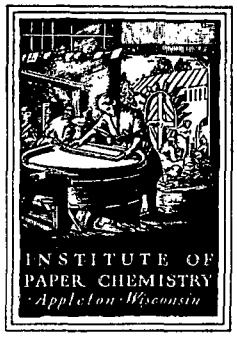

THE INSTITUTE OF PAPER CHEMISTRY
Appleton, Wisconsin 54911
Phone 414/734-9251

January 17, 1972

Project 3020

Dear Dr. Hider:

Progress report number one for Project 3020 is enclosed. Your reactions at this stage would be appreciated, particularly if you are interested in certain sheet properties which we have not examined to date.

After some deliberation, I decided against placing any mycelial paper samples in the report itself. However, attached to this letter you will find a very small piece of paper composed of 50% Saprolegnia ferax mycelia and 50% unbleached aspen kraft pulp.

Sincerely yours,

Morris A. Johnson
Research Fellow
Division of Natural
Materials & Systems

Dr. Shibley A. Hider
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Toledo, Ohio 43601

MAJ/kmb

Copies to: Dr. R. B. Valley
Mr. F. A. Adams
Dr. T. R. Santelli

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

UTILIZATION OF PULP MILL EFFLUENT IN THE PRODUCTION OF PAPERMAKING MYCELIA

Project 3020

Report One

A Progress Report

to

MEMBERS OF GROUP PROJECT 3020

January 7, 1972

MEMBERS OF GROUP PROJECT 3020

Green Bay Packaging Inc.

The Mead Corporation

Olinkraft, Inc.

Owens-Illinois, Inc.

St. Regis Paper Company

The Weston Paper and Manufacturing Company

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THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

UTILIZATION OF PULP MILL EFFLUENT IN THE PRODUCTION OF PAPERMAKING MYCELIA

SUMMARY

Gram quantities of mycelia of Phytophthora cinnamomi, Phytophthora parasitica, Mucor rouxii, Fusarium sp., and Pythium debaryanum were grown in suspension culture on a single defined medium. Harvested mycelia were killed by steaming, then washed and lyophilized for weight determinations. These mycelial "pulps" were combined with unbleached aspen kraft pulp at several percentages and made into standard handsheets which were subjected to some common paper evaluation tests. Mycelia of Saprolegnia ferax were evaluated in the same manner; however, in this case the organism failed to grow on the defined medium in use and required supplementation with yeast extract.

Trends in some of the paper evaluation data for various organisms through 25% incorporation into aspen kraft handsheets are tabulated below:

Organism	Test				
	Burst	Tensile	Tear	Stretch	Porosity
P. parasitica	No effect	Decrease	Decrease	No effect	Decrease
P. cinnamomi	Decrease	Decrease	Decrease	No effect	No effect
Fusarium sp.	Increase	No effect	Decrease	Increase	Decrease
M. rouxii	Decrease	Decrease	Decrease	No effect	No effect
S. ferax	Increase	Increase	Decrease	Increase	Decrease
P. debaryanum	No effect	Decrease	Decrease	No effect	Decrease

Results in some cases are more complex than indicated in this table. At the 50% level (half mycelia) all test values show decline or no change with the exception of a porosity increase by M. rouxii. The actual data plus further information on basis weight, thickness, density, and moisture are presented in the report along with other observations.

INTRODUCTION

This research effort attempts to address two substantial concerns of the paper industry simultaneously. The possiblity exists that one of these concerns, the disposal of waste effluents with high biochemical oxygen demand (B.O.D.), could be coupled to the solution of the other, the provision of source materials for the papermaking process. Several years ago a process for making sheets from fungal mycelia was developed and patented by The Institute of Paper Chemistry (1). Drawing upon that patent and recent literature reports which enhance our understanding of the chemistry of the cell walls of fungi, this project probes the feasibility of growing papermaking mycelia on the metabolites found in waste effluents of the paper industry.

Phase I in this investigation is now well under way. It consists of growing mycelia in suspension culture (hopefully on defined media) and evaluating their performance when incorporated into handsheets. Fungi used in Phase I are selected on the basis of reported cell wall compositions. Fungi judged desirable by their behavior in Phase I will be tested for their capacity to grow on waste effluents in Phase II.

MATERIALS AND METHODS

FUNGI

Phytophthora cinnamomi (ATCC No. 11928), *Phytophthora parasitica* (ATCC No. 13614), *Saprolegnia ferax* (ATCC No. 10396), *Mucor rouxii* (ATCC No. 4855), and *Pythium debaryanum* (ATCC No. 9998) were purchased from the American Type Culture Collection. The *Fusarium* sp. has originated in our own laboratories as a contaminant in cultures of *Mucor* remaining from the old project on which the patent was based. The *Mucor* organism of the patent description is no longer extant.

MEDIA

With the exception of *S. ferax* all organisms were grown on the following asparagine-glucose medium (2):

KH_2PO_4	3.0000 grams
$CaCl_2$	0.0034 grams
$FeSO_4 \cdot 7H_2O$	0.0010 grams
Citric acid	0.0014 grams
$ZnSO_4 \cdot 7H_2O$	0.0018 grams
$MnSO_4 \cdot H_2O$	0.0003 grams
$CuSO_4 \cdot 5H_2O$	0.0004 grams
$(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$	0.0003 grams
$MgSO_4 \cdot 7H_2O$	0.0005 grams
Thiamine $\cdot$ HCl	0.0010 grams
L-asparagine	2.0000 grams
Glucose	20.0000 grams

Final volume of the medium was brought to one liter with distilled water. The glucose (40% in distilled water) was autoclaved separately and then added to the rest of the medium which previously had been adjusted to pH 6.0 with KOH and autoclaved for 20 minutes at 121°C. For the culture of *S. ferax* the above medium was made to contain 0.1% yeast extract.

CULTURE TECHNIQUES

With the exception of some Fusarium which was grown in a minifermentor, all fungi were grown in 3-liter Erlenmeyer flasks containing 400 ml. of the asparagine-glucose medium. Growth periods ranged from 5-19 days depending upon the growth rate of the organism. The Erlenmeyers were inoculated, placed on a reciprocating shaker (50 cycles per minute) and incubated at 29°C. in the dark. Inocula were 1 or 2 ml. of mycelial suspension from small suspension cultures maintained for this purpose; mycelia were dispersed by shaking with glass beads before withdrawing the inoculum. Growth in the minifermentors (Mini-Ferm Model M-1000, Fermentation Design, Inc., Allentown, Pa.) entailed greater agitation and aeration with a sparger (discussed later).

When the growth period was complete, the shake flasks were transferred to a steam oven and steamed for one hour. Steamed mycelia were cooled, filtered[1] through qualitative filter paper on a Buchner funnel and resuspended in 300 ml. of distilled water for washing. This slurry was beaten for 30 seconds in a Waring Blendor or 200 counts in the British disintegrator (this latter now adopted and preferred but any beating may be undesirable — see Discussion). Following filtration, the washing procedure was repeated once more. Finally, the mycelia were filtered, washed with distilled water on the filter, removed, and lyophilized from a distilled water slurry. Freeze-dried weights were obtained prior to handsheet formation.

[1] Filtering problems occasionally encountered with some organisms.

HANDSHEET PREPARATION AND EVALUATION

Experimental unbleached aspen kraft pulp identified as T2-S6 (TA-14-58 in the case of S. ferax and P. debaryanum) was beaten 15 minutes in a Valley beater under TAPPI Standard Conditions. Freeze-dried mycelia were slurried and mixed with the aspen pulp in various proportions (usually 0, 5, 10, 20, 25, and 50% on an o.d. fiber basis). The mixtures were given 600 counts on the British disintegrator prior to making 1.2-g. handsheets according to TAPPI Standard T 205 m-58. Freeness and drainage times on the handsheet mold were recorded. Handsheets were examined visually and evaluated for basis weight, thickness, density, moisture, burst, tensile, stretch, and tear by TAPPI Standard Method T 220 m-60. Bendtsen porosity was determined in ml./min. for a 10 $cm.^2$ area at air pressure for 150 mm. of water.

RESULTS

GROWTH

Some appreciation for the magnitude of growth rates that have been encountered in this study to date may be gleaned from Table I. These results apply only to growth conditions used. Early efforts to grow Fusarium and the two Phytophthora species in the minifermentors were successful with Fusarium but only marginal with P. parasitica and P. cinnamomi. The Phytophthoras, particularly P. cinnamomi, had a tendency to cling to surfaces; as a result, they did not grow well in highly agitated systems and plugged the spargers (air intake) of the minifermentors if grown under static or near static conditions. These and the other organisms, including Fusarium, have grown satisfactorily in simple shake flasks at a slow shaking speed. As can be seen in Table I, it is possible that Fusarium may grow more rapidly when aerated; it does not plug the sparger since it grows well when agitated. A complete investigation of growth conditions has not been conducted. Note that rates calculated from a short growth period may be low because of an initial lag phase while those from a long growth period may be low because log phase growth has tapered off.

The size of the liquid volume in the 3-liter shake flasks was investigated briefly with P. parasitica. From the results in Table II, it was decided to use 400 ml. liquid volumes. The steaming procedure was also examined for its capacity to kill P. parasitica. Mycelia steamed for the usual one hour and used as inocula failed to show any growth over a period of 4 days or longer in shake culture.

TABLE I

GROWTH OF VARIOUS FUNGI

Organism	Culture	Medium	Days	Yield, g.	Growth Rate, g./l./day
F. sp.	Minifermentor	Standard plus antifoam	2	1.96	1.63
F. sp.	Shake	Standard	6	5.35	0.37
F. sp.	Shake	Standard	4	5.08	0.63
P. debaryanum	Shake	Standard	9	13.00	0.30
S. ferax	Shake	Standard plus yeast extract	5	12.30	0.51
M. rouxii	Shake	Standard	7	3.67	0.32
M. rouxii	Shake	Standard	5	12.23	0.51
P. cinnamomi	Shake	Standard	19	11.28	0.30
P. cinnamomi	Shake	Standard	14	6.77	0.48
P. cinnamomi	Minifermentor	Standard	15	1.97	0.22
P. parasitica	Shake	Standard	8	5.925	0.37
P. parasitica	Shake	Standard	8	1.125	0.35
P. parasitica	Minifermentor	Standard	15	1.30	0.15

TABLE II

P. parasitica GROWTH AT DIFFERENT LIQUID VOLUMES

Inoculum, ml.	Liquid Volume, ml.	8-Day Freeze-Dried Weight, g.
10	200	0.418
20	400	1.125
30	600	1.211
40	800	1.246

HANDSHEET DATA

The freeness and the handsheet mold drainage time data for the several organisms at various pulp-mycelia mixtures are shown in Table III. Some scanning electron micrographs of a handsheet containing 20% P. parasitica mycelia are shown in Fig. 1. Table IV contains all the paper evaluation data. Trends in the burst, stretch, tensile, tear, and porosity data can be visualized more readily in Fig. 2-6. All other observations not quantitated are covered in the Discussion.

TABLE III

FREENESS AND DRAINAGE FOR VARIOUS PULP-MYCELIA MIXTURES

Percentage Mycelia	Freeness, C.s., ml.						Drainage Time, sec.					
	P.p.[a]	P.c.	F. sp.	M.r.	S.f.	P.d.	P.p.	P.c.	F. sp.	M.r.	S.f.	P.d.
0 (control)	500	500	490	490	490	490	5.7	5.5	5.6	5.6	5.3	5.3
5	400	480	405	445	415	425	7.0	5.8	7.0	6.3	6.5	6.7
10	300	450	330	435	365	375	8.7	6.2	8.7	6.8	8.3	8.3
20	215	410	220	380	275	240	13.0	6.8	12.7	7.2	12.2	14.2
25	195	380	195	360	240	195	15.2	7.4	17.0	7.6	16.1	18.5
50	120	275	90	310	140	75	26.6	9.1	27.0	N.D.[b]	34.0	53.0

[a] Organism abbreviations.

[b] N.D. = not determined.

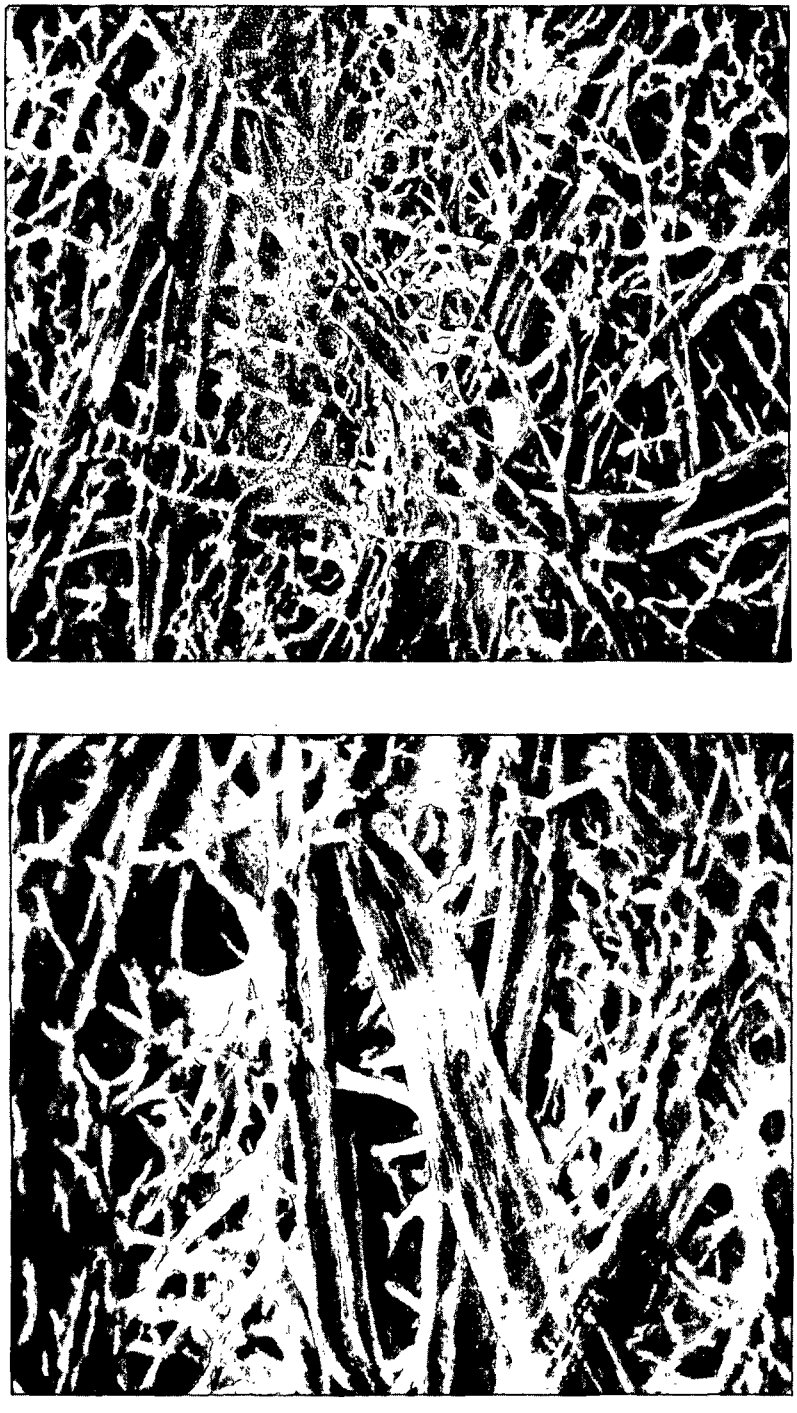

Figure 1. Scanning Electron Micrograph of an Aspen Kraft Handsheet Containing 20% P. parasitica Mycelia. Top: 300X. Bottom: 600X

TABLE IV

MYCELIAL PAPER EVALUATION DATA

Organism	Mycelia, %	Basis Weight, g./m.[a], o.d.	Thickness, µm.	Density, g./cc.	Moisture, %	Burst Factor	Bendtsen Porosity, ml./min.	Tensile, km.	Stretch, %	Tear Factor
P. parasitica	0	60.7	85.6	0.709	7.6	49.7	221	9.37	1.6	79.7
	5	61.6	86.1	0.715	7.7	48.2	169	9.43	1.9	75.3
	10	59.7	82.3	0.725	8.0	48.5	120	8.93	1.8	65.7
	20	59.9	81.5	0.735	8.3	45.1	64	8.22	1.8	59.4
	25	61.6	80.9	0.761	8.3	46.6	41	7.14	1.5	55.2
	50	61.4	77.7	0.790	8.8	37.2	<12	6.37	1.4	43.6
P. cinnamomi	0	61.3	86.7	0.707	7.9	49.1	218	9.63	1.7	80.3
	5	61.2	88.5	0.692	8.0	46.6	171	9.29	1.8	74.5
	10	60.5	89.7	0.674	8.2	43.5	157	8.44	1.6	70.7
	20	59.7	90.5	0.660	8.5	37.4	161	7.57	1.5	62.3
	25	60.7	92.7	0.655	8.6	36.5	137	7.23	1.6	61.3
	50	60.8	97.1	0.626	9.7	26.5	108	5.20	1.6	42.1
Fusarium sp.	0	61.1	86.3	0.708	7.7	49.9	183	9.87	1.8	78.6
	5	62.0	85.8	0.723	7.8	50.9	133	9.90	1.8	72.9
	10	60.1	80.2	0.749	8.0	55.1	76	9.97	2.0	66.6
	20	60.6	80.0	0.758	8.3	55.0	42	8.75	1.8	62.0
	25	60.8	79.6	0.764	8.4	57.1	32	9.36	2.1	52.6
	50	62.2	80.3	0.775	9.2	45.2	<12	7.11	1.6	34.1
M. rouxii	0	61.1	86.3	0.708	7.7	49.9	183	9.87	1.8	78.6
	5	60.4	84.4	0.716	8.0	46.6	168	9.02	1.6	75.5
	10	60.5	84.2	0.719	8.2	43.6	160	8.74	1.7	72.7
	20	61.3	86.6	0.708	8.7	38.3	160	7.87	1.6	62.6
	25	60.7	84.3	0.720	9.0	36.1	155	7.24	1.5	60.0
	50	59.9	84.4	0.710	10.1	27.0	193	5.32	1.3	40.1
S. ferax	0	60.4	84.5	0.715	8.1	48.2	331	8.87	2.1	82.1
	5	61.0	84.0	0.726	8.3	48.3	211	9.02	2.3	80.0
	10	61.9	83.0	0.746	8.3	53.5	108	9.19	2.3	72.4
	20	60.2	78.1	0.771	8.9	55.6	31	9.36	2.4	63.1
	25	60.6	77.3	0.784	9.0	51.4	20	9.34	2.3	58.1
	50	60.0	70.3	0.853	10.0	40.3	<5	8.36	1.8	37.3
P. debaryanum	0	60.4	84.5	0.715	8.1	48.2	331	8.87	2.1	82.1
	5	60.2	82.8	0.727	8.2	43.2	231	8.75	2.2	76.4
	10	60.7	81.6	0.744	8.3	43.1	134	8.30	2.1	71.2
	20	60.1	75.5	0.796	8.6	46.8	39	7.91	2.0	59.2
	25	60.0	73.5	0.816	8.7	48.2	20	7.87	2.2	54.7
	50	60.7	68.7	0.884	9.3	36.7	<5	5.49	1.5	34.9

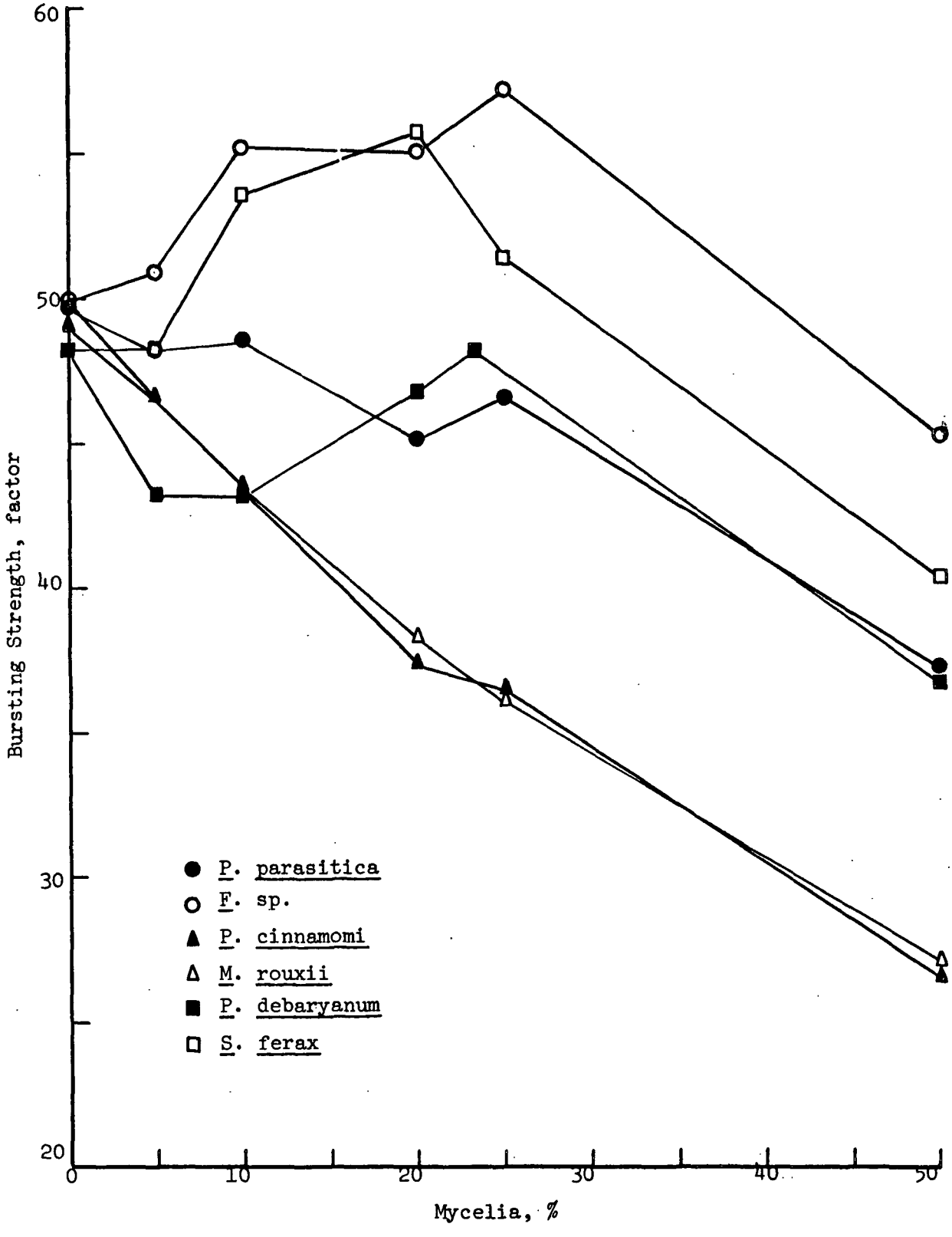

Figure 2. Bursting Strength of Mycelial Paper

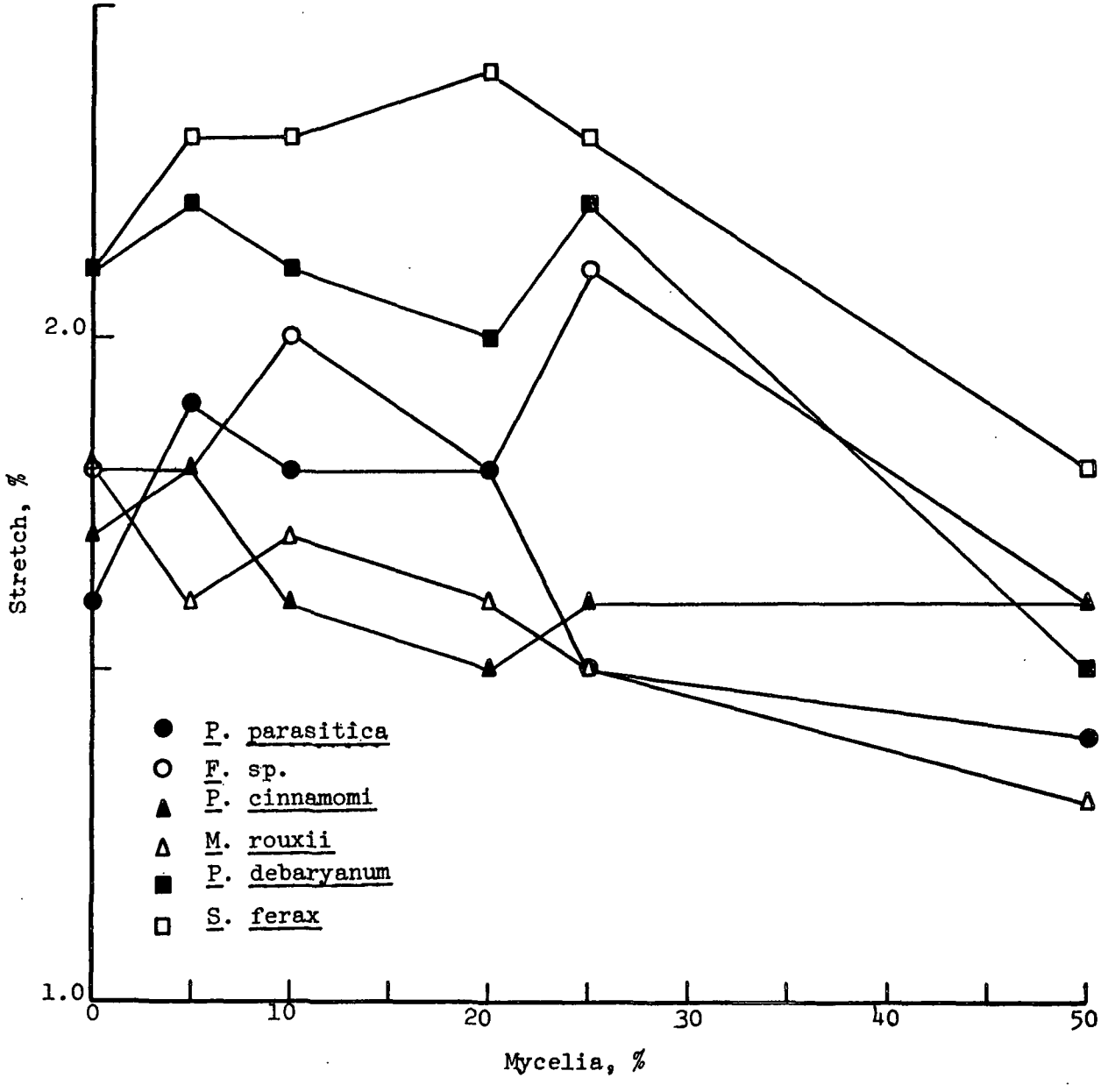

Figure 3. Stretch of Mycelial Paper

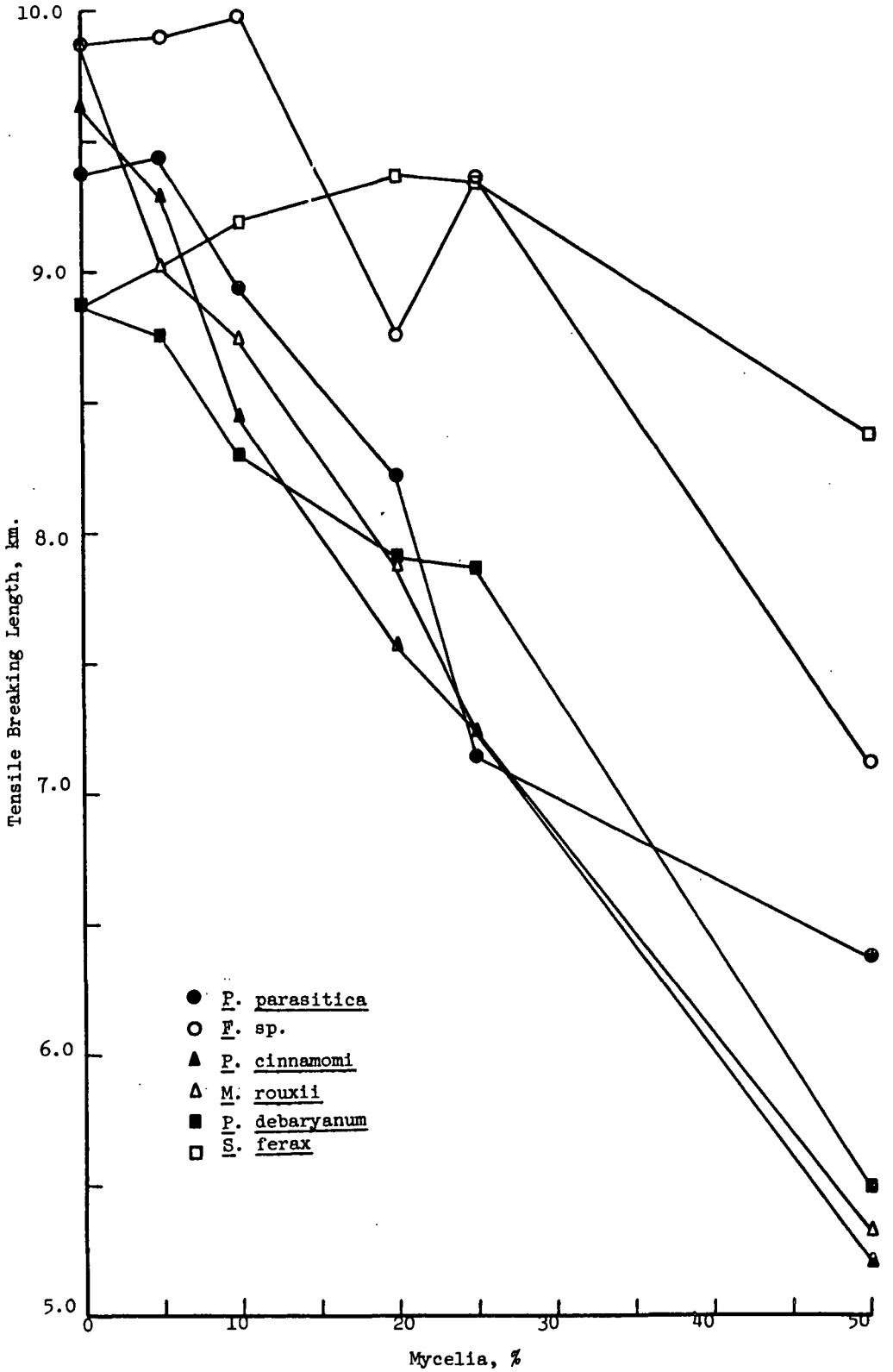

Figure 4. Tensile Strength of Mycelial Paper

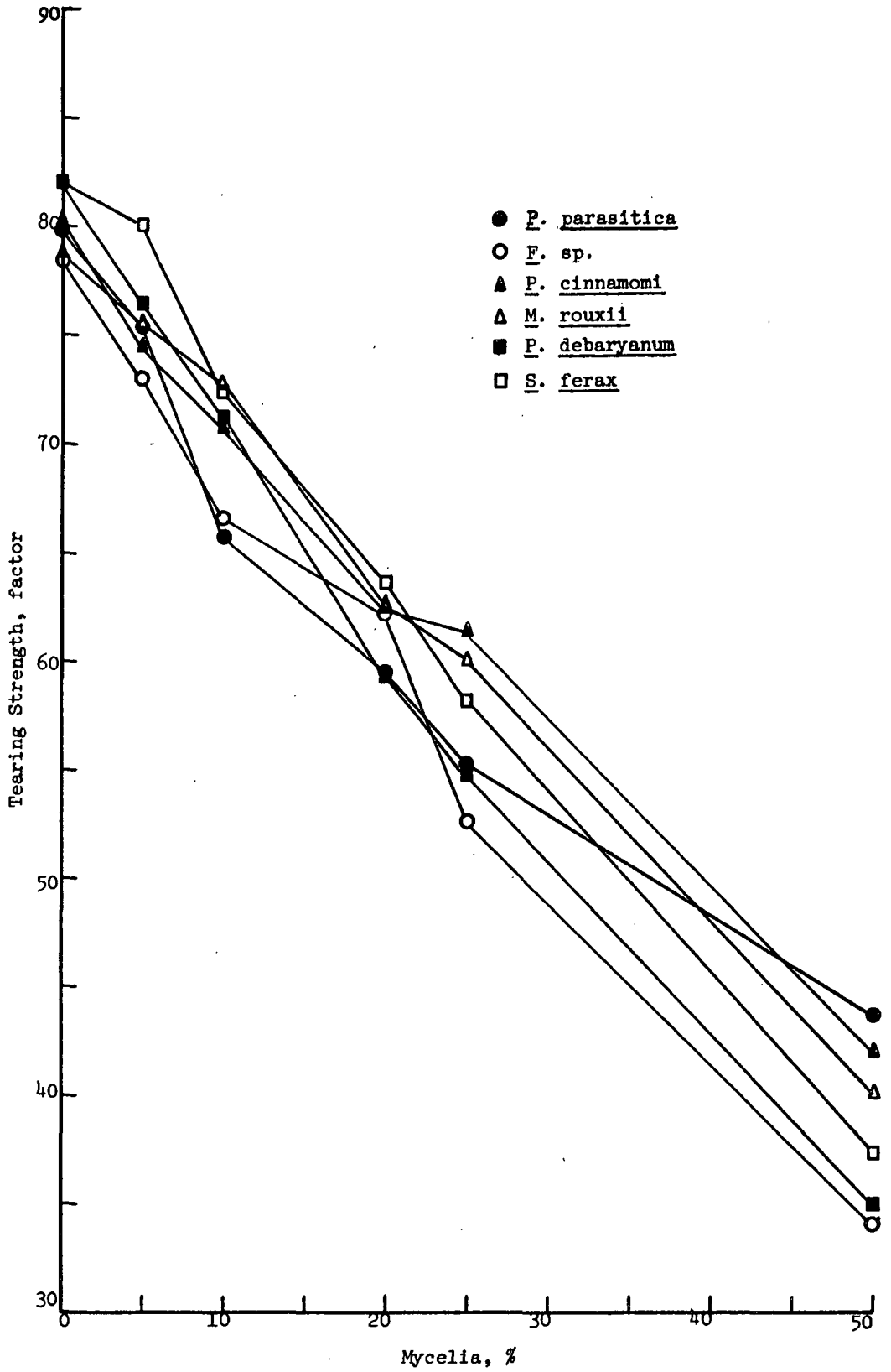

Figure 5. Tearing Strength of Mycelial Paper

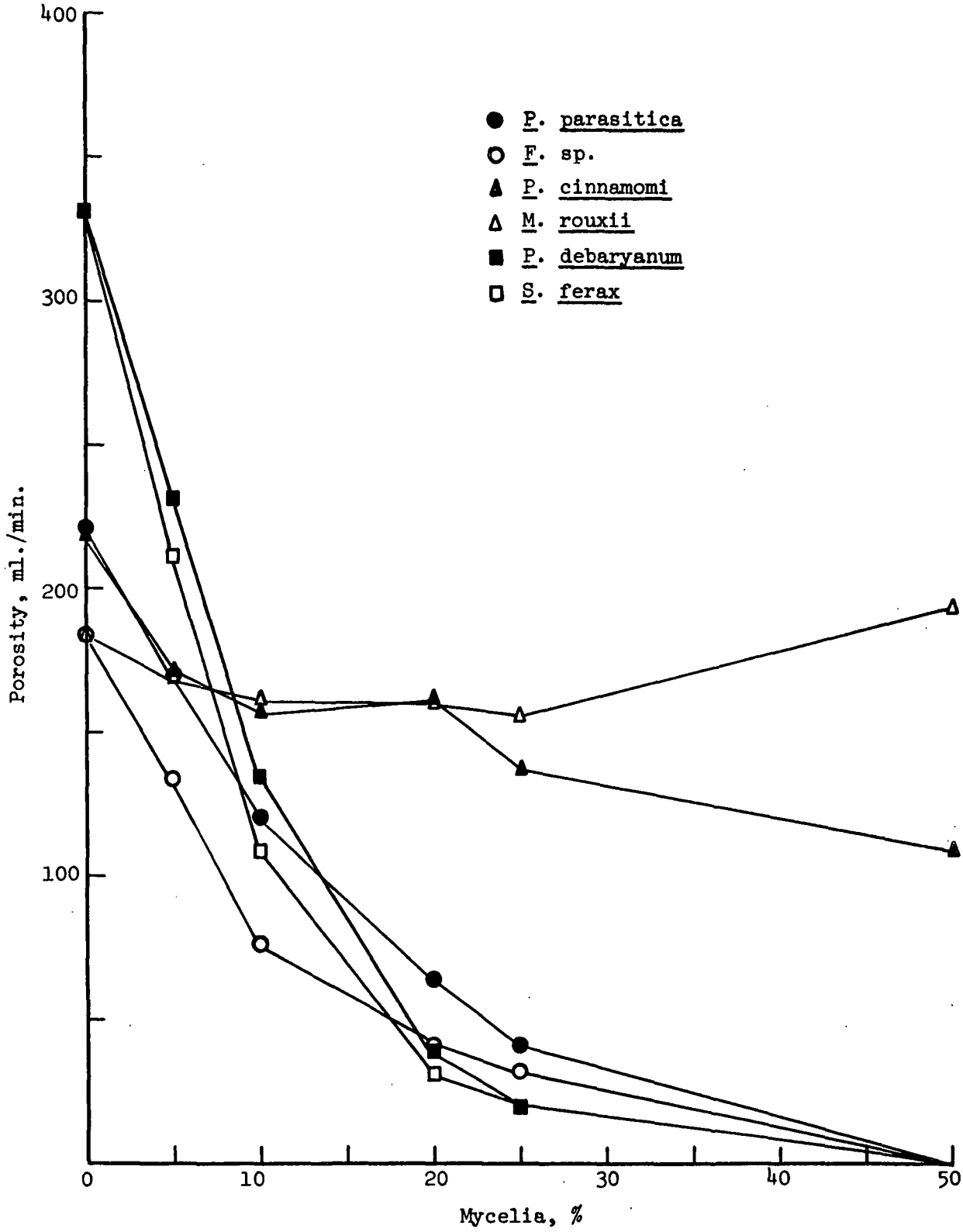

Figure 6. Porosity of Mycelial Paper

DISCUSSION

The growth data presented in this report are intended to serve only as a marker for future work when attempts are made to use waste effluent as a substrate. In this initial report period, the yield data have guided our efforts to produce enough mycelia from each organism to conduct the handsheet evaluations. To collect and compare growth rates properly will require that growth curves be constructed for each organism of interest; log phase growth rates can then be used. The complications of nonlogarithmic growth make it unfair to compare one organism's rate of growth against another with the data in Table I.

If there are organisms like Fusarium which thrive under high agitation conditions, they also may grow more rapidly in the minifermentors than in the standardized shake flask system employed. The liquid volume of 400 ml. used in the 3-liter shake flasks was based on the performance of a single organism arbitrarily selected. There are perhaps two primary factors to consider here, aeration and mechanical disruption of the mycelial growth. In Table II the different liquid volumes result in different surface-to-volume ratios for the medium. This ratio increases as the volume is lowered allowing better aeration of the system. However, if the volume is decreased too much at the shaking rate employed, then sloshing and frothing of the liquid appears to be detrimental to growth. Therefore, the 400 ml. is a compromise value for P. parasitica; it conceivably could be a lesser value for an organism like Fusarium which tolerates agitation.

One of the first problems encountered was a need for a simple procedure to kill the fungi; if this were not done, viable organisms could escape during handsheet preparation. Since the Phytophthoras were examined first and they are

important plant pathogens, it was necessary at the outset to control this hazard. The federal and state quarantine permit under which we obtained the Phytophthoras required that these organisms be destroyed before disposal. Fortunately, fungi are not as heat resistant as some bacteria and simple steam treatment met the objective. Incidentally, the other organisms studied are not under strict regulation like the Phytophthoras.

The results in Table III show that, in most cases, the addition of mycelia to the aspen pulp is much like a substitute for beating. Freeness drops and drainage resistance increases. The extreme case of this is P. debaryanum while M. rouxii and P. cinnamomi are least effective. Bear in mind that the control pulp for S. ferax and P. debaryanum is slightly different than that for the other organisms. The scanning electron micrographs of P. parasitica (Fig. 1) indicate the filamentous nature of the mycelia and an apparently bonded network with the larger aspen fibers. The small diameter of the mycelial filaments relative to wood fibers undoubtedly plays a role in resultant sheet properties.

The paper evaluation results (Fig. 2-6 and Table IV) suggest uniformity of all organisms with regard to a general loss of tearing strength as the percentage of mycelia is increased. Where bursting strength increases (F. sp. and S. ferax) there seems to be a corresponding definite increase in the stretch. On the other hand, where burst values are maintained or decline, the stretch data fluctuate and definite trends are not established. S. ferax is the only organism causing an increase in tensile strength; Fusarium shows little change while all other organisms lead to decreases in tensile. Porosity decreased rather rapidly for all mycelial additions except those of P. cinnamomi and M. rouxii which were poor performers in the strength tests. Basis weight values are quite uniform, but to insure this uniformity, it is important that the mycelia be steamed before washing

and determination of freeze-dried weights. Thickness, density, and moisture values seem to be consistent with other properties of the sheets. For example, where the porosity did not exhibit a rapid decrease (P. cinnamomi and M. rouxii), the sheet density decreased or held relatively constant; density increased in the four other cases.

Other interesting sheet properties were observed which are not evident from the data presented. Often sheets are characterized by a somewhat offensive odor which eventually dissipates upon storage. At the higher percentages of mycelia incorporation many handsheets become almost like glassine. Smoothness and opacity (light scattering) measurements would be of interest. Since initial tests aimed mainly at strength characteristics, an unbleached pulp was used for blending. By themselves (a 100% mycelial sheet has not been made in this project) or mixed with bleached wood pulp, the mycelial sheets should have desirable brightness characteristics.

Organisms included in this report fall into three general classes in terms of cell wall composition. The two Phytophthoras, Saprolegnia, and Pythium are all claimed to contain at least some cellulose in their walls, perhaps as high as 30-40%. Mucor belongs to a noncellulosic group where the biopolymers predominating are chitosan and chitin. Fusarium is a member of the higher fungi classed in the chitin-glucan group; most of this glucan appears to be other than β-1-4 linked although there is an unconfirmed report of cellulose in Fusarium (3). If one were to classify these organisms on the basis of their papermaking potential, the groupings might be somewhat different. For example, P. cinnamomi and S. ferax behaved quite differently in these tests even though one might expect similar performances. The pretreatment of these mycelia (steaming, washing, beating, etc.)

prior to handsheet formation must have some effect on their subsequent performance in paper evaluation. How significant such variables might be is not known now; we have processed all mycelia by a somewhat arbitrary regimen which might be improved or adjusted for individual cases. Note, for example, that at high percentages of Phytophthora incorporation, sheet formation appears uneven with blotches of material scattered throughout the handsheet; obviously this is contributing at least to data scatter if not to overall performance in paper evaluation tests. A very gentle dispersion method would be desirable. One might be tempted to single out S. ferax for further study based upon current observations; however, it must be remembered that this is the organism that required yeast extract for any appreciable growth, the benefit of which was not available to the other organisms studied. If the economics were right, it would appear that low levels (up to 20% or so with aspen kraft) of even the "weak" mycelia might be added to wood pulp paper without seriously affecting strength properties.

OUTLOOK

Certainly many possibilities for future investigation now present themselves. We have a representative of at least one more class of organisms which will be grown and tested soon under Phase I. Although currently of low priority, Phase I could be expanded to include more organisms related to those studied to date or even possibly to include other classes. It is now imperative to spend some time investigating the effects of pretreatments of the mycelia upon the quality of the resultant handsheets. Scanning electron microscopy may be of considerable value in this regard. Different control pulps may also be used for blending. Further tests such as brightness, opacity, and smoothness should be run on the handsheets plus perhaps other tests if suggested by cooperators; results of these additional tests may be of considerable interest and should be a first order of business in our own estimation.

Although it would be jumping ahead of the original timetable, some work on Phase II should begin soon. It is not known how much difficulty may be experienced in Phase II, so it would be desirable to get an early start on the waste effluent work. As stated earlier, it is rather pointless to conduct further growth rate and yield studies until we are working with an actual system. Therefore, some effluent from a neutral sulfite semichemical (NSSC) operation will be obtained in the near future to permit an assessment of what will be faced in Phase II proper.

Higher levels of mycelia incorporation, including 100% mycelial sheets, could be investigated. If there is only interest in strength properties, the data collected thus far indicate such efforts would be futile although investigation of pretreatment effects could change that outlook. Nevertheless, other properties might be examined more readily with pure mycelial sheets.

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THE INSTITUTE OF PAPER CHEMISTRY

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