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Effects of Varying Nutritional and Cultural Conditions on Growth of the Ectomycorrhizal Fungus *Pisolithus tinctorius* SMF

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The culture conditions and nutritional requirements for enhanced mycelial growth of the ectomycorrhizal fungus *P. tinctorius* SMF were determined in flask scale experiments. Optimum culture conditions for growth of *P. tinctorius* SMF in a further modified Melin-Norkrans broth were as follows; temperature 25~27°C, agitation 120 rpm, and pH 4.0. *P. tinctorius* SMF utilized various carbon sources including monosaccharides, disaccharides, and polysaccharides. D-Glucose and mannitol were respectively the first and second most suitable carbon sources for mycelial growth. With D-Glucose as the principal carbon source, supplementation of modified Melin-Norkrans (MMN) broth with Lysine (800 mg/*l*), Glutamic Acid (500 mg/*l*), or Proline (50 mg/*l*) enhanced mycelial yields 63%, 34%, and 22% respectively as compared to growth in medium lacking amino acids. Thiamin·HCl+biotin+pyridoxine supplementation also enhanced growth. As compared to mycelial growth in the MMN medium, growth of *P. tinctorius* SMF was enhanced 120% in MMN broth when the carbon/nitrogen ratio was 25/1 in citrate buffer at pH 4.5, and growth was 50% greater in MMN broth of carbon/nitrogen ratio with a 10/1~20/1 without using the buffer. Standard conditions established for growth of *P. tinctorius* SMF in MMN broth were 25~27°C, agitation 120 rpm, buffered to pH 4.0 with citrate, in MMN medium containing 10 g/*l* D-glucose supplemented with 800 mg/*l* lysine. In this medium the carbon/nitrogen ratio was 20/1~25/1, and the maximal mycelial yield ($Y_{x/s}$) was 0.472 (4.72 mg/m*l*) after 7 days of incubation, as compared to 0.214 (2.14 mg/m*l*), when the fungus was grown in standard MMN broth.

For tree species of pine, an inoculum consisting of vegetative mycelia of the ectomycorrhizal fungus *P. tinctorius* has proven to more fully colonize seedlings and to better stimulate seedling growth than an inoculum of basidiospores (10). In another study, mycelial inoculum produced nearly twice as many ectomycorrhizae as did a spore inoculum during colonization studies (12). Recently, it was shown that *P. tinctorius* mycelium can readily be produced by submerged culture fermentation (21). Different *P. tinctorius* strains have been reported to exhibit growth temperature optima varying between 21 and 32℃ (1, 4, 11, 19). In general, ectomycorrhizal fungi are acidophilic (16). Culture pH typically drops to values below 3 in minimal salts-glucose media lacking buffer, probably due to uptake of ammonium ions at an excessive rate as compared to anions (5). Such pH drops can inhibit growth before exhaustion of the carbon supply (16). More dry weight growth of the ectomycorrhizal fungus *C. geophilum* in pure culture was observed (2) when the nutrient solution was buffered to maintain a pH above 5 versus the use of unbuffered solution where pH dropped to values as low as 2.7. There have also been studies on the effects of different carbohydrates, amino acids, vitamins and other factors on the growth of ectomycorrhizal fungi in culture (3, 6, 8, 14, 15, 17). Laboratory studies with *P. tinctorius* characteristically report the growth period for maximal mycelial growth of this organism requires an incubation period of up to several weeks (7, 8, 13). The objective of this study

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was to determine the nutritional requirements and optimal culture conditions for *P. tinctorius* SMF growing in mycelial cultures. Another objective was to increase the biomass yield as a percentage of available carbon source, while achieving these yields over incubation times of days rather than weeks.

MATERIALS AND METHODS

Microorganism

P. tinctorius SMF (21) was incubated and maintained on Potato Dextrose Agar (PDA) plates (pH 5.6~5.7) at 25℃ in darkness.

Preparation of Stock Culture

Mycelium of *P. tinctorius* SMF, previously grown on PDA plates, was inoculated into 1.0 liter baffled flasks containing 300 m*l* further modified Melin-Norkrans (FMMN) (21) liquid medium (pH 5.5~5.7). After inoculation, flasks were incubated with shaking at 120 rpm and 25℃ for 4 days in darkness. The final pH of this stock culture was typically pH 2.9. All following studies (see below) used this mycelium as inoculum.

Optimum Conditions for the Mycelial Growth of *P. tinctorius* SMF

The culture conditions optimal for mycelial growth of *P. tinctorius* SMF were determined in flask cultures by varying cultural parameters in an organized set of experiments. Parameters examined included 1) growth temperature (22℃ to 30℃ in FMMN medium supplemented with 10.0 g/*l* of glucose or mannitol), 2) initial medium pH (3.0 to 7.0, at 1.0 unit intervals in FMMN medium), and 3) agitation rate (105 to 150 rpm; 100 m*l* FMMN medium in 250 m*l* baffled flasks; pH 5.5~5.7; 25℃; 7 days incubation; in a rotary shaker). Incubation temperature effects on growth of *P. tinctorius* SMF was first determined on the PDA plates (15~37℃) for 30 days, and then further investigated at incubation temperatures between 22℃ and 30℃ for 7 days in the FMMN broth.

In all of these experiments, cultures were typically grown in 250 m*l* flasks using 100 m*l* of medium and 10 m*l* of mycelial inoculum. Incubation times were for 7 days at 25℃ (except for the temperature study), pH 5.5~5.7 (except for the pH study), shaking at 120 rpm (except for the agitation study). Mycelial growth was measured by recovering, washing, drying, and weighing the biomass present in each flask. Values were recorded as averages of 3 replicates± standard deviation.

Investigation of Suitable Carbon Source for the Mycelial Growth of *P. tinctorius* SMF

The stock culture mycelium was first washed with ten volumes of sterile distilled-water. Then, 10 m*l* of the washed stock culture was transferred into 250 m*l* Erlenmeyer flasks containing 100 m*l* of FMMN (pH 5.5~5.7) supplemented with different carbon source. Only sugar sources were varied in the carbon utilization study. All soluble carbon sources were sterilized by millipore syringe filtration (0.22 μm pore size) and then added to the sterilized FMMN medium. Insoluble carbon sources were added to 250 m*l* Erlenmerer flasks containing other medium components, and they were then sterilized together. After inoculation, flasks were incubated shaking at 120 rpm at 25℃ for 10 days. Final fungal cell mass was measured as described above.

Amino Acid Effect on the Mycelial Growth of *P. tinctorius* SMF

The study was carried out in two steps. The first step consisted of a rapid screening of amino acids (100 mg/*l*) for possible stimulation of mycelial growth of *P. tinctorius* SMF. It involved measurement of biomass after 7 days growth in MMN (9) liquid medium containing a specific amino acid. In the second step. In the second step, selected amino acids [glutamic acid, proline, and lysine (50~100 mg/*l*)], selected on the basis of results from the screening test, were further investigated in the MMN liquid medium, varying the amounts of amino acids to find to optimum content for each amino acid.

Ten m*l* volumes of the stock culture mycelial inoculum in distilled water were inoculated into 250 m*l* Erlenmeyer flasks containing 100 m*l* MMN supplemented with each amino acid or other medium components, [thiamin·HCl, biotin, thiamin·HCl+biotin, thiamin+biotin+inositol, and thiamin+biotin+pyridoxine (0.10 mg/*l*)], which were added after filter sterilization, to study the effect of these supplements on cell growth. Amino acids were supplied as the L-configuration. The flasks were incubated with shaking (120 rpm) at 25℃ for 7 days. Fungal growth and pH changes were measured over time.

Carbon/Nitrogen Ratio and Citrate Buffer Effects on the Growth of *P. tinctorius* SMF

Ten m*l* of the mycelial inoculum was inoculated into 250 m*l* Erlenmeyer flasks containing 100 m*l* MMN supplemented to different carbon/nitrogen ratios. Various carbon/nitrogen (Glucose/$(NH_4)_2HPO_4$) were studied to find the optimum ratio for mycelial growth of *P. tinctorius* SMF. The ratio was adjusted by adjusting for 80% carbohydrate in malt extract (23) which was added at 3 g/*l*. The carbon/nitrogen ratios in the medium were adjusted by increasing the amount of ammonium phosphate relative to a constant 10 g/*l* glucose.

To determine pH effects on the growth of *P. tinctorius* SMF, another series of media differing, in carbon/nitrogen ratio were prepared with citrate buffer (18), which maintained the pH of the culture medium constant over the entire incubation period. The flasks were incubated with shaking (120 rpm) at 25℃ for 7 days. Fungal cell

mass and culture broth pH were measured over time as described above.

RESULTS AND DISCUSSION

Optimum Conditions for the Mycelial Growth of *P. tinctorius* SMF

The optimal conditions for the mycelial growth of *P. tinctorius* SMF, based upon the variables examined, was determined to be 25~27℃, initial pH 4.0, and 120 rpm, in FMMN medium containing glucose (10 g/*l*) as principal carbon source (Table 1). *P. tinctorius* SMF showed mycelial growth at temperatures as low as 15℃ and as high as 37℃. *P. tinctorius* SMF was highly acidophilic, and grew over a pH range of pH 3.0 to pH 6.0 (Table 1B). At pHs below 3.0 or above 7.0 growth was poor. The final culture broth pH was always quite acidic, ranging from pH 2.6~2.8 regardless of the initial pH of the medium. Isolates like *P. tinctorius* SMF which grow well over a broad pH range would be preferred for inoculation in tree nurseries. The pH optimum and pH tolerance of candidate isolates are among several important criteria commonly used in the selection of ectomycorrhizal fungi for use in inoculating soils in tree nurseries (22).

Table 1. Effects of cultural parameters, including temperature, initial pH, and agitation speed, on the mycelial growth of *P. tinctorius* SMF in FMMN liquid medium

Parameters	Cell mass (mg/m*l*)[1]	
	Glusose	Mannitol
A. Temperature (℃)		
22	1.86± 0.08	1.66± 0.06
25	2.14± 0.08	1.85± 0.06
27	2.12± 0.08	1.92± 0.06
30	1.82± 0.08	1.71± 0.06
B. Initial pH		
3.0	2.09± 0.06	1.82± 0.05
4.0	2.31± 0.06	2.09± 0.05
5.0	2.19± 0.06	2.01± 0.05
5.7	2.12± 0.06	1.92± 0.05
6.0	2.06± 0.06	1.90± 0.05
7.0	1.71± 0.06	1.67± 0.05
C. Agitation (rpm)		
105	1.86± 0.08	1.79± 0.07
120	2.13± 0.86	1.86± 0.07
135	1.73± 0.08	1.45± 0.07
150	1.66± 0.08	1.38± 0.07

[1]Cell mass determinations were carried out at the end of the active growth phase, which required harvesting cultures after specific incubation periods as described in the materials and methods.

Investigation of Suitable Carbon Source for the Mycelial Growth of *P. tinctorius* SMF

Although glucose was the preferred carbon source, *P. tinctorius* SMF utilized numerous carbon sources (Table 2). It metabolized monosaccharides more efficiently than derivatives, disaccharides, trisacchrides, or polysacchariedes (Table 2). Glucose and mannitol appeared to be the best sources of carbon for mycelial growth, of those tested. Different medium compositions and culture conditions significantly changed the growth pattern of *P. tinctorius* SMF which should be importantly considered with respect to oxygen supply for large scale cell mass production of *P. tinctorius* SMF. In particular, the fungus exhibited changes in growth habits with changing carbon sources and agitation speed. It grew as a smooth pellet with glucose as carbon source but as rough pellets bearing visible filamentous, when mannitol was the carbon source (all incubations at 120 rpm and at 25℃).

Amino Acid Effect on the Mycelial Growth of *P. tinctorius* SMF

Glutamic acid, proline, and lysine (100 mg/*l*) each stimulated mycelial growth of *P. tinctorius* SMF (Table 3). Lysine (800 mg/*l*), Glutamic Acid (500 mg/*l*), and Proline (50 mg/*l*) enhanced mycelial yields 63% (from 2.147 mg/m*l* to 3.49 mg/m*l*), 34% (from 2.14 mg/m*l* to 2.87 mg/m*l*), and 22% (from 2.14 mg/m*l* to 2.61 mg/m*l*), respectively as compared to growth in medium lacking amino acids (Fig. 1). Slight inceases (6.0%) in mycelial growth (from 2.17 mg/m*l* to 2.30~2.31 mg/m*l*), were

Table 2. Investigation of suitable carbon sources for mycelial growth of the *P. tinctorius* SMF in FMMN broth containing different carbon sources (10.0 g/*l*) after shaking incubation at 120 rpm and at 25°C for 10 days

Carbon source	$Y_{x/s}$[1]
Glucose	0.217± 0.008
Fructose	0.172± 0.010
Galactose	0.141± 0.006
Xylose	0.160± 0.012
Arabinose	0.148± 0.015
Sorbose	0.154± 0.011
Rhamnose	0.140± 0.014
Manitol	0.194± 0.007
Maltose	0.169± 0.008
Lactose	0.163± 0.011
Sucrose	0.166± 0.009
Starch	0.165± 0.013
Raffinose	0.157± 0.014

[1]Grams of cell mass/grams of carbon source

Table 3. Amino acid supplementation effects on mycelial growth of *P. tinctorius* SMF in MMN liquid medium

Amino acid added[1]	Cell mass[2] (mg/ml)	Final culture pH
Arginine	2.03± 0.08	2.53
Glutamic acid	2.46± 0.06	2.41
Proline	2.38± 0.10	2.77
Phenylalanine	1.65± 0.07	3.05
Tryptophan	0.37± 0.05	5.29
Tyrosine	2.07± 0.09	2.42
Cystine	0.53± 0.11	3.98
Glycine	0.73± 0.10	3.76
Serine	1.81± 0.07	2.68
Alanine	1.81± 0.05	2.81
Leucine	1.94± 0.08	2.62
Valine	1.89± 0.10	2.71
Aspartic acid	1.83± 0.07	2.47
Isoleucine	1.58± 0.11	2.98
Lysine	2.81± 0.07	2.63
Methionine	0.71± 0.07	3.45
Threonine	0.69± 0.06	4.23
Histidine	2.01± 0.10	2.63
Control-MMN	2.09± 0.08	2.43

[1]Amino acids were supplied at a concentration of 100 mg/l.
[2]Mg cell mass per ml culture medium, after growth in an MMN medium containing 10 mg/ml glucose

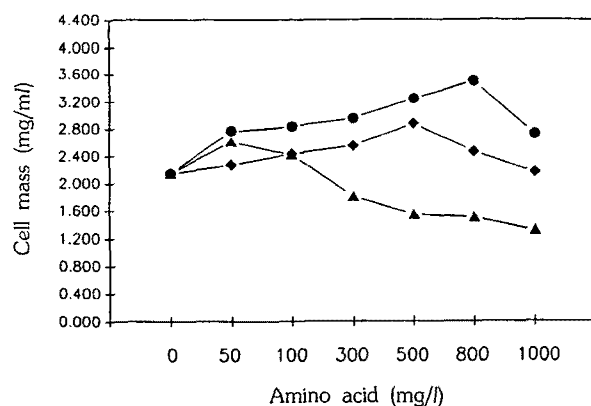

Fig. 1. Selected amino acid effects on mycelial yields of *P. tinctorius* SMF after growth in MMN liquid medium for 7 days at 25°C.
Cell mass (mg/ml) after the addition of glutamic acid (◆), proline (▲), and lysine (●) at amino acid concentrations varying between 0 and 1000 mg/l.

observed when *P. tinctorius* SMF was grown in the MMN liquid medium supplemented with thiamin·HCl+biotin and thiamin·HCl+biotin+pyridoxine (Table 4).

Table 4. Thiamin · HCl, biotin, inositol, and pyridoxine effects on mycelial growth yields of *P. tinctorius* SMF in MMN liquid medium

Component[1]	Cell mass (mg/ml)[2]	Final pH
Control-MMN	2.17+0.08	2.54
Biotin	2.15+0.08	2.49
Thiamin·HCl+Biotin	2.30+0.06	2.48
Thiamin·HCl +Biotin+Inositol	2.20+0.07	2.56
Thiamin·HCl +Biotin+Pyridoxine	2.31+0.08	2.49

[1]Added at a concentration of 0.10 mg/l; [2]Cultures were harvested after 7 days of incubation at 25C.

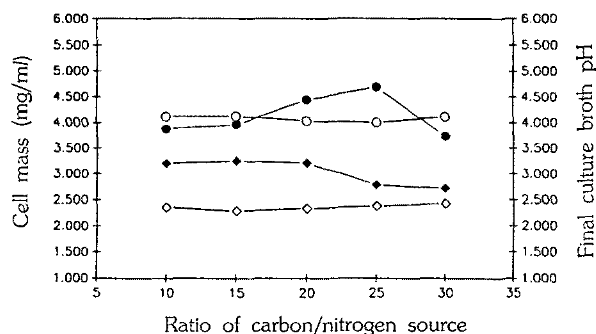

Fig. 2. Comparison of P. tinctorius SMF biomass yields as a function of carbon/nitrogen ratio, with or without 0.1 M citric acid/sodium citrate buffer (pH 4.5).
(◆), Cell mass (mg/l); (◇), Final culture broth pH without citrate buffer. (●), Cell mass (mg/ml); (○), Final culture broth pH with citrate buffer. Cells were harvested after 7 days growth at 25°C.

Carbon/Nitrogen Ratio and Citrate Buffer Effects on the Growth of *P. tinctrorius* SMF

As compared to mycelial growth in the original MMN medium, the mycelial growth of *P. tinctorius* SMF was 120% greater (from 2.14 mg/ml to 4.72 mg/ml) in the MMN liquid medium when the carbon/nitrogen ratio was 25/1 using citrate buffer at pH 4.5. The mycelial growth of *P. tinctorius* SMF was 50% greater (from 2.14 mg/ml to 3.21 mg/ml) in an MMN liquid medium of carbon/nitrogen ratio 10/1~20/1, when sodium citrate buffer was not used (Fig. 2). It has been reported that the optimum for *P. tinctorius* biomass production was at a carbohydrate-nitrogen ratio in the 20/1 to 4/1 range (13), or in the 20/1 to 10/1 range (20). The optimum carbon/nitrogen ratio for mycelial growth of *P. tinctorius* SMF in the present study was between 10/1 and 25/1 (Fig. 2). However, the pH dropped rapidly in the unbuf-

fered medium to levels inhibitory to growth. It remained within a more favorable range in citric acid/sodium citrate buffered media. Thus, greater fungal biomass would be produced in buffered media as compared to unbuffered media, due to the unfavorable pH values reached after several days incubation.

Based upon the accumulated data, standard conditions for growth of *P. tinctorius* SMF were determined to be 25~27℃, initial agitation 120 rpm, buffered to pH 4.0 with citrate, in modified Melin-Norkrans medium supplemented with 800 mg/m*l* lysine. In this medium the carbon/nitrogen ratio was 20/1~25/1. Under these conditions the maximal mycelial yield, $Y_{x/s}$, was 0.472 (4.72 mg/m*l*) after 7 days of incubation, as compared to a $Y_{x/s}$ of 0.214 (2.14 mg/m*l*), when the fungus was grown in standard MMN broth. These results represent a 120% increase in cell yield as compared to the nonoptimized medium, and the incubation time to achieve these yields was a quarter that typically used by other workers.

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