The Phycomycete *Catenaria anguillulae*: Growth Requirements

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SUMMARY

The physical factors and nitrogen requirements for the growth of the aquatic phycomycete *Catenaria anguillulae* in liquid shaken cultures from measured zoospore inocula were examined. Growth was determined by measuring mycelial dry weight and rate of titratable acid production. The optimum pH range for growth was pH 8.2 to 8.7. The toxicity of the pH indicators used was assessed. The optimum temperature for growth was 25°. The organism was highly aerobic. Inorganic nitrogen sources were not utilized. When organic nitrogen sources were supplied individually, L-aspartic acid, L-asparagine, L-glutamic acid, L-arginine, DL-citrulline and L-ornithine were utilized. Asparagine was the best single source. When the N sources were supplied as Difco vitamin-free Casamino acids, only lysine, methionine and arginine were utilized during the lag phase. During the more rapid phase of growth, all of the amino acids detected in the Casamino acids were utilized. When the vitamin-free Casamino acids were replaced by an equivalent amino acid mixture, the subsequent decrease in yield was not corrected by adding NaCl, oleic acid or stearic acid. The optimum concentrations of various N sources were determined. The medium finally evolved contained L-asparagine + L-lysine as N sources.

INTRODUCTION

Catenaria anguillulae was classified as a member of the Order Chytridiales until Couch (1945) transferred it to the Order Blastocladales. This change was made on the basis of similarities between the genus *Catenaria* and members of the Order Blastocladales with respect to zoospore structure and behaviour, nature of hyphal septations, origin and germination of the resistant sporangium and life cycle. A review of nutritional studies of the aquatic Phycomycetes (Cantino & Turian, 1959) indicated that members of the Order Chytridiales could utilize inorganic nitrogen and sulphur sources and could either synthesize all vitamins or required thiamine for growth. Members of the Order Blastocladales could neither utilize inorganic sulphur sources nor synthesize thiamine. Some isolates had additional vitamin requirements and, whereas most isolates could utilize inorganic nitrogen sources, one had lost this ability.

The first purpose of the present work was to examine the nutritional requirements of *Catenaria anguillulae* and to determine its nutritional relationship to members of the Orders Chytridiales and Blastocladales. The second purpose was to analyse the nutritional behaviour of *C. anguillulae* to provide data for the interpretation of eco-

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logical information and for future studies on the enzymology of its amino acid metabolism. A brief abstract of earlier phases of this study has been published (Nolan, 1966).

METHODS

Origin and maintenance of the isolate used. All of the present work was done with an isolate of *Catenaria anguillulae* Sorokin (strain 58-12) obtained from Dr R. Emerson (Department of Botany, University of California, Berkeley, Calif., U.S.A.). The fungus had been isolated into axenic culture from material infecting nematodes (*Hemicycliophora arenaria*). The infected nematodes and their identification had been kindly supplied by Dr R. Mankau (Department of Plant Nematology, Citrus Experiment Station, Riverside, California). Stock cultures were maintained on slopes of a yeast-extract + soluble-starch medium (EYPSS; Emerson, 1941) and were transferred at intervals of 2 to 4 weeks. These cultures were incubated at $17 \pm 1^\circ$.

Inoculum preparation. The method used to obtain a zoospore inoculum was a modification of the technique used by Carlile & Machlis (1965) for obtaining male gametes of *Allomyces* species. The concentration of zoospores obtained was 1 to 5×10^6 /ml. One ml. of this suspension or a dilution of it was used to inoculate each flask in an experiment. Zoospore concentrations were determined with a haemocytometer. The viability of the zoospores used in each experiment was tested by adding 1 ml. inoculum to a small Petri dish containing EYPSS agar medium and by examination for subsequent germination and growth.

Chemicals and stock solutions. L-aspartic acid (A grade) was obtained from Calbiochem (Los Angeles, California), all other amino acids were obtained from Nutritional Biochemicals Corp., (Cleveland, Ohio). The pH indicators were obtained from Matheson, Coleman & Bell (Norwood, Ohio). The fatty acids were obtained from Nutritional Biochemicals Corp. The yeast extract and vitamin-free Casamino acids were obtained from Difco Laboratories (Detroit, Michigan). The micronutrients were of analytical grade where available.

All stock solutions were prepared with glass double-distilled water and stored at 4° in glass-stoppered bottles.

Preparation of glassware and media. All glassware was chemically cleaned (concentrated H_2SO_4 saturated with potassium dichromate) after having been machine-washed. The glassware was then rinsed several times with double-distilled water.

The experimental media were generally prepared and autoclaved in three portions: (a) the carbon source, (b) the calcium and magnesium salts, (c) the remainder of the medium. In some experiments, the nitrogen source was also autoclaved separately. All autoclaving was done for 15 min. and at 120° . The fatty acids were added in ether solution to each sterile experimental flask before adding the remainder of the separately sterilized medium. The combined volume of the components was 50 ml./flask. The initial pH value of a medium was always adjusted after mixing the separately autoclaved components and just before inoculation.

Growth of experimental cultures. All cultures were grown in 125 ml. Bellco flasks with stainless steel caps (Bellco Glass, Inc., Vineland, New Jersey). The inoculated flasks were placed on a horizontal rotary shaker (120 rev./min.) in a room maintained at $20 \pm 1^\circ$ (for exceptions see below and text). These conditions provided vigorous mixing and aeration.

Two experimental regimes were maintained in Case glass anaerobic jars (19.5 inches (50 cm.) high $\times$ 7.75 inches (20 cm.) diameter; Case Laboratories, Inc., Chicago, Illinois). Anaerobic conditions were obtained by evacuating a jar to 6.5 cm. and then filling with 5% (v/v) CO₂ + 95% (v/v) hydrogen mixture. A catalyst, palladium-coated alumina pellets in a wire gauze sachet (Heller, 1954), and a reduced methylene-blue indicator (Baltimore Biological Laboratory, Cockeysville, Maryland; see Brewer, Allgeier & McLaughlin, 1966) were included in the jar. The 0.2% (v/v) oxygen concentration was obtained by evacuating a jar twice to 7.5 cm. Hg and filling each time with 'tank nitrogen'. The jars were placed on a low-speed horizontal, rotary shaker (60 rev./min.).

Adjustment of pH value. The pH value of a culture was maintained by the addition of sterile 0.5 N-KOH from a calibrated syringe. The amount of titratable acid produced in each culture flask was recorded as the amount of base required to return the culture medium to the original pH value; the pH value of the cultures was adjusted every 8 to 24 hr.

Yields of organism: dry weight. Cultures were harvested by vacuum filtration through previously tared filter papers. The papers were then placed in an oven (90°) for 24 hr, allowed to come to room temperature in a desiccator and weighed on a Mettler balance (Type H 16, Mettler Instrument Corp., Princeton, New Jersey). All dry weights are given to the nearest mg. A constant dry weight (variation of less than 1 mg. for repeated weighings) was obtained for any single culture flask harvested.

Acid production and dry weight served as criteria for comparing the growth of the fungus under the different experimental conditions. By comparing the growth curve and the rate of acid production (Fig. 5, 6) in an experiment, two types of information were obtained by following acid production: (1) the initial observation of acid production indicated the end of the lag phase of growth; (2) the slight inflexion in the acid-production curve indicated when the peak of the growth curve had been reached.

The variation in dry weights between flasks in a single treatment was dependent upon the phosphate concentration in the medium. When the concentration of phosphate was 15 mM, the variation was $\pm 20\%$ or less; when the phosphate concentration was 10 mM, the variation was $\pm 10\%$ or less; at 1 mM phosphate the variation between the dry weights in a treatment was less than 1 mg.

Amino acid analyses. Culture filtrates were analysed for amino acid content according to the column chromatography technique of Moore & Stein (1963). All determinations were made on a Beckman-Spinco automatic amino acid analyser (Model 120B; Spinco Division, Beckman Instruments, Inc., Palo Alto, California) adjusted to a sensitivity such that 0.05 μ M of an amino acid could give a full scale deflexion. The filtrates were frozen at the time of harvest and then thawed and diluted with buffer before being added to the column. All amino acid concentrations were determined from the chromatograms by using the 'height-width' method of integration (see manual for analyser). The accuracy of the column data was $\pm 8\%$. The data for ammonium concentrations are not given because of sensitivity to external conditions.

Standard micronutrients. Many of the media used contained a group of micronutrients which were always added at a fixed concentration; these are referred to as standard micronutrients (Table 1). The concentrations of these ions were taken from Medium B (Machlis, 1953).

RESULTS

Initial experiments. Early attempts to grow *Catenaria anguillulae* at pH 7.0 on a defined medium containing ammonium-N (Medium B in Table 2) were unsuccessful even with incubation for 54 days. Subsequently Medium B was modified by decreasing the total phosphate to 1 mM and by changing the N source to $(\text{NH}_4)_2\text{SO}_4$. The possibility of ammonium-N utilization (at 1, 10, 30 mM-N) was then tested with this modified Medium B at pH 6.2, 7.2 and 8.2. After 25 days of incubation no growth had occurred.

Table 1. *Standard micronutrients of medium B (Machlis, 1953)*

		(mM)
Mn	As $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.009
Co	As $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.003
Zn	As $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.001
Cu	As $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.001
Fe	As $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.018
B	As H_3BO_3	0.046
Mo	As $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.002

Table 2. *The composition of experimental media*

The ratio of phosphates was adjusted to correspond to the initial pH value desired. All media contain the standard micronutrients (see Table 1), D-glucose (166 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.5 mM), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.5 mM) and thiamine.HCl (150 μg .).

Components	Medium					
	B	1	4	5	6	7
Nitrogen source (g. N/l.)						
Difco Casamino acids	.	0.16	0.16	(see text, p. 172)		
$(\text{NH}_4)_2\text{HPO}_4$	10 mM	.	.	.	.	.
L-Asparagine	.	.	.	(see text, p. 176)		0.24
L-Lysine	.	.	.	.	.	100 μM
Sulphur source (mM-S)						
DL-Methionine	0.67	0.67	0.67	.	0.67	.
L-Methionine	.	.	.	0.53	.	0.53
Phosphate source (mM-P)						
K_2HPO_4	.	.	.	1.0	.	1.0
$\text{K}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$	.	10	10	.	.	.
$\text{K}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4 + (\text{NH}_4)_2\text{HPO}_4$	15	.	.	.	15	.
pH indicator (mg./l.)						
Bromocresol purple	2.0	.	.	.	2.0	.
m-Cresol purple	.	.	2.0	.	.	2.0
Other (see text)	.	2.0	.	2.0	.	.
Na_2EDTA (mM)	.	.	0.032	0.032	.	0.032

Growth occurred on Medium B only when the medium was supplemented with an organic nitrogen source. When L-glutamic acid was added to Medium B at 0.5 to 10 mM, growth was noted after 20 days. The isolate was also able to grow on a glucose + yeast-extract + salts medium (GY 5) (Emerson, 1958). A comparison of yeast-extract and vitamin-free Casamino acids (each at 1 g./l.) as N sources in Medium B indicated that yields were higher with Casamino acids.

pH indicators. The indicators selected (Table 3) were added to Medium B + Casamino acids (1 g./l.). The mean dry weights obtained in the different treatments were compared by using Duncan's multiple-range test (5 % level, error degrees of freedom = 33; see Steel & Torrie, 1960). The means were in three distinct groups: indicators in group A (Table 3) were considered to be non-toxic; those in group B were slightly toxic; those in group C were highly toxic. There was no structural correlation among the members of any group; the chemical compounds present in the pH indicators used are far from being standardized and the possibility exists that phenolic intermediates might have been present in the products used (personal communication from Mr F. Green, Technical Director, Matheson, Coleman & Bell). To avoid the introduction of additional variables, the stock solutions of the non-toxic indicators which were prepared for this experiment were used throughout the subsequent work. A later experiment indicated that xylenol blue and alizarine yellow R were also non-toxic.

Table 3. *The effect of various pH indicators on the growth of Catenaria anguillulae*

The medium used was Medium B + Casamino acids (1.0 g./l.). Initial pH 7.0 maintained during the 7-day incubation period. The dry weights are the averages of 4 replicates.

Indicator (2.0 mg./l.)	Dry weight (mg.)
Group A	
Bromocresol purple	27
m-Cresol purple	23
Cresol red	23
Control (no added indicator)	22
Phenol red	21
Bromothymol blue	21
Group B	
Thymol blue	16
Bromophenol blue	14
Group C	
Methyl red	5
Bromocresol green	3
Chlorophenol red	1

Optimal pH range. Butler & Humphries (1932) studied the development of *Catenaria anguillulae* as it grew out from infected fluke eggs into various surrounding complex natural media, which varied from pH 6.5 to 6.9. These authors did not study the effects of pH value. I studied the effects of pH value on the growth of *C. anguillulae* in Medium 1 (Table 2). The results (Fig. 1) indicated that the optimum range for growth was pH 8.2 to 8.7.

Optimum temperature. The optimum temperature for the growth of *Catenaria anguillulae* strain 58-12 was determined using Medium 1 in static cultures. The results (Fig. 2) indicated that 25° was the optimum temperature under these conditions; above 25° and below 19° the growth rate decreased rapidly.

Oxygen relations. Buckley & Clapham (1929) investigated the viability of sporangia of *Catenaria* under a variety of conditions. From their experiments with the transfer of the growth of the fungus from infected fluke ova to previously uninfected ova in the presence of sewage with a high biological oxygen demand and in the presence of charcoal-treated water in paraffin-sealed tubes, they concluded that *Catenaria* could complete

its development under anaerobic or nearly anaerobic conditions. To study the effects of degree of aeration on the amount of growth *Catenaria anguillulae* was grown in shaken cultures and in static cultures, in Medium 1. An average dry weight of 112 mg./flask was produced in the shaken cultures, in contrast to an average dry weight of 28 mg./flask in the static cultures, after 9 days of incubation. This would seem to indicate *C. anguillulae* strain 58-12 is an aerobic organism.

Table 4. *Composition of the vitamin mixture*

Personal communication from Dr S. Hutner, Haskins Laboratories, New York, to Dr L. Machlis; 25 September 1965. The mixture is a dry mix, 50% of which is pentaerythritol. The concentrations listed below were obtained by adding 10 ml. of a stock solution to 1 l. medium.

Vitamin	Concentration ($\mu\text{g./l.}$)	Vitamin	Concentration ($\mu\text{g./l.}$)
Thiamine. NO_3	180	Putrescine. 2 HCl	60
Nicotinic acid	240	Spermidine phosphate. 3 H_2O	30
Ca pantothenate	300	Folic acid	12
Na riboflavin phosphate	60	DL-Carnitine. HCl	120
Riboflavin	30	Betaine. HCl	4800
Pyridoxamine. 2 HCl	60	Choline N_3 citrate	4800
Pyridoxal. HCl	60	Cystamine. 2 HCl	30
Inositol	3000	B_{12}	1.2
p-Aminobenzoic acid	30	Biotin	3

In another experiment two atmospheric regimes were produced to determine: (1) whether strain 58-12 could grow under more strict anaerobic conditions; (2) whether strain 58-12 could grow in the presence of 0.2% (v/v) oxygen. Medium 1 without the usual thiamine source but supplemented with the vitamin mixture listed in Table 4 was used. The flasks were incubated at 23° for 13 days for the anaerobic treatment and 7 days for the 0.2% (v/v) oxygen treatment. Five replicates were used in each treatment. After 13 days of incubation the anaerobic flasks showed no visible growth and microscopic examination of their contents indicated no trace of the inoculum. After 7 days of incubation the flasks under the 0.2% (v/v) oxygen treatment yielded an average dry weight of 2.0 mg./flask; microscopic examination of the contents of the flasks indicated that the fungus was growing, but very poorly. The 0.2% (v/v) oxygen treatment, because of the way it was obtained, also contained $3 \times 10^{-4}\%$ CO_2 . It is clear that *Catenaria anguillulae* is neither anaerobic nor microaerophilic.

The optimum concentration of vitamin-free Casamino acids. The optimum concentration of vitamin-free Casamino acids was determined in Medium 6 (Table 2) but without ammonium phosphate. The results (Fig. 3) indicate that the optimum concentration of Casamino acids in this medium was about 2.0 g./l., equivalent to 0.16 g. N/l. Medium without added glucose was included to test availability of the Casamino acids at 1 g./l. as carbon sources. After 7 days of incubation only 4 mg. dry weight was produced.

Growth on an artificial Casamino acids mixture. An attempt was made to replace the casein hydrolysate (Casamino acids) with a mixture containing the same amino acids and in the same proportions (Table 5). The concentration of methionine used in the mixture was a departure from its concentration in the hydrolysate, in order to maintain the concentration at which methionine had normally been included in other

media. Medium 4 (Table 2) was used in this experiment but with the new N sources. The N concentrations in the various media were adjusted to correspond to 0 (control), 0.5, 1, 2, 3 and 4 times the total N concentration provided by the Difco product at the optimal concentration of 2.0 g./l. (Fig. 4). The decrease in yield can be seen by comparing the dry weight of 35 mg./flask obtained after 9 days of incubation on the artificial mixture with the dry weight of about 120 mg./flask obtained after the same period of time with the Difco hydrolysate (Fig. 5).

Fig. 1

Fig. 2

Fig. 1. The effects of pH on the growth of *Catenaria anguillulae* in Medium 1. The incubation period of the experiment was 7 days. Each point on the growth response curve represents the average of five to six replicates.

Fig. 2. The effects of temperature on the growth of *Catenaria anguillulae* in Medium 1 adjusted to a pH of 8.7. The pH indicator was *m*-cresol purple. The incubation period of the experiment was 9 days. Each point on the growth response curve represents the average of five replicates.

Fig. 3. The relation between growth of *Catenaria anguillulae* and the concentration of Difco Casamino acids in Medium 6 without the ammonium phosphate. The total phosphate was 15 mM; pH 6.9. Each point on the curve is the average of four replicate flasks. The incubation period 7 days.

Growth as measured by titratable acid produced is shown in Fig. 6. In an attempt to determine what critical factor(s) had been omitted from the medium in the change to a defined amino acid mixture two approaches were tried.

Effects of various 'Casamino factors'

NaCl. The Difco product is an acid (HCl) hydrolysate of casein; and since the final pH value of the hydrolysate is adjusted with NaOH, the final product contains 38% NaCl (Difco Manual, 1953). Various concentrations of NaCl were added to Medium 4 adjusted to pH 8.7. In this experiment the artificial amino acid mixture was the N source. Low concentrations of NaCl (0.00325, 0.0065, 0.013 M) were stimulatory to the

Table 5. *The composition of Difco vitamin-free Casamino acids (Column A) and of the amino acid mixture (Column B)*

Data on the Difco product supplied by Mr J. M. Willett (Technical Service, Difco Laboratories). All nitrogen (N) concentrations are for the L-isomer, even when the amino acid was supplied as a DL-mixture. The concentrations for the amino acids in the mixture are calculated as a substitute for Casamino acids 2 g./l.

Amino acid	A (%)	B (g. N/l.)
Arginine	2	0.0064 (L)
Aspartic acid	5	0.0160 (L)
Glutamic acid	15	0.0480 (L)
Glycine	1	0.0032
Histidine	1.5	0.0048 (DL)
Isoleucine	4	0.0128 (DL)
Leucine	5	0.0160 (L)
Lysine	5	0.0160 (DL)
Methionine	1	0.0080 (DL)
Phenylalanine	2	0.0064 (DL)
Threonine	2	0.0064 (DL)
Tyrosine	1	0.0032 (L)
Valine	4	0.0128 (L)
Total	48.5	0.1600

growth of strain 58-12 on this medium but did not account for the differences between the two nitrogen sources (Fig. 7).

Fatty acids. Arai & Kuwahara (1961) found that *Streptococcus hemolyticus* (s-8 strain) grew well on Difco vitamin-free Casamino acids but poorly on the ether-extracted Casamino acids; they concluded that the ether treatment removed a factor important for the growth of this streptococcal strain. The factor remained active in the ether extract and was found to be stearic acid which was present in the Casamino acids at 0.001 to 0.01%. Only saturated fatty acids (palmitic, arachidic) were able to replace the stearic acid. Demain, Hendlin & Newkirk (1959) with a *Sarcina* species found that on a defined amino acid medium the final yield was increased to one-half of the maximum yield obtained on a casein hydrolysate medium when they added; oleic acid 0.13 µg./ml., linoleic acid, 0.16 µg./ml., linolenic acid 0.32 µg./ml. or Tween 80 5.5 µg./ml. Thus unsaturated and saturated fatty acids were active in replacing, or actually may be, the active factor(s) lost in changing from a medium containing casein hydrolysate to one with a defined amino acid mixture as the nitrogen source.

For the experiment with *Catenaria anguillulae* oleic acid was selected as the unsaturated fatty acid and stearic acid as the saturated fatty acid. Medium 4 with the amino acid mixture (0.16 g. N/l.) as the N source was used. All three concentrations of oleic acid which were tested and stearic acid at 0.1 and 1.0 µg./ml. were stimulatory to *C. anguillulae* strain 58-12 in the medium containing the artificial amino acid mixture

(Fig. 8). The twofold increase in final dry weights was not, however, sufficient to account for the nearly fourfold difference between these results and those on the medium containing the Difco Casamino acids.

Fig. 4

Fig. 5

Fig. 4. The relation between growth of *Catenaria anguillulae* and the concentration of the artificial amino acid mixture in Medium 4. The incubation period was 9 days, pH 8.7. Each point on the curve is the average of six replicates.

Fig. 5. The growth curve of *Catenaria anguillulae* on Medium 4. Each point on the curve is the average of three to four replicates.

Fig. 6. Total titratable acid produced by *Catenaria anguillulae* when grown on Medium 4. Each point on the curve represents data from 10 pH adjustments or observations. The arrows indicate end of lag phase and peak of growth curve.

Utilization of single amino acids. The results (Table 6) indicated that the compounds in the ornithine + urea cycle, including glutamic acid, were utilized along with asparagine and aspartic acid. Because of the way in which the medium was prepared, the concentration of L-aspartic acid in the final medium was equivalent to 0.065 g. N/l., or one-half of the N concentration provided by the other compounds tested. Of the single N sources tested, L-asparagine gave the highest yield of organism for the 11-day incubation period; and this yield compared well with the yield obtained on the Difco Casamino acids at the same N concentration. L-asparagine was therefore used in further experiments.

The optimum concentration of L-asparagine. The amide L-asparagine was incorporated into Medium 5 at concentrations from 0 (control) to 0.48 g. N/l. The medium was initially adjusted to pH 8.7. The results (Fig. 9) indicated that the optimum concentration

Fig. 7

Fig. 8

Fig. 7. The relation between growth of *Catenaria anguillulae* and concentration of NaCl in Medium 4 with the amino acid mixture as the nitrogen source (0.16 g. N/l.). The incubation period 12 days; results of each treatment are the average of five replicates.

Fig. 8. The relation between the growth of *Catenaria anguillulae* and the concentration of oleic acid and stearic acid in Medium 4 with the amino acid mixture as the nitrogen source (0.16 g. N/l.). Incubation for 9 days; results of each treatment average of three replicates.

Table 6. *The growth of Catenaria anguillulae on organic nitrogen sources in Medium 5 at pH 8.7*

The incubation period 11 days; the dry weights averages of duplicate flasks. The N concentration was 0.13 g. N/l. except for L-aspartic acid (0.065 g. N/l.). For DL-mixtures the concentration is given for the L-isomer only.

Nitrogen source	Yield dry weight (mg.)	Nitrogen source	Yield dry weight (mg.)
Control (no added nitrogen)	2	Glycine, DL-histidine, DL-isoleucine, L-leucine, DL-lysine, DL-methionine, DL-phenylalanine, L-proline, hydroxy-L-proline, DL-serine, DL-threonine, L-tryptophan, L-tyrosine, L-valine,	all 2
Difco Casamino acids	85		
DL-Alanine	2		
L-Arginine	22		
L-Asparagine	91		
L-Aspartic acid	47		
DL-Citrulline	7		
L-Cystine	3		
L-Glutamic acid	38		
L-Ornithine	12		

of L-asparagine for the growth of *Catenaria anguillulae* on this medium was 0.24 g. N/l. The inoculum used was small in this experiment and accounted for the lower yield in comparison with the 11-day dry weight obtained with asparagine in the single N source survey.

The use of amino acids from a mixture. In studying the use of amino acids by an organism, an alternative to the method of supplying single sources is to provide the organism with a mixture of amino acids and then record the effects of the removal of

amino acids from this medium. One problem which arises in such studies is the possibility that the organism may remove an amino acid from a mixture even though it will not grow on that amino acid when it is supplied as sole N source. Inhibitory interactions between amino acids in a mixture also present a problem. Prescott, Peters & Snell (1953) found that alanine inhibited the growth of *Lactobacillus delbrueckii* and that this inhibition was annulled by serine or some of its peptides. The role of peptides in the present work was not assessed; peptides do occur in Difco Casamino acids. The early amino acid analyses were allowed to run for 24 hr, long enough for peptides to be indicated in the column chromatography; their total N concentration was not calculated and no attempt was made to identify specific peptides.

Table 7. *Amino acid concentrations in Medium 4 with Difco vitamin-free Casamino acids (2 g./l.) as the N source after growth of Catenaria anguillulae*

The analyses were made with a Beckman-Spinco automatic amino acid analyser; concentrations in $\mu\text{M/ml}$.

Amino acid	Time of incubation (days)			
	0	5	6	8
	Amino acid concentration in culture			
Lysine	0.744	0.500	0.451	0.085
Histidine	0.152	0.158	0.143	0.080
Arginine	0.118	0.118	0.056	Trace
Aspartic acid	0.657	0.630	0.638	0.277
Threonine	0.363	0.406	0.363	0.083
Serine	0.481	0.484	0.506	0.133
Glutamic acid	1.491	1.372	1.430	0.542
Proline	1.094	1.216	1.078	0.640
Glycine	0.257	0.238	0.264	0.101
Alanine	0.363	0.392	0.407	0.160
Valine	0.456	0.518	0.517	0.083
Methionine	0.753	0.644	0.418	0.139
Isoleucine	0.344	0.378	0.352	0.027
Leucine	0.620	0.644	0.649	0.051
Tyrosine	Trace	Trace	Trace	Trace
Phenylalanine	0.109	0.098	0.094	Trace

The disappearance of an amino acid from a mixture may not always mean that it has been taken up by the organism. Kihara, Klatt & Snell (1952) and Peters, Prescott & Snell (1953) reported for different bacteria the presence of an extracellular tyrosine decarboxylase which partially destroyed the free amino acid in the media before any utilization. Gale (1945) reported an instance in which a *Streptococcus* species converted free arginine to ornithine, but that the latter compound was not utilized for growth. Even when an amino acid is taken up by the organism it does not mean that it will be utilized for growth. Halvorson & Cowie (1961) reported the uptake of various isomers and analogues of amino acids which were not utilized by a yeast.

For the present study, *Catenaria anguillulae* was grown in Medium 4 with Difco Casamino acids (2.0 g./l.) as the N source. The growth curve (Fig. 5) and the curve for the production of titratable acid (Fig. 6) are from the present experiment. Through the lag phase of growth (5- and 6-day harvests; Table 7) the only amino acids removed from the medium in significant amounts (exceeding $\pm 8\%$ of the concentration

given in the 0-day column of Table 7) were lysine, arginine and methionine. Methionine was the only sulphur source of those detected in the analysis and was known to be an excellent sulphur source for *C. anguillulae* (Nolan, 1966). From the studies on utilization of single amino acids, methionine was not shown to be a readily utilized N source. Arginine was readily utilized as a N source; but was not the only one of those compounds in the medium available to strain 58-12. Of the compounds detected in the analysis, arginine, aspartic acid and glutamic acid were the ones utilized when given as

Fig. 9

Fig. 9. The relation between the growth of *Catenaria anguillulae* and the concentration of L-asparagine in Medium 5 at pH 8.7. Incubation for 11 days. Each point on curve average of three replicates.

Fig. 10

Fig. 10. The relation between the concentration of L-lysine added to Medium 5 containing L-asparagine (0.24 g. N/l.) as the N source and the production of titratable acid by *Catenaria anguillulae*. Initial pH 8.7.

single sources; of these, arginine was the one utilized from the mixture. One anomaly, namely lysine, stands out in the early uptake from the medium; this amino acid was not utilized in the earlier study dealing with single sources. Once the organism had passed through the lag phase into the more rapid period of growth, all of the amino acids in the mixture disappeared from the medium in significant amounts and were presumably utilized by the fungus.

Effects of the addition of L-lysine to a medium containing L-asparagine as the N source. From the studies dealing with single N sources, L-asparagine emerged as the best source; this compound had the additional advantage that it was not utilized as a carbon source.

Because of the anomalous utilization of lysine from the Difco Casamino acids medium, the possibility that lysine might be a key to the problem of obtaining a shorter lag phase and a better yield was examined. Lysine might be a compound which was not utilized as a single source but which, when in a mixture with one or more other compounds which could be utilized as a N source, had a stimulatory effect. The medium used was Medium 5 adjusted to pH 8.7. L-asparagine was included in the medium at the optimum concentration (0.24 g. N/l.). L-lysine was added in concentrations varying from 0 (control) to 7 mM (Table 8). The concentrations of 0.1 and 0.5 mM were more in accord with the concentrations utilized during the lag phase on the Difco Casamino acids medium (0.244 and 0.293 mM during the first 5-day and 6-day harvests, respectively). The growth of these cultures was followed on the basis of

their production of titratable acid. The production of titratable acid was used to signify the end of the lag phase of growth, and hence the earlier production of such acid in the treatments to which 0.050 and 0.100 mM of L-lysine had been added was taken as evidence of the shortening of the lag phase under these conditions (Fig. 10). This was in comparison to the additional 24 hr required for the cultures in the control and other treatments (excluding the 1 and 7 mM treatments) to enter the phase of rapid growth. The rate of growth also appeared to be higher in all treatments with added lysine (except for the two highest concentrations) as compared to the control. Not only was the rate of growth higher, but the dry weights were considerably higher than those of the control (Table 8). From the dry weights at 240, 263 and 283 hr, it can be seen that growth in the flasks containing the lower concentrations (0.010, 0.050 and 0.1 mM) of added lysine had reached the peak of the growth curve after 10 or 11 days. At that time the control flasks were still growing rapidly and continued to do so through the 12th day of incubation when the experiment was terminated.

Table 8. *The effects of added L-lysine on the growth of Catenaria anguillulae in Medium 5 with L-asparagine (0.24 g. N/l.) as the nitrogen source*

Dry weights are the averages of duplicate flasks. (pH 8.7)

L-lysine level (mM)	Incubation period (hr)		
	240	263	283
	Yield dry weight (mg.)		
0	19	47	79
0.01	111	110	116
0.05	95	118	—
0.10	95	122	—
0.50	35	118	—
1.00	—	—	11
7.00	—	—	3

The final medium evolved. Medium 7 (Table 2) was adopted as best satisfying the growth requirements of the isolate strain 58-12 of *Catenaria anguillulae*.

DISCUSSION

Couch (1945) observed the morphological similarities between the genera *Catenaria* and *Blastocladiella*; representatives of these two genera are unique within the Order Blastocladales in their inability to utilize inorganic nitrogen. The high pH optimum of the isolate strain 58-12 of *Catenaria anguillulae* used in the present work is unique among aquatic Phycomycetes; this is emphasized by its occurrence in regions of relatively alkaline pH value. For example, the fungus is found in the shallow margins of Lake George, Uganda, where the pH values often reach pH 10 (Dr L. G. Willoughby, personal communication to Dr R. Emerson; 29 November 1966) and in some barnyards (personal communication from Dr F. K. Sparrow, Department of Botany, University of Michigan, Ann Arbor, 1967).

The early studies of Buckley & Clapham (1929) and Butler & Humphries (1932) on the growth and development of *Catenaria* were made by using one of its natural substrates, the eggs of the liver-fluke *Fasciola hepatica* L. Qualitative information is available on the amino acid composition of liver-fluke egg proteins (Krvavica, Maloseja, Wagner & Martincic, 1964). Of those amino acids utilized by *Catenaria*

anguillulae in the present study, the eggs appear to contain proteins with high concentrations of arginine and lesser concentrations of glutamic acid and methionine; the eggs also contain stearic and oleic acids.

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