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STUDIES ON EXTRA-CELLULAR CARBOXYMETHYL CELLULASE, β-GLUCOSIDASE AND AMYLASE PRODUCTION BY THREE EDIBLE MUSHROOMS

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The role of *p*H and incubation period on the production of extra-cellular CM-cellulase, β-glucosidase and amylase activity by the three edible mushrooms viz., ***Collybia diminuta, Tricholoma lobayense*** and ***Oudemansiella canarii*** in submerged culture were studied. The optimal *p*H and incubation periods for the production of CM-cellulase by ***C. diminuta, T. lobayense*** and ***O. canarii*** were 5.0 and 20 days, 4.0 and 20 days and 5.0 and 20 days respectively. The *p*H 6.0 for ***C diminuta*** and ***O. canarii*** and *p*H 5.0 for ***T. lobayense*** were the best for extra - cellular β-glucosidase synthesis. The optimal pH and incubation periods for the production of amylase by ***C. diminuta T. lobayense*** and ***O. canarii*** were 6.0 and 20 days, 4.0 and 20 days and 6.0 and 25 days respectively.

INTRODUCTION

Cellulose is one of the major constituents of plant cell walls which can be utilized as a renewable carbon source. The cellulose also available in large quantities in nature and it could be utilized for protein production by microbial processess.

Cellulolytic enzymes are a group of enzymes which acting together, hydrolyse cellulose. The cellulase activity resulted in cellobiose and/or probably in some oligosaccharides built up by three or more anhydroglucose units. The product (or products) in turn is splitted into glucose by β-glucosidase or cellobiase.

Starch is one of the most available natural polysaccharide which can be utilized in cultivation of mushrooms. A few works have been done regarding the starch splitting enzymes specially amylase in the mushrooms

The enzymatic activities of cellulase, β-glucosidase and amylase have been studied in several fungi by different investigators (Norkons 1957a, 1957b ; Mattison *et al.* 1966 ; Lee *et al.* 1967 ; Keilich *et al.* 1969, Heldt-Hansen *et al.* 1983, Bhumibhamon 1983, Hong *et al* 1984, Michelina and Castillo 1984).

Norkans (1957b) has studied the production of extracellular β-glucosidase by some strains of *Collybia velutipes* and have shown the stability of the enzyme at

various hydrogen-on concentrations, The β-glucosidase activity is not found to decrease after a treatment for 14 hours within a *p*H range of about 3.0 to 6.5 However, there has been a marked inactivation at *p*H 7.0 and complete inactivation is found at *p*H 2 0 and 8.0. Schwalb (1971) has stated that *Schizophyllum commune* is a potential source of amylase. Fedorou and Badyai (1973) have reported that maximum cellulolytic enzyme (C_1-C_2-Cx) activity of *Armillaria mellea* occurs at *p*H 4.6-5.6. El Zalaki *et al.* (1979) have studied the quantitative ability to hydrolyse starch by five varieties of mushrooms-*Absidia blakesleeana*, *Agaricus bisporus*, *Lentinus edodes*, *Peziza auluroaniv* and *Polyporus sulphureus*. According to Hong and Kim (1981) the maximal cellulase production from *Pleurotus ostreatus* 301 and *Lentinus edodes* 3-1 takes place at 30 and 35 days respectively. Mel'nichuk and Dynilyak (1981) have studied the β-glucosidase activity of some members of Agaricales species including *Flamulina velutipes* strain 10, *Pleurotus ostreatus* strain 10 and UVM, *Armillaria malea* strain fV1V and B-1 and *Lentinus tigrinus* strain 62. The optimal initial *p*H for production of cellulase and β-glucosidase has been found to be at *p*H 5 5 but the maximum production of the enzymes occur when *p*H is at 6.0 (Desai *at al* 1982). Madan and Bisaria (1983) have found that the *p*H and temperature optima for cellulase secred from *Pleurotus sajor-caju* are 5.0 and 45 C respectively. Hong *at al.* (1924) have isolated cellulase and β-glucosidase from *Pleurotus sajor-caju* and have noted that optimum *p*H and temperature for enzyme production are 7.0 and 35 C for C_1-cellulase, 5.0 and 26°C for Cx-cellulase and 7.0 and 20°C for β-glucosidase respectively.

In the present investigation an attempt has been made to study the effect of *p*H and incubation periods on the production of CM-cellulase, β-glucosidase and amylase in the culture filtrate of *C. diminuta*, *T. lobayense* and *C. canarii*.

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 Erlenmever 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 (±0.5°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 (*p*H 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 the inoculum.

Medium and growth conditions :

The glucose-asparagine medium (Lilly and Barnett 1951) was prepared first without any carbon source. Then CM-cellulose (CM-cellulose and β-glucosidase and soluble starch (amylase) were added separately as 1% (w/v) to the medium. With the aid of a mixed citrate-phosphate-borate buffer (Teorell and Stenhagen 1938) a wider *p*H range from *p*H 3.0 upto *p*H 8.0 at 1.0 unit interval were prepared for each carbon containing medium. The flask were then properly plugged and sterilized at 10 p.s·i for 20 minutes. Each set of flask with one *p*H grade was them separately inoculated with 1.0 ml of cell suspension of *C. diminuta*. *T. lobayense* and *C. canarii* and incubated (stationery) in complete darkness for 10. 15, 20, 25 and 30 days at 30 days at 30°C (± 0.05°C). Several such flasks were incubated in order to have three replicates for each text fungus.

Harvesting and crude enzyme preparation :

After the incubation periods, the mycelial mats were removed from the cellulose and starch cultures by filtering through a Jena Glass Filter (IG-3). The filtrate in each case was used in different enzyme assay experiment. The final *p*H of the filtrate was noted in each set.

Analytical procedure :

Standard accepted methods were used for determining the activities of CM-cellulase, β-glucosidase and amylase. The methods finally used were those which gave the most consistent results. CM-cellulase activity was measured by the use of dinitrosalicylic acid reagent for the determination of reducing sugar (Miller, 1972). β-glucosidase activity was measured by the method adopted by Hestrin *et al* (1955). Amylase activity was measured by the method of Bernfeld (1951).

Measurements were made in Specord UV/VIS spectrophotometer (Model Cl-24). In case of β-glucosidase activity, data are presented as change in O.D. value indicating the release of free nitrophenol which gives rise a yellow colour in alkaline solution from the substrate per minute at 420 nm. Here the β-glucosidase activities of *C. diminuta* *T. lobayense* and *C. canari* in culture filtrate were measured at their respective optimum incubation periods of 15 days, 20 days and 20 days.

RESULTS AND DISCUSSION

The experimental data are given in Tables 1, 2 and 3.

The data reveal that the three test fungi, *C. dimunita*, *T lobayense* and *C. canarii* are capable of synthesizing extra cellular cellulolytic enzymes, Among the three fungi tested, *C. canarii* has a very low cellulolytic activities in comparison with *C. diminuta* and *T. lobayense* The best source of carboxymethyl cellulase is *C. diminuta* followed by *T. lobayense* and *C. canarii*.

The extra-cellular CM-cellulase secreted by *C diminuta* in the culture media at 15 days growth and *p*H 4.0 (final *p*H 4.5) is found to be highest by the liberation of 0.800 mg of sugar pet ml of enzyme activity whereas the lowest activity is found at *p*H 9.0 (final *p*H 7.0) and 30 days growth by the liberation of 0.102 mg of sugar per ml of enzyme activity (Table 1).

In case of *T. lobayense*. the highest extra-cellular CM-cellulase activity is found at *p*H 4.0 (final *p*H 5.0) and 20 days incubation period by the liberation of 0.700 mg of sugar per ml of enzyme activity. But the lowest activity is found at *p*H 8.0 (final *p*H 7.5) and 10 days incubation period by the liberation of 0.025 mg of sugar activity (Table 1).

C. canarii shows the highest CM-cellulase activity at *p*H 5.0 (final *p*H 5 5) and 20 days incubation period by the liberation of 0.205 mg of sugar per ml of enzyme activity whereas ,the lowest activity is at *p*H 8.0 (final *p*H 7.5) and 10 days incubation period by ihe liberation of 0.015 mg of sugar per ml of enzyme activity (Table 1).

All the three test fungi favour the acidic *p*H for the better production of extra-cellular CM-cellulase than the alkaline *p*H.

The data obtained by the three test fungi strongly correlate with the findings of Fedorow *et al.* (1974) on *Armillaria mellea.* The results also coincide with the opinions of Madan and Bisaria (1983) and Hong *at al.* (1984) on *Pleurotus sajor-caju.*

With the crude enzyme sample, the highest extra-cellular β-glucosidase activities are found at *p*H 6.0, 5.0 and 6.0 for *C. diminuta*, *T. lobayense* and *C. canarii* respectively. In all the cases, almost inactivation of β-glucosidase activity is found at *p*H 8.0. All the three test fungi favour the initial medial *p*H range of 5.0-6.0 for the bettar production of extra-cellular β-glucosidase (Table 2). The results obtained by the three test fungi are very close to the findings of Norkans (1957b) on *Collybia velutipes.* The present findings also correlate with the opinion of Desai *at al* (1982). But Hong *at al.* (1984) have shown that *p*H optima of β-glucosidase is 7.0 for *P. sajor-caju.*

Table 1. *Data *(mg of sugar released per ml of enzyme) showing the effect of different H+ concentrations in the cultural media and incubation periods on the extra-cellular CM-cellulase activity of* C. diminuta, T. lobayense *and* O. canarii *in submerged culture*

	Initial pH	Final pH	10 days	Final pH	15 days	Final pH	20 days	Final pH	25 days	Final pH	30 days
C. diminuta	3.0	4.0	0.400±0.008	4.0	0.735±0.005	3.8	0.505±0.008	4.0	0.455±0.007	4.0	0.300±0.002
	4.0	4.5	0.420±0.007	4.5	0.800±0.008	4.0	0.555±0.003	4.5	0.440±0.015	4.5	0.315±0.005
	5.0	5.0	0.440±0.010	5.0	0.650±0.007	4.5	0.750±0.004	5.0	0.615±0.010	5.0	0.340±0.004
	6.0	5.5	0.351±0.004	5 5	0.420±0.010	5.0	0.655±0.007	5.5	0.455±0.007	5.0	0.355±0.003
	7.0	6.5	0.200±0.006	6.0	0.255±0.006	6.0	0.351±0.005	6.5	0.440±0.004	6.0	0.340±0.010
	8.0	7.0	0.125±0.007	6.5	0.200±0.002	7.0	0.315±0.004	7.0	0.351±0.006	7.0	0.120±0.002
T. lobayense	3.0	4.0	0.240±0.010	4.5	0.255±0.005	4.6	0.400±0.008	4.0	0.600±0.008	4.0	0.400±0.005
	4.0	4.5	0.225±0.005	5.0	0.600±0 008	5.0	0.700±0.007	4.5	0.650±0.007	5.0	0.550±0.003
	5.0	5.5	0.155±0.008	6.0	0.540±0.007	5.5	0.550±0.005	5.0	0.455±0.005	5.5	0.450±0.005
	6.0	6.0	0.105±0.003	6.5	0.150±0.004	6.8	0.255±0.006	6.0	0.265±0.004	6.0	0.305±0.004
	7.0	6.5	0.050±0 002	7.0	0.050±0.001	7.0	0.05 ±0.003	7.0	0.200±0.002	6.5	0.255±0.003
	8.0	7.5	0.025±0.007	7.5	0.025±0.001	7.5	0.030±0.002	7.2	0.155±0.002	7.0	0.200±0.001
O. canarii	3.0	4.0	0.100±0.001	4.0	0.155±0.002	4.5	0.100±0 005	4.0	0.050±0.004	4.0	0.045±0.005
	4.0	4.5	0.150±0.002	4.5	0.200±0 004	5.0	0.105±0.006	4.5	0.052±0.001	4.5	0.050±0.004
	5.0	5.0	0.110±0.003	5.0	0.150±0.002	5.5	0.205±0.004	5.0	0.150±0.002	5.0	0.150±0.003
	6.0	5.5	0.050±0.004	5.5	0.100±0.001	6.0	0.104±0.003	5.5	0.105±0.004	5.5	0.100±0.002
	7.0	6.5	0 025±0.001	6.5	0.050±0.002	6.5	0.055±0.001	6.0	0.101±0.002	6.0	0.090±0.001
	8.0	7.5	0.015±0 002	7.5	0.015±0.001	7.0	0.050±0.001	7.0	0.051±0.001	7.0	0.055±0.001

* Results are the average of three replicas.

Table 2. *Data (O.D. value) showing the effect of different H+ concentration in the culture media of* C. diminuta, T. lobavense *and* O. caparii *on the β-glucosidase activity at their respective optimum incubation periods*

Initial *p*H	Final *p*H	*Collybia diminuta* (15 days) O.D. value	Final *p*H	*Tricholoma lobayense* (20 days) O.D. value	Final *p*H	*Oudemansiella canarii* (20 days) O.D. value
3.0	4.5	0.203+0 005	4.5	0.478+0.004	4.5	0.342+0.003
4.0	4.8	0.287+0.008	5.0	0.503+0.007	4.8	0.404+0.004
5.0	5.0	0 327+0.003	6.0	0.645+0.006	5.0	0.457+0.005
6.0	5.5	0.850+0 010	6.5	0.312+0.005	5.5	0.499+0.008
7.0	6.0	0 530+0.003	7.0	0.105+0 002	6.0	0 209+0.006
8.0	7.0	0.069+0.001	7.5	0 010+0.001	7.0	0 031+0.002

Results are the average of three replicates.

The data from the Table 3 reveal that all the three test fungi are capable of synthesizing extra-cellular amylase enzyme. Among the three fungi tested. *C. diminuta* has the highest ability to degrade starch followed by *O. canarii* and *T. lobayense*.

The extra-cellular amylase synthesized by *C. diminuta* in the culture media at 20 days growth and *p*H 6.0 (final *p*H 5.0) is found to be highest by the liberation of 6.600 mg of sugar per ml of engyme activity. The lowest activity is found at *p*H 8.0 (final *p*H 6.5) and 10 days growth by the liberation of 1.520 mg of sugar per ml of enzyme activity (Table 3).

In case of *T. lobaynse* the highest extra-cellular amylase activity is found at *p*H 4.0 (final *p*H 4.0) and 15 days incubation period by the liberation of 5.280 mg of sugar per ml of enzyme activity Whereas, the lowest activity is found at pH 8.0 (final pH 7 3) and 10 days incubation period by the liberation of 0.220 mg of sugar per ml of enzyme (Table 3).

O. canarii shows the optimum extra-cellular amylase activity at pH 6.0 (final pH 5 0) and 25 days incubation period by the liberation of 6.000 mg of sugar per ml enzyme activity. The lowest activity is found at *p*H 8.0 (final *p*H 7.0) and 10 days incubation period by the liberation of 0.80 mg of sugar per ml enzyme activity (Table 3).

Very little works have been done on amylase activity of the higher fungi. The optimal pH for amylase production by *C. diminuta* and *O. canarii* correlate with the findings of Michelena and Castillo (1984) on *Aspergillus foetidus*. The pH optima for *T. lobayense* also similar with the findings of Bhumibhamon (1983) on several spp of *Aspergillus*.

Among the three fungi tested, it may be concluded that *C. diminuta* has the

Table 3. *Data *(mg of sugar released per ml of enzyme) showing the effect of different H+ concentrations in the culture media and incubation periods on the extra-cellular amylase activity of* C. diminuta, T. lobayense *and* O. canarii *in submerged culture*

	Initial *p*H	Final *p*H	10 days	Final *p*H	15 days	Final *p*H	20 days	Final *p*H	25 days	Final *p*H	30 days
C. diminuta	3.0	4.5	5.680±0.018	4.0	3.280±0.010	4.0	2.920±0.015	4.0	2.800±0.070	4.0	2.280±0.010
	4.0	4.5	6.200±0.030	4.3	4.080±0.020	4.0	4.400±0.080	4.5	3.480±0.030	4.5	2.800±0.030
	5.0	5.0	2.400±0.050	4.5	5.680±0.050	4.5	5.780±0.050	5.0	5.120±0.050	5.0	4.960±0.015
	6.0	5.5	2.000±0.040	5.0	5.120±0.060	5.0	6.600±0.070	5.5	6.000±0.040	5.5	5.120±0.017
	7.0	6.0	1.600±0.030	5.5	3.840±0.040	5.5	6.200±0.065	6.0	5.680±0.015	6.0	3.640±0.020
	8.0	6.5	1.520±0.070	6.0	2.400±0.010	6.0	6.000±0.020	6.3	5.520±0.020	6.5	2.120±0.020
T. lobayense	3.0	4.0	1.600±0.015	3.5	2.000±0.010	3.5	1.640±0.055	3.5	1.600±0.015	3.5	0.920±0.010
	4.0	4.3	3.400±0.025	4.0	5.280±0.025	4.0	2.800±0.030	4.0	2.200±0.016	3.8	1.080±0.030
	5.0	5.3	3.280±0.050	5.0	4.400±0.010	4.5	3.400±0.050	4.5	2.520±0.030	4.5	1.280±0.012
	6.0	5.8	1.800±0.010	6.0	3.680±0.050	5.0	2.000±0.070	5.0	1.800±0.040	5.5	1.640±0.050
	7.0	6.5	1.440±0.050	7.0	1.600±0.020	6.5	1.650±0.030	6.5	1.700±0.020	6.0	2.400±0.040
	8.0	7.3	0.220±0.003	7.5	1.160±0.010	6.5	1.280±0.040	6.5	1.440±0.020	6.0	1.600±0.030
O. canarii	3.0	4.0	1.820±0.060	4.0	1.920±0.030	4.0	2.120±0 050	3.5	3.120±0.055	3.5	1.280±0.010
	4.0	4.3	2.080±0.080	4.5	2.400±0 040	4.5	3.120±0.040	4.0	3.960±0.070	4.0	1.440±0.025
	5.0	5.0	1.160±0.030	5.0	2.000±0.050	5.0	2.400±0.018	4.5	5.320±0.080	4.5	2.050±0.010
	6.0	5.7	1.000±0.002	6.0	1.280±0.060	5.5	2.200±0.020	5.0	6.000±0.095	5.0	2.400±0.060
	7.0	6.5	0.880±0.010	7.0	0.980±0.010	6.0	1.440±0.015	5.5	3.600±0.070	5.5	1.600±0.030
	8.0	7.0	0.800±0.030	7.2	0.950±0.004	6.5	1.050+0.003	6.0	1.980±0.010	5.5	1.120±0.020

* Results are the average of three replicates.

highest ability to utilize cellulose and starch followed by *T. lobayense* (except the amylase activity) and *O. canarii*. It is clear from the above mentioned data that all the three fungi may be grown on ligno-cellulosic waste materials such as wood, saw-dust, straw and other agricultural plant residues.

REFERENCES

Bernfeld, P. (1951). Enzymes of starch degradation and synthesis. *Advances Enzymol. Related Subj. Biochem.* **12** : 379-428.

Bhumibhamon, Orapin (1983). Production of amyloglucosidase by submerged culture. *Thai J. Agric. Sci.* **16** (3) : 173-184.

Desai, J. D., Desai, A. J. and Patel, N. P. (1982). Production of cellulases and β-glucosidase by shake culture of *Scytalidium lignicola*. *J. Ferment. Technol.* **60** (2) : 117-124.

El Zalaki, M. Ezmat and M. A. Hamzt (1979). Edible mushrooms as producers of amylases. *Food Chem.* **4** (3) : 203-212.

Federov, N. I. and Badyai, S. V. (1973). Cellulolytic activity of *Armillaria mellea* (Fr.) Karst. *Prikl. Biokhim. Mikrobiol.* **9** (3) : 408-413.

Heldt-Hansen, Hans Peter, Aunstrup, Kund (1983). Acid-stableamylase composition and it use. *D. K. Appl.* **83 4** : 3.2.

Hestrin, S., Feingold, D S and Schramm, M. (1955). *Mcthods in Enzymology* **1** : 231.

Hong, Jai Sik and Kim, Dong Han (1981). Studies on the enzymes produced by Basidiomycetes. Part 1. The production of crude enzymes. *Hanguk. Nonghwa Hakhoe Chi* **24** (1) : 7-14.

Hong, J. S., Uhm, T. B., Jung, G. T. and Lee, K. B. (1984). Studies on the enzyme produced by *Pleurotus sajor-caju* (I) - the production of cellulolytic enzymes. *Han'guk Kyunhakhoe chi* **12** (2) : 59-64.

Keilich, G., Baily, P. J., Afting, E. G. and Liese, W. (1969). Cellulase (β - 1, 4-glucan 4 glucanohydrolase) from the wood degrading fungus *Polyporus schweinitizii* Fr. II. Characterization. *Biochim. Biophys. Acta.* **185** (2) : 392-401.

Lee, Ernest, Y. C., Larry D., Nielson, Edmond, H. Fischer (1967). A new glycogen-debranching enzyme system in yeast *[Saccharomyces cerevisiae]*. *Arch. Biochem. Biophys.* **121** (1) : 245-246.

Lilly, V. G. and Barnett, H. L. (1951). *Physiology of Fungi.* McGraw Hill Book Co., London, pp. 427.

Madan, M. and Bisaria, R. (1983). Cellulolytic enzymes from an edible mushroom, *Pleurotus sajor-caju*. *Biotechnol. Lett.* **5** (9) : 601-604.

Mattison, N. L., Nizkovskya, O. P. and Sivochub, O.A. (1966). The cellulase activity of culture fluid filtrates of *Fomitopsis pinicola* [Products of biosynthesis of higher fungi and their use]. Nauka : Moscow - Leningrad 21-28, 1966. From *Ref. Zn. Biol.* 1967 No. 4V161

Mel'nichuk, G. G. and Dynilyak, M. I. (1981). Dynamics of the activity of exocellular hydrolases of some species of Agricales mushrooms. *Ukr. Bot. Zn.* **38** (4) : 55-58.

Michelena, V. V., Castillo, F. J. (1984). Production of amylase by *Aspergillus foetidus* on rice flour medium and characterization of the enzyme. *J. Appl. Bacteriol.* **56** (3) : 395-407.

Miller, G. L. (1972). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31** : 426-428.

Norkans, B. (1957a). Studies of β-glucoside and cellulose splitting enzymes from *Polyporus annosus* Fr. *Physiol. Plant* **10** : 198-214.

Norkans, B. (1957b). Studies of β-glucoside and cellulose splitting enzymes from different strains of *Collybia velutipes*. *Physiol. plant* **10** : 454-466.

Schwalb, Narvin N. (1971). Developmental regulation of amylase activity during fruiting of *Schizophyllum commune*. *J. Bacteriol.* **108** (3) : 1205-1209.

Teorell, T. and Stenhagen, E. (1938). Ein Universalpuffer fur den *p*H - Bereich 2.0 bis 12.0. *Biochem. Zeitschr.* **299** : 416.