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Studies on submerged fermentation of *Pleurotus tuber-regium* (Fr.) Singer. Part 2: effect of carbon-to-nitrogen ratio of the culture medium on the content and composition of the mycelial dietary fibre

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Abstract

The effects of different carbon/nitrogen (C/N) ratios in liquid media with equal amounts of total organic matter used for submerged fermentation, on the yield of biomass, total dietary fibre (TDF) content and monosaccharide composition of the mycelium of *Pleurotus tuber-regium* were studied. The mycelial growth was best at a C/N ratio ranging from 18:1 to 36:1 with an optimum value at 24:1. On increasing the C/N ratio of the medium, both the amount of TDF and chitin content in the mycelium of *P. tuber-regium* decreased while glucan content increased. These results indicate that the proportions of the nitrogen and carbon source in a fermentation medium affect the biosynthesis of cell wall non-starch polysaccharide in mushroom mycelium. The potential use of the mycelium of *P. tuber-regium* produced by submerged fermentation, as a high fibre food ingredient, is discussed.

Keywords: *Pleurotus tuber-regium*; Mushroom; Submerged fermentation; Carbon/nitrogen ratio; Total dietary fibre; Monosaccharide composition

1. Introduction

The sclerotia and fruit bodies of the mushroom, *Pleurotus tuber-regium* (Fr.) Singer are nutritional foods (Ude, Ezenwugo, & Agu, 2001; Zoberi, 1973) and have been used in the treatment of many diseases, such as constipation, stomach pain, fever, and colds (Oso, 1977). Notably, this edible mushroom has an exceptionally high total dietary fibre content of over 80% in its sclerotial form (Cheung & Lee, 1998) which is a potential source of fibre for functional foods (Blackwood, Salter, Dettmar, & Chaplin, 2000). But cultivation of the sclerotia and fruit bodies takes about 4–6 months by the conventional solid compost method with a resulting variable quality (Fasidi & Ekuere, 1993; Huang, Guo, & Huang, 1996). It is generally recognized that growing mushroom mycelium in a defined medium by submerged fermentation is a rapid alternative method to obtain fungal biomass of consistent quality (Litchfield, 1967). Only a few studies on submerged fermentation of *P. tuber-regium* have been reported (Fasidi & Olorunmaiye, 1994; Oso, 1977). Our laboratories have recently reported the physical and chemical factors affecting the rate of mycelial growth and bioconversion efficiency of *P. tuber-regium* using submerged fermentation (Wu, Cheung, Wong, & Huang, 2003). Our previous study has shown that, among other things, the best carbon and nitrogen sources in the liquid culture media were monosaccharides (including glucose) and yeast extract, respectively (Wu et al., 2003). Fasidi and Olorunmaiye (1994) had reported that a C/N ratio of 4:1 seemed to sustain the best growth of *P. tuber-regium* mycelium but only with a yield of 0.2 g mycelial dry weight/100 ml liquid medium. However, it is still not known whether a wider range of C/N ratios might increase the yield and affect the chemical composition of the mycelium grown in the liquid medium, especially its content of dietary fibre and monosaccharides. In the present work, the submerged fermentation of mycelium of *P. tuber-regium* was investigated in media with

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different C/N ratios, ranging from 6:1 to 96:1, and the total dietary fibre contents as well as the monosaccharide composition of the mycelium, were determined.

2. Materials and methods

2.1. General

A culture of *P. tuber-regium* mycelium was provided by the Sanming Mycological Institute in the Fujian Province of Mainland China and maintained in potato dextrose agar (PDA) (Difco Laboratories). The optimal submerged fermentation conditions used were set according to our previous study (Wu et al., 2003) as follows: chemical composition of the basal medium was 30 g glucose, 4 g yeast extract, 1 g KH_2PO_4, 0.6 g $MgSO_47H_2O$/l of distilled water; 10 ml of mycelial inoculum/l of medium; orbital shaking speed of 200 ± 10 rpm at 30 ± 1 °C; 100 ml of medium/250 ml conical flask.

2.2. Effect of C/N ratio of liquid medium on mycelial growth

Based on the chemical composition of the basal medium as mentioned above, seven liquid media with different C/N ratios but the same total organic matter, were prepared, as shown in Table 1. Harvesting period was set at the 20th day, based on previous results (Wu et al., 2003). The mycelium in the conical flasks (in triplicate) was separated from the remaining liquid medium by centrifugation for 10 min at 10 °C and 4800 rpm, washed and freeze-dried to obtain the yield of biomass.

2.3. Effect of C/N ratio of liquid medium on the total dietary fibre content of mycelium

The content of total dietary fibre (TDF) in the mycelial samples collected in 2.1 was determined according to the enzymatic–gravimetric method (AOAC, 1995). In brief, mycelial samples (1 g of dry matter) were suspended in 50 ml 0.08 M sodium phosphate buffer (pH 6.0) and treated with two amylases, a heat-stable bacterial α-amylase (EC 3.2.1.1 from *Bacillus licheniformis*, catalogue no. A3306, Sigma Chemical Co., St. Louis, MO) for 30 min in a boiling water bath and a fungal amyloglucosidase (EC 3.2.1.3 from *Aspergillus niger*, catalogue no. A3513, Sigma) for 30 min at 60 °C to remove glycogen and a bacterial protease (from *Subtilisin carlsberg*, catalogue no. P3910, Sigma) to solubilize protein. The enzyme-treated mixture, containing the buffer solution and non-digestible materials, was precipitated with four volumes of 95% ethanol. The ethanol-insoluble residue was filtered with a Fiber-Tec System (Tecator 1023, Hoganas, Sweden). The residue recovered was washed successively with 78% ethanol (3×20 ml), 95% ethanol (2×10 ml), and acetone (2×10 ml), and oven-dried at 105 °C overnight to give the gravimetric yield of the mycelial TDF.

The weight of mycelial TDF was corrected for the content of ash and residual protein, as well as non-protein nitrogen, contributed by chitin as described previously (Cheung, 1996). The content of chitin in the mycelial TDF was calculated from the amount of glucosamime quantified by gas chromatography. The non-protein nitrogen content in the mycelial TDF was determined by dividing the content of chitin by a factor of 14.5, based on the fact that there is 6.9% N in the chitin. Residual protein was then calculated from the corrected nitrogen content (total nitrogen content −non-protein nitrogen content) multiplied by a factor of 6.25. The total nitrogen content in the mycelial TDF was determined by a CHNS/O Analyser (Perkin-Elmer 2400, CT). Ash content in the mycelial TDF was determined by the AOAC dry-ashing procedure (AOAC, 1995).

The corrected TDF content of the mycelium was calculated as follows:

Table 1
The chemical composition of the liquid media with different C/N ratios for the submerged fermentation of *P. tuber-regium* mycelium

Medium	Glucose (g/l)	C%[a]	Yeast extract (g/l)	N%[b]	C/N ratio	Total organic matter (g/l)[c]
PTR-6	22.1	8.85	11.9	1.47	6:1	34.0
PTR-12	26.9	10.8	7.10	0.88	12:1	34.0
PTR-18	28.8	11.5	5.17	0.64	18:1	34.0
PTR-24	30.0	12.0	4.00	0.50	24:1	34.0
PTR-36	31.2	12.5	2.80	0.35	36:1	34.0
PTR-48	31.8	12.7	2.15	0.27	48:1	34.0
PTR-96	32.9	13.3	1.10	0.14	96:1	34.0

[a] The carbon content of glucose was 40% based on the chemical formula of $C_6H_{12}O_6$. C (g/l) equals the amount of glucose (g/l)×40%.
[b] The nitrogen content of the yeast extract was determined by the Kjeldahl method to be 12.4%. N (g/l) equals amount of yeast extract (g/l)×12.41%.
[c] Total organic matter represents the total dry weight (g) of glucose and yeast extract in 1 l of medium.

$$\text{Corrected TDF \%} = \text{Mycelial TDF \%} \times[1 - \text{Ash \%} - 6.25 \times(N_{\text{total}}\ \% - N_{\text{chitin}}\ \%)]$$

2.4. Monosaccharide composition of mycelial TDF

The mycelial TDF was subjected to sequential acid hydrolysis (12 M sulfuric acid for 1 h, 30 °C, and then 2 M sulfuric acid for 1 h in a boiling water bath). Alditol acetates of the neutral and amino sugars in the acid hydrolysate were prepared according to the method described by Blakeney, Harris, Henry, and Stone (1983) with β-D-allose as the internal standard. Alditol acetates of the monosaccharides in the mycelial TDF were quantified by an HP6890 Series II gas chromatograph, using an Alltech DB-225 capillary column (15×0.25 mm i.d., 0.25 μm film) and the following oven temperature programme: initial temperature, 180 °C and hold time 4 min; temperature rise at 2 °C/min to 220 °C and final hold for 25 min. The carrier gas was helium and detection was by flame ionization. The amount of monosaccharides was expressed as polysaccharide residues (anhydrosugars) by multiplying the amounts of pentoses and deoxypentoses by a factor of 0.88 and 0.89, respectively and of hexoses by a factor of 0.90. Individual monosaccharides were corrected for losses during acid hydrolysis and derivatization, as well as the detector response.

2.5. Statistical analysis

Mean values of mycelial yield obtained from media with different C/N ratios (Fig. 2) were analysed by one-way ANOVA and tests of significant differences were determined by Turkey-HSD at $P<0.05$ (SPSS 9.0, 1999).

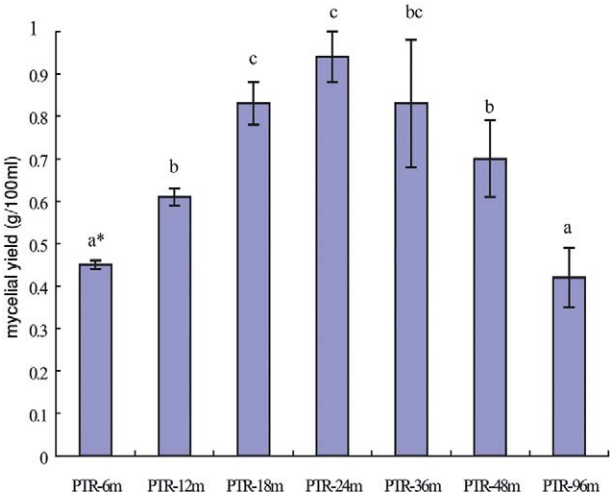

Fig. 1. The mycelia yield of *P. tuber-regium* on media with different C/N ratios on 20th day after inoculation. *Columns with different letters represent significant difference at $P<0.05$ (one-way ANOVA, Tukey-HSD).

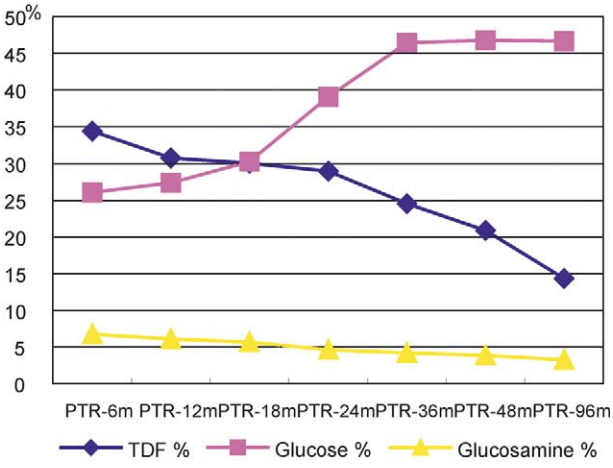

Fig. 2. The TDF content and amounts of glucose and glucosamine in mycelium of *P. tuber-regium* from media with different C/N ratios.

3. Results and discussion

Our previous study had shown that the growth curves of the mushroom mycelia were typical logarithmic functions with a maximum yield of biomass on the 20th day of fermentation (Wu et al., 2003). In the present study, the growth curves of mycelium in liquid media with different C/N ratios also showed a similar trend (data not shown). The yield of mycelium of *P. tuber-regium* grown in liquid media with different C/N ratios for a period of 20 days is shown in Fig. 1. An optimum C/N ratio of 24:1 was observed and a very high or low C/N ratio resulted in relatively lower yield of mycelial biomass (Fig. 1). However, the yield of mycelium at the C/N ratio of 24:1 was not statistically different from that of the C/N ratio of 18:1 and 36:1 (Fig. 1). This result showed that the C/N tolerable limit for the mycelial growth of *P. tuber-regium* was not too narrow. The present optimum C/N ratio of 24:1 represented 30 g glucose and 4 g yeast extract/litre of liquid medium and the yield of mycelial biomass of the medium with this C/N ratio was consistent with our previous result (Wu et al., 2003). A previous study showed that submerged fermentation of the mycelium of *P. tuber-regium* with an optimum C/N ratio of 4:1, using glucose and yeast extract as the sole carbon and nitrogen sources, gave a much lower yield of the mycelial biomass (0.2 g dry wt./100 ml of liquid medium) (Fasidi & Olorunmaiye, 1994) than our results (0.94 g dry wt./100 ml). This suggests that, among other chemical and physical factors, the C/N ratio is another important one that needs to be optimized in the growth of the mushroom mycelium.

The total dietary fibre (TDF) content of the mycelium grown in media with different C/N ratios is shown in

Table 2. Mycelial TDF content increased with a lower C/N ratio which suggested that a relatively larger amount of yeast extract (hence more nitrogenous material) in the medium was crucial to the biosynthesis of fungal cell wall materials, leading to a higher TDF content in the mycelium. The amount of mycelial TDF obtained from a low C/N ratio of 6:1 was twice more than that obtained from a C/N ratio of 96:1. On the other hand, a larger amount of glucose in the fermentation medium (higher C/N ratio) did not increase the TDF content in mycelium but increased the proportion of glucose residue in the TDF (Table 3). The C/N ratio of the fermentation medium also affected the relative amounts of other monosaccharide residues in the mycelial TDF. A relatively higher proportion of mannose and galactose as well as a higher level of glucosamine in mycelial TDF grown in media with lower C/N ratios was observed (Table 3). The major components of fungal TDF were non-starch cell wall polysaccharides, which include β-glucans, chitin, and mannans (Bartnicki-Garcia, 1968; Cheung & Lee, 1998). The principal monsaccharide found in the mycelial TDF of *P. tuber-regium* was glucose (66.3–84.5%), which indicated the presence of glucans. This was followed by glucosamine (5.96–17.2%), and mannose (4.36–8.66%), which implied the presence of chitin and mannans. In general, the monosaccharide profiles of the mycelial TDF obtained by submerged fermentation in media with different C/N ratios were consistent with our previous results (Cheung & Lee, 1998). Although the reason why a lower C/N ratio would increase the amount of TDF in the mycelial cell wall is unclear, the fact that less glucose and more nitrogen in the growing medium affected the amounts of glucans and chitin was very obvious, as shown in Fig. 2.

4. Conclusions

In liquid meda with equal amounts of total organic matter, *P. tuber-regium* mycelium grew best at a C/N ratio ranging from 18:1 to 36:1. The C/N ratio seems not only to affect the amount of TDF but also the non-starch polysaccharide components in the mycelium of *P. tuber-regium*. Besides its sclerotia and fruit bodies, the mycelia of *P. tuber-regium* produced by submerged fermentation are also rich in TDF (especially those that were cultivated at a low C/N ratio), offering potential for use as a food ingredient because of its efficiency and controllable quality. Investigations of the nutritional value and functional properties of this fungal mycelium are under way.

Table 2
Correction for non-protein nitrogen and ash content in the mycelial TDF of *P. tuber-regium* obtained by the AOAC method[a]

Mycelial TDF	Mycelial TDF (% dry matter of mycelium)	Chitin content[b]	Chitin N[c]	Total N[d]	Net protein N[e]	Ash content[f]	Corrected mycelial TDF[g]
PTR-6m	71.5	6.78	0.47	3.66	3.19	10.9	49.5
PTR-12m	62.9	6.13	0.42	3.96	3.54	9.83	42.8
PTR-18m	60.6	5.96	0.39	3.83	3.44	8.99	42.1
PTR-24m	58.6	4.61	0.32	3.26	2.94	8.67	42.8
PTR-36m	47.8	4.20	0.29	2.25	1.96	6.59	38.8
PTR-48m	41.9	3.89	0.27	2.21	1.94	7.95	33.5
PTR-96m	30.1	3.29	0.33	2.03	1.70	9.86	23.9

[a] Mean value of two measurements expressed as percent dry matter of TDF except mycelial TDF.
[b] Chitin content is the % dry matter of glucosamine in the mycelial TDF.
[c] Chitin content divided by 14.5.
[d] Nitrogen content of mycelial TDF as determined by a CHN/S elemental analyser.
[e] Net protein content = total N−chitin N.
[f] %dry matter of mycelial TDF.
[g] Corrected TDF content in mycelium was calculated according to the formula shown in Section 2.3.

Table 3
Monosaccharide composition of mycelial TDF of *P. tuber-regium* expressed as percentage of total polysaccharide sugars[a]

Mycelial TDF	Fucose	Xylose	Mannose	Galactose	Glucose	Glucosamine
PTR-6m	0.94	1.27	8.66	5.62	66.3	17.2
PTR-12m	0.98	1.58	6.28	4.55	70.7	15.8
PTR-18m	1.04	1.76	6.55	3.68	73.2	13.8
PTR-24m	0.92	1.65	5.56	2.87	79.6	9.38
PTR-36m	0.96	1.57	4.76	2.37	82.8	7.49
PTR-48m	0.81	1.52	4.51	2.37	83.8	6.97
PTR-96m	0.90	1.43	4.36	2.94	84.5	5.96

[a] Mean value of two measurements.

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