation. The composition of the inoculum medium was (g/l): glucose 10.0, beef extract 1.0, yeast extract 1.0, casein hydrolysate 2.0. The inoculum medium 25 ml in 300-ml conical flask was inoculated with a loop of mycelium from the agar slant. It was incubated at 30°C for 48 hr.

Fermentation Medium.—The composition of the basal medium was (g/l): starch 50, urea 4.8; $MgSO_4$. $7H_2O$ 5.0, K_2HPO_4 5.0, $FeSO_4$. $7H_2O$ 0.02, $ZnSO_4$ 0.02 and $MnSO_4$. 0.01. The concentration of the medium constituents was varied accordingly in the single variable and factorial experiments. All reagents were of analytical grade and glass distilled water was used for the preparation of solutions. All media unless otherwise stated were autoclaved at 121°C for 15 min. The initial pH of the medium was 6.8. For shake flask cultures 25 ml fermentation medium including 1 ml vegetative inoculum was held in 300-ml conical flask. The flasks were shaken on a rotary shaker rotated at 125 rev/min.

Analytical Method.—Cycloserine was estimated colorimetrically.[8] The growth of *Mycobacterium tuberculosis* was inhibited by the cycloserine produced by *S. roseochromogenus* NRRL B-2036.

Design of Factorial Experiments.—In most experiments, effect of two nutrients at three or four concentrations was investigated making twelve or sixteen fermentation experiments. Thus, the influence of each concentration of a nutrient was studied in three or four fermentations containing three or four different concentrations of the other nutrients.

Results

Single Variable Experiments with Trace Metals.—The effect of each of the trace metals such as iron, zinc and manganese on cycloserine formation by *S. roseochromogenus* was studied in the basal medium (Figs. 1-3). The concentration of one of the trace metals was changed while the other trace metals were held at the concentration of the fer-

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mentation medium. All experiments were carried out in duplicate and the results presented are mean value of the chemical analysis. The best yield of cycloserine was with 20 mg/l of iron.

The process of cycloserine synthesis was very sensitive to the iron level; since antibiotic formation was greatly affected by lowering and increasing the concentration of iron (20 mg/l). The mycelial growth, however, was not affected by changing iron concentration.

The amount of antibiotic produced was increased by increasing zinc concentration and optimum level of the trace metals was 25 mg/l. Further increase in its concentration decreased both the cycloserine formation and mycelial dry weight.

The optimum concentration of Mn was 10 mg/l and further increase in its concentration also greatly reduced the production of the antibiotic. Mycelial dry weight was also affected by increasing Mn.

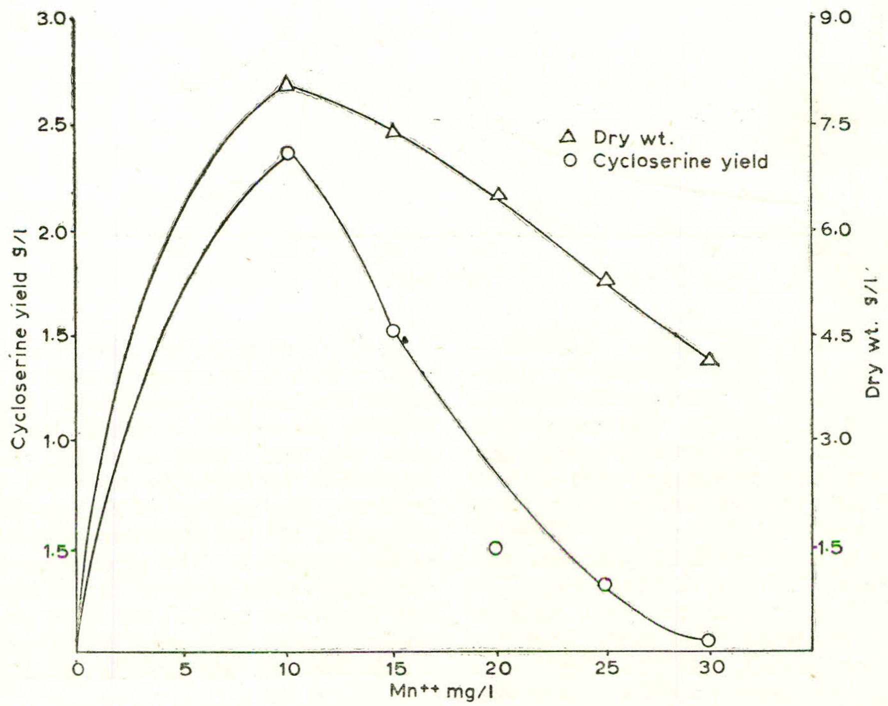

Fig. 1.

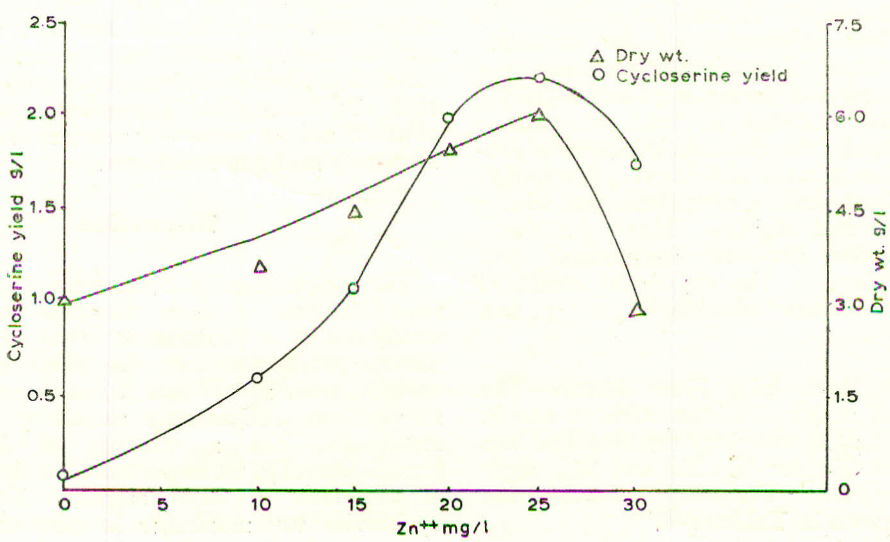

Fig. 2.

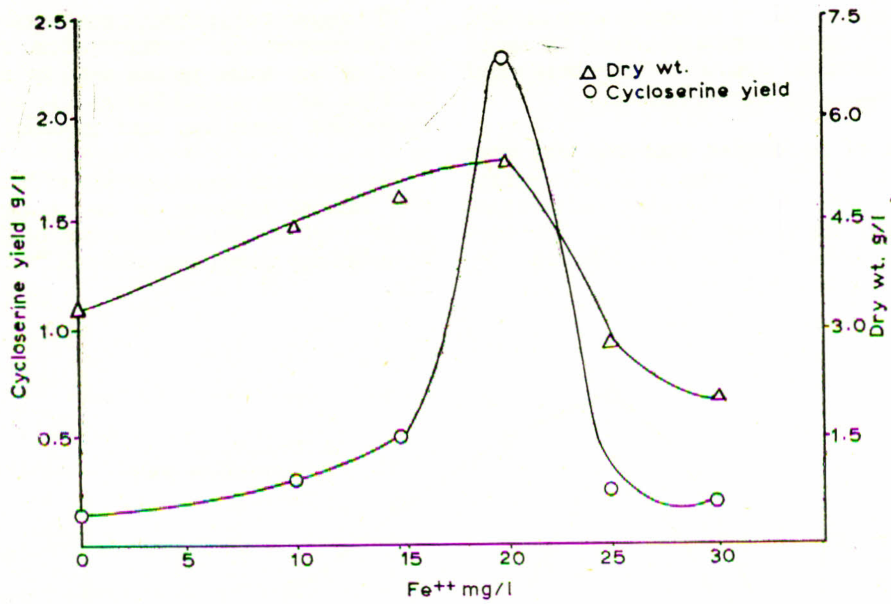

Fig. 3.

Factorial Experiments with Major Nutrients.—To determine the best concentrations for cycloserine synthesis of the major nutrients like nitrogen (urea), phosphorus (K_2HPO_4) and magnesium ($MgSO_4 . 7H_2O$) factorial experiments were designed to test different concentrations of each of these nutrients. The effect of interaction between two nutrients, e.g. urea, K_2HPO_4; urea, $MgSO_4$; K_2HPO_4, $MgSO_4$ on the yield of cycloserine was studied. The results are shown in Tables 1–3. The concentrations of the major nutrients used in the factorial experiments were urea (2.5–7.5 g/l), K_2HPO_4 or $MgSO_4$ (2.5–10.0 g/l) and like. Starch and trace metals, however, were kept constant. Duncan's multiple range test was applied in all factorial experiments to find out the best interaction of the nutrients. The optimum results of cycloserine synthesis (2.4 g/l) were obtained with urea 5.0 g/l and K_2HPO_4 5.0 g/l. Further increase in both constituents showed less stimulatory effect on cycloserine production. In case of urea and $MgSO_4$ interaction (Table 2) the best yield of cycloserine was with 5 g/l each of urea and $MgSO_4$. There was also a definite interaction between magnesium and phosphate (Table 3). The significant results of cycloserine synthesis were with $MgSO_4$ 2.5 g/l and K_2HPO_4 7.5 g/l.

Factorial Experiments with Trace Metals.—The concentration of major nutrients such as starch, K_2HPO4 and $MgSO4$ were kept constant and two trace metals out of three (Fe, Zn and Mn) were varied simultaneously. The results of cycloserine formation are given in Tables 4–6.

In the iron and zinc interaction (Table 4) 20 mg/l of both these metals showed significant effect on the biosynthesis of antibiotic. Further increase reduced the cycloserine yield. Both these metal gave the same result at 0 and 30 mg/l. The interaction between Zn and Mn was of great interest. The increase of Zn (10–30 mg/l) in the absence of Mn has shown an increase in the yield of the antibiotic. The production of cycloserine was improved at all levels in this interaction (Mn and Zn 10–20 mg/l). At all levels the yield of antibiotic was quite good as compared to other interaction (Fe, Mn and Zn, Fe).

A comparison of Tables 4–6 of the effect of these interactions Fe and Mn with others as Fe and Zn, and Zn and Mn was not significant. The yield of antibiotic was maximum with Fe (20 mg/l) and Mn (10 mg/l). The optimum concentration of trace metals were also confirmed by applying Duncan's multiple range test.

Discussion

The mechanism of cycloserine synthesis by *S. roseochromogenus* in large amounts under certain conditions is a problem of great interest. The present work describes the effect of (i) single variable trace metals and (ii) interaction of major nutrients as well as trace metals on the yield of antibiotics. The morphology of the mycelial growth was of great importance for the production of antibiotic in large amount. The yield of cycloserine was maximum in cultures with pellet

TABLE 1.—EFFECT OF INTERACTION BETWEEN UREA AND K_2HPO_4 ON CYCLOSERINE PRODUCTION BY *S. roseochromogenus* NRRL B-2036

K_2HPO_4 (g/l)	Mean yield of cycloserine (g/l) Urea concentration (g/l)		
	2.5	5.0	7.5
2.5	0.50	0.30	1.42
5.0	0.55	2.40	1.76
7.5	0.64	1.20	1.65
10.0	0.80	1.85	0.70

S.E. a. = 0.0207
S.E. b. = 0.0240

a, UREA; *b*, K_2HPO_4.

TABLE 2.—EFFECT OF INTERACTION BETWEEN UREA AND $MgSO_4.7H_2O$ ON CYCLOSERINE PRODUCTION *S. roseochromogenus.*

$MgSO_4$ (g/l)	Mean yield of cycloserine (g/l) Urea concentration (g/l)		
	2.5	5.0	7.5
2.5	1.67	2.15	0.10
5.0	0.32	2.35	1.60
7.5	0.50	1.62	2.45
10.0	0.25	0.40	2.23

S.E. a. = 0.0173
S.E. b. = 0.020

a, UREA; *b*, $MgSO_4.7H_2O$.

TABLE 3.—EFFECT OF INTERACTION BETWEEN $MgSO_4.7H_2O$ AND K_2HPO_4 ON CYCLOSERINE PRODUCTION BY *S. roseochromogenus* NRRL B-2036

K_2HPO_4 (g/l)	Mean yield of cycloserine (g/l) $MgSO_4.7H_2O$ concentration (g/l)			
	2.5	5.0	7.5	10.0
2.5	1.37	0.24	1.51	0.50
5.0	0.90	1.37	0.35	0.22
7.5	2.30	1.60	0.93	1.33
10.0	1.85	1.25	1.47	0.32

S.E. a. = 0.1131
S.E. b. = 0.1131

a, $MgSO_4.7H_2O$; *b*, K_2HPO_4.

TABLE 4.—EFFECT OF INTERACTION BETWEEN ZINC AND IRON ON CYCLOSERINE PRODUCTION BY *S. roseochromogenus* NRRL B-2036.

Fe conc (mg/l)	Mean yield of cycloserine Zn concentration (mg/l)			
	0	10	20	30
0	0.21	0.80	0.81	0.70
10	0.84	0.84	0.73	0.87
20	0.80	1.50	2.40	0.80
30	0.81	0.90	0.90	0.81

S.E. a. = 0.033
S.E. b. = 0.033

a, zinc; *b*, Fe

TABLE 5.—EFFECT OF INTERACTION BETWEEN Mn^{++} AND Zn^{++} ON CYCLOSERINE PRODUCTION BY *S. roseochromogenes* NRRL-B-2036.

Zn conc. (mg/l)	Mean yield of cycloserine (g/l) Mn^{++} concentration (mg/l)			
	0	10	20	30
0	0.32	0.50	0.40	0.30
10	2.15	2.24	2.05	2.08
20	2.31	2.18	2.50	2.13
30	2.82	2.31	2.15	2.05

S.E. a. = 0.0245
S.E. b. = 0.0245

a, Mu; *b*, Zn

TABLE 6.—EFFECT OF INTERACTION BETWEEN Fe^{++} AND Mn^{++} ON CYCLOSERINE PRODUCTION BY *S. roseochromogenus* NRRL B-2036.

Mn conc (mg/l)	Mean yield of cycloserine (g/l) Fe concentration (mg/l)			
	0	10	20	30
0	0.45	0.17	0.83	1.01
10	0.50	0.15	2.32	0.82
20	0.30	0.25	0.74	0.70
30	0.47	0.48	0.75	0.40

S.E. a. = 0.020
S.E. b. = 0.020

a, Fe; *b*, Mu.

type growth, due to (i) large surface volume ratio, and (ii) better aeration (oxygen supply) of the culture. The omission of single trace metals particularly manganese and lesser with Zn or iron affected both the production of cycloserine and mycelial growth. The optimum levels of iron, zinc or manganese in single variable experiments were 20, 25 or 10 mg/l respectively. Further increase in the concentration of trace metals particularly iron or manganese resulted in lowering the mycelial formation and the production of antibiotic. Thus the adequate supply of trace metals to the culture was essential for maximum antibiotic production.

The effect of the interaction of trace metals on the yield of antibiotic was quite interesting. The optimum levels of trace metals were (mg/l)

(i) 20 mg of both iron and zinc (ii) Mn (10 mg) and Zn (20 mg) and (iii) Zn (20 mg) iron (20 mg) and Mn (10 mg).

The interaction between (i) iron and zinc, (ii) zinc and manganese and (iii) iron and manganese was of great significance. The optimum levels of both iron and zinc was 20 mg/l. The higher interaction of both iron and zinc decreased the yield of cycloserine. The amount of cycloserine synthesised, however, was little affected at higher levels of manganese and zinc. The affect of iron and manganese was also like iron and zinc, that is the yield of antibiotic was decreased beyond the optimum levels of Fe (10 mg/l) and Mn (10 mg/l).

The effect of the interaction of major nutrients like (i) urea and K_2HPO_4, (ii) urea and $MgSO_4$, and (iii) $MgSO_4$ and K_2HPO_4 was also interesting. The yield of cycloserine was decreased by increasing urea in both interactions. The optimum concentrations of $MgSO_4$ and K_2HPO_4 were 7.5 g/l instead of 5 g/l in the normal medium.

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