A CHEMICALLY DEFINED MEDIUM FOR THE GROWTH OF CEPHALOSPORIUM SALMOSYNNEMATUM¹

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Cephalosporium salmosynnematum, strain 3590A, was found by Gottshall *et al.* (1951) to produce the antibiotic synnematin. Recent work (Olson and Jennings, 1954) indicates that this antibiotic may have future importance. The purpose of the present study was to determine the nutritional requirements of this organism and to devise a chemically defined medium which would allow the fungus to produce yields of mycelium comparable to those obtained with a good natural medium.

Synthetic media have been developed for submerged growth of several fungi (Jarvis and Johnson, 1947; Olson and Johnson, 1949; Stone and Farrell, 1946) and actinomycetes (Thornberry and Anderson, 1948), but to date none has been reported for the genus *Cephalosporium*. It seemed desirable to study the requirements of *C. salmosynnematum*, strain 3590A, for growth in a chemically defined medium since this information might eventually prove to be useful in obtaining better yields of synnematin from the fungus.

MATERIALS AND METHODS

Fermentation procedures. All fermentations were carried out on a Gump rotary shaker in wide-mouthed 500 ml Erlenmeyer flasks. The shaker operated at 250 rpm causing each flask to describe a two and one-quarter inch diameter circle. Each flask contained 50 ml of the fermentation medium, was plugged with cotton, and sterilized at 120 C for 25 minutes. Five ml of a water suspension of the culture growth from a slant of Czapek-Dox agar were used to inoculate each seed flask. The seed flasks were shaken for 72 hours at 30 C, and one ml of the growth was

used to inoculate each second stage seed flask. These seed flasks were shaken for 24 hours and then were used to inoculate the fermentation flasks. A 0.4 to 0.6 per cent inoculum was used. In all cases except in the study of mineral requirements, the inoculum was homogenized in a sterile cotton stoppered Waring blender.

Two different seed media were employed to prepare the inoculum. The compositions of these media are shown in table 1.

For the studies concerning the minerals which are required in gross amounts, no special purification of the medium was attempted; however, the level of the particular element under investigation was reduced in concentration in the seed medium. The inoculum for these experiments was not homogenized in the blender to eliminate the possibility of introducing metals into the medium from the blender itself.

The inoculum for the trace metal studies was prepared as usual except that the metal under investigation was omitted from the final seed flask. The fermentation medium for these studies was purified according to the methods of Olson and Johnson. These methods use dithizone extractions of the medium at appropriate pH to remove copper and zinc, respectively, and 8-hydroxyquinoline to remove iron. The following modification of these methods was used: All extractions were shaken intermittently by hand for a period of two hours instead of shaking on a reciprocating shaker overnight. All trace metals to be added were added after the purification procedure was completed.

All fermentations were run in duplicate and then repeated. For the analytical data of figure 3 only one run was made, but duplicate determinations were made on each of three flasks at each time of sampling.

All fermentations were run for 72 hours. At the end of this time the flasks were removed from the shaker and the mycelium collected by filtering the contents of each flask through a tared filter

¹ The data presented in this paper were taken from a thesis submitted in June, 1953, by Michael A. Pisano to Michigan State College in partial fulfillment of the requirements for the degree Doctor of Philosophy.

TABLE 1
Composition of the seed media

Medium A		Medium B	
Component	Concentration per liter	Component	Concentration per liter
Glucose	10 g	Glucose	10 g
(NH ₄) ₂ SO ₄	4 g	L-Glutamic acid	5 g
KH ₂ PO ₄	1 g	Potassium (as KCl)	0.2 g
MgSO ₄ ·7H ₂ O	1.5 g	Magnesium (as MgCl ₂ ·6H ₂ O)	0.04 g
L-Asparagine	1 g	Sulfur (as Na ₂ SO ₄)	0.1 g
Zinc (as ZnSO ₄ ·7H ₂ O)	4.58 mg	Phosphorus (as NaH ₂ PO ₄ ·H ₂ O)	0.2 g
Iron (as FeSO ₄ ·7H ₂ O)	2 mg	Zinc (as ZnSO ₄ ·7H ₂ O)	4.5 mg
Copper (as CuSO ₄ ·5H ₂ O)	1.25 mg	Iron (as FeSO ₄ ·7H ₂ O)	5 mg
		Copper (as CuSO ₄ ·5H ₂ O)	0.08 mg
Distilled water to 1,000 ml pH adjusted to 6.5 with NaOH		Distilled water to 1,000 ml pH adjusted to 7.0 with NH ₄ OH	

paper (Whatman no. 1, 7 cm) on a Buchner funnel. Filtrates to be saved for future study were stored at -20 C. The filter papers with the mycelium were placed in an oven (105 C) for eighteen to twenty-four hours. At the end of this time the mycelial weight was determined. The growth of the organism on the various media was evaluated by the following formula:

Per cent conversion of added carbon to dry

$$\text{mycelium} = \frac{\text{g dry mycelium} \times 100}{\text{g carbon compounds added}}$$

Analytical procedures. Glucose determinations were made by the method of Shaffer and Somogyi (1933). Their reagent no. 5 which contains 5 g of potassium iodide per liter was used.

The method used for the ammonia nitrogen determinations was that of Gailey *et al.* (1946) except that the ammonia was steam distilled rather than aerated.

Total nitrogen was determined by the semi-micro-Kjeldahl method of Ma and Zuazaga (1942).

RESULTS

Composition of the final fermentation medium. The composition of the fermentation medium finally developed is shown in table 2.

When grown on the final fermentation medium, *C. salmosynnematum*, strain 3590A, gave yields (dry weight of mycelium) of 45 per cent of the weight of glucose and L-glutamic acid added. The medium contained levels of minerals equal

TABLE 2
Composition of the final fermentation medium

Component	Concentration Per Liter
Glucose	10 g
L-Glutamic acid	5 g
Potassium (as KCl)	0.2 g
Magnesium (as MgCl ₂ ·6H ₂ O)	0.04 g
Sulfur (as Na ₂ SO ₄)	0.1 g
Phosphorus (as NaH ₂ PO ₄ ·H ₂ O)	0.2 g
Zinc (as ZnSO ₄ ·7H ₂ O)	4.5 mg
Iron (as FeSO ₄ ·7H ₂ O)	5 mg
Copper (as CuSO ₄ ·5H ₂ O)	0.08 mg
Distilled water to 1,000 ml pH adjusted to 7.0 with NH ₄ OH	

to twice those found necessary for maximum growth. Based on yields obtained, it is apparent that L-glutamic acid functions as a carbon source as well as a nitrogen source. The L-glutamic acid and glucose were not added in excess. The levels of sodium and chloride ions employed in the medium produced no toxic effects.

Carbon requirements. Fructose, glucose, maltose, and mannose were each found to serve equally well as the major carbon source. These sugars were utilized approximately 1.3 times more efficiently than sucrose and 7.7 times more than lactose. Since glucose was readily utilized by the fungus, it was chosen as the major carbon source for all test media as well as the final fermentation medium.

Nitrogen requirements. Nitrate nitrogen and

TABLE 3

Mycelial yields with pairs of amino acids in a chemically defined medium

Amino Acid	Concentration of Amino Acid	Dry Weight of Mycelium	$\frac{\text{g Dry Mycelium} \times 100}{\text{g Carbon Compounds* Added}}$
	g/L	g/L	
DL-Alanine L-Asparagine	2 1	4.54	34.9
DL-Valine L-Asparagine	2 1	4.38	33.6
L-Tryptophan L-Asparagine	1 1	4.04	33.6
L-Glutamic acid L-Asparagine	1 1	4	33.3
L-Proline L-Asparagine	1 1	3.96	33
L-Asparagine	2.5	4.02	32.1

* Sum of weights of glucose and amino acids.

ammonia nitrogen were found to be incapable of replacing organic nitrogen in the fermentation medium. Yields of dry mycelium in the final fermentation medium containing L-glutamic acid as the nitrogen source were approximately 7.8 times greater than those obtained in media containing NaNO_3 or $(\text{NH}_4)_2\text{SO}_4$.

It was found that several of the amino acids served as satisfactory sources of nitrogen. The effect of the presence of two amino acids on the growth of *C. salmosynnematum*, strain 3590A, was investigated first. Medium A (table 1) was used for these studies. The L-amino acids were added in a concentration of one g per liter while the DL-amino acids were added in a concentration of two g per liter since quite often only one of the optical forms is utilized by a fungus. It is to be noted that L-asparagine of medium A (table 1) served throughout as one component of the amino acid combinations. The following eleven amino acids gave highest yields: DL-alanine, L-glutamic acid, L-proline, L-tryptophan, DL-valine, L-arginine, DL-aspartic acid, glycine, histidine (free base), L-phenylalanine, DL-serine. The first five amino acids listed gave markedly higher yields. The results obtained with these five amino acids are shown in table 3.

TABLE 4

Mycelial yields obtained when combinations of amino acids serve as the nitrogen source

Amino Acid	Concentration of Amino Acid	Dry Weight of Mycelium	$\frac{\text{g Dry Mycelium} \times 100}{\text{g Carbon Compounds* Added}}$
	g/L	g/L	
DL-Serine L-Arginine L-Asparagine	2 1 1	5.24	37.4
L-Tryptophan L-Arginine L-Asparagine	1 1 1	4.76	36.6
DL-Alanine L-Proline L-Asparagine	2 1 1	5.02	35.8
L-Tryptophan Histidine (f.b.) L-Asparagine	1 1 1	4.60	35.3
L-Proline L-Arginine L-Asparagine	1 1 1	4.6	35.3
L-Proline DL-Serine L-Asparagine	1 2 1	4.94	35.0
DL-Valine L-Glutamic acid L-Asparagine	2 1 1	4.9	35
DL-Alanine L-Arginine L-Asparagine	2 1 1	4.9	35
L-Asparagine	5	4.84	32.2

* Sum of weights of glucose and amino acids.

When mixtures of 3 amino acids were employed, the best yields were obtained with the combinations shown in table 4. Again L-asparagine served as one of the amino acids.

When the concentrations of the amino acids being investigated were increased, it was found that highest yield was obtained with L-glutamic acid. Table 5 illustrates the results obtained.

Since table 5 shows that L-glutamic acid was the best source of nitrogen, it was employed as the nitrogen source in the final fermentation medium.

TABLE 5

Mycelial yields in a chemically defined medium with an amino acid concentration of 5 g/L

Amino Acid	Concentration of Amino Acid	Dry Weight of Mycelium	$\frac{\text{g Dry Mycelium} \times 100}{\text{g Carbon Compounds* Added}}$
	g/L	g/L	
L-Glutamic acid	5	6.78	45
L-Proline	4	6.22	41.5
L-Asparagine	1		
L-Glutamic acid	4	5.86	39.1
L-Asparagine	1		
DL-Aspartic acid	4	5.6	37.4
L-Asparagine	1		
L-Asparagine	5	4.84	32.2
DL-Valine	4	4.79	31.9
L-Asparagine	1		
DL-Threonine	4	4.75	31.6
L-Asparagine	1		
DL-Serine	4	4.70	31.3
L-Asparagine	1		
Glycine	4	4.69	31
L-Asparagine	1		

* Sum of weights of glucose and amino acids.

It is to be noted that the weight of D-amino acid added was included in calculations of conversion of carbon to cell substance although it is known that quite frequently the D form of the amino acids is not available to the fungi.

Gross mineral requirements. It may be seen from figure 1 that 100 mg/L of potassium as potassium chloride were needed to give maximum yields of cell substance. The flasks with no potassium added had only 0.3 per cent the amount of mycelium given by 100 mg/L of potassium. It was necessary to add 50 mg/L of potassium per flask of seed medium B to prepare the inoculum for the potassium requirements tests.

The magnesium added to the seed medium was restricted to 5 mg/L. Figure 1 shows that maximum yields of mycelium were obtained when 10 to 20 mg of magnesium as $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ were added to one liter of fermentation medium.

Sulfur was supplied as Na_2SO_4 . The medium

Figure 1. Effect of potassium, magnesium, sulfur, and phosphorus on yield of mycelium.

for this study did not require any purification when all the sulfate salts normally employed in the fermentation medium were replaced by the corresponding chlorides at the equivalent metal levels. Sulfur was added to the seed medium at a concentration of 25 mg/L. Maximum yield of mycelium occurred when sulfur was present in a concentration of 50 mg/L. Figure 1 demonstrates the growth response to increasing levels of sulfur.

Phosphorus as $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ was added to the seed medium at a level of 50 mg/L. Control flasks without added phosphorus gave only 3 per cent of maximum yield. The highest yield was attained when the concentration of added phosphorus was between 50 and 100 mg/L. Figure 1 shows these results.

No attempt was made to study the calcium requirements of *C. salmosynnematum*, strain 3590A, but it was noted that the addition of calcium to the fermentation medium did not affect mycelial yields.

Figure 2. Effect of zinc and iron on yield of mycelium.

Trace metal requirements. The media for these studies were prepared as described in the section of Materials and Methods. Figure 2 shows that 0.5 mg of zinc as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ per L gave maximum mycelial yields. The flasks with no added zinc gave only 7 per cent of this yield.

It may also be seen from figure 2 that 0.5 mg of added iron as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per liter of medium gave maximum yield. The control flasks with no added iron gave 7 per cent of the maximum yield.

Copper was added to the purified medium as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in concentrations of 0, 0.0005, 0.001, 0.0025, 0.005, 0.01, and 0.025 mg/L. (Zinc and iron were added in excess to the purified medium.) Flasks which contained no added copper served as controls. Maximum yield occurred at a concentration of added copper of 0.01 mg/L.

The addition of manganese to the fermentation medium did not affect cell yields. No detailed studies of any other trace metal requirements were made.

Vitamin requirements. The following vitamins were tested for a stimulatory effect on the growth of the fungus: thiamin, riboflavin, nicotinamide, pyridoxin, inositol, choline, calcium pantothenate, *p*-aminobenzoic acid, folic acid, and biotin. In all cases, the addition of single vitamins or of

mixtures did not result in any increased yield of mycelium.

pH requirements. An initial pH of 6.3 (after autoclaving) proved to be optimum for the growth of the fungus. The medium with a pH of 5.57 gave a decrease of 34.5 per cent of maximum yield. At pH 6.97, a decrease of 9.7 per cent of maximum yield was obtained.

Chemical changes during growth of C. salmosynnematum. Figure 3 shows some of the chemical changes occurring in shake flasks fermentation of *C. salmosynnematum*. Glucose was rapidly utilized after the first 24 hours. By 60 hours only 1.2 per cent of the glucose originally present remained in solution. After 72 hours no glucose remained.

The ammonia nitrogen present in the uninoculated medium came from the NH_4OH used to adjust the pH. This level of ammonia nitrogen remained fairly constant during the first 24 hours, but at 36 hours the ammonia nitrogen content of the medium was increased by 10 per cent over the zero hour level. This increase may have been due to concentration of the medium. Between 36 and 60 hours the ammonia nitrogen level fell to a value which was 81.6 per cent of the original concentration. After this time the ammonia nitrogen level rose until it reached a concentration which was 15 per cent above the

Figure 3. Chemical changes during growth of *Cephalosporium salmosynnematum*, strain 3590 A. — glucose g/L; ---- total soluble nitrogen mg/ml; pH; dry weight of mycelium g/L; ——— ammonia nitrogen mg/ml.

zero hour level. This liberation of ammonia is attributed to autolysis of the mycelium. The fluctuation of the ammonia nitrogen concentration indicates that, while the fungus prefers an organic nitrogen source, under some conditions it may use ammonia nitrogen.

The concentration of total soluble nitrogen began to decrease after 36 hours. The lowest level reached was at 60 hours when only 37.1 per cent of the initial concentration remained in solution. As the autolytic process began, the values for total soluble nitrogen returned to a level which was 59.3 per cent of the zero hour concentration.

The pH was 6.3 at the beginning of the fermentation. It began to increase at 36 hours and rose sharply between 48 and 60 hours. A peak of pH 8.85 was reached at 108 hours.

Mycelial weight did not increase appreciably during the first 12 hours. By 24 hours the organism had entered a period of rapid formation of mycelium. At 48 hours the yield of mycelium reached a value which was 67 per cent of maximum. Maximum yield occurred at 60 hours. Autolysis began between 60 and 72 hours and proceeded rapidly until at 108 hours only 38 per cent of the mycelium present at 60 hours remained. The maximum yield was 6.78 g of mycelium (dry weight) which was obtained from 10 g of glucose and 5 g of L-glutamic acid. This value represents a conversion of 45 per cent of added carbon compounds to mycelium (dry weight). Values for per cent conversion between 25 and 50 per cent have been reported for the fungi. Values over 50 per cent are rare.

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SUMMARY

A chemically defined medium was developed which supplied the nutritional requirements of *Cephalosporium salmosynnematum*, strain 3590A. A yield of dry mycelium of 6.78 g was obtained from 10 g of glucose and 5 g of L-glutamic acid present in one liter of medium.

Glucose served as the major carbon source. The organism required organic nitrogen to attain

maximum yield. A variety of amino acids was found to serve as suitable nitrogen sources as well as carbon sources. L-Glutamic acid was the best.

Maximum yields of mycelium were obtained when required minerals were added to the test medium at the following levels listed in mg/L: potassium 100, magnesium 20, sulfur 100, phosphorus 100, zinc 0.5, iron 0.5, and copper 0.01.

Under the conditions of these experiments the addition of vitamins to the medium did not affect mycelial yields.

An initial pH of 6.3 proved to be optimum for the growth of the fungus.

Analytical data compiled showed that practically no glucose remained at sixty hours, by which time the greatest yield of mycelium had been produced. It was further demonstrated that while organic nitrogen was preferred, some utilization of ammonia nitrogen did take place.

REFERENCES

- GAILEY, F. B., STEFANIAK, J. J., OLSON, B. H., AND JOHNSON, M. J. 1946 A comparison of penicillin-producing strains of *Penicillium notatum-chrysogenum*. *J. Bacteriol.*, **52**, 129-140.
- GOTTSHALL, R. Y., ROBERTS, J. M., PORTWOOD, L. M., AND JENNINGS, J. C. 1951 Synnematin, an antibiotic produced by *Tilachlidium*. *Proc. Soc. Exptl. Biol. Med.*, **76**, 307-311.
- JARVIS, F. G., AND JOHNSON, M. J. 1947 Role of constituents of synthetic media for penicillin production. *J. Am. Chem. Soc.*, **69**, 3010-3017.
- MA, T. S., AND ZUAZAGA, G. 1942 Micro-Kjeldahl determination of nitrogen. *Ind. Eng. Chem., Anal. Ed.*, **14**, 280-282.
- OLSON, B. H., AND JENNINGS, J. C. 1954 Effect of synnematin B treatment of *Salmonella* infections in mice and chicks. *Antibiotics & Chemotherapy*, **4**, 11-17.
- OLSON, B. H., AND JOHNSON, M. J. 1949 Factors producing high yeast yields in synthetic media. *J. Bacteriol.*, **57**, 235-246.
- SHAFFER, P. A., AND SOMOGYI, M. 1933 Copper-iodometric reagents for sugar determination. *J. Biol. Chem.*, **100**, 695-713.
- STONE, R. W., AND FARRELL, M. A. 1946 Synthetic media for penicillin production. *Science*, **104**, 445-446.
- THORNBERRY, H. H., AND ANDERSON, H. W. 1948 Synthetic medium for *Streptomyces griseus* and production of streptomycin. *Arch. Biochem.*, **16**, 389-397.