Applied and Environmental Microbiology, Oct. 2005, p. 6388–6389 Vol. 71, No. 10
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.10.6388–6389.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Simple Colorimetric Method for Detecting Degenerate Strains of the Cultivated Basidiomycete *Flammulina velutipes* (Enokitake)

Yumi Magae,[1]* Kobun Akahane,[2] Kimiyoshi Nakamura,[2] and Shigeyuki Tsunoda[2]

Department of Applied Microbiology, Forestry and Forest Products Research Institute, Tsukuba, Ibaraki 305-8687, Japan,[1] and Nagano Vegetable and Ornamental Experiment Station, Matsushiro 2206, Nagano 381-1211, Japan[2]

Received 16 December 2004/Accepted 28 April 2005

Degeneration of cultivated strains of *Flammulina velutipes* is a serious problem. We developed a simple colorimetric method to detect degenerate strains by using a liquid medium supplemented with bromothymol blue and lactose. The ability of a strain to develop normal mushrooms could be determined by the color of the medium.

The primary objective of this study was to develop a simple method for detecting degenerate *Flammulina velutipes* (Enokitake) cultures. Cultural degeneration of cultivated strains of Enokitake similar to the degeneration observed for *Agaricus bisporus* (1, 2) has become a serious problem in Japan. Previous efforts to evaluate the fruiting potential of Enokitake have been made using isozyme electrophoresis profiles, randomly amplified polymorphic DNA, and mitochondrial DNA restriction fragment length polymorphism, but there was no clear correlation between the results of these experiments and the degenerate symptoms. Normal and degenerate strains differ in the ability to decolorize synthetic dyes, such as bromothymol blue (BTB), which changes from blue-green to yellow. The underlying cause of degeneration is not known, so a simple bioassay that distinguishes degenerate and productive strains of Enokitake would be of use for both researchers and commercial spawn producers.

Commercial cultivation of Enokitake began in Japan in the 1930s with sawdust and rice bran as the substrate. In 2003, the total yield of Enokitake was 110,185 tons, which is much greater than the 65,363 tons of *Lentinula edodes* (Shiitake) or the 5,210 tons of *Pleurotus ostreatus* produced in Japan (annual statistics for mushroom production in Japan from the Forestry Agency [http://www.rinya.maff.go.jp/puresu/h16-8gatu/0805tokusan.htm]). The degenerate symptoms of Enokitake reported in the 1980s were malformed fruiting bodies, reduced numbers of primordia, and in some cases complete loss of fruiting body development. However, degenerate mycelia are morphologically indistinguishable from normal mycelia, and the symptoms of degeneration are not apparent until the final stage of mushroom cultivation, which may result in major financial losses.

Four cultivars (TK,YO, JB, and G5) developed at Nagano Vegetable and Ornamental Experiment Station were used in the present study. The first symptom of degeneration usually is the development of undifferentiated, callus-like tissue in normal fruiting bodies. TKd, YOd, and JBd were isolated from malformed portions of fruiting bodies. TKd mycelia can produce malformed abnormal fruiting bodies, but TKm and JBm are even more severely degenerate and can develop very few primordia. Field strains in our culture collection that produced normal fruiting bodies on potato dextrose agar (Nissui, Tokyo, Japan) (FV wild a, FV wild b, and FV wild c) or that formed no fruiting bodies (FV wild d and FV wild e) also were examined. None of the strains used in this study contained detectable double-stranded RNAs (3).

Each strain was inoculated onto malt extract agar (10 g/liter malt extract, 18 g/liter agar) in 9-cm petri dishes. Cultures were incubated for ~1 week at 24°C in the dark, and plugs were taken ~5 mm inside the edge of each colony with a cork borer and used for the assay. To compare the effects of sugars, six portions of colonies of TK and TKm were grown in 2 ml of basal medium (4.5 g/liter yeast extract, 7.5 g/liter Bacto peptone [Difco, Detroit, MI], and 0.025 g/liter BTB [Nacalai tesque, Kyoto, Japan]) supplemented with 5 g/liter of xylose, trehalose, glucose, sucrose, fructose, cellobiose, galactose, or lactose in a 24-well culture plate (Asahi Techno Glass, Tokyo, Japan). Each culture plate was incubated on a reciprocal shaker at 60 rpm at room temperature for 4 days, and then the A_{615}, the visible absorbance maximum of BTB, was measured. The decolorization ratio (D ratio) (expressed as a percentage) was calculated as follows: $[1 - (A_{615}$ of strain/A_{615} of blank$)] \times 100$. TK decolorized BTB regardless of the sugar in the medium (D ratio, 91% ± 1%), while TKm did not decolorize BTB when it was cultured with fructose (D ratio, −4% ± 14%), cellobiose (D ratio, −10% ± 7%), galactose (D ratio, −93% ± 15%), or lactose (D ratio, −113% ± 6%). Specifically, the blue color of the lactose-containing medium of TKm was significantly more intense ($P < 0.001$, as determined by Student's *t* test) than the blue color of the medium containing any of the other sugars. Thus, lactose was selected as the most suitable sugar for this assay, and the assay medium was designated YBLB.

When Enokitake was grown in YBLB, it decolorized the medium in proportion to the ability to produce normal fruiting bodies (Table 1). Mycelial transfers from a single colony of strain G5 produced various colors in 24-well YBLB microplates, showing that the original colony was heterogeneous (data not shown). Mycelia were isolated from degenerate fruiting bodies and from the culture bed that produced the degen-

* Corresponding author. Mailing address: Forestry and Forest Products Research Institute, Tsukuba, Ibaraki 305-8687, Japan. Phone: 81-298-733211. Fax: 81-298-743720. E-mail: ymagae@ffpri.affrc.go.jp.

TABLE 1. Results of YBLB assay

Strain	D ratio (%)[a]	Color
Control	0	Green
Commercial strains[b]		
TK	70 ± 8	Yellow
TKd	56 ± 7	Green
TKm	−53 ± 1	Blue
YO	85 ± 14	Yellow
YOd	64 ± 5	Green
JB	73 ± 4	Yellow
JBd	31 ± 9	Green
JBm	−18 ± 1	Blue
Wild strains[c]		
FV wild a	93 ± 2	Yellow
FV wild b	95 ± 1	Yellow
FV wild c	92 ± 2	Yellow
FV wild d	−57 ± 5	Blue
FV wild e	−100 ± 6	Blue

[a] Mean for six colony pieces. Assays were performed three independent times, and representative data are shown.
[b] "Pure white" cultivars developed at Nagano Vegetable and Ornamental Experiment Station.
[c] FV wild a, FV wild b, and FV wild c develop fruiting bodies. FV wild d and FV wild e do not develop fruiting bodies.

erate fruiting bodies for a culture of strain G5 with degenerate symptoms on two separate occasions. Mycelia also were isolated from fruiting bodies and a culture bottle from the same spawn lot that had no symptoms of degeneration. Ten samples of each colony were assayed in microplates containing YBLB. None of the transfers from the degenerate subcultures turned YBLB yellow, but if a subculture had at least one mycelial transfer that decolorized BTB, the strain could produce normal fruiting bodies.

To determine the degree of fruiting body productivity reflected in the YBLB assay, mycelial portions of TKd that were blue, green, or yellow in the assay were cultivated in bottles on a commercial scale. Fruiting bodies were harvested in bulk from each culture bottle and weighed without separating the malformed and normal fruiting bodies. Fruiting body quality was assessed numerically by determining the proportion of malformed stipes or pilei (Table 2). The quality of fruiting bodies produced by isolate 5 was very low. Isolates 3 and 4, which turned YBLB green, produced normal numbers of primordia, and the final yield was similar to that for isolates 1 and 2, but a high proportion of the fruiting bodies were malformed.

Overall, the YBLB assay was very efficient for identifying degenerate isolates with quantitatively low fruiting ability that turned YBLB blue. If a culture turns YBLB green, then individual growers must decide whether the culture can yield enough normal fruiting bodies to be worth growing.

This assay may be applicable to other edible mushroom species for the identification of degenerate strains and could be useful in Enokitake breeding programs to select stable, high-yielding strains.

This work was supported by a grant from the Agriculture, Forestry and Fisheries Research Council.

TABLE 2. Characterization of fruiting bodies produced by TKd subcultures after the YBLB assay

Isolate	Color of YBLB[a]	D ratio (%)	Fruiting bodies		
			Yield (g)	Malformation scores[b]	
				Stipe	Pilei
1	Yellow	95	135	1	1
			154	1	1
			145	0	0
			163	0	1
			135	1	1
			141	2	1
			156	1	1
			(147)[c]	(0.9)	(0.9)
2	Yellow	91	135	0	1
			127	1	2
			129	1	1
			132	0	1
			126	1	1
			127	0	1
			127	1	1
			(129)	(0.6)	(1.1)
3	Green	72	134	1	1
			142	1	1
			152	1	1
			138	1	1
			145	2	2
			139	3	3
			144	1	2
			(142)	(1.4)	(1.6)
4	Green	15	144	1	2
			162	2	2
			141	1	1
			134	1	1
			142	3	3
			141	3	2
			144	1	1
			(144)	(1.7)	(1.7)
5	Blue	−20	116	1	4
			100	2	5
			123	4	3
			108	1	5
			114	3	4
			123	5	5
			126	4	5
			(116)	(2.9)	(4.4)

[a] Color of YBLB after mycelia grew for 7 days.
[b] Malformation scores: 1, 1 to 20%; 2, 21 to 40%; 3, 41 to 60%; 4, 61 to 80%; 5, 81 to 100%.
[c] The values in parentheses are averages.

REFERENCES

1. **Horgen, P. A., D. Carvalho, A. Sonnenberg, A. Li, and L. J. L. D. Van Griensven.** 1996. Chromosomal abnormalities associated with strain degeneration in the cultivated mushroom, *Agaricus bisporus*. Fungal Genet. Biol. **20:**229–241.
2. **Li, A., M. Begin, K. Kokurewicz, C. Bowden, and P. A. Horgen.** 1994. Inheritance of strain instability (sectoring) in the commercial button mushroom, *Agaricus bisporus*. Appl. Environ. Microbiol. **60:**2384–2388.
3. **Magae, Y., and N. Hayashi.** 1999. Double-stranded RNA and virus-like particles in the edible basidiomycete *Flammulina velutipes* (Enokitake). FEMS Microbiol. Lett. **180:**331–335.