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Production of Enniatins by *Fusarium sambucinum*: Selection of High-yield Conditions from Liquid Surface Cultures

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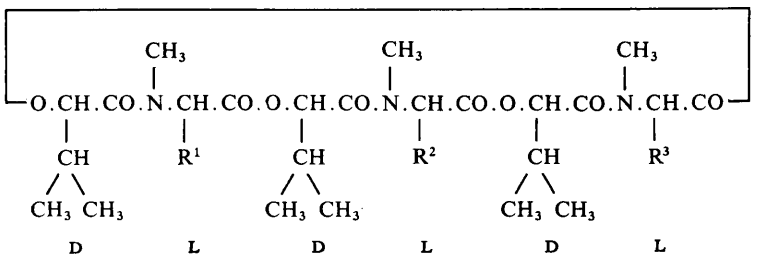
SUMMARY

Enniatins were determined in the mycelium of *Fusarium sambucinum* grown in liquid surface culture on semidefined or undefined media. Light was required for significant enniatin production, which was also favoured by moderate growth temperature. Production was supported by various carbon and nitrogen sources. Growth on a medium containing (w/v) 5 % lactose and 0·8 % tryptone, at 20 °C, with daily 12 h photoperiods, yielded 1·7 g enniatins/l (10 % of mycelial dry wt). Glucose inhibited sporulation of *F. sambucinum*.

Enniatin, named by Gäumann *et al.* (1947), is an antibiotic isolated from the mycelium of *Fusarium orthoceras* App. var. *enniatinum*. Lateritiins I and II, avenacein, fructigenin, and sambucinin (Cook, Cox, Farmer & Lacey, 1947), obtained from various Fusarium isolates (Lacey, 1950), were thought to be similar but chemically distinct antibiotics (Cook, Cox & Farmer, 1949), as was baccatin A obtained from *Gibberella baccata* (Guérillot-Vinet *et al.* 1950). It is now known that all these substances are mixtures (Plattner & Nager, 1948*a*; Hall, 1960; Audhya & Russell, 1973*a*; A. H. Cook, personal communication) of isomeric and homologous cyclodepsipeptides (Russell, 1966; Taylor, 1970) to which the general name 'enniatins' is applied.

Enniatins are cyclohexadepsipeptides of the general structure:



The only pure enniatin known to have been isolated from natural sources is enniatin B ($R^1 = R^2 = R^3$ = isopropyl) obtained from *Fusarium* strains ETH4363 and ETH1574 (Plattner & Nager, 1948*b*; Plattner, Nager & Boller, 1948) and from *F. avenaceum* IMI49894 (Tirunarayanan & Sirsi, 1957); it contains three residues of *N*-methyl-L-valine. Enniatin (Gäumann *et al.* 1947), subsequently called enniatin A (Plattner & Nager, 1948*c*; Plattner *et al.* 1948) is a mixture of 'true' enniatin A ($R^1 = R^2 = R^3$ = s-butyl) containing three residues of *N*-methyl-*erythro*-L-isoleucine (Shemyakin, Ovchinnikov, Kiryushkin & Ivanov, 1963*b*; Plattner *et al.* 1963) together with homologues (Kiryushkin, Rozynov & Ovchinnikov, 1968) and stereoisomers (Audhya & Russell, 1973*a*) in which different R groups may be present in the same molecule. The presence of enniatin C ($R^1 = R^2 = R^3$ = isobutyl;

Ovchinnikov, Ivanov, Mikhaleva & Shemyakin, 1964) in many Fusarium isolates has been inferred (Plattner & Nager, 1948*a*) from chromatographic evidence of *N*-methyl-leucine in enniatin hydrolysates.

Enniatins are powerfully bioactive, apparently by interaction with membrane components. These properties are shared to some extent by the related cyclo-octadepsipeptide valinomycin (Brockmann & Schmidt-Kastner, 1955; MacDonald, 1969), but there are differences, notably with regard to ion-selectivity (Shemyakin *et al.* 1969). Valinomycin is available commercially, but enniatins are not; enniatins A and B have been synthesized (Shemyakin *et al.* 1963*a*, *b*; Quitt, Studer & Vogler, 1963; Plattner *et al.* 1963) but the syntheses require expensive materials and are technically demanding. This probably explains the relative neglect of enniatins in membrane research, and prompted us to reinvestigate fermentation as a cheap method of producing them in quantity (Audhya & Russell, 1973*b*). This paper describes enniatin production by a *Fusarium sambucinum* isolate in surface culture; the chemistry of these enniatins is described by Audhya & Russell (1974).

METHODS

Organism. Fusarium sambucinum Fuckel, strain HLX316, was obtained from the Atlantic Regional Laboratory, National Research Council, Halifax, Nova Scotia, Canada, where it was maintained on 2% malt agar slants (20 g malt extract and 15 g agar/l). In our laboratory it was subcultured on to potato+sucrose agar slopes (extractive from 200 g unpeeled potatoes, plus 20 g sucrose and 20 g agar/l), grown at 20 °C for 21 days, then stored at 4°C. Subcultures were made at six-month intervals.

Preparation of inoculum. Ten days before inoculum was required, subcultures were made into Petri dishes containing potato+sucrose agar. A slope culture was flooded with sterile water, and the surface was rubbed gently with a stout platinum loop to obtain a mycelial suspension; 0·2 ml was distributed evenly over the medium. Plates were incubated for 7 to 10 days at 20 °C. Four discs, 1·2 cm in diam, were cut from the culture with a sterile cork-borer and added to 500 ml sporulation medium in a 2·8 l Fernbach flask. After vigorous magnetic stirring for 65 to 70 h at 20 °C the culture consisted almost entirely of spores, $25–35 \times 3–4\ \mu m$, with 3 or 4 transverse septa. The suspension was strained aseptically through a single layer of cellulose tissue (Kimwipes type 900S; Kimberley-Clark of Canada Ltd) and centrifuged at 2000 *g* for 10 min, and the pellet was suspended in sterile water. Spores were counted in a haemocytometer, and the suspension was adjusted to contain 5×10^6 spores/ml.

Media. Culture media were sterilized by heating at 121 °C for 25 min in an autoclave.

Sporulation medium contained: sodium acetate trihydrate, 2 g; Bacto-tryptone, 2 g; KCl, 0·25 g; $MgSO_4.7H_2O$, 0·25 g; KH_2PO_4, 0·5 g; $FeSO_4.7H_2O$, 5 mg; $Ca(NO_3)_2.4H_2O$, 30 mg; trace-metal solution (Butler, Russell & Clarke, 1962), 5 ml; and distilled water to 1 l. To determine the effect of carbon source and concentration on sporulation, the sodium acetate was altered in concentration or replaced by another carbon compound.

Semidefined medium contained: glucose, 30 g; Bacto-tryptone, 3·8 g; KCl, 0·5 g; $MgSO_4.7H_2O$, 0·5 g; KH_2PO_4, 1 g; $FeSO_4.7H_2O$, 10 mg; $Ca(NO_3)_2.4H_2O$, 60 mg; trace-metal solution, 10 ml; and distilled water to 1 l. The pH (6·0 before sterilization) was not adjusted. To determine the effects of carbon and nitrogen sources and concentrations on enniatin yield, the glucose and/or tryptone were altered in concentration or replaced by other carbon and/or nitrogen sources.

Standard potato+carrot extract (Done, Mortimer, Taylor & Russell, 1961) was prepared

as described by Okotore & Russell (1972); the pH was adjusted to 5·5 with N-H_2SO_4 before sterilization.

Other media used in preliminary experiments were the ones of Gäumann *et al.* (1947), Cook *et al.* (1949) and Cappellini & Peterson (1969); in the latter, two sodium acetate concentrations were used.

Cultivation of organism. The medium was dispensed in 167 ml portions into 1 l Pyrex Roux bottles, which then were closed with non-absorbent 'diSPo'-plugs (1·5 × 2 in; American Hospital Supply Co.) and sterilized. The cooled vessels were incubated at 20 °C for 3 days. Then, if no contamination was apparent, the media were inoculated with 2 ml spore suspension, which was stirred magnetically to ensure a uniform inoculum. The vessels were incubated at 20 °C.

Each shelf (115 × 60 cm) was equipped with two parallel 4 ft fluorescent lamp holders, 20 cm apart, bearing Deluxe cool-white lamps (General Electric of Canada Ltd). The lamps were fastened 45 cm above the shelf and were controlled by a common timer. The Roux bottles were laid horizontally in a single layer on the shelves; the intensity of illumination at their surface was approximately 1080 lux. Culture temperature was maintained at ± 1 °C of the reported value, by air blown gently across each shelf. Unless otherwise stated, all cultures (including slopes, plates, and spore suspensions) were grown at 20 °C with a daily 12 h photoperiod.

Cultures were filtered in batches of six on a Büchner funnel, by using Whatman No. 5 filter paper. The mycelium was washed with two 500 ml portions of distilled water, and left under suction for 4 h. The combined filtrate and washings were mixed, and their volume was measured. Samples were stored at −16 °C until analysed. The moist mycelium was weighed, then homogenized for 5 min in methanol (2 ml/g). The suspension was centrifuged at 2500 ***g*** for 10 min, and the supernatant extract was clarified by passage through a coarse sintered-glass filter. The pellet was re-extracted twice in the same way, and the combined clarified extracts were evaporated to dryness *in vacuo* at 50 °C. The residue and mycelial pellet were dried to constant weight *in vacuo* over NaOH flakes, and the weights were added to obtain the mycelial dry weight. The extractive was dissolved in chloroform and treated with cellulose, charcoal, and aluminium oxide (Audhya & Russell, 1973*b*) to give a mixture of enniatins and neutral lipids. All enniatin analyses were made on material brought to this stage of purification.

Analyses of medium constituents. Nitrogen was determined by a micro-Kjeldahl method (Chibnall, Rees & Williams, 1943); reducing sugars by the method of Hanes (1929), using a standard of the appropriate sugar; and sucrose by the method of Dubois *et al.* (1956), after hydrolysis in 0·5 N-HCl at 100 °C for 15 min. Glycerol was determined by a semi-micro adaptation of the method of Lambert & Neish (1950). To the sample (1 ml) containing glycerol (1 to 10 μg) was added 0·5 ml N-H_2SO_4 and 0·2 ml 0·05 M-$NaIO_4$, followed 10 min later by 0·2 ml 0·5 M-$NaAsO_2$. After a further 10 min, 5 ml of 0·2 % (w/v) chromotropic acid was added and the mixture was heated in a boiling water bath for 45 min. The solution was cooled to room temperature; absorbance was measured at 570 nm, and the glycerol concentration was determined by reference to a standard curve. Analyses of medium constituents were in duplicate and results were averaged.

Enniatins were determined as enniatin A in the (enniatin + neutral lipid) mixtures. Each sample (15 to 25 mg) was hydrolysed in a sealed tube with a mixture of concentrated hydrochloric and glacial acetic acids (1:1, v/v; 1 ml) for 24 h at 110 °C, and excess acid was removed *in vacuo* over NaOH flakes. The residue was dissolved in 1 ml water, and total *N*-methylamino acids were determined by quantitative paper chromatography, or by spectro-

Table 1. *Formation of macroconidia by Fusarium sambucinum in shake-flask culture at* 20 °C *with a daily* 12 *h photoperiod*

Medium	Carbon source (mg C/ml)	Macroconidia ($\times 10^{-5}$/ml) at 24 h	48 h	72 h	96 h
1*	Na acetate (6)	0·5	1·2	1·8	2·9
	Na acetate (0·6)	1·1	3·4	3·9	4·1
2†	Na acetate (6)	2·3	4·9	6·7	7·9
	Glucose (6)	0·4	0·5	0·6	0·5
	Na acetate (0·6)	—	—	8·0	—
	Na citrate (0·6)	—	—	9·5	—
	Na pyruvate (0.6)	—	—	30·5	—
	Glycerol (0·6)	—	—	6·0	—

* The medium of Capellini & Peterson (1969) containing sodium acetate at the carbon concentrations indicated.

† The sporulation medium described in the text containing carbon sources at the concentrations indicated.

photometry using 2,4,6-trinitrobenzenesulphonic acid and ninhydrin (Audhya & Russell, 1973*c*). The result (mg of *N*-methylisoleucine/mg of sample) was multiplied by the theoretical factor 1·57 to give total enniatins as enniatin A. Analyses were in duplicate and the results averaged.

RESULTS

Fusarium sambucinum HLX316 formed many macroconidia when grown on the medium of Cappellini & Peterson (1969) in shake flasks at two concentrations of sodium acetate. At half strength the semidefined medium gave fewer spores, but when glucose was replaced by the sodium salt of an organic acid there was abundant sporulation (Table 1). Inhibition of sporulation by glucose accords with the similar observation of Cappellini & Peterson (1969) using *Gibberella zeae*. Sodium pyruvate was an excellent carbon source for sporulation by *F. sambucinum*, but for routine inoculum preparation we used the cheaper sodium acetate.

Gäumann *et al.* (1947) obtained enniatins from the mycelium of *Fusarium orthoceras*, and Plattner *et al.* (1948) found very small amounts in Fusarium culture filtrates. We determined enniatins in the mycelium only. In our early trials, Soxhlet extraction of dried mycelium with methanol or other solvents removed enniatins only slowly. However, extraction of enniatins from moist mycelium with cold methanol, as described by MacDonald (1969) for isolating valinomycin from Streptomyces, was rapid; the last extract of *F. sambucinum* mycelium thrice extracted in this way contained only traces of enniatins, and no more was obtained by overnight Soxhlet extraction of the residue.

Initially, *Fusarium sambucinum* was grown for 21 days at 25 °C with a daily 8 h photoperiod. Enniatin yields (μg/ml) on four media were: 0 (Cook *et al.* 1949); 25 (Gäumann *et al.* 1947); 72 (semidefined medium); and 147 (potato + carrot extract). The last two media were selected for further study. Enniatins appeared only when growth had almost ceased and the nitrogen in the medium had become minimal (Table 2); once formed, they appeared stable for several weeks. The amounts formed were considerably greater on potato + carrot than on the semidefined medium, but considerably less than reported by Plattner *et al.* (1948).

Effects of temperature and lighting

(i) The organism was grown on potato + carrot medium with a daily 8 h photoperiod at three temperatures, and the residual nitrogen in the medium was determined every 3 days. Cultures were harvested after about 1·5 times the time required to reach minimal residual

Table 2. *Growth, utilization of medium constituents, and enniatin production by Fusarium sambucinum in surface culture at 25 °C with a daily 8 h photoperiod*

Time after inoculation (days)	Medium analyses*						Mycelium analyses			
	pH		Reducing sugar (mg/ml)		Nitrogen (mg/ml)		Dry weight (mg/ml)		Enniatins (mg/l)	
	A	B	A	B	A	B	A	B	A	B
8	4·6	7·8	0·65	1·42	0·24	0·15	5·8	8·4	0	0
16	6·1	8·4	0·55	0·67	0·16	0·05	8·2	7·1	41	163
22	6·9	8·7	0·46	0·60	0·13	0·28	6·2	6·4	47	159
30	7·2	8·8	0·55	0·70	0·15	0·26	5·5	6·1	66	152
38	7·65	8·9	0·45	0·63	0·23	0·29	5·0	5·4	57	72
45	7·6	9·0	0·40	0·55	0·25	0·25	4·9	5·1	15	23

A, on semidefined medium; B, on potato+carrot extract.

* Initial values, for A and B respectively: pH, 6·0 and 5·5; reducing sugar, 30 and 10 mg/ml; nitrogen, 0·475 and 0·53 mg/ml.

Table 3. *Effects of temperature on enniatin production by Fusarium sambucinum grown in surface culture on semidefined medium with a daily 8 h photoperiod*

Incubation temperature (°C)	Minimal N* (mg/ml)	Time to minimal N (days)	Time of harvest (days)	Dry wt (mg/ml)	Enniatins (mg/l)
20	0·08	24	37	5·75	376
25	0·10	15	24	5·9	66
30	0·10	6	11	4·6	47

* Initially, 0·485 mg/ml.

Table 4. *Effect of photoperiod on enniatin production by Fusarium sambucinum grown in surface culture for 21 days on potato+carrot medium at 25 °C*

Light (h/day)	Dry wt (mg/ml)	Enniatins (mg/l)
0	4·8	41
8	6·5	158
12	6·2	170
24	5·6	125

nitrogen (standard growth period). Enniatin production was very much greater at 20 °C than at 25 or 30 °C (Table 3). This experiment was repeated, with similar results.

(ii) The organism was grown at 25 °C on potato+carrot medium with a varied daily photoperiod and was harvested after 21 days. Enniatin production was much less in darkness than in light, but the length of the photoperiod was not critical in the range studied (Table 4). This experiment was repeated, with similar results.

Effects of carbon and nitrogen concentrations and sources on enniatin yield

In these experiments the more reproducible semidefined medium was used as a standard. All cultures were grown at 20 °C with a daily 12 h photoperiod.

Effect of glucose concentration. The organism was grown on semidefined medium containing various concentrations of glucose and cultures were harvested after the standard growth period. Good enniatin yields were obtained in the range 12·5 to 30 mg glucose/ml;

Table 5. *Effect of glucose concentration on enniatin production by Fusarium sambucinum in surface culture on semidefined medium at* 20 °C *with a daily* 12 *h photoperiod*

Initial glucose (mg/ml)	Minimal N (mg/ml)	Time to minimal N (days)	Time of harvest (days)	Enniatins (mg/l)
6·25	0·15	9	14	79
12·5	0·11	13	22	360
25	0·10	22	34	490
30	0·10	29	47	440
50	0·09	37	57	187
60	0·09	41	63	110

Table 6. *Effect of carbon source on enniatin production by Fusarium sambucinum in surface culture for* 34 *days on semidefined medium at* 20 °C *with a daily* 12 *h photoperiod*

Carbon source added	No. of expts	Mean dry wt (mg/ml)	Enniatin yield (mg/l) Mean	Range
	Concentration 1*			
Glucose	5	6·7	354	320–382
Galactose	2	6·0	305	295, 316
Lactose	8	8·9	797	750–865
Glucose + galactose†	2	5·3	299	289, 310
Maltose	2	5·4	302	290, 315
Sucrose	2	6·5	357	330, 384
Glycerol	3	7·7	600	572–640
	Concentration 2‡			
None	2	3·0	25	17, 33
Na acetate	2	4·9	451	410, 492
Na citrate	2	4·1	482	459, 506
Na lactate	2	7·8	546	510, 582

* Carbon compound at a concentration equivalent to 10 mg C/ml and 0·5 mg tryptone N/ml.
† Each sugar was added at a concentration equivalent to 5 mg C/ml.
‡ Carbon compound at a concentration equivalent to 6 mg C/ml and 1 mg tryptone N/ml.

higher concentrations were markedly inhibitory. The yield appeared optimal with a glucose concentration of 25 mg/ml (Table 5), an observation confirmed in two further experiments. The yields were similar when the glucose was autoclaved separately.

Effect of carbon source. A concentration of 25 mg glucose/ml corresponds to a carbon concentration of 10 mg/ml. The organism was grown for 34 days on semidefined medium with this C concentration in various chemical forms. All sources were completely utilized except for lactose, 20% of which remained in the medium at harvest, and all supported enniatin production. Of the common sugars tested, lactose gave more than twice as much enniatin as any other; glycerol also was an excellent source (Table 6).

When similar comparison of the sodium salts of organic acids was attempted, the media rapidly became alkaline and growth ceased. Therefore, these compounds were used at a lower concentration (6 mg C/ml), and the amount of tryptone was doubled for extra buffering. The results (Table 6), although not directly comparable to those obtained with carbohydrates and glycerol, showed that organic acids are good carbon sources for enniatin production.

Effect of nitrogen concentration. As enniatin production was greatest with lactose, this

Table 7. *Effect of tryptone concentration on enniatin production by Fusarium sambucinum grown for* 34 *days on semidefined medium* at* 20 °C *with a daily* 12 *h photoperiod*

Tryptone (mg/ml)	Unused lactose† (mg/ml)	Enniatins (mg/l)
2	13·1	444
4	2·9	797
6	0·7	1187
8	0·4	1208
12	14·2	253

* Glucose replaced by lactose (25 mg/ml). † Remaining in the medium at harvest.

Table 8. *Effect of nitrogen source on enniatin production by Fusarium sambucinum grown in surface culture for* 34 *days on semidefined medium at* 20 °C *with a daily* 12 *h photoperiod*

Nitrogen source*	No. of expts	Mean dry wt (mg/ml)	Enniatins (mg/l) Mean	Range
NH_4NO_3	2	2·2	61	54, 69
$NaNO_3$	2	7·8	910	879, 941
Casein hydrolysate, enzymic	2	7·1	1080	1040, 1120
Tryptone	4	9·2	1147	1070–1190
Glutamic acid	2	1·1	0	
Glutamic acid†	2	3·1	0	
Asparagine	2	0	0	
Asparagine†	2	5·8	627	620, 635
Urea‡	3	6·1	1062	865–1381

* 1 mg N/ml. † 0·5 mg N/ml. ‡ Sterilized separately by filtration.

sugar was used in place of glucose in the semidefined medium, with tryptone at various concentrations. Enniatin yield and sugar utilization after 34 days' incubation were maximal with 8 mg tryptone per ml (Table 7). More than 95 % of the nitrogen was utilized except with the highest concentration, at which 60 % remained in the medium at harvest.

Effect of nitrogen source. A tryptone concentration of 8 mg/ml corresponds to 1 mg nitrogen/ml. *Fusarium sambucinum* HLX316 was grown for 34 days on semidefined medium containing 10 mg lactose C/ml, with 1 mg N/ml in various chemical forms. Media containing NH_4NO_3 rapidly became very acidic and produced little enniatin, but those containing $NaNO_3$ or protein hydrolysates gave high yields. Tryptone was consistently the best source tested for enniatin production (Table 8).

Effect of C/N ratio. Since lactose and tryptone, respectively, were the best carbon and nitrogen sources tested, the effect of varying their proportion was studied. Expt 1: semidefined medium containing a standard amount of lactose (equivalent to 10 mg C/ml) was used with various tryptone concentrations. Expt 2: the initial tryptone concentration (equivalent to 1 mg N/ml) was kept constant and that of lactose varied. Expt 3: tryptone equivalent to 0·5 mg N/ml was used with various glucose concentrations. The batch of tryptone used in these experiments contained 46·0 % C and 12·5 % N. C/N ratios were calculated from the expression

$$\frac{C}{N} = \frac{0{\cdot}46\, C_{\text{tryptone}} + 0{\cdot}42\, C_{\text{lactose}}}{0{\cdot}125\, C_{\text{tryptone}}},$$

where C is initial concentration (mg/ml). Cultures were harvested 34 days after inoculation and were analysed.

Table 9. *Effects of C/N ratio on enniatin production by Fusarium sambucinum grown in surface culture for* 34 *days on semidefined medium at* 20 °C *with a daily* 12 *h photoperiod*

Nutrient* (mg/ml)					Enniatins (mg/g nutrient*)	
Tryptone	Sugar	C/N	Dry wt (mg/ml)	Enniatins (mg/l)	Tryptone	Sugar
			Experiment 1			
2	Lactose, 25	45·8	5·8	440	220	17.6
3·8		25·8	10·0	900	237	36·0
6		18·0	12·2	1150	191	46·0
8		14·2	12·5	1200	150	48·0
12		10·7	7·8	253	21	10·1
18		8·4	4·8	180	10	7·2
25		7·1	3·2	95	4	3·8
			Experiment 2			
8	Lactose, 12	8·7	4·6	581	73	48·4
	24	13·7	9·2	1147	143	47·8
	36	18·8	14·6	1514	187	42·1
	50	24·7	16·3	1724	216	34·5
	60	28·9	18·2	1622	202	27·0
			Experiment 3			
3·8	Glucose, 6·25	9·0	2·4	79	21	12·6
	12·5	14·2	3·0	360	95	28·8
	25	24·7	5·1	490	128	19·6
	50	45·8	9·5	187	49	3·7

* In medium at time of inoculation.

In all three experiments, lactose conversion to enniatins was maximal at C/N = 14, and tryptone conversion was greatest at C/N = 25. The highest absolute yield was from a medium containing 50 mg lactose and 8 mg tryptone per ml, corresponding to C/N = 25 (Table 9).

DISCUSSION

The results recorded here establish physical, physiological, and nutritional conditions conducive to the accumulation of large amounts of enniatins in the mycelium of *Fusarium sambucinum* HLX316. More than 95% of enniatins in mycelial extracts could be obtained crystalline (Audhya & Russell, 1973*d*) by simple fractionation (Audhya & Russell, 1973*b*).

No attempt was made to determine enniatins in the culture filtrate. Plattner *et al.* (1948) found 99% of enniatin A in the mycelium of *Fusarium orthoceras*, but Cook *et al.* (1949) obtained enniatins by extraction of the spent medium and found little activity in the mycelium. Both groups grew Fusaria at 25 °C; neither specified the conditions of illumination, but it seems unlikely that light was totally excluded. Tirunarayanan & Sirsi (1957), who studied antibiotic production by *F. avenaceum* on glucose+sodium nitrate, observed that, when the C/N ratio was low, antibiotic activity attributed to enniatin B appeared in the culture filtrate; when the C/N ratio was > 7·5, all activity was in the mycelium. The media used by Plattner *et al.* (1948) and Cook *et al.* (1949) had C/N ratios of approximately 4 and 11 respectively; this difference may account for their seemingly conflicting results. Tirunarayanan & Sirsi (1957) attributed to cell lysis the ability of low C/N ratio to promote the release of enniatin B into the medium, a suggestion supported by our finding of low dry weights and mycelial enniatin yields in cultures grown for 34 days on media with a low C/N ratio (Table 9).

The effects of light on microbial metabolism have received little attention. Many Fusaria require light for pigmentation (Zachariah, Hansen & Snyder, 1956) and Bermingham, Deol & Still (1971) reported a striking correlation between pigment (prodigiosin) formation and cyclodepsipeptide (serratamolide) synthesis by *Serratia marcescens*. Light absorption by pigment may be the first step in the chain of metabolic events leading to the formation of certain highly reduced compounds, such as enniatin and serratamolide.

The beneficial effect of low temperature on enniatin production is only slightly less obscure. It may be related to slower carbohydrate utilization favouring the pentose phosphate pathway over glycolysis (Saltero & Johnson, 1953; Hošťálek, 1964) and thereby generating more NADPH to promote reductive biosynthetic processes. Consistent with this suggestion is the fact that lactose, which supported high enniatin yield, was the only carbon source not completely utilized in the comparative experiments.

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