result from other techniques, such as those requiring aeration of cultures. The exhaust air from such operations must be sterilized and the most reliable means of doing this is incineration. The 1 cfm capacity of the incinerator described above is sufficient for most laboratory scale operations.

If provision is made for cooling the exit air, the apparatus may be employed as a source of sterile air which is sometimes needed for certain laboratory procedures.

Although the minimum sterilizing temperature was 400 F, the incinerator is routinely operated at 500 F or above. This stipulation allows for variations which may occur in the air flow and provides a safety factor in case of power failure during operation. This operating temperature will also permit a reduction in the size of the transformer used on future models.

SUMMARY

A small-volume air incinerator is described which is capable of sterilizing air at flow rates up to 1 cfm. The apparatus is a resistance-type heater utilizing a 750 watt transformer and a coil of stainless steel tubing as the resistance and air channel. The incinerator can be used for sterilizing air that is contaminated by some laboratory techniques or may be used to produce sterile air for various laboratory procedures.

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Chemically Defined Media Supplemented with Precursors for Synnematin Production

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Synnematin has been shown to have many of the attributes of a valuable antibiotic. It has an acute toxicity of only one-seventh as much as penicillin (Hwang and Primack, 1956–7). It is effective *in vivo* against a variety of experimental infections (Hobby *et al.*, 1957; Olson and Jennings, 1954; and Rickher *et al.*, 1957) and is also effective clinically (Benavides *et al.*, 1955). Abraham *et al.* (1955) found cephalosporin N to be the same as synnematin B. Therefore, the characteristics of cephalosporin N (Newton and Abraham, 1954) apply to synnematin also. The reasons for the present lack of commercial availability are most closely related to cost and difficulty of isolation and purification. Published data (Olson *et al.*, 1954) show that the only medium which would allow synnematin production of 400 units per ml or more is one which contains 4 per cent corn meal, 4 per cent soybean meal, 1 per cent calcium carbonate, 0.1 per cent ammonium sulfate, and 0.1 per cent antifoam (lard oil with 3 per cent octadecanol). This medium becomes so heavy with growth that some industrial fermentors are not capable of providing sufficient aeration. Also, the harvested filtrate obtained with this medium contains a high percentage of impurities, thus adding to the difficulty of recovering synnematin. To obviate some of these difficulties a study was initiated to develop media with the following characteristics: (a) Sufficient chemical definition to allow the evaluation of substances for stimulation of synnematin production and selection of substances which might be precursors of the synnematin molecule; (b) less viscous, so that the less efficient industrial fermentors could be used; and (c) lower percentage of solids in the harvested filtrate with high antibiotic potency in order to facilitate recovery.

MATERIALS AND METHODS

Culture. Cephalosporium salmosynnematum MDH 3590AUV48B3 was used in all experiments. The genus of this culture has now been changed by Grosklags and Swift (1957) to *Emericellopsis*. The culture was maintained on Czapek-Dox agar slants and transferred every 30 days.

Seed production. A portion of mycelium approximately 50 sq mm removed from an agar slant was used to start the seed flask. The seed flasks were grown 2 days in a natural medium which consisted of 2 per cent soybean meal, 2 per cent corn meal, 0.5 per cent calcium carbonate, and 0.1 per cent antifoam (lard oil plus 3 per cent octadecanol) (Olson *et al.*, 1954). After 2 days of incubation, 0.5 ml was used to seed 50 ml of the medium B of Pisano *et al.* (1954) and allowed to grow 24 hr before use. All seed flasks were shaken at 30 C.

Fermentation. All natural media used were autoclaved

1 hr at 121 C. Chemically defined and semi-chemically defined media were autoclaved 30 min at 121 C. Calcium carbonate when used in other than natural media was autoclaved separately and added prior to inoculation.

Production flasks were seeded with 2 per cent inoculum and grown at 34 C unless otherwise stated. Flasks other than those used to determine growth curves were harvested at 72 hr. Fermentation results were obtained from duplicate flasks in a minimum of two runs.

Cotton stoppered wide-mouthed 500 ml Erlenmeyer flasks containing 50 ml of medium were used. Fermentations were carried out on a Gump[1] rotary shaker operated at 255 rpm and describing a 2¼-in. diameter circle.

Assay. A *Bacillus subtilis* disc assay similar to that described by Loo *et al.* (1945) was used to determine synnematin potency of the filtrates. The synnematin unit as used in this paper is based on the *Salmonella typhimurium* dilution unit (Gottshall *et al.*, 1951).

Results

Carbon sources. Pisano *et al.* (1954) showed that in their chemically defined growth medium, glucose, fructose, maltose, and mannose served equally well as a carbon source, but sucrose was slightly less effective. In addition to the sugar present, L-glutamic acid was always available to be used as a source of carbon. In the present work when DL-asparagine replaced the L-glutamic acid, the following substances could be used interchangeably as the carbon source for growth: glucose, fructose, maltose, sucrose, dextrin, corn starch, and potato starch. When these same carbon sources were evaluated for synnematin production it was found, as is shown in table 1, that mono- and di-saccharides gave 25 to 50 per cent of the antibiotic activity that was obtained with dextrin or starch. The improved yield appeared to be due to slow breakdown of the starch to glucose, thus, the starch or dextrin provided a slow feed of the glucose to the mold.

[1] B. F. Gump Co., Chicago, Illinois.

TABLE 1

Effect of carbon source on synnematin activity

Carbohydrate Component of Medium	Synnematin Activity	
	Carbohydrate at 4 g per 100 ml*	Carbohydrate at 6 g per 100 ml†
	units/ml	*units/ml*
Soluble starch (Difco)	280	400
Corn starch (Argo)	315	
Dextrin (Difco)	265	
D-Fructose	58	
Maltose	77	
Sucrose	132	
Glucose	137	100

Salts and precursors were present as given in medium A, table 3.

* DL-Asparagine at a concentration of 0.6 g per 100 ml was present.

† DL-Asparagine at a concentration of 0.9 g per 100 ml was present.

Except for specific experiments, Difco soluble starch has been used throughout the course of this study. It was selected in place of corn starch since Difco soluble starch yields a completely clear solution at the concentrations used.

Nitrogen source. As mentioned earlier, DL-asparagine can serve as the sole source of nitrogen. Other amino acids can also provide the nitrogen needed for growth and antibiotic production, but they are not used equally well. DL-Asparagine has been shown to be best; other promising amino acids are glycine, L-arginine, and DL-alanine.

Under modified conditions, ammonium salts, such as the sulfate, nitrate, acetate, lactate, and citrate, can also serve as the sole source of nitrogen for growth. When ammonium sulfate or ammonium nitrate is used, calcium carbonate must be added after sterilization to prevent the pH from dropping too low during the fermentation. In media with inorganic nitrogen, ammonium sulfate gave the best antibiotic activity. The activity obtained was equal to that produced when DL-asparagine was the sole source of nitrogen.

While studying the available nitrogen sources it was found that the ratio of the carbon to nitrogen was very important. Figure 1 shows that the activity produced falls off steeply on either side of the optimum ratio. Both an organic and inorganic source of nitrogen were used to establish the optimum ratio. When determining the effect of carbon to nitrogen ratios, the nitrogen source was varied and the initial starch concentration kept at 6 per cent. Ammonium sulfate was used as the inorganic nitrogen source and DL-asparagine as the organic source. With these exceptions the media from table 3 were used. In both cases the optimum ratio was found to be approximately 15:1.

Mineral requirements. With the exception of sulfur, the quantities of minerals found in Pisano's medium B (1954) were sufficient for maximum activity in our

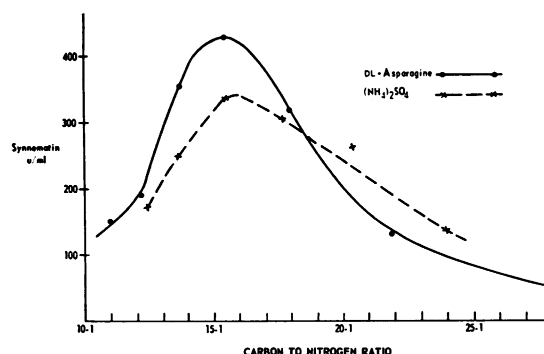

Figure 1. Effect of carbon to nitrogen ratio on synnematin production when nitrogen concentration is varied.

TABLE 2

Medium used for testing compounds for stimulation of synnematin production

Component	Concentration g per L
Soluble starch (Difco)	20
L-Glutamic acid	5
Na_2SO_4	0.90
KCl	0.38
$MgCl_2 \cdot 6H_2O$	0.33
$NaH_2PO_4 \cdot H_2O$	0.20
$FeSO_4 \cdot 7H_2O$	0.025
$ZnSO_4 \cdot 7H_2O$	0.020
$CuSO_4 \cdot 5H_2O$	0.00032

Distilled water to 1000 ml, pH adjusted to 7 with NaOH.

TABLE 3

Composition of semi-chemically defined media which allow synnematin yields of more than 400 units per ml

Component	Concentration g per L
Medium A	
Soluble starch	60
DL-Asparagine	9
Betaine	0.50
Na_2SO_4	0.90
KCl	0.38
$MgCl_2 \cdot 6H_2O$	0.33
$NaH_2PO_4 \cdot H_2O$	0.20
$FeSO_4 \cdot 7H_2O$	0.025
$ZnSO_4 \cdot 7H_2O$	0.020
$CuSO_4 \cdot 5H_2O$	0.00032
Medium B	
Soluble starch	60
Ammonium sulfate	7.5
Calcium carbonate	5
Betaine	0.50
KCl	0.38
$MgCl_2 \cdot 6H_2O$	0.33
$NaH_2PO_4 \cdot H_2O$	0.20
$FeSO_4 \cdot 7H_2O$	0.025
$ZnSO_4 \cdot 7H_2O$	0.020
$CuSO_4 \cdot 5H_2O$	0.00032

Distilled water to 1000 ml; pH adjusted to 7 with NaOH.

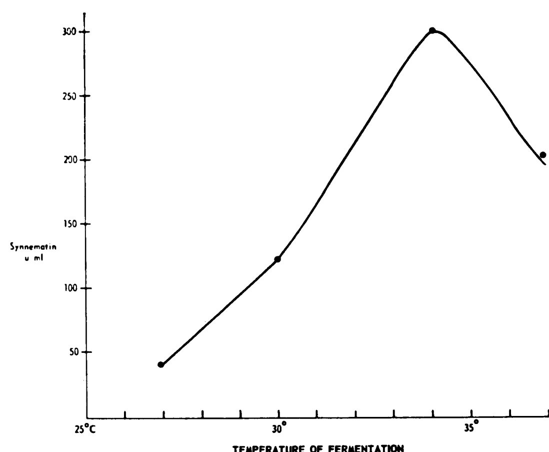

Figure 2. Effect of temperature on synnematin production.

media. It was found that 50 mg of sodium sulfate per 100 ml was the minimum amount needed to produce 400 unit per ml. In order to have an excess, 90 mg of sodium sulfate per 100 ml have been used throughout this study.

It was necessary to decrease the amount of sodium di-hydrogenphosphate from 89 to 20 mg per 100 ml in order to obtain maximum activity. The amount of phosphate has been found to be critical. The activity dropped rapidly on either side of the optimum amount. The reason for this is not clear at this time.

Temperature. It may be seen from figure 2 that the best temperature range for the production of synnematin was 33 to 35 C. The points indicated on the figure represent maximum activity obtained in the fermentation at the specified temperature. The medium used had 4 per cent soluble starch and 0.6 per cent DL-asparagine. Other ingredients were the same as medium A in table 3.

Aeration. Olson *et al.* (1954) showed that synnematin production in a natural medium is dependent upon effective aeration. We have found this to be true for the chemically defined medium also. There was no difference found in the amount of activity produced with media volumes of 25 and 50 ml per flask, but when the volume was increased to 100 ml the decrease in aeration cut the antibiotic production to only 60 per cent of that with 50 ml. When 150 ml of media were used in a flask the production was cut further.

Compounds that stimulate synnematin production. The medium presented in table 2 was found to provide excellent mycelial production, but only small amounts of synnematin (6 to 8 units per ml). To increase this low antibiotic production, individual compounds were added in various concentrations to this medium to test for stimulation. All of the amino acids were tested as well as a number of related compounds. It was found in this medium that the following compounds gave increases in synnematin production over the control: DL-asparagine, L-asparagine, DL-alanine, DL-isoleucine, DL-tyrosine, choline chloride, hydroxy-D-proline, betaine, L-proline, glutathione, histidine, and glycine. The stimulation due to the addition of DL-asparagine was so pronounced that it was soon used to replace the L-glutamic acid entirely. It would seem that D-asparagine served as the stimulant in this medium, because the addition of DL-asparagine gave an increase of two and one-half-fold over the use of L-asparagine alone. Further tests for stimulation were carried out in a medium of 4 per cent soluble starch, 0.6 per cent DL-asparagine, and salts as in table 2. Betaine and choline chloride each showed an increase of 25 per cent in synnematin production. It was found that their stimulations were not additive and that they could be used interchangeably.

D-Asparagine, betaine, and choline chloride were

found to stimulate antibiotic production consistently by 10 to 20 per cent when added to the natural medium of Olson *et al.* (1954). Again the betaine and choline chloride could replace each other, but not the asparagine.

Betaine and choline chloride have served as precursors or stimulants in all media tested. D-Asparagine has stimulated synnematin production except when ammonium sulfate was used to supply the required nitrogen.

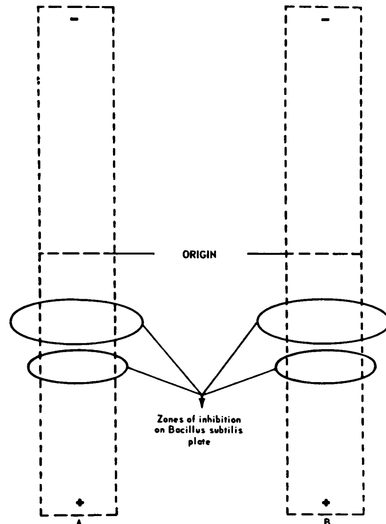

Figure 3. Separation of synnematin by paper electrophoresis. Eighty units in 0.01 ml were added to each strip and developed in 0.03 M sodium phthalate buffer (pH 5.9) with a constant current of 21 ma. *A*, Purified synnematin from natural medium; *B*, Synnematin culture filtrate from medium A of able 3.

Identity of the antibiotic produced in the presence of betaine and D-asparagine. The synnematin which is produced in the media listed in table 3 has been compared with synnematin produced in a natural medium and found to be identical. The synnematin from these media is inactivated by penicillinase and also separates into two components in the paper chromatography system developed for the separation of synnematin A and B (Olson *et al.*, 1953). A separation into two antimicrobially active bands has also been accomplished in the following paper electrophoresis system. The two bands were identical to those found with the synnematin from a natural medium. Eighty units were applied to the center of the paper strip which is Beckman[2] 3 mm, 3 cm wide and 30 cm long. A Spinco model R[2] series D Durrum type cell[2] with 0.03 M pH 5.9 sodium phthalate buffer was run with eight strips for 2 hr at a constant current of 21 ma. The initial voltage was 300 and the final voltage was 180. Figure 3 shows the separation and degree of movement of the bands.

Mycelial growth and antibiotic production. The growth and activity curves shown in figure 4 were obtained with medium A of table 3 using DL-asparagine. The data were obtained with and without the addition

[2] Beckman, Spinco Division, Palo Alto, California.

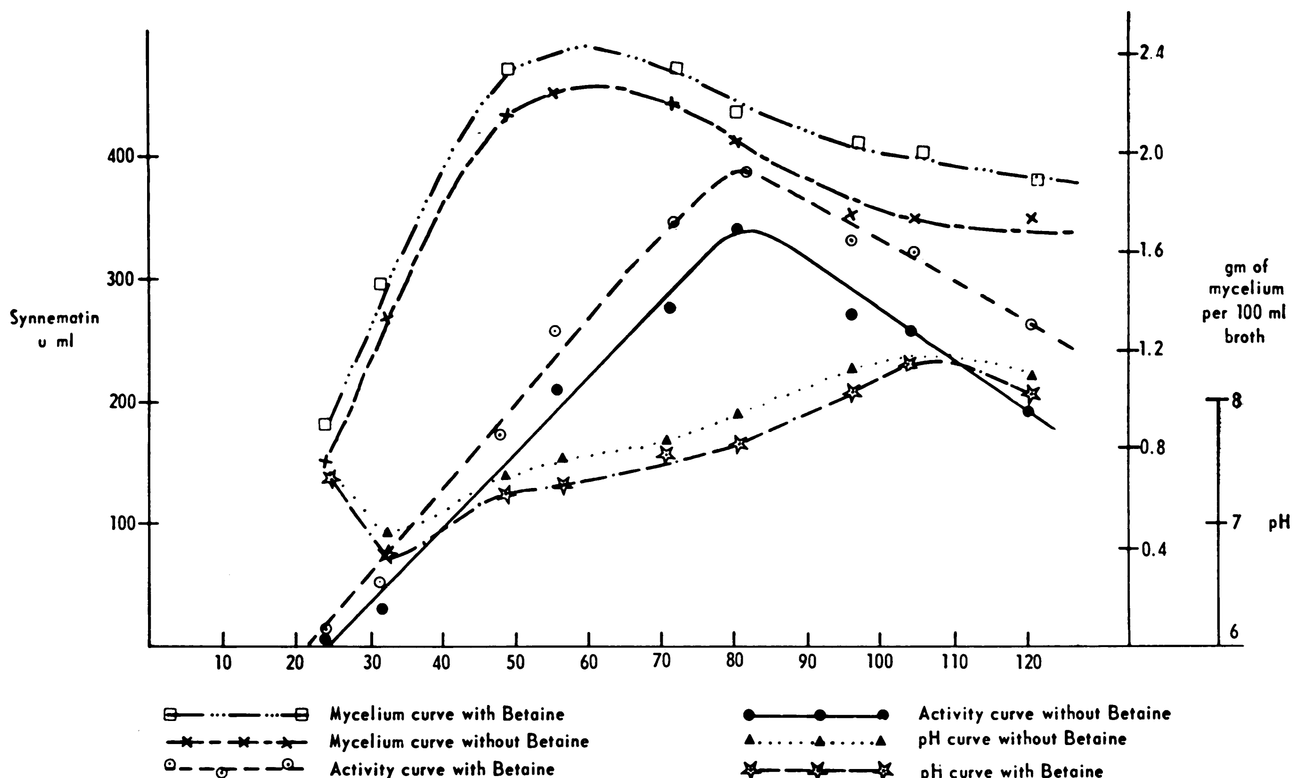

Figure 4. Growth and activity curves obtained with medium A of table 3 with and without betaine.

of betaine. The growth and activity reached a maximum at the same time in both media. However, the medium with betaine gave more activity and better growth. The maximum antibiotic activity was reached at 80 hr. The activity first appeared between 20 and 24 hr and increased at a uniform rate up to 80 hr. After 80 hr the synnematin was destroyed at a faster rate than it was produced.

Since the medium was a clear solution, the mycelial weight was determined by filtering, drying the pad overnight at 100 C, and weighing the residue. It was found that the mycelium was produced most rapidly in the first 48 hr, at which time the starch had disappeared and the rate of growth began decreasing. The maximum amount of mycelium was produced in the first 60 hr. After 60 hr the mycelium began to break down. Figure 4 shows that the pH of the fermentation dipped briefly in the early hours and then started a slow climb until at 80 hr it was about 7.7 to 7.9.

Table 3 describes two media which can be used for production of synnematin with yields of 400 units per ml or better. These yields have been substantiated in 15 gal fermentors. Both media are sufficiently defined to allow further study of precursors and, if need be, the starch can be replaced by sucrose or glucose to give a completely chemically defined medium. The two media are not as heavy or viscous as the natural medium and are easier to agitate and aerate. The culture filtrate is low in solids. Evaporation of the harvested filtrate gives a dry solid which has 55 to 85 units per mg. A filtrate from the natural medium of the same potency per ml treated similarly yields a dry solid with 14 to 18 units per ml.

Acknowledgments

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Summary

Media have been developed that give synnematin activities of 400 units per ml. This is equal to the activity obtained in a natural medium. The synnematin in the filtrate of the presently described media is three times as active on a dry weight basis as that in the filtrate of the natural medium.

Soluble starch, corn starch, potato starch, or dextrin may serve as the carbon source. Glucose or sucrose can replace the starch but the yield of antibiotic activity decreases to 100 to 200 units per ml. However, in the latter case the medium is completely chemically defined.

It was found that the nitrogen source may be either organic or inorganic. When an inorganic nitrogen source such as ammonium sulfate is used, calcium carbonate is needed for the control of pH. The optimum ratio of carbon to nitrogen was shown to be 15:1 for both organic and inorganic nitrogen.

Betaine and choline chloride have served as stimulants to antibiotic production in all media tested including a natural medium. D-Asparagine has likewise served as a stimulant, except in media with ammonium sulfate as the nitrogen source.

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