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Effect of liquid mycelial culture used as a spawn on sawdust cultivation of shiitake (*Lentinula edodes*)

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Mycelium of shiitake (*Lentinula edodes*) was cultured continuously in liquid medium. The liquid culture was carried out for the production of liquid spawn in the cultivation of this mushroom on synthetic sawdust substrate, and its performance was compared with that of the solid spawn. The initial colonization in culture bags was faster with the solid spawn than with the liquid spawn, but after this stage CO_2 production was higher with the liquid spawn than with the solid spawn. For harvesting sufficient amount of good quality mushrooms, 120 d of incubation in bags was needed with the solid spawn, but this was reduced to 90 d for the sawdust blocks using liquid spawn of less than 50 d old. If continuous culture of the liquid spawn was prolonged over 50 d, immature fruit-bodies or their initials formed during the period of bag incubation. The solid subcultures of the liquid spawns retained the fruiting characteristics acquired in the liquid culture. Liquid culture could be a useful tool for breeding of mushrooms.

Key Words——breeding; early fruiting; *Lentinula edodes*; liquid spawn; shiitake.

Shiitake (*Lentinula edodes* (Berk.) Pegler) is one of the five most cultivated edible mushrooms in the world (Chang, 1993). This mushroom is mainly cultivated in East Asia, including Japan and China, but is now of expanding interest in the world (Chang, 1992). The cultivation method is improving rapidly (Furukawa, 1992). In Japan most shiitake is cultivated on logs of oak or other broad-leaved deciduous tree species but the cultivation on synthetic sawdust substrate is rapidly increasing, accounting for a 25% share in 1994 (Ohashi, 1995). Short fruiting cycle, large harvests and ease of labor are the main benefits of the sawdust growing (Chang and Miles, 1989; Wuest, 1989). The cultivation process involves: (1) manufacture of sawdust substrate, (2) inoculation of spawn, (3) spawn run, and (4) fruiting. In the spawn run stage, the substrate is completely permeated with the mycelium, covered with a mycelial coat and pigmented (Chang and Miles, 1989). If the spawn run is inadequate, the block will produce malformed or abnormal fruit-bodies, e.g., without pileus, with irregularly shaped pileus, with short, stunted or swollen stipes, etc. (Chang and Miles, 1989). Kirchhoff and Lelley (1991) reported that the use of liquid spawn for the sawdust substrate cultivation induced a higher fruit-body yield than grain spawn. We established the continuous liquid culture of shiitake mycelium for over 70 d without contamination (Fukushima et al., 1993). When the liquid mycelial spawn thus produced was inoculated on the artificial sawdust substrate, the incubation period needed for harvesting sufficient amount of good quality mushrooms was greatly reduced. The culture period of liquid spawn had to be within 50 d, and prolongation of cultures over 50 d formed immature fruit-bodies in the bag cultivation. We studied the adaptation of liquid culture for spawn production in comparison with solid culture spawn.

Materials and Methods

Strain *Lentinula edodes* L1052, a stock culture of Kikkoman Corporation, was used throughout this study.

Liquid culture conditions and the liquid spawns Culture conditions were basically the same as in the previous paper (Fukushima et al., 1993). The jar fermentor was a Chemap series 3000 (Chemap AG, Volketswil, Switzerland). Five L of medium was cultured in a 7.5-L fermentation vessel (SG-type) with a FUNDASPIN agitation system. The batch culture medium contained 30 g of glucose, 2.5 g of polypeptone, 2.5 g of yeast extract, 2 g of KH_2PO_4, 1 g of $CaCl_2 \cdot 2H_2O$, 1 g of $MgSO_4 \cdot 7H_2O$, 1 ml of soy sauce oil, 0.5 ml of silicone (KM-72F, Shin-Etsu Chemical, Tokyo) and 1 L of tap water. The feeding medium contained 4 ml soy sauce oil, 0.2 ml Tween 80, and 1 ml CH_3COOH besides the batch culture medium. The dilution rate of continuous culture was 0.01 h^{-1} and the agitation rate was set at 300–600 rpm, as shown in Fig. 2. The exhausted gas was monitored by use of a CO_2 Gas Analyzer IRA 107, URA 107 (Shimadzu, Kyoto). Figure 1 shows the continuous liquid culture system. The liquid mycelial culture products were directly used as liquid spawns (L1-4). The incubation times were 23, 42, 34, and 10 d for the production of L1, L2, L3, and L4, respectively. L1 and L2 were prepared from the same culture, and the others were independent cultures. The liquid culture mycelia were subcultured on PDA slants to

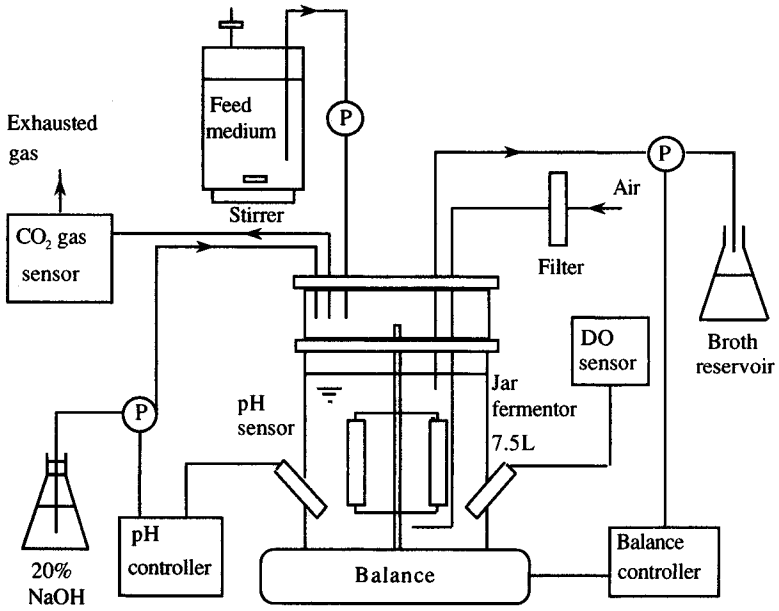

Fig. 1. Schematic drawing of continuous liquid culture system.

make stock cultures.

Solid spawn and liquid solid spawn The stock culture of L1052 or the solid stock cultures of liquid culture mycelia indicated above were inoculated on a sawdust substrate (w/w: beech wood sawdust 32% (dry), rice bran 4% (dry), corn bran 4% (dry), water 60%) in a polypropylene bag. The bag was capped with Celgard No. 2500 (Hoechst Celanese, Charlotte, USA) for aeration and cultured at 20°C for 30 d to make the solid spawns (S1-5) or the liquid-solid spawns (LS1-4). Solid spawns (S1-5) were the same as each other except that they were cultured independently. Liquid-solid spawns (LS1, LS2, LS3, and LS4) were prepared from liquid spawns (L1, L2, L3, and L4, respectively).

Cultivation of Shiitake The sawdust substrate (1.9 kg) was sterilized at 120°C for 120 min in a polypropylene bag. After cooling, the substrate was inoculated with 30 ml of the liquid culture or 30 g of the solid or the liquid-solid spawn, mixed by shaking, and packed into another bag made of two-ply polyethylene sheets with many small pores (diam: 0.1-0.2 mm) to form a cylindrical block. O_2 transmission of the two-ply sheets was 20 ml cm^{-2} h^{-1} atm^{-1}. The bag was incubated at 22 ±1°C for 80, 90, 100, 110, or 120 d under limited daylight (50-100 lx). The colonized substrate block was then removed from the plastic bag and placed in a room with a day temperature of 20±2°C, a night temperature of 12±2°C, and a relative humidity of 80-95%. The block was watered intermittently to maintain the moisture of the substrate within the range of 50-70% for 20 d. Fruit-bodies formed during this period were harvested when the cap was 50% open, weighed, and classified according to the five standards stated later. The block was dried at 20±1°C for 5 d, soaked in cold water overnight, then returned to the fruiting room to produce a second harvest. This procedure was repeated 8-10 cycles of fruiting. The five standards for weight of fruit-body were: L≧20 g, 20 g>M≧15 g, 15 g>S≧10 g, 10 g>SS≧5 g, and others (abnormal shape or <5 g). Normal fruit-bodies had short stipes (less than the diameter of cap), circle caps (the major axis/the minor axis <1.2), and without any contamination and the abnormal one is the other.

Twenty (S3, L3) or ten (other spawns) sawdust substrate blocks were used for each experimental section. None of blocks were spoiled and discarded in the term of this experiment. Biological efficiency (BE) was determined as the weight ratio of fresh mushrooms harvested per unit of dry substrate and expressed as a percentage (Royse and Bahler, 1986). Most of the harvested fruit-bodies were not assayed for dry ratio, but four samples which looked to be of average moisture content for all the harvests were examined by lyophilization and found to exhibit a dry ratio of 0.112±0.008.

Respiration assay Three blocks just inoculated were placed in a sealed box (45×45×45 cm) supplied with air at a constant rate of 500 ml/min, the air inside the box was mixed by a fan, and the outlet air was analyzed every 2 h by use of an Exhaust Oxygen Carbon Dioxide Meter EX-1562-P (Able, Tokyo). The data were processed by use of the software EXSAMP61.EXE (Able, Tokyo). The assay was done in a room controlled at 20±2°C.

Results

Liquid culture of shiitake Exhausted carbon dioxide is a parameter for evaluating the metabolic activity of aerobic microorganisms. Figure 2 shows the culture conditions, the concentration of the exhausted CO_2 and the dry weight during the liquid culture of shiitake mycelium. The release of CO_2 and mycelium concentration were nearly constant (mycelium dry weight: 7-9 mg/ml) from

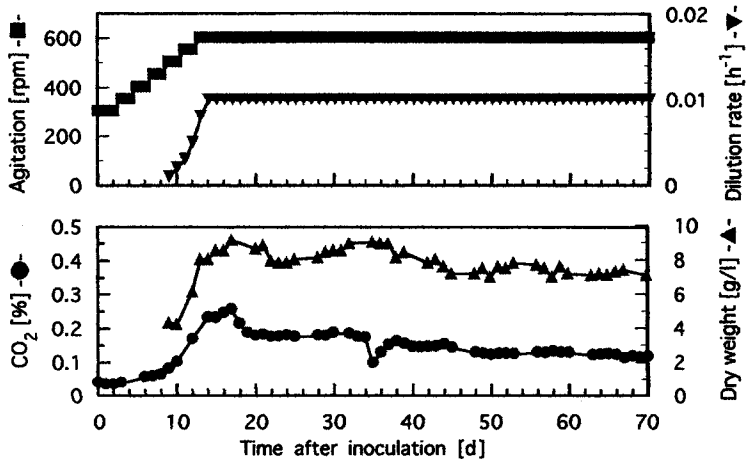

Fig. 2. Time course of a continuous liquid culture.
This culture was used as L3.

days 16 to 70 of culture. The sharp decrease in exhausted CO_2 on day 34 was due to the extraction of 3 L of the 5 L of culture broth as inoculum for 100 blocks of sawdust substrate.

Colonization of the blocks The initial colonization was faster with solid spawn than with liquid spawn, but the growth indicated by CO_2 release reversed at around the day 6 after inoculation (Fig. 3). At around the time of the maximal CO_2 release, the mycelium had covered all the surface of the substrate. The oscillation of the exhausted CO_2 was correlated with temperature deviation of the room caused by poor control of the air-conditioning system. The blocks were fully colonized by the mycelium and the surface became white by around day 14 (Fig. 4).

Pigmentation of the blocks The colonized blocks began pigmenting at around day 30 and became completely brown after day 60 (Figs. 4, 5A). The blocks inoculated with long-term (more than 50 d) liquid-culture mycelium were only partially pigmented even after 90 d of incubation, and often formed immature fruit-bodies or initials in the bag cultivation period (Fig. 5B). All these blocks were spoiled and discarded at the early stage of the fruiting period.

Maturation of the blocks The sawdust blocks inoculated with the solid or liquid spawn were incubated in bags

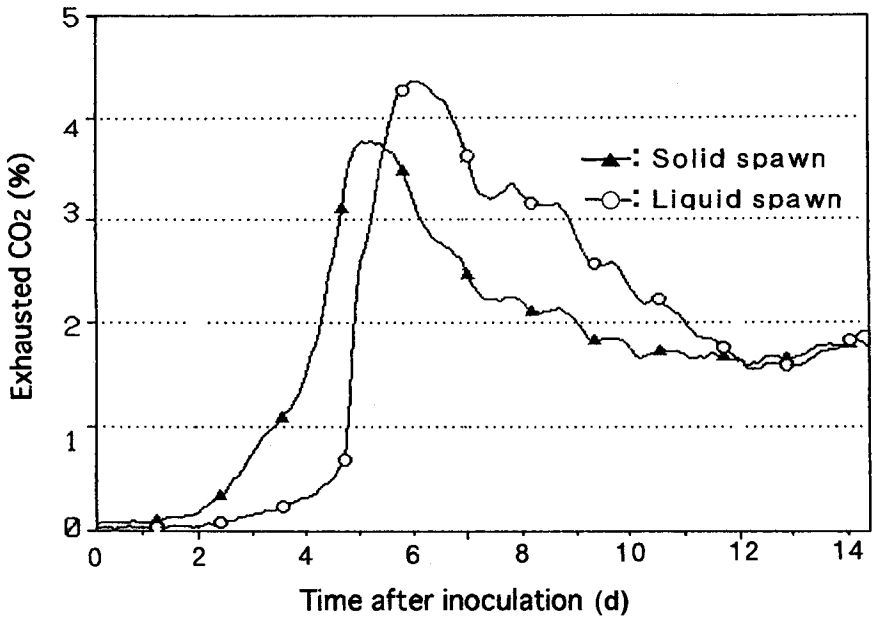

Fig. 3. Exhausted carbon dioxide from the colonizing sawdust substrate in bags.
Three blocks just inoculated with the solid (S3) or the liquid spawn (L3) were placed in a sealed box (45 × 45 × 45 cm) supplied with air at a constant rate of 500 ml/min, the air was mixed by a fan inside the box, and the outlet air was analyzed every 2 h as indicated in the text.

Fig. 4. Pigmentation of the colonized blocks.
The blocks inoculated with solid spawn incubated for from left to right: 0, 14, 30, 45, 60 d.

for 80 to 120 d, and fruited for 200-250 d thereafter. The first harvest from the blocks inoculated with the solid spawn was less than 10% BE even with the 120-d incubation period, and the second harvest was also small (Fig. 6). On the other hand, the blocks inoculated with the liquid spawn gave a first harvest of 11% BE with the 90-d incubation. Total harvests from the blocks inoculated with liquid spawn (160±7% BE) were higher than those with solid spawn (135±10% BE). Table 1 shows the data of the first to eighth harvests of four independent cultivations following 90 d of bag cultivation. The yields of first harvest were always greater in the blocks inoculated with the liquid spawn than in those inoculated with the solid spawn. The total harvests from the blocks with liquid spawn were greater than those with solid spawn. The probabilities that the two observations represented the true picture were more than 99% and 98%, respectively, by t-dependent analysis of variance. The other harvests varied widely. The environmental conditions such as temperature, humidity, and air exchange in the cultivation room and in different parts of the room were not exactly the same in each experiment, although the harvests might be influenced greatly by the environment. The mushrooms produced by both the solid and the liquid spawn were of good quality: large cap, thick and dense in pileus trama. Liquid culture did not reduce the quality of mushrooms (Table 1).

Preservation of early fruit nature of liquid spawn after solid subculture The liquid spawns (L1-4) were inoculated on agar medium to make stock cultures and the stocks were subsequently inoculated on sawdust substrate to make the liquid-solid spawn (LS1-4). Figure 7 shows the harvests of shiitake mushrooms from the blocks with the solid spawn and the solid-liquid spawn. The first harvests of blocks inoculated with liquid-solid spawn were remarkably better than those inoculated with the solid

Table 1. Harvests of mushrooms from sawdust blocks inoculated with liquid and solid spawn.

Section [a]	Harvest (BE, %)									Standard (%)				
	1	2	3	4	5	6	7	8	1~8	L	M	S	SS	Oth.
S1	6	13	28	16	8	21	11	10	114	65	20	7	5	3
L1	25	13	21	22	19	6	11	7	123	73	18	4	1	3
S2	2	5	22	8	13	34	16	7	107	64	19	12	3	3
L2	14	14	18	14	15	15	17	13	120	64	20	10	4	2
S3	1	11	24	5	23	34	22	17	137	60	22	11	4	4
L3	11	36	21	11	17	23	14	12	146	54	29	11	5	3
S4	3	2	18	17	8	9	39	14	111	60	24	8	2	5
L4	29	3	8	19	11	10	11	22	114	58	25	9	2	4
S mean	3	8	23	11	13	25	22	12	117	62	21	10	3	4
SD	2	5	4	6	7	12	12	4	14	3	2	2	1	1
L mean	20	16	17	17	15	13	14	13	126	62	23	9	3	3
SD	8	14	6	5	3	7	3	6	14	8	5	3	2	1

[a] S1-4: Solid spawn, L1-4: Liquid spawn, SD: standard deviation.
Four independent cultivation tests were done to compare the effects of solid and liquid spawn. S3 and L3 were the same in Fig. 6. One experimental section for the other three sets of tests consisted of 10 blocks. The incubation in bags was for 90 d and the harvesting was done in 8

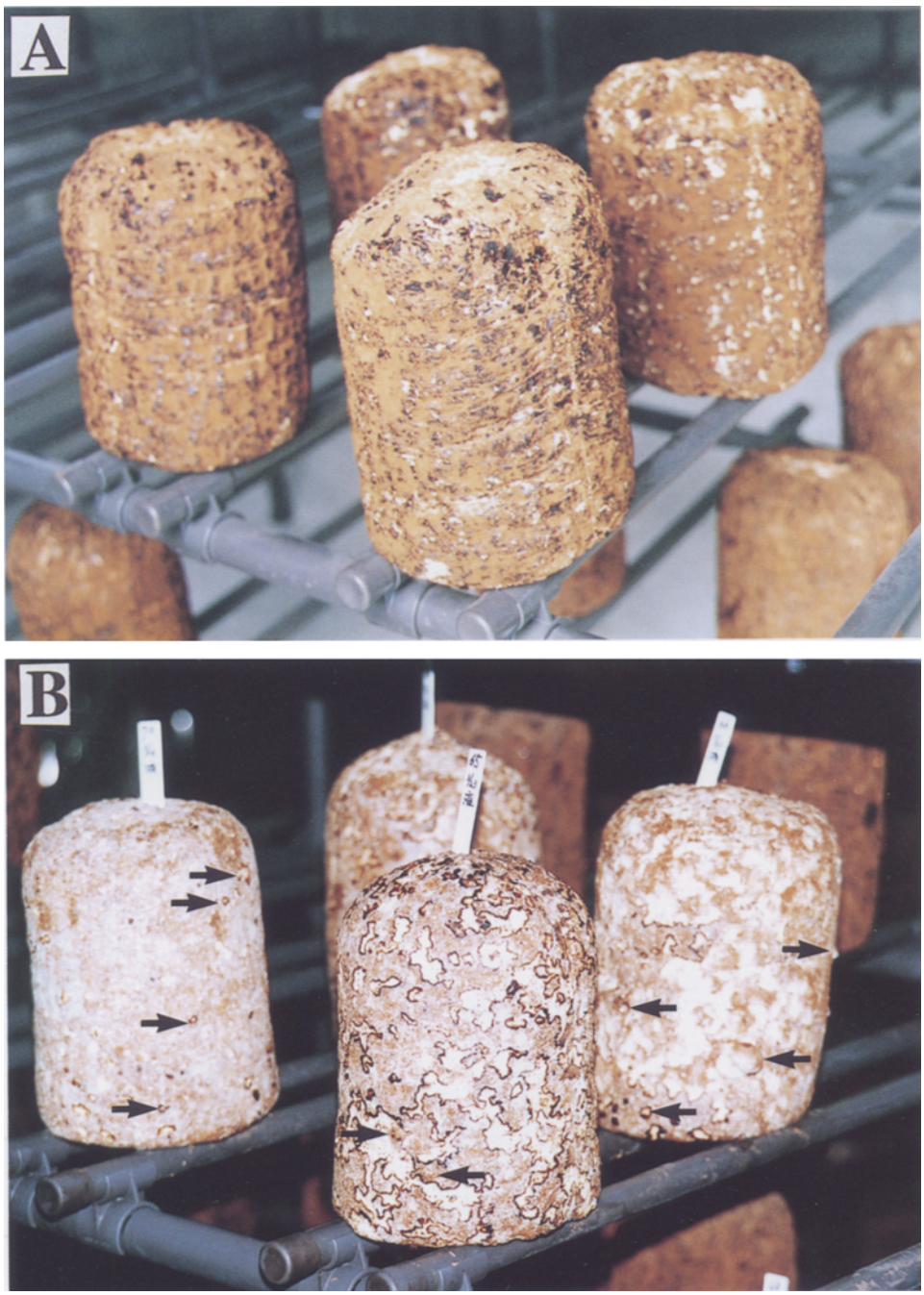

Fig. 5. Appearance of the colonized blocks just removed from the polyethylene bags.
A. 60-d incubated blocks inoculated with a solid spawn. B. 90-d incubated blocks inoculated with a liquid spawn cultured for 53 d.
Arrows: Fruit-body initials.

spawn. The total harvests from the blocks with the liquid-solid spawn were better than those with the solid spawn. The early fruit nature of the liquid spawn was preserved by subculturing mycelia on agar medium and sawdust substrate. Lack of pigmentation and immature fruit-body formation during bag-incubation were also observed on the blocks with the solid subcultured spawn of the long-term liquid culture.

Discussion

In the blocks inoculated with liquid spawn, the first colonization was one day behind that of blocks inoculated with the solid spawn, but the maximal formation of carbon dioxide was higher (Fig. 3). Kinugawa and Tanesaka (1990) suggested that the production of carbon dioxide largely correlates with the area of newly

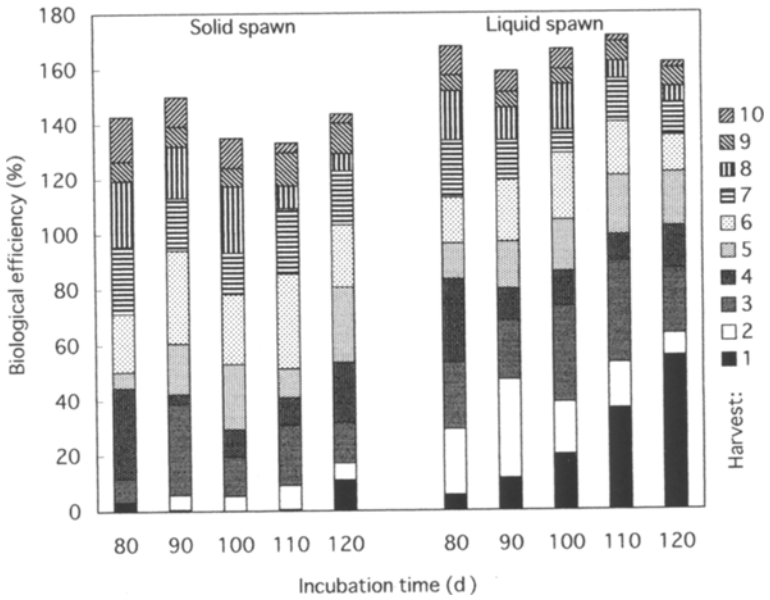

Fig. 6. Effect of incubation period on mycelial colonization and maturation prior to the fruiting stage.
The blocks inoculated with the solid (S3: left) or liquid spawn (L3: right) were incubated for the indicated periods, and harvesting was done in 10 cycles of 25 d in the fruiting stage as indicated in the text. One experimental section consisted of twenty blocks, and the average harvest per block is shown. The liquid spawn (L3) was cultured for 34 d.

growing mycelium. The liquid spawn might have acquired higher growth rate and that might shorten the incubation period needed for normal fruiting.

Royse (1989) reported that the biological efficiency (BE) of a synthetic substrate was from 85 to 135%, depending on the strain used, length of spawn run, and length of harvest. Diehle and Royse (1991) reported a higher value of 154%. Our data (BE: 105-169%) are on the high side. The fruiting period was reported to be 105 d by Kirchhoff and Lelly (1991), 120 d by Jong (1982), or 200 d by Diehle and Royse (1991). This mushroom cultivation needs a rather long time of 200-250 d. The test strain (L1052) had a good tolerance against injuries by rough handling and allowed the long fruiting period.

The incubation period may vary from 60 to 160 d depending on the strain, the substrate, temperature, humidity, aeration, and so on (Jong, 1992; Wuest, 1989).

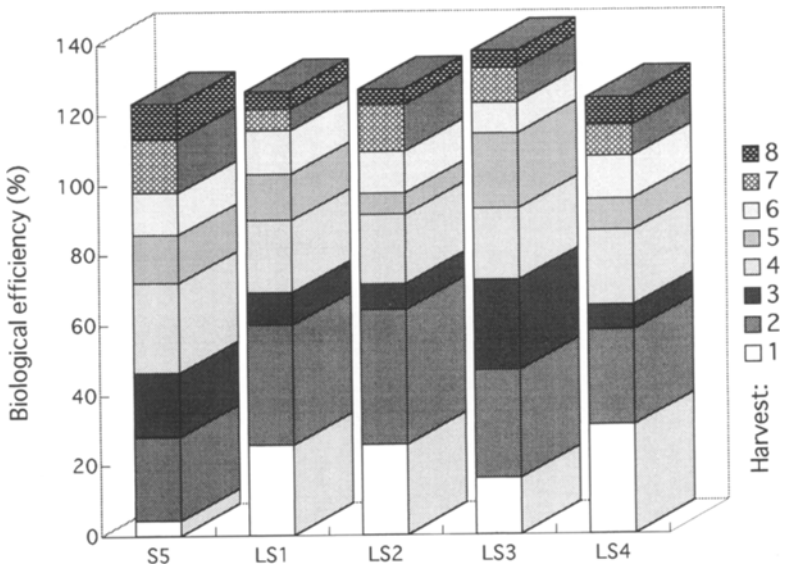

Fig. 7. Harvests of shiitake mushroom from sawdust blocks inoculated with the solid subcultured liquid spawn.
The liquid spawns used in the test in Table 1 were also inoculated on an agar slant and preserved at 5°C. Then the culture was inoculated onto sawdust medium to make the liquid solid spawn (LS1-4). Culture conditions were the same as in Table 1.

The solid spawn needed more than 120 d. The strain (L1052) was not an early fruiter. Early fruiters usually produce poor quality mushrooms. The mushrooms produced by the solid spawn of the test strain were of good quality. The sawdust blocks with the liquid spawn incubated in bags for less than 90 d produced good quality mushrooms.

The solid subcultured spawn of the liquid cultured mycelium retained the early fruit nature (Fig. 7). We assume that the early fruit nature might be a hereditary phenomenon. It is supposed that the liquid culture might change the organism into early fruit. Solid culture did not have such an effect. Rustchenko et al. (1993) reported that *Candida albicans* and *Saccharomyces cerevisiae* accumulated high number of rDNA units per cell in rapidly growing cells. It was also reported that repeat sequences such as rDNA (Iwaguchi et al., 1992) and sub-telomeric sequences (Corcoran et al., 1988) were frequently deleted, amplified, and recombined in the culture. It is possible that such a DNA change was induced by the liquid mycelial culture and retained in the solid culture. Identifying such mutant genes is needed to prove this speculation.

This paper reported only on strain L1052. Another strain of *L. edodes* was also induced into early fruiting by the liquid culture (data not shown). Liquid culture could be a useful tool for breeding mushrooms, especially *L. edodes*.

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