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EFFECTS OF pH, TEMPERATURE, AND MINERAL NUTRITION ON  
CELLULOLYTIC FUNGI

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DURING THE LAST war with the gigantic military procurement of over 2½ billion square yards of cotton duck and other cloth by the United States Army alone (U. S. War Department, 1945) the prevention of the microbiological breakdown of cellulose assumed vast importance. Extensive research programs have since been initiated in many laboratories, particularly under the stimulus of the U. S. National Defense Research Committee, the U. S. Department of Agriculture, and the U. S. Army Quartermaster Corps (Heimsch, 1946; Siu, 1947).

One of the important preliminary questions is the effect of environmental and nutritional factors on fungi growing on cellulose. Heretofore, most of the physiological studies on fungi have been carried out using non-cellulose-destroying species growing on sugars as carbon source (Foster, 1939; Steinberg, 1939). The relationship between nitrogen and carbon assimilation in the formation of humus from cellulose in the soil have also been the subject of much research (Jensen, 1931; Waksman, 1938). Greathouse and Ames (1945) investigated the effects of different nitrogen sources for sixteen species of *Chaetomium* and Basu (1948) studied the same problem with *Aspergillus terreus*, *Aspergillus fumigatus*, *Penicillium islandicum*, *Stachybotrys atra*, and *Chaetomium indicum*. Although a few scattered studies exist on the mineral nutrition of cellulose-destroying fungi (Galloway, 1934; Greathouse *et al.*, 1942; Heukelekian and Waksman, 1925; Vartiavaara, 1935), there are no literature records of a complete survey of the effects of various minerals and environmental conditions on the growth of mesophilic fungi on cellulose. The present paper reports results of studies on representative cellulolytic fungi isolated from deteriorated cotton fabrics.

**EXPERIMENTAL METHODS AND RESULTS.**—*Fungi studied.*—Five species of fungi from the Quartermaster Culture Collection were used. These have been isolated from deteriorated cotton fabrics exposed in the tropics and are listed below in descending order of cellulolytic capacity: 1) *Myrothecium verrucaria* USDA 1334.2, 2) *Gliomastix convoluta* QM 4c, 3) *Curvularia lanata* QM 1204, 4) *Penicillium luteum* Aust. 41, and 5) *Aspergillus flavipes* Fla. A-14.

*Determination of cellulolytic activity.*—All determinations on the rate of cellulose decomposition were made with 4 oz. de-sized bleached cotton sheeting. This was cut into strips 3.0 cm. wide. The excess yarns were unravelled to a strip width

of 2.5 cm., and the strips finally cut into 7.5 cm. lengths.

Petri dishes were used as culture vessels. Two microscope slides were supported on the bottom of each culture dish on a U-shaped glass rod of 3 mm. diameter. This allowed the cloth to be wetted without being submerged when 20 ml. of liquid were added. Culture dishes thus equipped were sterilized in an electric oven at a temperature of at least 165°C. for a minimum of 3 hr. After cooling, autoclaved strips of cloth were transferred, one pair to each culture dish, by means of sterile forceps. To each dish were added 20 ml. of sterile medium pipetted from the sterile flask by means of the Brewer Automatic Pipette.

Spore suspensions containing about 100,000 spores per ml. were atomized over the surface of the cloth strips using air filtered through cotton plugs. Approximately 10,000 spores were delivered per dish.

After inoculation the plates were incubated at 29.5°C., except in those cases where the effects of temperature were being determined.

Harvesting was accomplished by first transferring the strips to 70 per cent ethanol for several minutes after which they were washed in distilled water, squeezed by hand to remove the excess water, and laid on paper toweling to dry. The dried strips were conditioned at 60 per cent relative humidity and 21°C. for 24 hr. and broken on a Scott Tester. The decrease in tensile strength of the cloth strips was taken as the index of cellulolytic activity. This loss in tensile strength has been shown to be closely correlated with the loss in weight and therefore is a good criterion of cellulolytic activity. The coefficient of variability of inoculated strips was about 10 per cent and of uninoculated controls about 8 per cent. Ten strips of cloth were used for each determination.

*Effect of pH of media.*—The respective media contained mineral salts in that concentration determined to be most favorable for the respective species of fungi in nutritional experiments where the pH was held between 5.8 and 6.5. The pH was adjusted by the addition of 0.5N NaOH and 0.5N HCl in this series.

For studies on the relation between cellulose degradation by *M. verrucaria* and cultural pH, the following salts were used per liter of medium:  $\text{NH}_4\text{NO}_3$ , 0.6 g.;  $\text{NaNO}_3$ , 6.46 g.;  $\text{KH}_2\text{PO}_4$ , 0.425 g.;  $\text{K}_2\text{HPO}_4$ , 0.164 g.;  $\text{Na}_2\text{HPO}_4$ , 0.975 g.;  $\text{NaH}_2\text{PO}_4$ , 1.149 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.246 g.;  $\text{MnSO}_4$ , 0.024 mg.;  $\text{ZnSO}_4$ , 5.00 mg.;  $\text{CuSO}_4$ , 0.005 mg.;  $\text{MnSO}_4$ , 0.055 mg.; and  $\text{FeSO}_4$ , 0.540 mg.

The percentage loss in cloth strength caused by *M. verrucaria* in this pH series is plotted in fig. 1. Similar results were obtained when pH was ad-

justed by means of phosphate buffers instead of by hydrochloric acid and sodium hydroxide.

*Curvularia lanata* was grown on a medium containing the following weights of salts per liter:  $\text{NH}_4\text{NO}_3$ , 0.6 g.;  $\text{NaNO}_3$ , 3.85 g.;  $\text{KH}_2\text{PO}_4$ , 1.70 g.;  $\text{K}_2\text{HPO}_4$ , 0.657 g.;  $\text{Na}_2\text{HPO}_4$ , 0.620 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.246 g.; and minor elements as in the previous medium. The purpose in using the relatively high concentration of sodium nitrate was to decrease the rate of change of pH in the medium especially in the alkaline range. A preliminary test had shown that when only half as much nitrate was present the pH had declined in 7 days from 7.4 to 6.7 vitiating the results of that test.

*Aspergillus flavipes* was grown on a medium similar to that used for *C. lanata* except that the sodium nitrate was present in only half the concentration.

For *G. convoluta* the following salts were used per liter:  $\text{KH}_2\text{PO}_4$ , 1.70 g.;  $\text{K}_2\text{HPO}_4$ , 1.28 g.; KCl, 0.95 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 g.; yeast extract, 1.0 g.; and minor elements as before.

*Gliomastix convoluta* showed by far the greatest tolerance of hydroxyl ions, being able to start growth and decompose cellulose at an initial pH of 11, which declined rapidly to 8.8. However, it is probable that if some method of maintaining a high pH were devised the growth might have been very slow.

*Effect of temperature.*—With the initial pH of the media adjusted at 6.35, four species of fungi were incubated at different temperatures. Results of the effects of temperature on the rate of cloth decomposition are presented in table 1.

TABLE 1. Effect of temperature on degradation of cellulose by fungi.

Organisms	Average percentage loss in breaking strength of cloth strips at					
	15°C.	19°C.	21°C.	25°C.	29°C.	35°C. 40°C.
<i>M. verrucaria</i> (9 days)	0	34	52	57	70	67
<i>G. convoluta</i> (8 days)	0	9	18	21	46	7
<i>C. lanata</i> (9 days)	3	10	24	23	56	7
<i>A. flavipes</i> (13 days)	5	11	19	30	44	15

*Effect of nitrogen.*—Media in these studies contained, besides the nitrogen source, 1.702 g.  $\text{KH}_2\text{PO}_4$ , 0.657 g.  $\text{K}_2\text{HPO}_4$ , 0.520 g.  $\text{Na}_2\text{HPO}_4$ , 0.25 g.  $\text{MgSO}_4$ , per liter and minor elements as in previous experiments.

The nitrogen content was first maintained constant while the proportion of nitrate to ammonium ion was varied by proper combinations of ammonium chloride, sodium nitrate, ammonium nitrate and ammonium phosphate. The total nitrogen content was 1.681 g. per liter. Fig. 2 gives

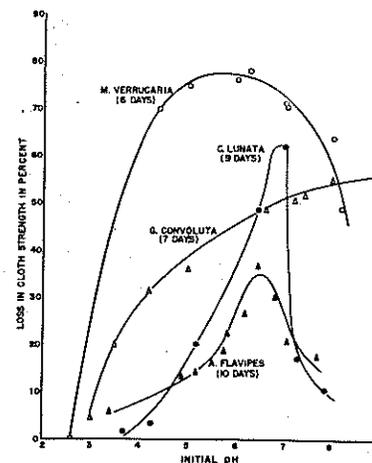


Fig. 1. Effect of pH on cellulolytic activity of fungi.

the concentrations of ammonium and nitrate ions in the respective solutions as well as their effect on the cellulolytic activity of fungi and the attendant changes in pH of the media.

Other experiments were conducted to determine the effect of variation in the concentration of ammonium nitrate. The same three fungi responded to differences in concentration of ammonium nitrate, as shown by results recorded in table 2.

Studies were also conducted to establish the optimal level of ammonium nitrate and sodium nitrate in the media. The two salts were added to different media in the same ratio in decreasing amounts. No substitutions of ions were made in these experiments. This resulted in a decrease of the total ionic concentration in the solution as smaller amounts of salts were used. Results are given in fig. 3.

*Effect of potassium ion concentration.*—In order to vary the potassium ion concentration without disturbing the concentration of other nutritional elements, sodium phosphates were used in equivalent concentrations in the low potassium media. Ten different concentrations of potassium ion were employed ranging from 0-3.128 g. per liter. From the solution containing the lowest concentration to that with the highest, the concentration was increased in a two to one progression. The concentration of the phosphate ion and the pH were maintained constant by combining the various sodium and potassium phosphates. The media all contained ammonium nitrate at 4.803 g. per liter, magnesium

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TABLE 2. Effect of ammonium nitrate concentration on cellulolytic activity of fungi.

Organism	Days growth	Loss in cloth strength in percentage in media with molar concentrations						
		0.0	0.0019	0.0037	0.0075	0.015	0.030	0.060
<i>M. verrucaria</i>	7	0	62	70	74	78	88	86
<i>C. lanata</i>	8	5	42	45	44	35	39	39
	8	-	-	-	66	52	46	49
<i>A. flavipes</i>	10	1	28	34	48	35	37	26

sulfate at 0.49 g. per liter, and the minor elements as previously used. The pH of the original solutions varied from 5.9-6.5 with most of the solutions approximating 6.2. At the end of the experiments, the final pH was, in most instances, not more than 0.1 lower than the original. In the few cases where the final pH was lower, it was still within the range of good growth for all the organisms. In no case was the pH a limiting factor in the rate of decomposition or the growth of the fungus. The results of these investigations are presented in fig. 4.

**Effect of magnesium ion concentration.**—Since

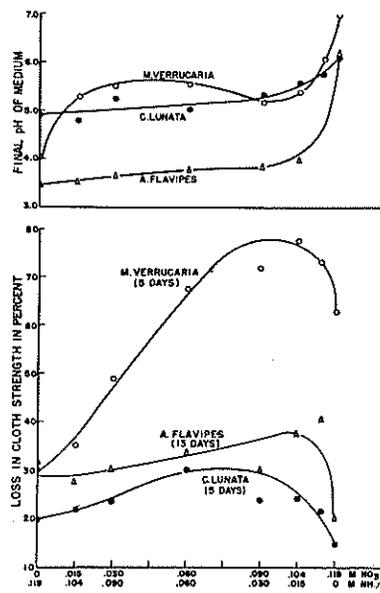


Fig. 2. Effect of nitrogen source on pH and cellulolytic activity of fungi.

magnesium was added to the medium as the sulfate ion when variation in concentration of this element was undertaken, it was necessary to make up the deficiency of the sulfate ion from another source. Sodium sulfate was used for this purpose. The concentrations of other nutrients per liter of media were:  $\text{KH}_2\text{PO}_4$ , 1.7 g.;  $\text{K}_2\text{HPO}_4$ , 0.657 g.;  $\text{Na}_2\text{HPO}_4$ , 0.520 g.;  $\text{NaNO}_3$ , 8.855 g.;  $\text{NH}_4\text{NO}_3$ , 0.60 g.; and minor elements as before. For *C. convoluta* 0.2 per cent yeast extract was used in place of the inorganic nitrogen salts. Sulfate was added to a total of 0.192 g. per liter.

No significant differences were observed in the loss of tensile strength of cloth strips exposed to

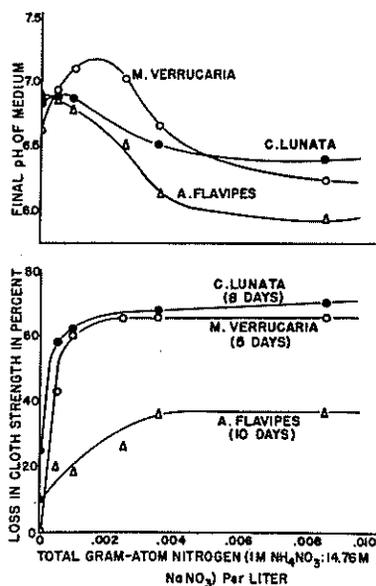


Fig. 3. Effect of nitrogen on cellulolytic activity of fungi.

attack by all five species of fungi supplied with varying amounts of magnesium from 0.0-0.073 g. per liter of medium. The amount of mycelial growth, however, was greatly influenced by the magnesium concentration. In lower amounts there was practically no visible mycelial growth at all. Only small bits of spindling mycelium and thin, short chlamydo-spores were seen on the cloth under the microscope. Despite this extremely reduced growth of the fungi, the rate of cellulolytic breakdown was just as rapid as that with samples in media containing higher concentrations of magnesium and luxuriant mycelial growth. There seems to be no direct relationship between mycelial growth and cellulolytic processes.

**Effect of phosphate ion concentration.**—Phosphate was supplied as the potassium hydrogen salt. In all cases, the potassium was maintained at a constant value. In order to reduce the concentration of phosphate ions with the potassium at a constant value, the level of  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$  was reduced through a substitution by KCl. In addition to these salts, the medium contained 0.06M  $\text{NH}_4\text{NO}_3$ ; 0.002M  $\text{MgSO}_4$ ; and minor elements. The effect of these various solutions are given in fig. 5.

**Effect of sulfate ion concentration.**—Sulfate was added to the medium as the magnesium salt. In order to determine the most favorable concentration and the effect of omission of this radical, it was necessary to substitute magnesium chloride for the magnesium sulfate in certain cases so as to main-

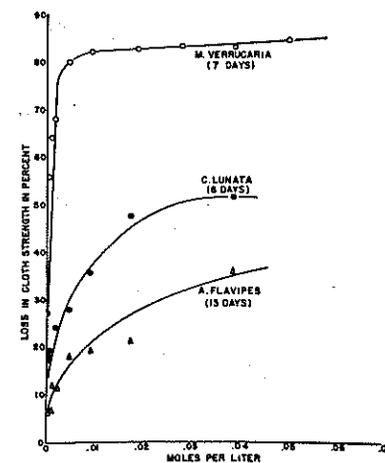


Fig. 5. Effect of phosphate on cellulolytic activity of fungi.

tain a constant concentration of magnesium. Where the sulfate ion was increased above the amount used in the basic solution, sodium sulfate was used as the substitution compound. The basic medium used for *M. verrucaria*, *C. lunata* and *A. flavipes* was constituted of the following ingredients per liter:  $\text{KH}_2\text{PO}_4$ , 1.78 g.;  $\text{K}_2\text{HPO}_4$ , 0.657 g.;  $\text{Na}_2\text{HPO}_4$ , 0.520 g.;  $\text{NH}_4\text{NO}_3$ , 0.60 g.;  $\text{NaNO}_3$ , 8.855 g.;  $\text{FeSO}_4$ , 0.540 mg.;  $\text{MnO}_2$ , 0.024 mg.;  $\text{CuSO}_4$ , 0.005 mg.;  $\text{MnSO}_4$ , 0.055 mg.;  $\text{ZnSO}_4$ , 5.00 mg. The medium used in the first experiment with *C. convoluta* contained yeast extract as the nitrogen source. As was the case with studies on other elements with this organism the results showed no significant differential response to the presence or absence of added sulfates. This was probably due to the sulfur present in the yeast extract. A second experiment was therefore performed in which the nitrogen source was glutamic acid instead of yeast extract. The medium for this second experiment with *C. convoluta* contained 1.7 g.  $\text{KH}_2\text{PO}_4$ , 1.28 g.  $\text{K}_2\text{HPO}_4$ , 0.54 g. KCl, 2.0 g. glutamic acid per liter, and minor elements as above. In all of the media used the magnesium content was kept at 0.0486 g. per liter. The response of the various species is plotted in fig. 6.

**Effect of organic compounds.**—The effects of an added source of carbon besides cellulose were studied with *M. verrucaria* using xylose, glucose, and cellobiose. The sugars were added to the mineral solutions and the loss in tensile strength

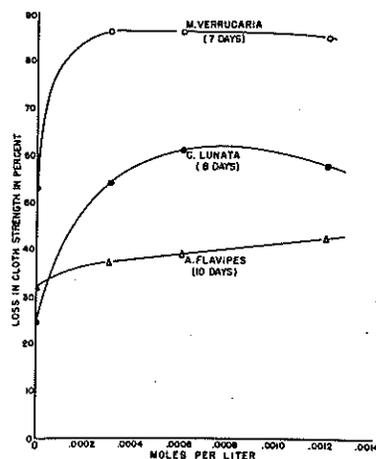


Fig. 4. Effect of potassium on cellulolytic activity of fungi.

TABLE 3. Cellulolytic activity of *M. verrucaria* in the presence of various concentrations of sugars.

No. of Days	No sugar		Xylose		Glucose		Cellulose	
	Repli- cation	% strength retention						
2	10	92	—	—	—	—	—	—
4	10	68	10	93	10	100	10	75
6	10	51	10	90	10	95	10	54
7	10	13	—	—	—	—	—	—
8	—	—	10	86	10	39	10	46
14	10	2	10	—	10	95	10	27
18	—	—	10	86	10	7	10	13
22	—	—	10	82	—	—	—	8
28	—	—	10	75	—	—	—	—

of the cloth attacked by the fungus growing at 30°C. on cloth in the various mineral-sugar solutions are given in table 3.

Similar experiments were carried out using 0.0, 0.01, 1.0 and 10.0 per cent sucrose solutions. At the same time a similar series of Petri dishes was prepared without the cloth strips. The mycelial growth in the latter set was measured after different periods of incubation. The rate of cellulolytic action and mycelial growth in these solutions are given in fig. 7.

Another interesting effect is that of organic nitrogen compounds, particularly in the case of *G. convoluta*. This organism has been largely omitted from previous discussions since it showed no response to any of the variants involving inorganic nitrogen to which it was subjected. To all media on which this fungus was grown, however, 2 g. of

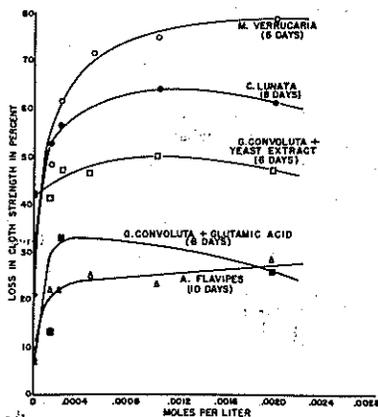


Fig. 6. Effect of sulphate on cellulolytic activity of fungi.

yeast extract per liter have been added. Apparently, this amount of yeast extract contained sufficient nitrogen to satisfy the nutritional requirements of the species as far as nitrogen is concerned. Inorganic nitrogen appears to be unavailable to this fungus since in the presence of inorganic nitrogen alone no cellulose breakdown occurred.

The effect of varying concentrations of yeast extract on the rate of cellulose decomposition of *G. convoluta* was studied with the results given in table 4.

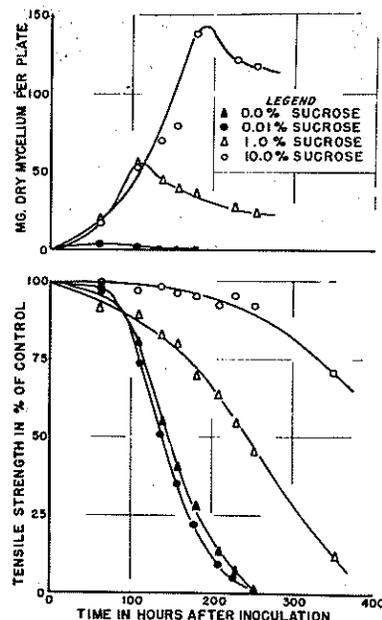
TABLE 4. Effect of yeast extract on degradation of cloth by *G. convoluta* in 7 days.

Yeast extract (per cent)	Loss in cloth strength (per cent)
0.2	35
0.1	37
0.05	21
0.025	23
0.0125	20
0.00625	13

In a study on the organic nitrogen requirements several sources of nitrogen were added to the medium to determine their availability. Media with different sources of organic nitrogen were divided into three parts. To these were added respectively, 1.0 g., 0.1 g. and 0.0 g. of yeast extract per liter. Table 5 shows the results.

TABLE 5. Relation of organic nitrogen to degradation of cloth by *G. convoluta* in 5 days.

Nitrogen source	g. per liter	Percentage loss in cloth strength with yeast extract		
		0.0 g./l.	0.01 g./l.	0.1 g./l.
Casein	1.2	—	—	—
hydrolysate	—	26	25	35
Peptone	1.25	23	26	44
Urea	0.433	10	24	28
Asparagine	0.943	24	27	36
Glutamic acid	2.1	29	32	26

Fig. 7. Effect of sucrose on rates of mycelial growth and cellulolytic activity of *M. verrucaria*.

From the results it is apparent that the nitrogen furnished by yeast extract may be at least partially replaced by other organic sources of nitrogen, such as glutamic acid.

Following the discovery that yeast extract was able to supply necessary nitrogenous substances for the development of this fungus, attempts were made to determine whether or not growth factors were also involved. Vitamins were first tested. Thiamin, riboflavin, pantothenic acid, and inositol were added individually and together without stimulating its development on filter paper on agar.

Biotin was tested singly against the three other fungi with the results given in table 6.

TABLE 6. Effect of biotin on cellulolytic activity of fungi.

Organism	Percentage loss in cloth strength in 7 days	
	2 gammas biotin/l.	No biotin
<i>M. verrucaria</i>	69	69
<i>C. lunata</i>	44	41
<i>A. flavipes</i>	30	18

## SUMMARY

The effects of pH, temperature, and various inorganic and organic compounds on the cellulolytic activity of four species of fungi were studied. *Gliomastix convoluta* proved distinctly different from the rest in its nutritional requirements. *Curvularia lunata* and *Aspergillus flavipes* resemble each other in general. *Mycrothecium verrucaria* was characterized by a uniformly high rate of cellulose decomposition between relatively wide limits of physical and nutritional conditions. The pH optima for the last three species fell within pH 6.0-7.0. Preliminary studies indicate a higher tolerance of hydroxyl ions by *G. convoluta*, which was able to initiate growth in media with an original pH of 11. The optimum temperature for the four species is about 29°C. With the possible exception of magnesium, omission of macro nutritional elements resulted in marked decrease in cellulolytic activity. Added magnesium was not found to be essential for the degradation of cellulose although mycelial growth was greatly suppressed by its absence. Sulfate was optimal at 0.0012 M, phosphate between 0.01-0.4 M, potassium at 0.0006 M and nitrogen at about 0.0036 M. In the last instance the molar ratios of ammonium to nitrate ions found best for *M. verrucaria*, *C. lunata*, and *A. flavipes* were 1:7, 1:1, 1:7, respectively. *Gliomastix convoluta* apparently requires nitrogen in an organic form, such as glutamic acid or yeast extract. The other three species were able to attack cotton fabric vigorously in an otherwise purely inorganic medium. Added biotin, however, stimulated the cellulolytic activity of *A. flavipes* in concentrations of 2 gammas per liter. The presence of soluble carbon sources, such as glucose, xylose, cellobiose, and sucrose, inhibited the simultaneous utilization of cellulose by *M. verrucaria*. It appears that the fungus does not degrade the cellulose until it is no longer capable of growing on the more easily accessible sugars as a source of carbon.

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