

EFFECT OF NITROGEN SOURCES ON THE GROWTH AND PROTEIN PRODUCTION OF *COLLYBIA DIMINUTA*, *TRICHOLOMA LOBAYENSE* AND *OUDEMANSIELLA CANRII* IN SUBMERGED CULTURE

BY

A. K. SAHA AND N. SAMAJPATI

Mycology Laboratory, Department of Botany
University of Calcutta, Calcutta-700 019

Three species of edible mushrooms, *Collybia diminuta*, *Tricholoma lobayense* and *Oudemansiella canarii* were grown on defined liquid media containing different nitrogen sources separately to determine the optimal nitrogen source for growth and protein production. Yeast extract was found to be best nitrogenous source for the growth of three test mushrooms. *C. diminuta* and *O. canarii* showed the highest protein production in the 5-days old mycelia grown in the medium containing yeast extract and peptone respectively. Whereas, *T. lobayense* showed the highest protein production in 15-days old mycelia grown in the medium containing casein hydrolysate.

INTRODUCTION

Mushrooms can utilize different forms of inorganic, organic and complex nitrogenous compounds although they differ in their ability to utilize them. The different sources of nitrogen utilized by the mushrooms include urea (Humfeld, 1948; Humfeld and Sugihara, 1948), nitrates (Block *et al.*, 1953) and ammonium salt (Reusser *et al.*, 1958). Rangaswami (1956) reported that *Volvariella volvacea* grew best in peptone containing synthetic medium as nitrogen source. The organic nitrogen was better than inorganic nitrogen for the growth of *Pleurotus ostreatus* (Usef and Magid, 1967). Protein nitrogen (egg albumin) was the sole nitrogen source for the maximum mycelial growth of *Boletus luteus* (Luppi *et al.*, 1974-75). Safari and Geribova (1975) found that proline and asparagine were the best nitrogen sources for the 14 strains of eleven species of genus *Agaricus*, *Serpula lacrimans* grew better when both sucrose and aspartate were given in high concentration (Watkinson, 1975). Ghosh and Sengupta (1977) reported that *Volvariella volvacea* preferred most KNO_3 as the nitrogen source for its maximum mat yield. Data on the response of eight species of *Morchella* to thirty different sources of nitrogen were reported by Kaul (1977). Goto and Noriko (1978) reported the utilization of glutamate as a sole source of nitrogen by

Lentinus edodes. Duvnjak *et al.* (1980) observed that *Morchella hortensis* grew well in undiluted whey when sodium nitrate, ammonium chloride and ammonium sulphate were added. Peptone was the best source of nitrogen for the growth of both *Agaricus bitorquis* and *Pleurotus ostreatus* in submerged culture (Hong *et al.*, 1981). Koserice and Nabuo (1981) reported that peptone and yeast extract were the best nitrogen sources for the growth of morel mushrooms. Rao (1983) reported that L-asparagine was the best for growth of *Agaricus trisulphuratus* while yeast extract was the best for both *Rhodocybe subgliva* and *Agrocybe praecox*. In the present investigation an attempt has been made to find out the optimal nitrogen source for the growth and protein yield by the three edible mushrooms.

MATERIALS AND METHODS

Test Organisms

The tissue cultures of *Collybia diminuta* (Berk. & Br.) Sacc., *Tricholoma lobayense* Heim. and *Oudemansiella canarii* (Jungh.) Hohn were used in the present investigation.

Preparation of Inoculum :

A small portion of the actively growing mycelium from a agar slant of each test fungus was aseptically transferred to sterile Erlenmeyer flask (250 ml) containing 50 ml of glucose asparagine medium (Lilly and Barnett, 1951) and was incubated on a shaking incubator (120 r.p.m) at 30°C ($\pm 0.5^\circ\text{C}$) for 7 days in complete darkness. After 7 days, the mycelial mat was aseptically fragmented into small pieces with the help of a waring blender. The fragmented mycelium was washed several times with distilled water to remove any trace of adhering medium and then suspended in a phosphate buffer medium (pH 5.5) for 24 hours to overcome the shock encountered during blending. An aliquot of 1.0 ml of the mycelial cell suspension was used as inoculum.

Medium and growth conditions

To test the effect of different nitrogen sources, the standard glucose asparagine medium was first prepared without any source of nitrogen. After preparation, only one source of nitrogen was added to each set of medium in several flasks. The different organic nitrogen sources used in this experiment were glycine, β -alanine, L-arginine, DL-tryptophan, DL-methionine, L-asparagine and tyrosine while the inorganic sources were urea and ammonium nitrate. Among the complex

nitrogen sources used were peptone, casein hydrolysate and yeast extract. The yeast extract was made vitamin free by treating it with activated charcoal powder (5 g/l) before incorporating in the medium.

Each of the organic, inorganic and complex nitrogen sources added to the glucose-asparagine medium was as mg of nitrogen present per litre of original basal liquid medium. The pH of the media of the three test fungi, *C. diminuta*, *T. lobayense* and *O. canarii* were adjusted to their respective optimum values of 6.5, 6.5 and 6.0 with 0.2M phosphate buffer before sterilization. An aliquot of 50 ml of the medium was dispensed in each Erlenmeyer flask (250 ml), plugged and sterilized at 10 p.s.i. for 20 minutes. However, urea was sterilized by filtration. The flask without any source of nitrogen were treated as control. Sufficient number of flasks were prepared in order to have three replicates for each treatment. Each set of sterilized flask with one type of nitrogen source was inoculated separately with 1.0 ml of cell suspension of the mycelia of the three test fungi, *C. diminuta*, *T. lobayense* and *O. canarii* and incubated (stationary) separately in complete darkness for 5 days, 10 days, 15 days and 20 days at their respective optimal temperatures of 30°C, 20°C and 20°C ($\pm 0.5^\circ\text{C}$),

Table 1. Fungal mycelium (g Dw/L) produced on different nitrogen sources

Nitrogen source	<i>Collybia diminuta</i>	<i>Tricholoma lobayense</i>	<i>Oudemansiella canarii</i>	Average
Control	2.483	1.161	2.105	1.916
Glycine	12.488	2.471	4.000	6.320
β -Alanine	12.022	2.872	3.600	6.165
L-Arginine	8.480	1.748	3.250	4.493
DL-Tryptophan	7.253	1.780	3.219	4.084
DL-Methionine	9.782	3.118	4.033	5.644
L-Asparagine	13.047	2.636	4.004	6.562
Tyrosine	10.760	2.519	3.622	5.634
Ammonium nitrate	11.273	2.156	3.773	5.734
Urea	10.219	1.537	3.946	5.234
Casein hydrolysate	17.892	1.786	6.917	8.865
Peptone	17.947	3.939	7.658	9.848
Yeast extract	19.331	4.582	8.737	10.183
Average	11.767	2.485	4.527	

L. S. D. at 5% level Fungus (F) = 0.031 ; Nitrogen (T) = 0.064 ; F x T = 0.111

Table 2. *Effect of incubation period on production of fungal mycelium (g Dw/L)*

Incubation (days)	<i>Collybia diminuta</i>	<i>Tricholoma lobayense</i>	<i>Oudemansiella canarii</i>	Average
5	7.512	1.615	2.714	3.947
10	10.631	2.336	3.743	5.570
15	13.865	2.771	5.151	7.262
20	15.061	3.218	6.502	8.260
Average	11.767	2.485	4.527	

L. S. D. at 5% level Fungus (F) = 0.031 ; Incubation (I) = 0.036 ; F x I = 0.062

Table 3. *Data (g Dw/L) showing the interaction between different nitrogen sources and incubation period on the growth of the mushrooms*

Nitrogen source	5-days	10-days	15-days	20-days	Average
Control	1.808	2.374	2.127	1.356	1.916
Glycine	3.922	5.204	7.249	8.903	6.320
β -Alanine	4.012	5.351	6.990	8.304	6.165
L-Arginine	5.255	4.452	4.094	4.153	4.493
DL-Tryptophan	2.773	3.952	4.658	4.952	4.084
DL-Methionine	4.548	5.382	6.022	6.623	5.644
L-Asparagine	4.438	5.078	7.730	9.003	6.562
Tyrosine	2.543	4.540	7.074	8.377	5.634
Ammonium nitrate	3.551	5.153	6.822	7.407	5.734
Urea	2.579	4.358	6.122	7.876	5.234
Casein hydrolysate	3.998	7.508	11.279	12.674	8.865
Peptone	5.755	9.444	11.209	12.982	9.848
Yeast extract	6.110	9.616	13.033	14.775	10.883
Average	3.947	5.570	7.262	8.260	

L. S. D. at 5% level Nitrogen (T) = 0.064 ; Incubation (I) = 0.036 ; T x I = 0.129

Measurement of growth

Every fifth day, three flasks of each nitrogen source for each fungus were harvested. The medium and mycelium were separated by filtration through a tarred sintered funnel (Jena IG-3). The separated mycelium was washed free of medium and dried

Table 4. Data (mean)* showing the effect of different nitrogen sources on the protein content (%) of *Collybia diminuta*, *Tricholoma lobayense* and *Oudemansiella canarii* at different incubation periods under submerged condition

source	<i>Collybia diminuta</i>					<i>Tricholoma lobayense</i>					<i>Oudemansiella canarii</i>				
	5 Days	10 Days	15 Days	20 Days	5 Days	10 Days	15 Days	20 Days	5 Days	10 Days	15 Days	10 Days	5 Days	10 Days	15 Days
ne	9.855±0.07	8.440±0.05	7.140±0.08	5.140±0.07	16.375±0.05	14.930±0.11	10.150±0.13	8.390±0.06	13.578±0.04	10.264±0.08	8.760±0.05	10.264±0.08	13.578±0.04	10.264±0.08	8.760±0.05
ophan	19.230±0.09	18.520±0.10	16.185±0.05	13.930±0.08	26.718±0.12	25.185±0.14	24.528±0.09	23.652±0.07	20.673±0.08	15.330±0.09	13.145±0.10	15.330±0.09	20.673±0.08	15.330±0.09	13.145±0.10
ionine	12.955±0.12	10.330±0.08	8.568±0.07	6.750±0.09	18.140±0.10	15.955±0.09	12.855±0.08	10.570±0.10	19.350±0.10	16.080±0.14	14.185±0.10	16.080±0.14	19.350±0.10	16.080±0.14	14.185±0.10
ngine	18.710±0.11	16.695±0.14	13.170±0.15	11.593±0.10	26.280±0.15	22.778±0.12	16.425±0.10	13.578±0.12	21.180±0.05	16.644±0.08	14.518±0.12	16.644±0.08	21.180±0.05	16.644±0.08	14.518±0.12
	17.315±0.07	14.985±0.15	12.110±0.03	9.075±0.11	22.410±0.11	17.934±0.08	13.355±0.07	9.855±0.10	23.520±0.10	17.081±0.06	16.380±0.11	17.081±0.06	23.520±0.10	17.081±0.06	16.380±0.11
um	19.978±0.13	17.065±0.12	15.580±0.07	12.670±0.08	27.375±0.11	23.652±0.07	22.995±0.10	17.082±0.09	20.805±0.09	17.214±0.10	14.985±0.07	17.214±0.10	20.805±0.09	17.214±0.10	14.985±0.07
	17.390±0.11	15.330±0.07	11.815±0.09	8.295±0.05	22.338±0.09	15.330±0.07	10.512±0.15	9.884±0.01	15.264±0.14	9.885±0.05	8.090±0.10	9.885±0.05	15.264±0.14	9.885±0.05	8.090±0.10
	18.215±0.06	15.370±0.06	12.775±0.07	10.150±0.08	23.214±0.10	18.396±0.05	14.235±0.08	10.512±0.11	25.850±0.06	19.710±0.10	17.087±0.03	19.710±0.10	25.850±0.06	19.710±0.10	17.087±0.03
	21.375±0.14	19.873±0.07	15.320±0.09	12.730±0.08	37.230±0.07	35.040±0.10	28.470±0.05	20.385±0.09	35.610±0.10	29.915±0.07	26.718±0.05	29.915±0.07	35.610±0.10	29.915±0.07	26.718±0.05
	18.390±0.11	15.650±0.07	12.265±0.08	9.805±0.11	11.338±0.11	12.264±0.15	22.995±0.09	14.673±0.12	27.380±0.07	24.210±0.10	20.367±0.08	24.210±0.10	27.380±0.07	24.210±0.10	20.367±0.08
	24.095±0.13	23.510±0.08	20.710±0.11	18.325±0.15	21.024±0.17	31.755±0.14	39.420±0.11	30.660±0.05	32.420±0.04	30.660±0.15	28.900±0.10	30.660±0.15	32.420±0.04	30.660±0.15	28.900±0.10
ate	22.870±0.14	19.795±0.04	17.530±0.07	16.970±0.12	20.805±0.07	30.660±0.11	32.193±0.08	28.032±0.10	37.230±0.10	38.153±0.08	30.050±0.10	38.153±0.08	37.230±0.10	38.153±0.08	30.050±0.10
tract	25.150±0.13	24.075±0.08	22.430±0.11	19.475±0.09	28.396±0.10	30.718±0.06	32.412±0.10	27.375±0.14	29.127±0.12	28.404±0.10	26.681±0.09	28.404±0.10	29.127±0.12	28.404±0.10	26.681±0.09

are the average of three separate replicates.

to a constant weight in a vacuum oven at 60°C for 24 hours, cooled in a desiccator and weighed.

Measurement of Protein

Total nitrogen content of dried mycelium was determined colorimetrically (Folin & Wu 1919; Vogel 1961) using a photo-electric colorimeter (Model AE-II, Tokyo, Erma Optical Works Ltd., Japan). A conversion factor ($N \times 4.38$) was used to estimate mushroom protein content (Crisan and Sands 1978).

RESULTS AND DISCUSSION

All the tested nitrogenous sources had a stimulatory effect on the growth of each of the three test fungi but each test fungus individually showed a diverse nature of utilization of different nitrogen sources. Yeast extract when used as the sole nitrogenous source showed the optimum growth of each test fungus. DL-tryptophan showed the least effect on growth of *C. diminuta* and *O. canarii* whereas *T. lobayense* grew least in medium containing urea as nitrogen source (Table 1). Next to yeast extract, peptone and caesin hydrolysate were found to increase the mycelial yield of *C. diminuta* appreciably. Among the amino acids, L-Asparagine was best for growth and this might be due to its better buffering capacity. Glycine and β -alanine came next and which was followed by ammonium nitrate, tyrosine, urea, DL-methionine, L-arginine and DL-tryptophan. In case of *T. lobayense*, peptone was the best source after yeast extract which was followed by DL-methionine, β -alanine, L-asparagine, tyrosine, glycine, ammonium nitrate, caesin hydrolysate, DL-tryptophan, L-arginine and urea. In case of *O. canarii*, yeast extract was followed by peptone, caesin hydrolysate, DL-methionine, L-asparagine, glycine, urea, ammonium nitrate, tyrosine, β -alanine, L-arginine and DL-tryptophan. The statistical analysis of the data on the effect of nitrogen sources on the growth of all the test fungi were significant individually in comparison with the control (without any nitrogen source) at 5% level. The effects of incubation periods had a significant role on the growth of all the test fungi individually at 5% level (Table 2). Of the four incubation periods tried, 20 days was found to yield maximum amount of mycelial biomass in all the three test mushrooms. This was followed by 15 days, 10 days and 5 days in order of preference. The growth of *C. diminuta* was maximum in all the treatments and next to it was *O. canarii* followed by *T. lobayense*. It was also evident that all the tested nitrogen sources had a significant role on the growth of the test fungi in various incubation periods (5, 10, 15 and 20 days) at 5% level in comparison with the control (Table 3). In the present investigation the data revealed that in all the three test-mushrooms there is possibly

separate transport system for acid, basic and neutral amino acids (Pall, 1969 ; 1970 a,b).

The data in Table 4 revealed that all the tested nitrogenous sources had stimulatory effect on protein production although they showed a varied nature of protein production in different nitrogenous sources. *C. diminuta* and *O. canarii* showed their highest protein yield at 5 days old mycelia grown in medium containing yeast extract and peptone respectively. *T. lobayense* showed the highest yield of protein at 15 days old mycelium grown in medium containing casein hydrolysate as nitrogen source (Table 4). *C. diminuta* and *O. canarii* in all tested nitrogenous sources showed a gradual decline of protein yield from 5th day to 20th day. In these two mushrooms, the trend of protein production was same in all inorganic, organic and complex nitrogen sources tested although the individual treatment had differences in quantitative protein production among themselves. In *T. lobayense* exceptions were urea and other complex nitrogen sources where gradual rise of protein content was found from 5 days to 15 days after which a fall of protein content occurred in 20 days old mycelium. However, all the test fungi preferred most complex nitrogenous sources for protein production other than inorganic and organic nitrogenous sources.

The data of present investigation showed similarly with the findings of Kosaric and Nabuo (1981) on morel mushrooms and on *Rhodocybe subsliva* and *Agnocybe praecox*, reported by Rao (1983). Whereas, the present findings showed dissimilarly with Safari and Garibova (1975) on *Agaricus spp.* where protein and asparagine were the best nitrogen sources. The abilities of the test mushrooms to grow well on yeast extract, caesin hydrolysate and peptone showed that all the fungi were able to secrete proteases which could hydrolyzed these macromolecules into smaller subunits (Cohen, 1973 a, b). The hydrolysed products must had been further cleaved into individual amino acids by the extracellular proteases (Wolfenbarger and Marzluf, 1974 ; 1975) or proteases covalently bound to the cell wall (Frey and Roehm, 1978). The protein of these mushrooms could be utilized as a source of vegetable protein or supplemented with other food to combat the acute protein malnutrition.

ACKNOWLEDGEMENTS

The authors are grateful to Prof. A. K. Chandra, Head of the Department of Botany University of Calcutta, for his keen interest and also to the Department of Planning and Development, Govt. of West Bengal for the financial support.

REFERENCES

- Block, S. S., Stearn, T. W., Stephens, R. L. and McCandless, R. F. (1953). Mushroom mycelium experiments with submerged culture. *J. Agric. Food. Chem.* **1**, 890-891.
- Cohen, B. L. (1973a). The neutral and alkaline proteases of *Aspergillus nidulans*, *J. Gen. Microbiol.* **77**, 521-528.
- Cohen, B. L. (1973b). Regulation of intracellular and extracellular neutral and alkaline proteases in *Aspergillus nidulans*. *J. Gen. Microbiol.*, **79**, 311-320.
- Crisan, E. V. and Sands, A. (1978). Nutritional Values, in *The Biology and Cultivation of Edible Mushrooms*, (Eds. Chang, S. T. & Hayes, W. A.) Academic Press. Inc., London, pp. 137-168.
- Duvnjak, Z., Ivka, S. and Gavro, T. (1980). Effect of organic salts on the growth of the mushroom *Morehella hortensis*. *Acta. Bot. Croat.* **39**, 79-84.
- Folin, O. and Wu, H. (1919). Determination of non-protein nitrogen. *J. Biol. Chem.* **38**, 87-91.
- Frey, J. and Roehm, K. (1978). Subcellular localization and levels of amino peptidases and dipeptidase in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta*, **527**, 31-41.
- Ghosh, A. K. and Sengupta, S. (1977). Studies on biochemistry of higher fungi. I. Submerged growth of *Volvariella volvacea* in synthetic medium. *J. Fd. Sci. Technol.* **14**, 6-10.
- Goto, M. and Noriko, K. (1978). Utilization of glutamate as a sole source of nitrogen by the isolates of shiitake mushroom (*Lentinus edodes*) *Trans. Mycol. Soc. Japan.* **19**, 151-160.
- Hong, Jaesik, Kep Sang Lee and Dong Sung Choi (1983). Studies on Basidiomycetes - I. Mycelial growth of *Agaricus bitorquis* and *Pleurotus ostreatus*. *Korean J. Mycol.* **9**, 19-24.
- Humfeld, H. (1948). The production of mushroom mycelium (*Agaricus campestris*) in submerged culture. *Science*. **107**, 373.
- Humfeld, H. and Sugihara, T. F. (1949). Mushroom mycelium production by submerged propagation. *Food Tech.* **3**, 355-356.
- Kaul, T. N. (1977). Physiological studies on *Morehella* Sp. II. Nitrogen utilization. *Mushroom Journal.* **58**, 328-332.
- Kosaric, N. and Nabuo, M. (1981). Growth of morel mushroom mycelium in cheese whey. *J. Dairy Res.* **48**, 149-162.
- Lilly, V. G. and Barnett, H. L. (1951). *Physiology of Fungi*. McGraw Hill Book Co., London. 27.
- Luppi, M., Anna, M. and Anna, F. (1974-75). On protein nitrogen utilization of *Boletus luteus* L. *Allionia (Turin)*. **20**, 47-51.
- Pall, M. L. (1969). Amino acid transport in *Neurospora crassa*: I. Properties of two amino acid transport system. *Biochim. Biophys. Acta*, **173**, 113-127.
- Pall, M. L. (1970a). Amino acid transport in *Neurospora crassa*: II. Properties of a basic amino acid transport system. *Biochim. Biophys. Acta*, **203**, 139-149.
- Pall, M. L. (1970b). Amino acid transport in *Neurospora crassa*: III. Acidic amino acid transport. *Biochim. Biophys. Acta*. **211**, 513-520.
- Rangaswami, G. (1956). Studies on *Volvariella diplasia* Brek. & Br. the paddy straw mushroom. *Madras Agric. J.* **43**, 182-191.