Table II. Comparison of Mole Fractions of Cations Obtained on Amberlite IR-120 Ion Exchange Resins After Equilibrating with Solutions with a Composition Calculated to Give a Resin with the Desired Mole Fraction of Cations

Ion	Mole Fraction Desired	Mole Fraction Obtained (Column) I	II	III
Na	0.10	0.113	0.101	0.103
K	0.41	0.461	0.418	0.450
Ca	0.44	0.390	0.428	0.398
Mg	0.05	0.036	0.052	0.049

Therefore;

$$\mu = C_{Na^+} + C_{K^+} + 3C_{Ca^{+2}} + 3C_{Mg^{+2}}$$

From Figure 2, the following equilibrium constants are obtained for reactions 1a through 1f, respectively: 1.20×10^2; 5.81×10^2; 2.34; 7.10×10^3; 3.20×10^2; and 2.35. Since millimoles are used, $\mu = 366$, or

$$366 = C_{Na^+} + C_{K^+} + 3C_{Ca^{+2}} + 3C_{Mg^{+2}}$$

$$K_{K^+}^{Na^+} = \frac{[X_{K^+}]_R[Na^+]}{[X_{Na^+}]_R[K^+]} = 2.34 \quad (3)$$

Substituting the desired mole fraction in equation 3,

$$\frac{0.44[N_a^+]}{0.10[K^+]} = 2.34$$

$$[Na^+] = 0.533[K^+]$$

By successive substitutions, a quadratic equation is obtained, the solution of which yields the concentrations desired in the charging solution. For example, considered here these values are:

Na = 79 millimoles per liter
K = 149 millimoles per liter
Ca = 36 millimoles per liter
Mg = 9.6 millimoles per liter

Table II shows the mole fractions obtained for three different columns which were charged with a solution having this composition.

This method of determining apparent equilibrium constants involving exchange reactions between a strong sulfonic acid resin and a solution of potassium, sodium, calcium, and magnesium chlorides is shown to be a function of ionic strength. If the equilibrium constants and the mole fraction of ions which is desired on the resin are known, it is possible to calculate the solution composition which will equilibrate the resin with the desired mole fraction. This procedure has application for charging a resin so that it will be in equilibrium with milk when it is desirable to remove specific cations, such as strontium-90 or other cationic radionuclides, while leaving the other components unchanged, or for similar research on other liquids. In applying this procedure to milk systems, the equilibrium ratio of cations on resin with those in milk must first be determined. At the normal pH of milk, about $^2/_3$ of calcium is bound, and therefore the total cationic ratio in milk is not a measure of the equilibrium values obtained for salt solutions. A future paper will consider the cationic composition of milk as affected by ion exchange resins.

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MUSHROOM CULTURE

Factors Affecting the Growth of Morel Mushroom Mycelium in Submerged Culture

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During the past decade, there has been considerable interest in the submerged culture production of mushroom mycelium for food and fodder. The potential value of mushroom mycelium as a source of protein has been pointed out in reviews by Block (*2*), and Robinson and Davidson (*17*). In addition, the mycelia of certain species of mushrooms have desirable flavors which make them more attractive as food than other microorganisms such as algae and yeasts proposed as sources of protein.

Gray and Bushnell (*10*) have studied the biosynthetic activities of a number of Ascomycetes and Basidiomycetes which included various species of mushrooms. Species of a number of genera of mushrooms have been grown successfully in submerged culture (*3, 5, 11–13, 15, 16, 19–21*). However, only *Agaricus campestris* (*11, 13, 19*), *A. blazei* (*2*), *Lepiota rachodes* (*19*), *Coprinus comatus* (*8*), and *Tricholoma nudum* (*15, 16*) of the Basidiomycetes, and *Morchella* (*17, 20, 21*) species of the Ascomycetes have been reported to have a satisfactory flavor. Of the *Morchella* spp. that have been studied in the authors' laboratory, *Morchella hortensis*, *M. esculenta*, and *M. crassipes* were found to have a desirable aroma and flavor. Some of the factors affecting the growth of these organisms in submerged culture have been investigated, and the results of this work are presented in this paper.

Materials and Methods

Cultures. The stock cultures of *Morchella crassipes* and *M. esculenta* were obtained by culturing spores from the ascocarps of those organisms as collected from natural habitats. These cultures were maintained on a synthetic medium and on a tryptone–glucose–yeast extract medium (TGYE) whose compositions are shown in Table I. Slants were inoculated with a portion of the mycelium, at least 0.5 sq. cm., to minimize the probability of selecting variant types. The synthetic agar cultures were incubated for 6 days and the TGYE cultures for 4 days at 25° C. and then were stored at 4° C. Fresh transfers were made every 2 weeks.

Cultural Procedures. The media used for shake flask cultures are shown in Table II. Glucose was autoclaved separately to prevent excessive browning which occurred when all ingredients were autoclaved together. Flasks were in-

Submerged culture growth of mycelia of *Morchella hortensis, M. crassipes,* and *M. esculenta* was investigated with glucose, maltose, or lactose as substrates in a corn steep liquor–ammonium phosphate basal medium. Similar yields of mycelium of all three organisms were obtained from glucose or maltose. Yields of *M. hortensis* and *M. crassipes* from lactose were comparable to those obtained from glucose or maltose; yields of *M. esculenta* from lactose were less than half of those obtained from glucose and maltose. The highest yields of *M. hortensis* from glucose were obtained with carbon to nitrogen ratios of 5:1 to 10:1 in the medium, pH 5.5 to 6.5, and an aeration rate of 0.08 mmole of oxygen per liter per minute. It was concluded that *M. hortensis* warrants further consideration for producing protein from wastes containing glucose, maltose, or lactose.

Table I. Composition of Stock Culture Media for Morel Mushrooms

Constituent	Quantity, Grams/Liter	
	Synthetic	TGYE
Glucose (anhydrous)	12.5	5.0
$(NH_4)_2HPO_4$	4.2	...
NH_4Cl	3.0	...
KH_2PO_4	0.5	...
Trace elements solution[a]	10.0 ml.	...
Tryptone (Difco)	...	10.0
Yeast extract (Difco)	...	5.0
Agar	15.0	15.0

[a] Trace elements solution contained per liter: 100 grams $MgSO_4.7H_2O$; 20 grams NaCl; 2.0 grams $CaCl_2$; 5.0 grams $MnSO_4.H_2O$; 0.50 gram $FeCl_3.6H_2O$; 0.005 gram $CuSO_4.5H_2O$; 0.15 gram $ZnCl_2$.

Table II. Composition of Shake Flask Media

Constituent	Quantity, Grams/Liter	
	Complex	Synthetic
Glucose	24.0	25.0
$(NH_4)_2HPO_4$	2.0	4.0
KH_2PO_4	...	1.0
Corn steep liquor	10.0	...
Trace elements solution[a]	...	10.0 ml.
pH adjusted to	6.5	6.5

[a] Trace elements solution as shown in Table I.

Figure 1. Culture vessel used for submerged growth of morel mycelium

oculated by transferring the entire growth from an agar slant (0.15 to 0.25 gram dry weight) to a sterile micro-Waring Blendor jar containing 50 ml. of sterile water and blending for 30 seconds. Each flask was inoculated with a 10-ml. sample of this suspension. Flasks containing synthetic medium were inoculated with cultures grown on synthetic agar. Flasks containing the complex medium were inoculated with cultures grown on TGYE agar. All flasks were incubated in triplicate on a rotary shaker at 120 oscillations per minute at 25° C.

In previous investigations in the authors' laboratory, impeller-agitated vessels were found to be unsatisfactory for submerged culture of morel mushrooms since the mycelium grew in amorphous strands around the impeller, eventually fouling it and obstructing uniform agitation and aeration. The culture vessel shown in Figure 1, consisting of a 2.5-gallon borosilicate glass bottle and a 2000-ml. Erlenmeyer flask, overcame these difficulties and was used for growing mushroom mycelium in larger quantities than possible in shake flasks.

The bottle was fitted with a No. 12 rubber stopper having 8-mm. I.D. stainless steel inlet and outlet tubes. The flask served as a reservoir for the sugar and calcium carbonate, thus allowing these ingredients to be autoclaved separately from the nitrogenous constituents. The neck of the flask and the outlet tube of the bottle were fitted with cotton air filters. All tubing connections and rubber stopper were clamped before autoclaving. The tubing connecting the flask and the bottle was closed with a pinch clamp to prevent backsiphoning of the medium from the bottle to the flask after autoclaving.

The protocol shown in Table III was followed in preparing media for the culture apparatus. The entire assembly was autoclaved for 30 minutes at 121° C. The final pH of the combined medium after autoclaving was 6.5.

The inoculum for the culture vessels was prepared in a similar manner as in the procedure used for shake flasks.

The growth in one shake flask was used to inoculate one culture vessel after comminution in a sterile Waring Blendor jar. The culture vessel was inoculated by removing the stopper from the Erlenmeyer flask and adding the entire inoculum slurry under aseptic conditions. The stopper was then replaced, and the flask was swirled vigorously to suspend the inoculum and the calcium carbonate. The contents of the flask were allowed to drain into the culture vessel by opening the pinch clamp on the outlet tube. Then, the tube was disconnected from the flask tubulature under aseptic conditions and attached to the cotton filter which was used previously to stopper the flask. This filter was then connected to a second sterile cotton filter which was in turn connected to the outlet of a needle valve on the laboratory air supply line. Any oil droplets in the compressed air supply were thus trapped in the first filter which allowed the second filter to remain fully effective in trapping airborne microorganisms throughout the growth period.

Table III. Media Used in Culture Vessels

Constituent	Quantity, Grams/Liter Flask	Quantity, Grams/Liter Bottle
Carbon source		
Glucose monohydrate (Cerelose)[a] or	175	...
Lactose monohydrate or	80	...
Maltose monohydrate	80	...
$CaCO_3$	14	...
$(NH_4)_2HPO_4$	...	14
Corn steep liquor[a]	...	70
Antifoam (Dow Corning AF)	...	3.5
Water, ml.	1000	6000

[a] Product of Corn Products Co., Argo, Ill.

Aeration Measurements. The volume of air supplied to each culture vessel per volume of medium was determined by attaching a C-Mar rotameter (The C-Mar Corp., Manasquan, N. J.) to the air outlet tube. Sulfite oxygen absorption values were determined by the procedure of Cooper, Fernstrom, and Miller (*6*), as modified by Corman *et al.* (*7*).

Analytical Procedures. Reducing content was determined by the Shaffer-Somogyi (*18*) copper-iodometric procedure, as modified by Neish (*14*) with glucose, maltose, or lactose as the standard depending upon the sugar used as the substrate in the culture medium. Nitrogen was determined by the official Kjeldahl procedure of the Association of Official Agricultural Chemists (*1*). Mycelial protein was assumed to contain 16% nitrogen, and consequently a factor of 6.25 was used to convert nitrogen values to protein content. Mycelial yield was determined as grams dry weight per 100 ml. by drying a washed mycelium sample for 4 hours in a forced draft oven at 105° C. This value was found to be equivalent to that obtained by drying the sample in a vacuum oven at 60° C. for 24 hours. The fat contents of samples of dried mycelium were determined by continuous ether extraction for 24 hours.

Results and Discussion

Effect of pH, Carbon to Nitrogen Ratio, and Aeration on Growth of *Morchella hortensis*. In these studies, *M. hortensis* was chosen as the organism for investigation since its growth characteristics had not been reported pre-

Table IV. Effect of pH on Growth of *Morchella hortensis* in a Complex Medium[a]

Initial pH	Final pH	Net Yield of Mycelium (Grams/Liter, Dry Basis)	Glucose Used, Grams/Liter	Yield, Grams Mycelium per 100 Grams Glucose: Used	Supplied
7.0	3.80	7.11	16.8	42.3	28.5
6.5	3.10	8.08	18.0	44.9	32.3
6.0	3.10	7.90	17.5	45.1	31.6
5.5	2.80	7.82	17.5	44.7	31.3
5.0	2.65	7.15	16.9	42.3	28.6
4.5	2.55	6.31	15.7	40.2	25.2
4.0	2.50	5.26	13.8	38.1	21.0
3.5	2.60	3.45	9.6	36.0	13.8
3.0	2.60	1.38	4.7	29.4	5.5
2.5	2.55	0.25	0.0	0.0	1.0

[a] Shake flasks were incubated for 6 days at 25° C. Values are averages of triplicate determinations.

Table V. Effect of Carbon to Nitrogen Ratio on Growth of *Morchella hortensis* in Synthetic and Complex Media[a]

Carbon to Nitrogen Ratio	Final pH: Synthetic medium	Final pH: Complex medium	Net Yield of Mycelium (Grams/Liter Dry Basis): Synthetic medium	Net Yield of Mycelium (Grams/Liter Dry Basis): Complex medium
30:1	5.10	3.80	2.05	4.90
25:1	4.80	3.70	3.20	5.25
20:1	4.00	3.40	3.65	6.14
15:1	3.65	3.10	4.60	6.62
10:1	3.45	3.05	4.95	7.97
5:1	3.40	3.00	5.11	8.12

[a] Flasks of synthetic medium were incubated for 8 days, flasks of complex medium for 6 days. Values are averages of triplicate determinations.

Table VI. Growth of Morel Mycelium in Culture Vessels with Glucose, Maltose, or Lactose as Substrate

Organism	Final pH	Net Yield of of Mycelium, Grams/Liter (Dry Basis)	Fat Content of Mycelium % (Dry Basis)	Protein Content of Mycelium % (Dry Basis)	Amount of Substrate Used, Grams/Liter	Yield of Mycelium, Grams/100 Grams of Substrate: Used	Supplied	Protein efficiency, Grams/100 Grams of Substrate: Used	Supplied
				Glucose					
M. crassipes	5.20	8.02	3.07	30.6	16.5	48.6	33.6	14.9	10.3
M. esculenta	5.20	7.85	1.88	31.1	16.3	48.1	32.8	15.0	10.2
M. hortensis	5.10	8.20	1.38	34.8	16.8	48.8	34.3	17.0	11.9
				Maltose					
M. crassipes	5.20	3.55	3.35	30.1	7.44	47.8	31.4	14.4	9.5
M. esculenta	5.30	3.40	1.32	29.6	7.20	47.5	30.1	14.1	8.9
M. hortensis	5.20	3.75	1.29	32.7	7.67	49.0	33.2	16.0	10.9
				Lactose					
M. crassipes	5.35	3.30	3.72	29.8	7.12	46.3	29.2	13.8	9.9
M. esculenta	5.90	1.65	1.93	30.0	3.80	43.4	14.6	13.0	4.4
M. hortensis	5.20	3.60	1.82	32.2	7.51	47.9	31.8	15.4	10.2

viously. Shake flasks of the complex medium with glucose as the substrate were adjusted to initial pH values ranging from 6.5 to 2.5 at 0.5 pH unit intervals. A temperature of 25° C. was used on the basis of the results of previous studies in the authors' laboratories.

Table IV shows that the net yield of mycelium was essentially constant over the initial pH range of 6.5 to 5.5. However, the yield dropped off sharply in the range of pH 4.5 to 3.0 and above pH 6.5. No growth took place at pH 2.5.

It is interesting to compare these results with those reported for *Tricholoma nudum* by Reusser *et al.* (*15*). These investigators obtained the highest yields of *T. nudum* mycelium at pH values of 4.5 and 5.0. The yields decreased on either side of this pH range to no growth at pH 2.0 and only slight growth at pH 6.5. Brock (*4*) obtained the highest yield of *M. esculenta* in a glucose–ammonium chloride medium at pH 8.4. However, this value was obtained with static cultures and under conditions that differed from those employed in the authors' work.

Reusser *et al.* (*15*) reported that the yield of *M. hybrida* Gray 149 practically doubled when the carbon to nitrogen (C:N) ratio decreased from 73.3:1 to 16:1. Accordingly, the effect of C:N ratio of the yields of *M. hortensis* in synthetic and complex media was investigated. On the basis of the results of the pH study, both types of medium were adjusted to an initial pH of 6.5.

Table V shows that yields of *M. hortensis* as a function of C:N ratios followed the same pattern in both synthetic and complex media. However, the yields in the synthetic medium were less than those obtained in the complex medium. The improved yields in the latter undoubtedly reflect the effects of vitamins and other growth factors in the corn steep liquor component. Yields increased markedly as the C:N ratio was decreased from 30:1 to 10:1. However, the values obtained with a C:N ratio of 5:1 were only slightly greater than those obtained with a C:N ratio of 10:1, and were probably not significantly different. These results are quite similar to those obtained by Reusser *et al.* (*15*) with *M. hybrida* and by Moustafa (*13*) with *Agaricus campestris*.

The results of previous studies gave a qualitative indication that intense aeration was undesirable in the submerged culture of morel mycelium since high aeration rates gave slimy filamentous growth rather than the desired round pellets as shown in Figure 2. To determine the effects of aeration rate on growth of *M. hortensis,* culture vessels of the glucose–corn steep liquor medium were inoculated and aerated at rates of 0.25, 0.50, and 0.75 liter of air per liter of medium per minute. These rates were equivalent to sulfite oxidation values of 0.08, 0.15, and 0.20 mmole of oxygen per liter per minute. All culture vessels were incubated at 25° C.

Identical yields of 47 grams of mycelium (dry weight) per 100 grams of glucose utilized were obtained with aeration rates of 0.08 and 0.15 mmole of oxygen per liter per minute, but only 31 grams of mycelium (dry weight) was obtained per 100 grams of glucose utilized at an aeration rate of 0.20 mmole of oxygen per liter per minute.

The mycelium produced at the 0.15 mmole aeration rate was somewhat filamentous as compared with the firm round pellets ranging from 0.5 to 0.8 cm. in diameter obtained at the 0.08 mmole aeration rate. The mycelium produced at the 0.20 mmole aeration rate was filamentous, slimy, and difficult to harvest.

Maximum yields were obtained at the time when the pH had reached its lowest point. This could be determined visually by observation of the clearing of the medium which occurred when all of the calcium carbonate and insoluble components of the corn steep liquor went into solution. Beyond this point, the pH began to rise and excessive foaming was observed, which could be attributed to the autolysis of the mycelium with the accompanying liberation of cellular proteins which have strong foam producing characteristics. Also, protein degradation products, such as basic amino acids and ammonia formed during autolysis, would tend to increase the pH of the medium.

Utilization of Glucose, Maltose, and Lactose by Morel Mushrooms. Szuecs (*20, 21*) and Brock (*4*) reported that *M. esculenta* grew well in both glucose and maltose media. Szuecs did not mention lactose utilization in his patent claims, and the strain studied by Brock did not utilize lactose to any appreciable extent. The authors have obtained strains of *M. esculenta* and *M. crassipes* from natural habitats which utilize lactose in addition to glucose and maltose. Cultures which do and do not utilize lactose can be derived from the same sporophore. The *M. hortensis* strain used in this study utilized lactose, but there is no evidence in the literature concerning the prevalence of this characteristic.

On the basis of these observations and the importance of glucose, maltose, and lactose as sugar components of agricultural and dairy wastes, it was considered desirable to investigate the utilization of these sugars by the three *Morchella* cultures in submerged culture. An aeration rate of 0.08 mmole per liter per minute was used throughout these investigations; the incubation

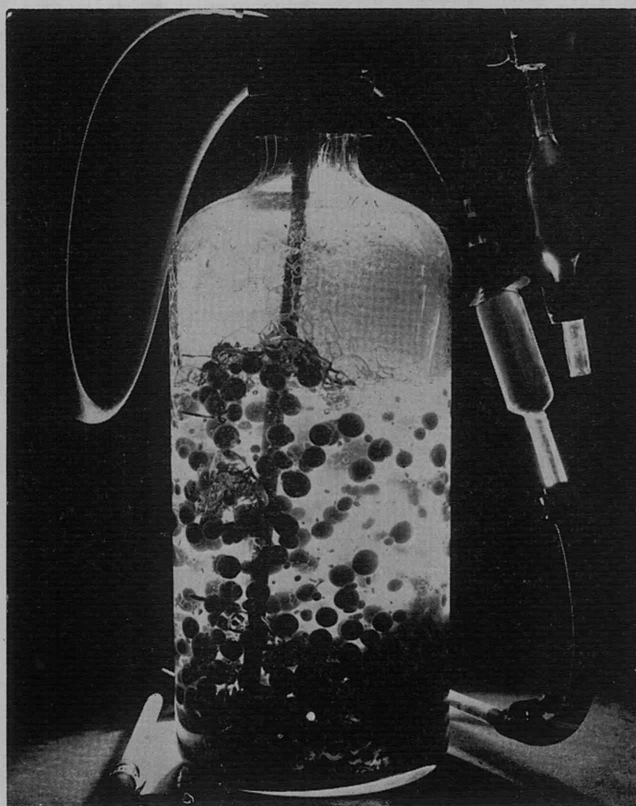

Figure 2. Submerged culture of *Morchella hortensis* showing mycelial pallets

temperature was 25° C. All results given in Table VI are averages of triplicate determinations.

The glucose medium used in the culture vessels was determined to have a C:N ratio of 9.75:1 on the basis of reducing sugar and nitrogen analyses. The nonreducing sugar carbon component of the corn steep liquor used was not considered in this calculation. To test the validity of this assumption, control culture vessels containing the basal growth medium without any sugar, were inoculated with the three morel cultures and aerated under the same conditions as culture vessels containing glucose. Yields of less than 0.5 gram of dry mycelium per liter were obtained in the corn steep liquor medium without glucose; these yields were less than 6% of the values obtained with glucose as the substrate.

The yields, protein contents, and fat contents of *M. crassipes*, *M. esculenta*, and *M. hortensis*, grown with glucose as the substrate, are shown in Table VI. *M. hortensis* grew at a slightly faster rate than *M. esculenta;* a 4.5-day incubation period was required for maximum yields of the former as compared with 5 days for the latter organism. *M. crassipes* required a 7-day incubation period at 25° C. for maximum yields.

Yields and protein contents of *M. esculenta* and *M. crassipes* were similar. Somewhat higher yields, protein contents, and efficiences of conversion of glucose to protein were obtained with *M. hortensis*. The latter values are comparable to those obtained by Reusser *et al.* (*15*) for *M. hybrida*. The protein contents should be multiplied by 1.36 if the value of 11.79% nitrogen determined for purified mushroom protein by Fitzpatrick *et al.* (*9*) is used. It was found that the protein content of *M. hortensis* increases with increasing nitrogen content of the medium, but approaches a limit of 37%.

As shown in Table VI, the patterns of maltose utilization by the three organisms were quite similar to those of glucose utilization. Comparable yields were obtained with both of these sugars, and growth rates were approximately the same.

M. hortensis utilized lactose to a greater extent than either *M. crassipes* or *M. esculenta* (Table VI). The significantly poorer yields of *M. esculenta* mycelium with lactose as the substrate would eliminate this organism from consideration for use in the treatment of wastes containing lactose, such as whey. Also, all three organisms grew more slowly in the lactose medium than in the glucose or maltose medium. *M. hortensis* grew at the fastest rate, requiring 5.5 days to produce a maximum yield of mycelium while *M. esculenta* and *M. crassipes* required 6 and 7.5 days, respectively, for maximum yields.

M. crassipes has the highest fat content of the three cultures regardless of substrate. All three organisms had higher fat contents when lactose was the substrate than when glucose or maltose were used.

Samples of the dry, powdered mycelium of the organisms described here have been subjected alone and in soup formulations to taste panel evaluation. The flavor of *M. esculenta* was the mildest and *M. crassipes* the strongest. However, the longer growth time required by *M. crassipes* as compared with *M. hortensis* makes the former less attractive economically than the latter, which has a pleasant flavor intermediate between the two extremes.

M. hortensis merits consideration as a means of producing protein from carbohydrate waste materials containing glucose, maltose, or lactose. The yields and efficiencies of conversion of sugar to protein by this organism approximate the values obtained with other fungi, and its pleasant aroma and flavor are other desirable attributes. Additional studies are in progress on the utilization of carbohydrate waste materials by *Morchella* species, and the results of this work will be presented in a subsequent report.

Acknowledgment

The *Morchella hortensis* culture used in this investigation was originally obtained from Joseph Szuecs.

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