OPTIMIZATION OF GROWTH CONDITIONS OF THE WILD-TYPE AND MUTANT STRAINS OF THE PLEUROMUTILIN-PRODUCING *Clitopilus passeckerianus* (PILÁT) SING. NRRL 3100

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Abstract

The most suitable substrates for the growth of C. passeckerianus wild-type and mutant strain HP76 were found to be similar for both strains based on the extent of mycelial growth. Mycological agar (MA) was selected as a suitable agar medium while wheat bran was determined to be a suitable supplement for mycelial growth. Vegetative growth was also most vigorous on sawdust compared to the other solid substrates tested. The optimum physical conditions, in terms of temperature and lighting condition, were also similar for both strains when grown on wheat bran-supplemented sorghum grains. Air-conditioned temperature (24°C) was found to support better growth in both strains compared to room temperature (29-30°C). Lighting condition did not affect mycelial growth. Hyphal strands were the only structures noted in the growth of the wild type and HP76 when microscopically observed for five weeks. Oidia formation was observed on two other mutant strains, LP1 and LP2, grown using previously selected substrates and conditions.

Introduction

Pleuromutilin is an antibiotic produced by several species of the genus *Pleurotus*, by *Clitopilus passeckerianus* and *Drosophila subatrata* (Kavanagh *et al.*, 1951). Its activity is mainly against penicillin- and streptomycin resistant staphylococci and strains of *Streptococcus*, *Enterococcus*, *Bacillus subtilis*, *Escherichia coli*, *Eberthella typhi* and *Vibrio cholerae*.

C. passeckerianus is a therapeutically significant basidiomycete in that it produces pleuromutilin through fermentation (Knauseder and Brandl, 1975). Through mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine, high-yielding mutants were obtained (Papa, 1999); on the other hand, treatment with acridine-orange resulted in the emergence of low-producing mutants (Raymundo *et al.*, 1998).

The fungus is used for antibiotic production in its vegetative form (Papa, 1999). Mycelial growth optimization is significant to the ensuing fruiting and basidiospore formation, which can contribute to the easy and effective handling and storage of the fungus. Fruit body formation can also pave the way for investigations into the presence or production of antimicrobial compounds in basidiocarp tissues.

This study was conducted to determine the most suitable substrate and physical conditions for mycelial growth of the *C. passeckerianus* wild-type and high producing mutant strain HP 76; to compare, microscopically, the mycelia of the two *C. passeckerianus* strains; and to compare the growth of low-producing *C. passeckerianus* mutants LP1 and LP2 with that of the wild-type and HP76 strains using selected substrates and physical conditions.

Materials and Methods

Preparation of Stock Cultures

Cultures of *C. passeckerianus* (Pilát) Sing. NRRL were obtained from the National Institutes of Molecular Biology and Biotechnology (BIOTECH), UPLB, College, Laguna. Stock cultures were prepared by inoculating agar blocks of *C. passeckerianus* in tubes of mycological agar (MA) slants. Incubation was done at 30°C for two weeks. Agar plugs were prepared using a 10 mm cork borer from two-week old plate cultures.

Unless otherwise specified, incubation was at 28-30°C for one month.

Selection of Substrate

- Agar Medium

Agar plugs of *C. passeckerianus* wild-type and high-producing mutant HP76 were inoculated onto plates containing each of the following media: mycological agar (MA), corn meal agar (CMA), and potato dextrose agar (PDA) (DIFCO, 1984; Pañales, 1998). Incubation was done for two weeks. The growth of the organism on different media was compared qualitatively in terms of area covered by the mycelia. The agar medium observed to be the most suitable for mycelial growth was used in the succeeding experiments.

- Supplement

Boiled sorghum grains mixed with 20% (w/w) of each of the supplements (wheat bran, rice bran and soybean meal) were placed in three sets of test tubes. They were sterilized and inoculated on the surface with agar plugs of *C. passeckerianus* wild-type and mutant HP76. A set of control tubes containing sorghum grains alone was also inoculated with agar plugs. Mycelial growth was qualitatively noted.

- Solid Substrate

The procedures formulated by Quimio (2001) for solid substrate preparation were followed. The substrates utilized were sawdust, rice straw, rice hull, water hyacinth and sugarcane bagasse. Twenty percent (w/w) of the best supplement obtained from the previous result was also used. The succeeding steps were the same as above.

Selection of Physical Conditions

- Temperature

C. passeckerianus wild-type and HP76 agar plugs were inoculated onto two sets of tubes containing boiled sorghum grains and 20% (w/w) of the best supplement as per result of the previous experiment. One set was incubated in a room where the air-conditioning unit was turned on for a month, while the other was incubated at a room which was not air-conditioned. Temperature was monitored in the two sites during the incubation period. Comparison of growth was done qualitatively in terms of extent of proliferation mycelium in the test tube.

➢ Lighting Condition

C. passeckerianus wild-type and mutant HP76 agar plugs were inoculated onto three sets of tubes containing supplemented sorghum grains. The first set was incubated under continuous lighting provided by an 18-watt, white fluorescent lamp. The second set was exposed to day light (alternate light and darkness), while the third one was covered with carbon paper and incubated in a dark corner. Comparison of mycelial growth in the three sets of tubes was done qualitatively.

Microscopic Observation

The mycelia of *C. passeckerianus* wild-type and mutant strain HP76 grown for five weeks on sorghum grains with selected supplement (20% w/w) were microscopically observed. Lactophenol blue was used as the mounting medium.

Growth and Observation of Low-producing Mutants

C. passeckerianus low pleuromutilin-producing mutants LP1 and LP2 were grown employing the substrates and physical conditions selected in the preceding experiments. Agar blocks of both strains were inoculated in tubes containing boiled sorghum grains and 20% (w/w) of selected supplement. The cultures were incubated for three weeks. Weekly microscopic observation was similarly done as in the wild-type and mutant strain HP76.

Results and Discussion

Selection of Substrate

➢ Agar Medium

Among the three agar media used, mycelial growth of both *C. passeckerianus* wild-type and mutant strain HP76 was found to be most extensive on mycological agar (MA) (Table 1).

Table 1. Mycelial growth of wild-type and high-producing mutant (HP76) of *Clitopilus passeckerianus* on different media grown for two weeks, on sorghum grains with different supplements and on different solid substrates grown for one month at 30°C.

Medium/Supplement/ Substrate	Strain	
	Wild type	HP76
Agar Medium*		
Mycological Agar	++	++
Potato Dextrose Agar	+	+
Corn Meal Agar	+	+
Supplement**		
Wheat Bran	+++	+++
Soybean Meal	++	++
Rice Bran	++	++
Control	++	+
Solid Substrate**		
Sawdust	+++	+++
Rice straw	++	+
Water hyacinth	+	+
Sugarcane bagasse	+	+
Rice hull	+	+

* + Mycelial growth covers less than one-fourth of plate diameter
++ Mycelial growth covers more than one-fourth of plate diameter

** + Mycelial growth covers less than one-half of the tube length
++ Mycelial growth covers more than one-half but less than the whole tube length
+++ Mycelial growth covers the whole tube length

The result concurs with the findings of Trovela (1995) that mycological agar is the best growth medium for *C. passeckerianus*. The medium contains glucose which is a readily utilizable carbon source, and soybean meal extract, a rich source of protein, carbohydrates and vitamins. The growth of HP76 on all three agar media did not show any difference compared to the wild type. The conjecture of Barnes (2001) regarding the growth similarities of the strains was that the genes encoding for enzyme biosynthesis needed for glucose utilization were not affected by the mutagenic treatment of the organism.

- Supplement

Among the three commonly employed supplement in mushroom production, wheat bran was found to be the most suitable supplement for the growth of the two *C. passeckerianus* strains (Table 1). Mycelial growth on soybean meal- and rice bran-supplemented sorghum showed almost same extent in terms of length covered by mycelia.

Wheat bran is an excellent supplement in that it augments the nutritional properties of the growth substrate. It is known to have a high protein content and contains Ca, P, N and K which are essential requirements for mushroom growth (Flegg 1998).

- Solid Substrate

Based on the previous results, all substrates were supplemented with 20% (w/w) wheat bran. Wheat bran-supplemented sawdust was found to be the most suitable substrate for mycelial growth of both *C. passeckerianus* strains (Table 1). The growth of wild type and mutant strain HP76 in rice straw, water hyacinth, sugarcane bagasse and rice hull (all supplemented with wheat bran) was comparatively less extensive.

Singer's (1962) description that *Clitopilus* grows naturally on woody materials explains the organism's vigorous colonization on sawdust. Enzymes are activated to break down cellulose, hemicellulose and lignin into simpler substances to be used by mycelia for growth and propagation (Chen, 2001). Sawdust is commonly used for mushroom cultures because it is capable of absorbing and holding a physiologically sufficient amount of water (Tonomura, 1978). In addition, the substrate is highly dispersed, which is favorable for supporting vegetative mycelia (Stamets, 1993).

Selection of Physical Conditions

- Temperature

The ambient temperatures of incubation sites were monitored. The temperature reading at the air-conditioned site was 24°C. Room temperature was recorded as 29-30°C. Air-conditioned temperature was found to be more favorable for mycelial growth in both *C. passeckerianus* wild-type and high pleuromutilin-producing mutant HP76 than room temperature when the strains were grown on wheat bran-supplemented (20% w/w) sorghum grains (Table 2).

The global distribution of *Clitopilus* may have a lot to do with its temperature requirement for mycelial growth. The fungus thrives in boreal temperate regions during summer and autumn (Gulden, 1998).

- Lighting Condition

No difference was noted in the extent of mycelial growth of the wild type and mutant strain HP76 when the cultures were exposed to different lighting conditions (continuous light, alternate light and darkness, continuous darkness) (Table 2).

Table 2. Mycelial growth of wild-type and high-yielding mutant (HP76) of *Clitopilus passeckerianus* on wheat bran-supplemented sorghum grains incubated at different temperatures and under different lighting conditions for one month.

	Incubation temperature*		Lighting condition*		
strain	Air-conditioned temperature (24°C)	Room temperature (29-30°C)	Continuous light	Alternate light and darkness	Continuous darkness
Wild-type	+ +	+	+ +	+ +	+ +
HP76	+ +	+	+ +	+ +	+ +

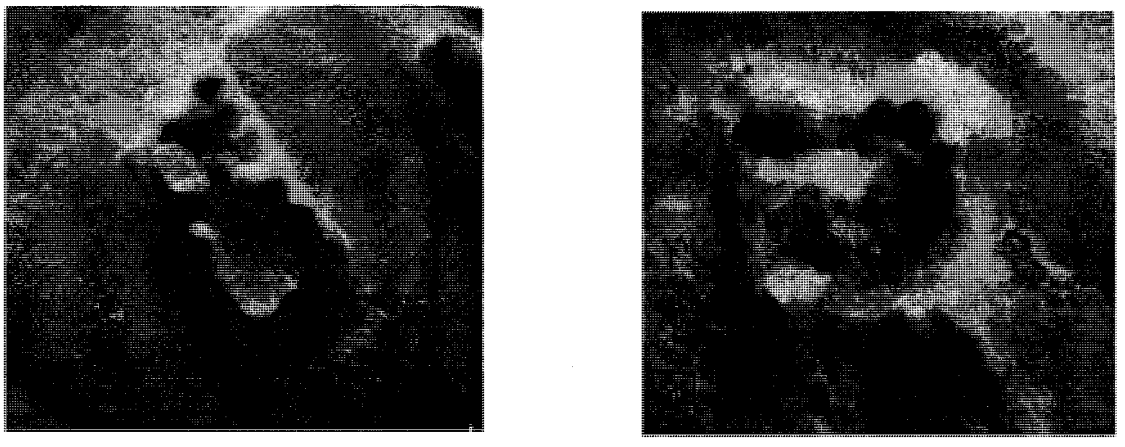
* + Mycelial growth covers less than one-half of the tube length
++ Mycelial growth covers more than one-half but less than the whole tube length

It was observed in a study conducted by Panales (1998) on *Flammulina velutipes* that light was not necessary for mycelia formation. The same results were obtained by Mangonon (1998) regarding *Ganoderma* mycelial response to different light conditions. Exposure of the mushroom to light did not confer significant effect to mycelial growth. Light can bring about different fungal physiological responses (Jennings and Lysek, 1996). Although basidiomycetes are less sensitive to illumination compared to lower fungi, light may directly or indirectly affect their growth and differentiation. It was established that light has a greater effect on the reproduction of fungi than on mycelial growth (Griffin, 1994).

Primordia Formation on Mycological Agar

As early as two weeks after inoculation, formation of primordia or fruit body initials in *C. passeckerianus* wild type and mutant HP76 was observed on MA plates incubated at 24°C (Fig.1). Such an observation indicates the possibility that the fungus is capable of producing fruitbodies. The premature formation of primordia is considered a strain characteristic and is pointed out as a possible consequence of physical shock (Chen, 2001).

Figure 1 Primordia formation of (A) wild-type and (B) high-producing mutant (HP76) of *Clitopilus passeckerians* on mycolological agar after two weeks of incubation at 240C



Wild Type HP76

Basidiocarp Formation on Supplemented Sorghum Grain

Fruit body formation was observed when *C. passeckerianus* wild type was allowed to grow on wheat bran-supplemented sorghum grains for four months (Fig. 2). The basidiocarps formed were, however, abnormal, possibly due to the limited spatial environment where the strain was grown.

Figure 2 Basidiocarp formation of wild-type of *Clitopilus passeckerianus* in wheat bran-supplemented sorghum grains after four months of incubation at 24 °C. High-producing mutant HP76 of the same age did not produce basidiocarp when grown on the same temperature

Mutant strain HP76 did not show basidiocarp formation after four months using the same substrate. This may have been the effect of mutagenesis (Papa, 1999) where regions in the fungal DNA governing basidiocarp formation may have been affected. It was not established, however, if a basidiocarp would have been formed if growth on MA was allowed for a longer period since primordia formation was observed.

Microscopic Observation

Microscopic observation of mycelia of the two *C. passeckerianus* strains revealed that there was no hyphal structural change as far as emergence of reproductive structures is concerned.

Mutation-induced variation in antibiotic production instead of morphological difference had been the basis for considering *C. passeckerianus* parent and HP76 as separate strains (Papa, 1999). In the light of this, it is important to note that genetic improvement involving antibiotic synthesis does not necessarily involve morphological changes (Barnes, 2001; Zulaybar, 1992)

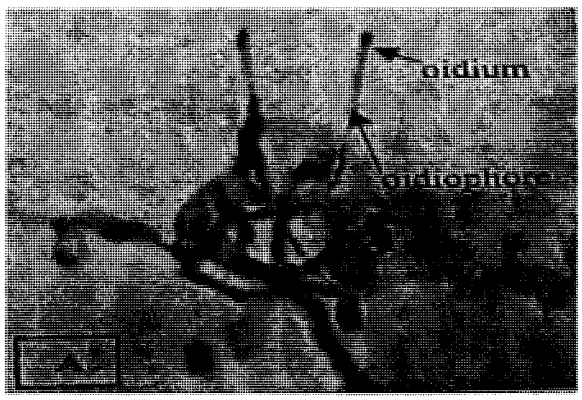
Growth and Observation of Low-producing Mutants

The suitability of the selected substrates and physical conditions to the growth and development of other *C. passeckerianus* mutant strains was tested by growing acridine orange-mutagenized (Raymundo *et al.*, 1998) *C. passeckerianus* LP1 and LP2. Both mutants exhibited cultural characteristics remarkably different from that of the wild-type and HP76 strains when grown on MA. Upon transfer from MA to wheat bran-supplemented sorghum grains, the mycelial growth of both strains showed similarity to that of the wild-type and HP76.

Rajak (2000) pointed out that the substrate type used in growing *Pleurotus* directly affected the mushroom's composition by varying the concentrations of essential amino acids and lipids. Such biochemical changes might have occurred on both mutants and were manifested by changes in morphology.

The mycelia of the two *C. passeckerianus* mutants were observed microscopically under the high power objective. Allowing the mycelia to grow for one week revealed oidia formation in LP1 and LP2. These asexual spores, oval to cylindrical in shape, appeared in small clusters at the apex of the supporting oidiophores (Fig. 3). Oidiophores were also noted to be arising terminally and intercalarily from the branched mycelia.

Figure 3 Photomicrographs of *Clitopilus passeckerianus* low-producing mutants LP1 and LP2 on wheat bran-supplemented sorghum grains after one week.



Oidia are asexual spores produced by some basidiomycetes from monokaryotic hyphae (Alexopoulos *et al.*, 1996). They may fuse with somatic hyphae to give rise to a heterokaryotic mycelium. Oidia are borne by oidiophores and are enveloped by a droplet of mucus. It was inferred that acridine-orange mutagenesis resulted in the induction of gene expression which favored oidia formation.

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