Further Studies on a Filamentous Saprophyte from Wookey Hole

By

A. J. WILLIS, B.Sc., Ph.D., F.L.S.

(Department of Botany, University of Bristol)

INTRODUCTION

Preliminary observations on an unusual, branched, filamentous saprophyte found under water in Wookey Hole Caves, near Wells, Somerset, were reported by Round and Willis (1956). The results of further investigations on this organism are given here, although no final conclusion can be reached as to its identity. It was felt that it would, however, be useful to record the observations made on its structure and growth, in view of the great plasticity of form of the organism and as it may well be found elsewhere. On some media it shows a superficial resemblance to certain yeast-like fungi, whereas on others its filaments are somewhat similar to those of *Cladophora* but lack chlorophyll. It is of interest that this aquatic organism may be a rather primitive type as it has some algal features but others which are characteristic of the fungi.

OCCURRENCE OF THE ORGANISM

The saprophyte was found by Dr. R. E. Davies in December, 1955, growing as a fairly thick felt on the insulation of a reel of cable in the ninth chamber of Wookey Hole. The line reel had been in this chamber, in complete darkness, for more than two years, under about 10 ft. of water. The water level of the chamber, which is controlled by a dam at the entrance, is fairly constant, increasing temporarily under flood conditions. The current here is very slow (1 in. in many minutes), and the organism was growing some 30 ft. from the main streamway in a rather stagnant backwater. The temperature of the water varies only very slightly, and on average is about 10·0° C. (minimum of 9·5° C. on Jan. 31st, 1953, and maximum of 11·0° C. on June 26th, 1954). The water of the chamber contains appreciable amounts of calcium, as a result of percolation through the Carboniferous Limestone of the Mendips (outflow water from the Caves was found to contain *c.* 80–100 p.p.m. of calcium). Measurements of the *p*H of water from the Caves indicated, however, only slight alkalinity (values of 7·2–7·3).

Iron bacteria and sulphur bacteria were associated with the filaments of the organism when brought from the cave, some of the filaments having a strand-like coating of ferric hydroxide. Some filamentous blue-green algæ were also present. It was not, however, difficult to isolate a pure culture of the saprophyte, which was used in subsequent nutritional studies and for detailed investigation of its structure.

STRUCTURE

Some of the chief structural features of the organism have already been recorded and illustrated (Round and Willis, 1956). Other features are shown in *Plate* 12 and *Fig.* 36. The branching filaments are septate and range in diameter from *c.* 30μ to less than 5μ (*Plate* 12, Nos. 1 and 7). Branches arise just beneath the cross walls (*Plate* 12, No. 7; *Fig.* 36, No. 5) in the young filaments, but subsequent development is often such that the branches ultimately appear lateral to the cells of the main filaments (*Plate* 12, No. 2). In young filaments the individual cells are of fairly uniform diameter throughout (*Plate* 12, Nos. 4 and 7), but in the old filaments the cells become vesicle-like, being appreciably extended except at the cross walls, and the filaments develop a catenulate, constricted appearance (*Plate* 12, No. 9; *Fig.* 36, Nos. 4 and 8). The cytoplasm is minutely granular, but contains no obvious chromatophores, nor do any develop after prolonged exposure to light. No photosynthetic pigments have been detected, but on some culture media blackening of the filaments (probably due to melanins) is pronounced. The cells of the mature, large filaments are strongly vacuolated (*Fig.* 36, No. 3), and on certain media produce a considerable amount of oil, either as a number of small droplets in the cells (*Plate* 12, Nos. 2, 3 and 8) or as one large globule (*Plate* 12, No. 12). Septate, fine, finger-like filaments may be produced from the ends of broken filaments (*Fig.* 36, No. 1), and the protoplasts of some cells readily grow through adjoining dead or dying ones, producing at first a peg-like structure (*Plate* 12, Nos. 5 and 6; *Fig.* 36, Nos. 2 and 3). Growth appears to be chiefly localized towards the ends of the filaments, but occasionally cell division occurs in the larger cells further back (*Plate* 12, No. 8).

The nuclei, which are very small and not easy to observe, have been investigated by several techniques. Staining carried out on alcohol-treated material by Dr. Kihlman indicates that the young cells have one nucleus each and the older ones several; the large, vesicle-like cells are, however, stained more or less throughout as if the desoxyribosenucleic acid is dispersed in the whole cytoplast. Further studies of the nuclei by Mrs. A. von Hofsten, who used a staining procedure with Azure-A (Hofsten, 1959), give evidence that the number of nuclei per cell varies from two to several.

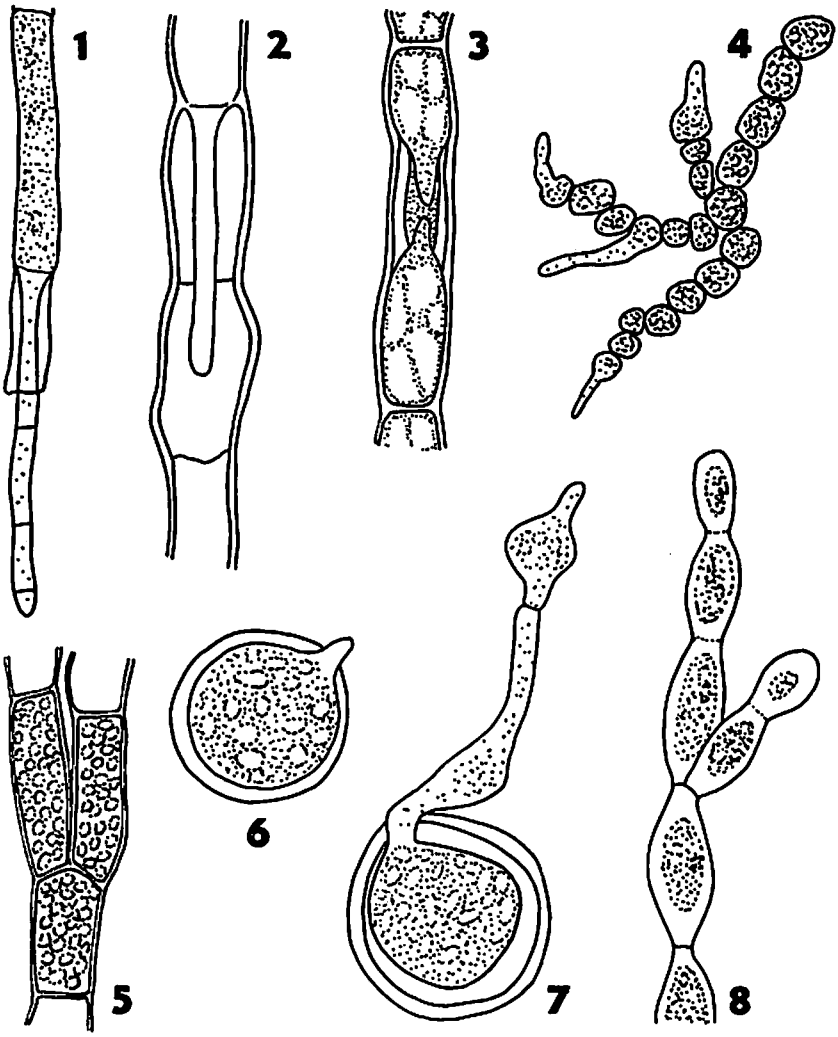

Fig. 36.—Features of the structure of the filamentous saprophyte.

All the drawings were made by means of a camera lucida. (1) Production of new filament at end of broken filament (×175). (2) Peg-like process growing through two dead cells (×405). (3) Peg-like processes approaching each other and surrounded by the contracted protoplast of a dying cell (×175). (4) Chains of spherical cells from a culture on 1 per cent sucrose–minerals–agar medium (×185). (5) Branching of filament (×175). (6) Production of new filament from spore-like body in hanging drop culture (×465). (7) Later stage in production of new filament (×465). (8) Catenulate growth on 1 per cent glucose–minerals–agar medium of *p*H 6·9 (×465).

The cell walls do not give the typical reactions of cellulose; their nature has been further examined by Dr. E. Nicolai. Material extracted by boiling with dilute hydrochloric acid (hydrolysed at 100° C. for 1 hr. with 2 per cent HCl) give X-ray diagrams indicating marked crystallinity very different from the unhydrolysed material which does not give clear diagrams because of impurities associated with the wall. The spacings of the X-ray diagrams are not those of *Cladophora* (which are distinctive), but are similar to those found in the native (untreated) walls of some other algæ, such as *Ulothrix*; the diagrams also resemble those produced by Houwink and Kreger (1953) for the cell walls of yeasts after pretreatment with acid and alkali. In the yeasts the high crystallinity results from yeast-glucan and yeast-hydro-glucan.

No structures which could be recognized as sexual reproductive organs have been seen, although filaments occasionally develop to give a shortly branching, somewhat contorted, structure. Anastomosis between filaments is not uncommon; the cross connections may occur between fairly young cells (*Plate* 12, No. 4) or older ones (*Plate* 12, No. 3). Old, slow-growing colonies may produce thick-walled chlamydospore-like bodies at the ends of catenulate filaments (*Plate* 12, No. 10), sometimes several such bodies being formed in a row. These structures have been shown to germinate (in hanging drop cultures) to form a septate filament (*Plate* 12, No. 11; *Fig.* 36, Nos. 6 and 7). Germination may occur *in situ* (*Plate* 12, No. 10).

GROWTH ON DIFFERENT MEDIA

The organism will grow in the light and in the dark on a range of media, both solid and liquid. It is, however, slow growing even under the most favourable conditions which have been found.

With a view to elucidating some of its requirements and establishing the conditions of temperature and *p*H at which the organism makes good growth, a series of cultures was set up and growth rates measured; observations were also made on morphological features. It was hoped that reproductive stages would be formed on some of the media, and to try to promote the development of these stages various vitamins and nitrogen bases were added to some cultures.

The essential features of the composition of the solid media (1·5 per cent agar) on which growth was investigated are given in *Table I*, together with details of growth rates and the appearance of the organism. The minerals used in the media included, in usual proportions, potassium dihydrogen phosphate, magnesium sulphate, ammonium sulphate and ferric chloride (made up in tap water); the vitamins included thiamin, riboflavin, calcium pantothenate, nicotinic acid, pyridoxal, *p*-amino-benzoic acid, biotin and folic acid; and the nitrogen bases included cytosine, thymine,

Table I.—THE GROWTH OF THE ORGANISM ON VARIOUS SOLID MEDIA

Except where otherwise stated the media were of *p*H 6·0 and the temperature 16° C.

Culture Medium	Mean Diameter of Colony (mm.)			Oil Content of Filaments	Growth Characters
	14 Days	34 Days	69 Days		
3 per cent malt-agar	10·3	21·8	40·0	High	Catenulate; a few anastomoses
3 per cent malt–2·5 per cent glucose	9·6	20·0	38·0	High	Catenulate; thick-walled cells
1 per cent glucose–minerals, *p*H 3·7	9·0	13·9	15·8	Medium	Very catenulate; globular cells with fine outgrowths; no anastomoses
*p*H 5·9	11·8	18·6	27·8	Medium	
*p*H 6·9	6·8	18·5	33·5	Medium	
+vitamins	12·4	18·6	21·0	Medium	
+nitrogen bases	10·0	15·5	19·3	Medium	Fairly catenulate
+vitamins+bases	10·3	15·7	24·1	Medium	
0·2 per cent glucose–minerals	13·7	23·1	36·5	Low	Not catenulate; many anastomoses
5 per cent glucose–minerals	11·3	19·1	22·0	Medium	Very catenulate; thick-walled cells
1 per cent sucrose–minerals	11·2	20·8	31·4	Medium	Very catenulate
1 per cent mannose–minerals	12·2	25·3	41·3	Medium	Catenulate
1 per cent glucose–nitrate–minerals	12·9	24·0	35·5	Low	Not catenulate
1 per cent glucose–potato-agar, 6° C.	4·8	6·3	8·8	Medium	Not catenulate
20° C.	15·9	39·2	73·0	High	Catenulate
25° C.	17·0	39·0	77·0	High	Catenulate

xanthine, guanine, uracil and adenine. The media were usually of *p*H 6·0, but for the investigation of the effect of *p*H the 1 per cent glucose–minerals–agar medium was adjusted with either tartaric acid or sodium carbonate to give initial *p*H values of 3·7, 5·9, 6·9, 8·0 and 9·9. The media were poured into Petri dishes, inoculated and incubated in darkness at 16° C. except for the 1 per cent glucose–potato-agar cultures which were kept at 6° C., 20° C., 25° C. and 30° C. For study of growth rates, the dimensions of the colonies were measured along two axes at right angles to each other. The values shown in *Table I* are the average of the means of these dimensions for three replicate cultures.

Growth of the organism occurred on most of the media tested. Cultures at 30° C. and in media of *p*H 8·0 and 9·9 did not, however, develop at all. There was fairly good growth on 1 per cent glucose–minerals–agar medium at *p*H 5·9 and at *p*H 6·9, although the latter cultures grew poorly at first. On the other hand, the cultures at *p*H 3·7 made moderate growth initially, but later slowed up appreciably. On high glucose and medium-level glucose media, moderate amounts of oil were found in the cells of the filaments which became very catenulate (*Fig.* 36, No. 8); especially on the high glucose medium, growth slowed up after about 5 weeks and large, thick-walled, spore-like cells were formed, some of which produced narrow, finger-like outgrowths. When old, these cultures were intensely black at the centre of the colonies, and lighter towards the periphery. Cultures on a low (0·2 per cent) glucose medium, however, were slow to blacken, sectored freely, formed woolly aerial filaments, did not show ballooning of the cells, contained little oil and the fine filaments showed frequent anastomoses (*Plate* 12, No. 4). The addition of vitamins and of nitrogen bases to the medium had no marked effect, although old colonies showed some doming.

Sucrose and mannose were readily utilized. Cultures on media containing either of these sugars had a diffuse edge, whereas the margin of the colony was fairly sharp on media containing glucose (except under conditions as acid as *p*H 3·7). The cells rounded off almost to the point of separation in old cultures on a sucrose medium (*Fig.* 36, No. 4), and the organism looked very different from the original isolate. However, these rounded cells used as an inoculum gave rise to filaments indistinguishable from those of the original isolate when cultured on appropriate media.

When nitrate rather than an ammonium salt was used as a source of nitrogen, blackening of the culture was slow, only little oil was formed, and the cells did not become appreciably rounded. Malt-agar and potato-agar were also found to be suitable media.

A little growth was made on 1 per cent glucose–potato-agar at temperatures as low as 6° C., but it is clear that the most favourable temperatures are about 20–25° C. In its habitat at Wookey Hole the saprophyte clearly

is not growing under optimum conditions of temperature. Warmer and perhaps very slightly acidic conditions would probably lead to better growth.

The organism can also be cultured successfully on liquid media of slightly acidic or neutral reaction, growth usually being rather faster than on solid media.

THE SYSTEMATIC POSITION OF THE ORGANISM

As no sexual reproductive organs have been found it is not possible to classify the saprophyte with certainty. Its simple organization suggests a relatively primitive fungus or alga.

Truly saprophytic algæ with branching filaments are uncommon. Coker and Shanor (1939) have described a "saprophytic fungoid alga", *Saprochæte saccharophila*, resembling a water mould, which is like the present organism in several features such as cell size, mode of branching and nutrition. One major difference, however, between the Wookey Hole organism and *S. saccharophila* concerns their nuclei; whereas the latter has a large, obvious nucleus (5·6–8·6μ diameter) with a conspicuous nucleolus in the large cells, the former appears usually to have several tiny, obscure nuclei in the cells. *S. saccharophila* is referred to the Saprochætaceæ near the Chætophoraceæ in the Ulotrichales. The Wookey Hole organism is, however, in some respects more like members of the Cladophorales, and it might be suggested that it is a colourless *Cladophora*. There are, nevertheless, serious objections to this view, as, for example, apart from the saprophytic nature of the Wookey Hole organism and its lack of chromatophores, the cell walls and nuclei are different from those of *Cladophora*; the rounded cells of the old filaments, the thick-walled spore-like bodies, the storage of large quantities of oil and the plasticity of form have no parallel in *Cladophora*.

The nutrition of the organism appears to indicate fungal affinities, as do a number of its morphological features, although such large cell size is unusual in fungi. Information derived from X-ray studies of the cell wall and from the examination of the nuclei is consistent with the view, held by Professor Nannfeldt, Professor Skuja and Dr. Santesson, that the organism is a fungus. Some support for this opinion is also gained from the wide range of form, the frequent anastomoses found in some cultures, and the production of copious oil. The chlamydospore-like bodies are also paralleled in many fungi.

It may be provisionally concluded that the organism could be an aquatic fungus (perhaps a rather primitive Ascomycete or Phycomycete). The true relationship and systematic position of the saprophyte must, however, remain uncertain unless sexual structures are found. It seems unlikely that sexual stages will be seen in the present culture, as treatments known to induce the onset of sexual reproduction in primitive organisms have been

tried and shown to have been ineffective in this instance. There is, however, the possibility that other samples of this organism may be found in Wookey Hole or elsewhere, which may lead to a more complete elucidation of its nature.

Whether the organism is a natural inhabitant of the specialized habitat of the Caves at Wookey or whether it was introduced from outside (perhaps with the cable) and survived in the cave water but in an unusual growth form is unknown. Although moulds have been recorded from other caves of Mendip, such as Read's Cave (Walton, 1944) where *Stemphylium botryosum* and a monoverticillate *Penicillium* were found, and some for caves in Wales (Mason-Williams and Benson-Evans, 1958), no organism comparable with that from Wookey Hole has apparently ever been reported.

SUMMARY

The structure, growth and nutrition of a filamentous saprophyte from Wookey Hole Caves have been investigated. The organism grows on a variety of media, showing considerable plasticity of form, ranging from filaments resembling those of *Cladophora* to rounded somewhat yeast-like cells. Oil is formed extensively in old cultures; thick-walled bodies capable of germination are also produced. The affinities of the organism are uncertain, but the evidence suggests that it may be an aquatic fungus rather than an alga.

ACKNOWLEDGEMENTS

I am indebted to Dr. F. E. Round and Dr. P. Hunt for considerable assistance with the culture and examination of the organism, to Dr. E. Nicolai (Leeds University) for X-ray studies of the cell walls, to Dr. Kihlman and Mrs. A. von Hofsten (University of Uppsala, Sweden) for the examination of nuclei, and to Mr. H. Joyner for photography. Thanks are also due to Mrs. L. Fries, Dr. E. K. Tratman, and to many mycologists and phycologists who have examined the organism.

REFERENCES

Proc. = *Proceedings, University of Bristol Spelæological Society*

COKER, W. C., and SHANOR, L., 1939, "A Remarkable Saprophytic Fungoid Alga", *J. Elisha Mitchell Sci. Soc.*, Vol. 55, 152–165.

HOFSTEN, ANGELICA v., 1959, "The Vegetative Nuclei of the Fungus *Ophiostoma*", *Hereditas, Lund*, Vol. 45, 573–578.

HOUWINK, A. L., and KREGER, D. R., 1953, "Observations on the Cell Walls of Yeasts. An Electron Microscope and X-ray Study", *Antonie van Leeuwenhoek, Jour. Microbiol. Serol.*, Vol. 19, 1–24.

MASON-WILLIAMS, A., and BENSON-EVANS, K., 1958, "A Preliminary Investigation into the Bacterial and Botanical Flora of Caves in South Wales", *Publ. No.* 8, *Cave Research Group of Great Britain*, Berkhamsted, Herts.

ROUND, F. E., and WILLIS, A. J., 1956, "A Filamentous Saprophyte from Wookey Hole Caves", *Nature, Lond.*, Vol. 178, 215–216.

WALTON, G. A., 1944, for 1943, "The Natural History of Read's Cave", *Proc.*, Vol. 5, 127–137.

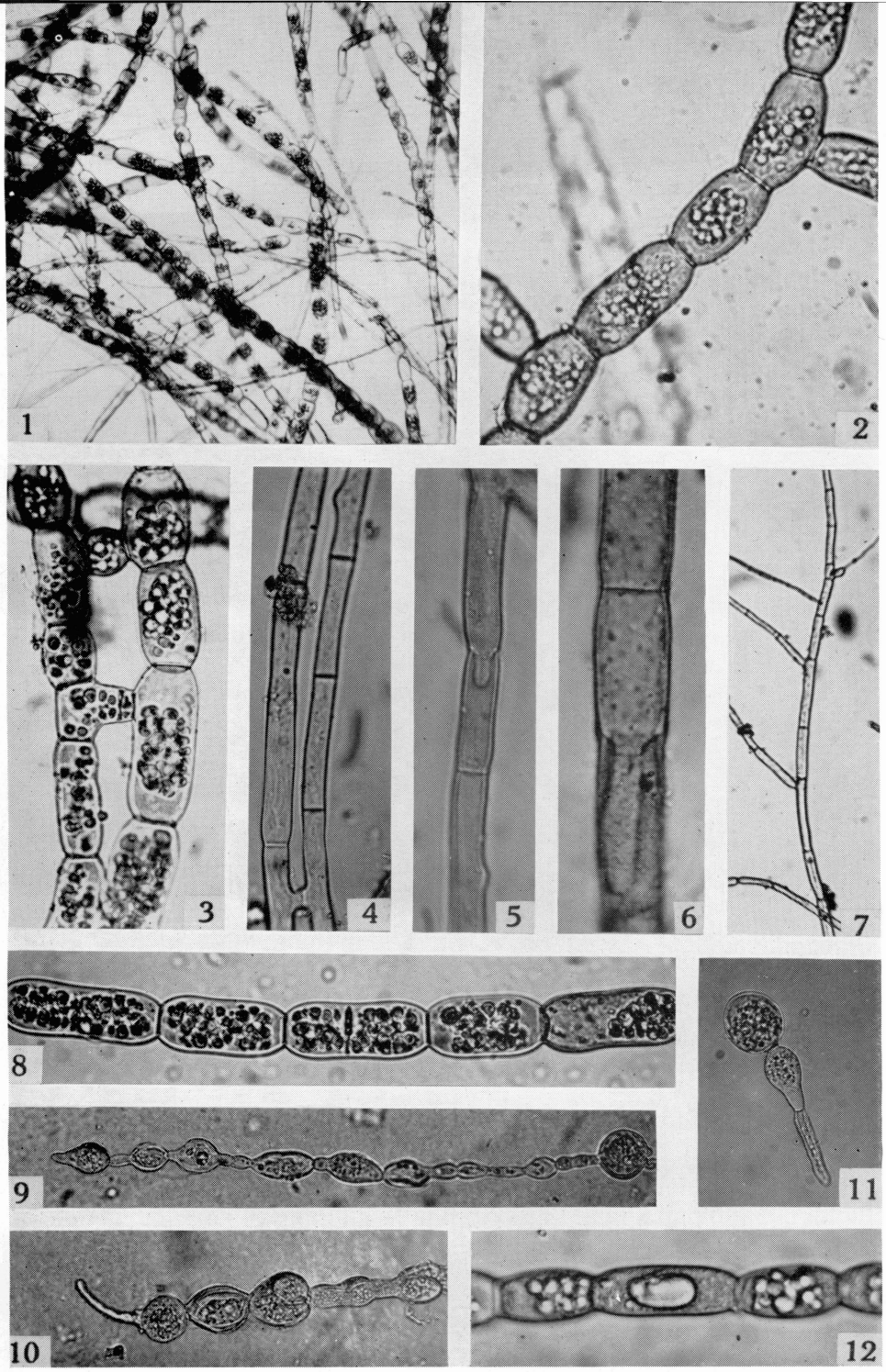

PLATE 12

Some features of the structure of the Wookey Hole organism.

The photomicrographs are as follows: (1) Filaments of a fairly old culture on nutrient agar, showing oil droplets in the cells of the large filaments (× 120). (2) Branching in a fairly old filament (× 480). (3) Cross connection between two old filaments (× 480). (4) Cross connection between young filaments (× 480). (5) Peg formation (× 480). (6) Peg formation, large filament (× 480). (7) Fine filaments of a young culture (× 480). (8) Old filament showing division of one of the cells; oil plentiful (× 480). (9) Catenulate filament (× 240). (10) Thick-walled spore-like bodies at end of filament; terminal "spore" producing outgrowth (× 240). (11) Germination of "spore" (hanging drop culture) (× 240). (12) Oil in cells of old filament (× 480).