Part I Shiitake

Chapter 2

Shiitake Spawn and Strain

SHIITAKE SPAWN PREPARATION CHIEFLY WITH SAWDUST

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Introduction

Common characteristics

Shiitake, *Lentinula edodes*, is a saprophyte and white rot fungus which feeds on dead oak tree species in nature. In its life cycle, each basidium under the gill of a fruitbody produces four basidiospores which sporulate under a certain condition to become a primary mycelium and then a secondary mycelium by fusion between compatible mycelia. Shiitake spawn is a medium, colonized by the secondary mycelia. The secondary mycelia will eventually form fruitbodies.
Factors regarding mycelial growth are shown in the table below.

Table 1. Spawn run parameters of shiitake and others

	Lentinula edodes	*Coprinus comatus*	*Pleurotus ostreatus*	*Volvariella volvacea*	*Ganoderma lucidum*
Temperature (℃)	21-27	21-27	24	24-35	21-27
R.H. (%)	95-100	95-100	85-95	80-95	95-100
Duration (days)	35-70	12-14	12-21	5-10	10-20
CO_2 (ppm)	> 10,000	5,000-20,000	5,000-20,000	> 5,000	< 50,000
Fresh air exchange	0-1 / hour	0-1 / hour	1 / hour	1	0-1 / hour
Light (lux)	50-100	n/a	n/a	No light	n/a

Source: Paul Stamets, 2000

Classification

Strain categorized by fruiting temperature

Shiitake strains are classified into three kinds by fruiting temperature: high, mid and low temperature strains. Strains fruiting best at 15±5℃ belong to mid-temp. strain range; lower than that are called a low-temp. strain; strains fruiting at higher temperatures are called a high-temp. strain. Optimum incubation temperature for all strains is around 25℃(Fig. 1). It is reasonable for a grower to have an assortment of 2-3 strains.

1) Low-temperature (cold-weather) strains usually grow slowly and produce thick-fleshed big caps. In cold areas where the climate is not good for mass production, growers can rather produce high quality shiitake by growing low-temp. strain shiitake on logs. In log cultivation, it takes long time for incubation, from 16 to 20 months. However, logs may produce fruitbodies for four or more years, much longer than substrate bags.
2) High-temperature (warm-weather) strains grow fast, are easily browned, but produce thin-fleshed small caps. Under

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the warm climate, growers can combine high-temp. strains with the bag cultivation method to shorten the production period and compete well in terms of producing amount. In case of bag cultivation, these warm strains require 1-2 months for the spawn run and will produce for several months after production begins.

3) With wide-temperature (all-weather, wide-range) strains, incubation takes nearly the same time as warm-weather strains. They can be grown at a wide range of temperatures and they can be good choice to beginning shiitake growers. In case of log cultivation, with the wide-range strain, spring inoculation may produce the following fall and fall spawning may produce the following spring. Logs are expected to produce fruitbodies for 3-4 years after pinning. The production period depends on substrate material, log size, spawn type, climate, management and so forth (D. B. Hill, 2002).

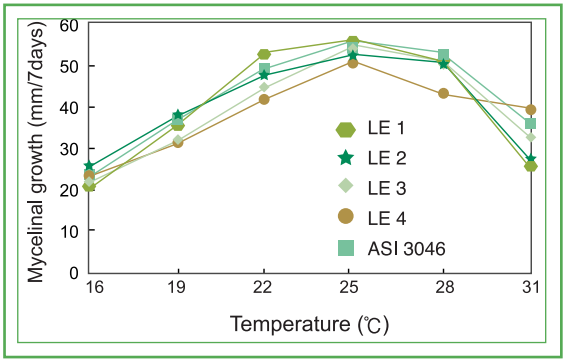

Figure 1. Mycelial growth of Lentinula edodes PDA medium for 7 days after inoculation. Fruiting temperature: LE1 (16-24℃, mid-high), LE2 (18-20℃, mid), LE3 (14-24℃, mid-high), LE4 (5-16℃, mid-low), ASI3046 (10-25℃, mid-high) (Hongkyu Kim, 1999)

Spawn types by spawn media

Sawdust from various trees, grains, woods, and other agricultural by-products can be used for preparing shiitake spawn media. J. Poppe (2000) reported several research results showing a variety of substrate materials for suitable shiitake spawn, including hammer-milled corncobs (Chang and Miles, 1989); cottonseed hulls (Chang and Miles, 1989); peanut hulls (Chang and Miles, 1989); sawdust with rice bran as additive (Singer, 1961); and sawdust, mixed with rice bran (Singer, 1961). A spawn maker has the choice of at least thirty kinds of sawdust but some sawdust is not digested as well by the mushroom enzymes. In general, the broad-leaved trees deliver the best sawdust for shiitake spawn. In very exceptional cases, lubricant oil from chain saw can disturb the mycelial growth (Chang and Miles, 1989; Poppe, 1995). Tea leaves have also been used as a spawn substrate in Asia, mostly when mixed with grains or cotton waste (Chang and Miles, 1989). Wheat bran is also used as an additive for sawdust based spawn (Chang and Miles, 1989).

1) Dowels (wood plugs): Dowels, made of hard wood, are resistant to dry conditions and can be stored longer than sawdust spawn. They are easy and fast to use when inoculating as the farmer needs only hammer them or push to insert them into the inoculation holes and then seal.
2) Sawdust spawn: Being composed of small sawdust particles, it has a larger surface area than the same volume of dowels. Sawdust spawn incubates quickly, and can also dry out quickly unless proper conditions are maintained. In case of log cultivation, a spawning tool and sealing wax are required for performing inoculation. Some modern spawning machines use compressed air to insert the spawn into the hole and cover the hole with a styrofoam cap at the same time. In case of bag cultivation, a farmer cuts a piece of the spawn mass and puts it into the inoculation hole. Like a wood dowel, sawdust spawn can be made into sawdust plugs (thimbles). They have the merits of both dowels and sawdust spawns. Thimbles are easy to use and colonize substrate well. However, they are more expensive than the other spawn types. They also dry easily unless stored properly, and should be used as soon as they arrive (D. B. Hill, 2002).
3) Grain spawn: It is used for bag cultivation. This spawn may attract rodents, but is easier to handle than sawdust spawn.

How to Make Spawn

Shiitake spawn can be made using high quality fresh shiitake or wild picked specimens as the inoculum. A fruitbody is cut into two pieces vertically, and then a small piece is taken from a cut surface of the fruitbody and transferred to PDA media. This is then incubated. Though some growers certainly make their own spawn by themselves using this method, the development of a pure, high quality strain needs sophisticated equipment, time and money. It is, therefore, recommended to buy the desired strains from reliable sources.

The starting point for most spawn producers is the acquisition of a test tube containing strain from an institution such as universities or extension centers where the strains are developed and maintained. They will then transfer the strain to PDA medium in petri dishes. After multiplying the culture by repeating the process several times, they will incubate strain among a spawn substrate such as sawdust, wood plugs or grains. When they acquire sufficient quantities, the spawn is ready for distribution. There are commonly five generations of the spawn, they being the F1 (strain in tubes), the F2, F3, F4 (strain in petri dishes or spawn bottles), and the F5 (spawn in spawn substrate).

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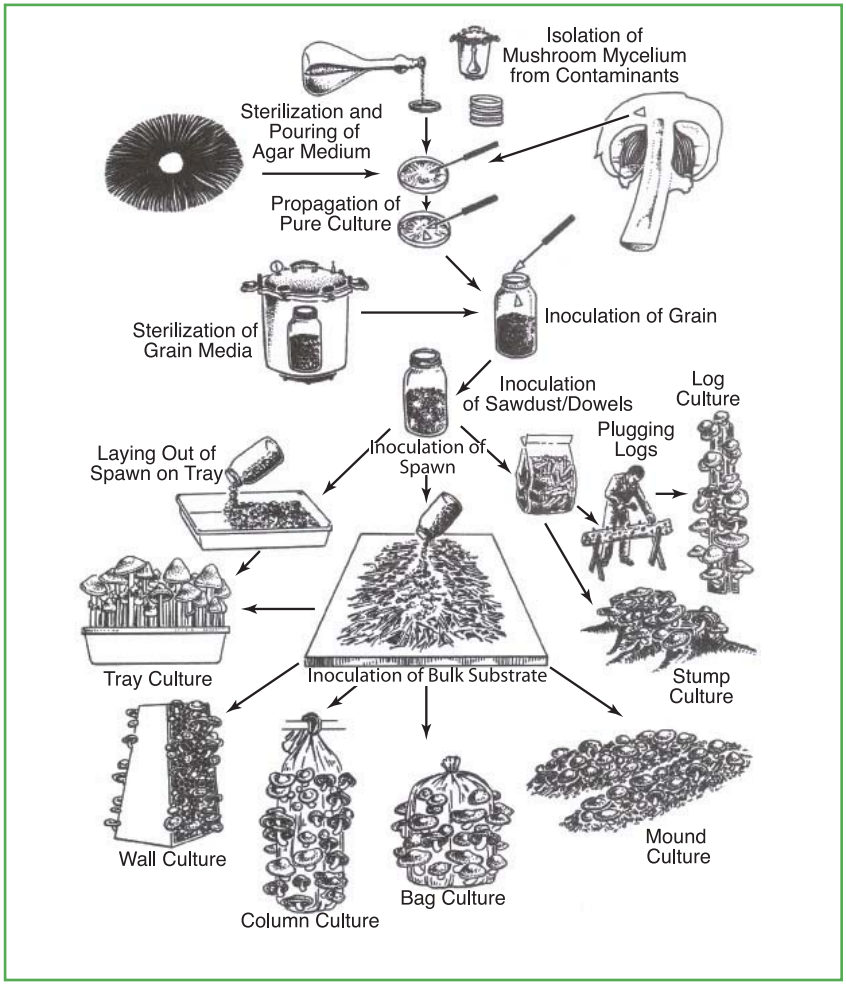

Figure 2. Overview of techniques for growing mushrooms (Paul Stamets, 2000)

Mycelial transfer from PDA in tube (strain) to PDA in petri dish

Materials

a) Strain (mother culture in tube)
b) 500㎖ PDA media: 1,000㎖ distilled water, potatoes, 10g agar, and 10g sugar
c) Clean bench equipped with air filter, alcohol lamp, petri dishes, transferring tools, 70-75% alcohol (ethanol) and UV lamp
d) Autoclave, glass jars, scale, tin foil, incubator and etc.

Preparation of PDA media (500㎖)

Figure 3. Preparation of 500㎖ PDA media **A**: Wash potatoes; peel; remove buds; and cut them into cubes (1×1×1cm each). **B**: Weigh on the balance (100g). **C**: Boil 100g of cube in 500㎖ water for about 30 minutes on a small flame to extract the starch.

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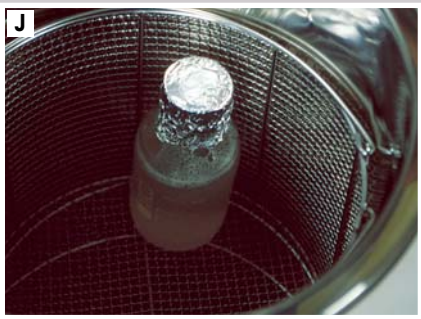
D: During boiling, prepare exactly 10g agar and 10g sugar for 500㎖ PDA media. **E** and **F**: Some water vaporizes during boiling. After boiling, add distilled water to make the volume (potato extract water) 500㎖ again.

G: Stir well to melt the agar & sugar, and pour the mixed extract into a bottle for autoclaving. **H** and **I**: Close the jar lightly and cover it with tin foil. The air volume inside the jar is going to decrease during cooling after sterilization; and outside pathogens may invade the jars via the incoming outside air. Tin foil is to keep out contamination.



J: Autoclave the jars at 121 ℃ for 20 minutes. Time should be measured from the point that the temperature inside the autoclave reaches 121 ℃; so, the total autoclaving time takes more than 20 minutes. **K**: Disinfect the clean bench before using by spraying 70-75% alcohol on the clean bench and on the transferring tools. In the hot season when contamination increases, a UV lamp can also be used. Operators should wash their hands with 70-75% alcohol. After autoclaving, let the jars cool down to 65-70 ℃ on the clean bench. **L**: Place the petri dishes in a column; open them from the bottom one and pour 30-35㎖ of the PDA broth quickly and then let those dishes solidify. By staking the dishes, less water drops will form inside the each petri dish. The agar will solidify at around 45 ℃ and after it does then dishes are ready to use.

Transferring

Figure 4. Transferring from PDA in tube (strain) to PDA in petri dish **A**: Confirm that the mother culture is good. Before transferring, sterilize the tool until it becomes red-hot in the flame. **B**: One hand grabs tube and the other hand grabs tool; cool the tool by touching it on the media. **C**: Hang the tube mouth on the flame for a while; open the cap with a little finger.

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D: Cut a sample (2 × 2mm) from the mother culture. Tube mouths and tools are flamed on the alcohol lamp before each opening and action. **E**: Slightly open the petri dish and place the sample to the center of the PDA media; close the cap; wrap up the complete dishes with plastic **F**: Record the required information such as date and strain name. Immediately after the inoculation the petri dishes are kept at a slightly (2-3 ℃) higher temperature for several days. It will quite visible soon whether they are contaminated or not (Hyunjong Kwon, 2002).

Problems and solutions

Problems which can occur during the above mentioned stages and the solutions to those problems are described in the following table.

Table 2. Problems and solutions

Problems	Causes	Solutions
Agar medium is very soft and does not solidify	- Incorrect ratio of agar and liquid - Variation in pH	- Follow the correct ratio - Adjust the pH using HCl or NaOH
Growth of contaminants on the surface of the medium in test tube before inoculation	- Medium has not been properly sterilized - Plug (tube cap) may not be airtight - Incorrect method of pouring medium	- Sterilize at required pressure for required time - Plug the cotton tightly - Pour the medium into clean dry sterilized dish
No growth on transferred mycelial bits/discs	- Use of incorrect medium - Variation in pH - Medium is too hot - Death of mycelial tissues in the bit during transfer	- Use correct medium - Adjust the pH - Pour the medium at a lower temperature - Cool the hot inoculation needle before contact with the tissue
Contaminated mycelial plug / contaminated surface after 2-3 days of transfer	- Inoculation location is not aseptically maintained - Use of contaminated culture	- Laminar flow chamber and clean inoculation chamber may be used for inoculation - Clean pure-culture (without contamination) should be used
Growth of mycelium is very slow and fluffy	- Degenerated or virus infected mycelium - Non viable culture	- Use medium size, first harvested mushrooms for pure culturing - Isolate the mushroom from the tissues at the junction of stipe and the pileus

Source: G. Arjunan *et al*, 1999

Mycelial transfer from PDA in petri dish to spawn substrate

When enough petri dishes are acquired by repeating the above process several times, they are transferred to spawn substrates.

Materials

a) Mother culture in petri dish
b) Substrate materials for shiitake spawn
c) Container (spawn bottle), sterilizer (autoclave), mixer and spawning room (clean booth or bench)
d) Incubation room with environmental control system is recommended

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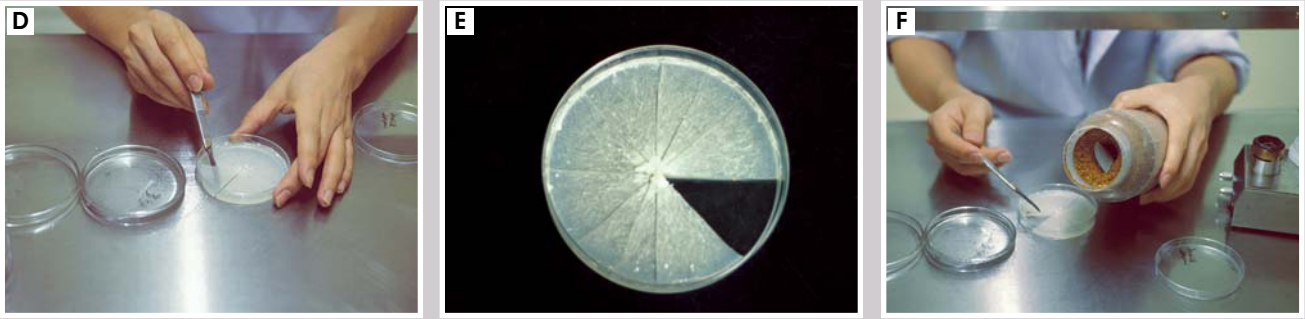
Transferring from PDA in petri dish to sawdust

Figure 5. Transferring from PDA in petri dish to sawdust **A** and **B**: Fill the heat-resisting spawn containers (bottles, jars or bags) with substrate mixture. 850cc bottle is usually filled with 550cc substrate. Compact the surface of the substrate and make the inoculation hole (1.5-2cm diameter). Close the cap and remove the substrate particles around the bottle mouth. **C**: Sterilize the bags just after filling at 121 ℃ for 60-90 minutes. Time should be measured from the point that the temperature inside reaches 121 ℃; so, the total autoclaving time takes more than 60-90 minutes practically. During sterilization, keep discharging small amount of steam to raise the temperature of substrate evenly. When it goes up to 121 ℃ inside the substrate, keep the temperature for 60-90 minutes.



D, **E** and **F**: After cooling, when the substrate is prepared in the bottles, disinfect the clean bench; transfer the mycelia from incubated mother culture in petri dish above into the inoculation hole of the substrate in the bottles. Record any needed information. Completed spawn is kept at low temperature around 4-5 ℃. After two days of spawning, growers should raise them to a normal temperature. When enough quantity is acquired by this multiplication, the bottle contents can be used as sawdust spawn (Hyunjong Kwon, 2002).

Transferring from sawdust into thimble

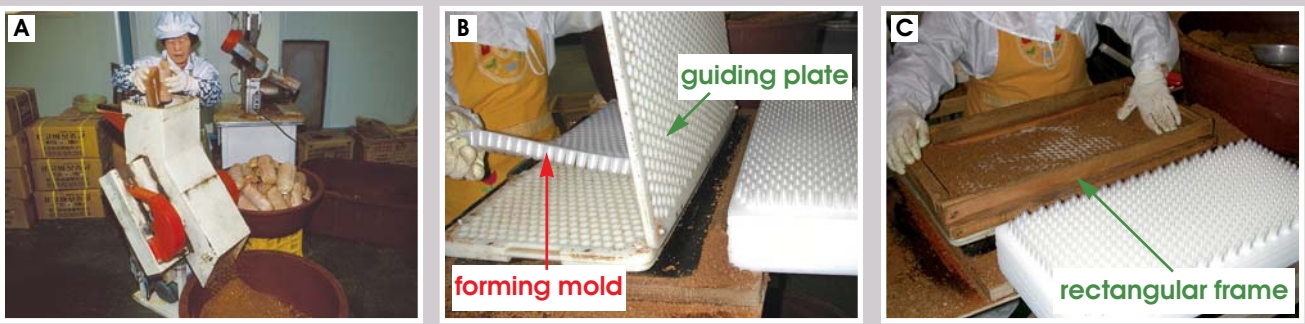


Figure 6. Transferring from sawdust into thimble **A**: Get rid of plastic bottles and crush sawdust spawn. **B**: Put forming mold between two layers of guiding plate. Guiding plate has many perforations. **C**: Place rectangular frame on the guiding plate and pour crushed spawn into the frame.

D: After filling each hole of forming mold with crushed sawdust spawn, remove surplus spawn from the plate. **E**: Place styrofoam on the plate and press to seal each hole of the mold; then remove the guiding plate. **F**: Stack completed forming mold, and move them to incubation room.

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Transferring from PDA in petri dish to grain (by Wanchai Pale, Thailand)

After washing the grains in clean water to remove decayed seeds or dirt, the grains are soaked in clean water for 8 hours at room temperature. Both wheat and sorghum grains can be used, but sorghum is more common. Boil or steam the grains until the grain testae expand and began to crack open. Discard the water and air-dry the steamed grains on clean paper or clean linen cloth.

Fill the clear-flat bottles with the grains to the halfway mark. Close them with cotton plugs, wrap over the plugs with paper. Newspaper is in common due to its low cost. Tie the bottle necks with rubber bands. Plastic bags also can be used as the containers. Sterilize them in an autoclave at 15 psi for 30-40 min and allow the grains to cool. In a transfer chamber, inoculate the cooled grains aseptically with the mycelia from incubated mother culture in petri dish above. It takes about 30 days for the mycelia to fully colonize the grains.

Figure 7. Sorghum spawn **A**: Incubation **B**: Fully colonized ones

Problems and solutions

Table 3. Problems and solutions

Problems	Causes	Solutions
Spawn substrate contaminated after sterilization and before inoculation	- Spawn substrate not completely sterilized - Damaged container	- Proper sterilization for the required period - Remove
Mycelial growth may not develop or continue to the bottom	- Excess moisture in the spawn substrate - Over cooking of spawn substrate	- Add sufficient quantity of $CaCO_3$ (20g/kg) for proper moisture content of spawn substrate - Adopt a correct sterilization period
Mycelial spread in the spawn substrate is slow	- Spawn substrate too dry - Prevalence of high/low temperature in the room	- Boiling the spawn substrate for specified period - Maintain the required temperature
Irregular mycelial growth in the spawn substrate	- A virus infected culture	- Isolate and use better high yielding culture
Contamination appears on the surface of the spawn substrate / on the mycelial disc	- Contamination during inoculation - Contaminated culture	- Inoculation has to be done in aseptic conditions in a culture room - Use fresh culture free from contaminant
No growth from the mycelial disc on surface layer of spawn substrate	- Dehydrated surface layer - Sterilized bottles or poly-bags not inoculated in time	- Tightly plug the mouth with cotton wool and cover with aluminum paper and tie with a twine - Use the sterilized spawn substrate within 3 days

Source: G. Arjunan *et al.*, 1999

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Spawn Quality

Maintenance of quality

The criteria for spawn quality are mycelial vigor and the quantity of fruitbodies produced. High quality spawn should also be microbiologically pure and not mixed with other microorganisms and other mushroom strains. The strain should be genetically stable without variation, showing the consistent characteristics of the strain such as color, shape and pinning habit. Mushroom mycelia lose vitality through a series of transfers. Producers should keep the substrate materials clean and meet the sterilization standards. Both cooling and spawning must be done in a clean room. Incubated spawns should be stored in a cool, dark location and shipped in less than a month. Completed spawns should be cultivated at several locations in order to know, when and where any problem has occurred, and to be able to determine whether the problem arose in the spawn production or in the growing process.

Visual method to select high quality spawns

It is not easy to tell whether the spawn is good or bad by viewing only the outside of the mass. High quality spawn should be fully colonized by thread-like white mycelia that appear highly active and sometimes show a gloss on the surface. There should be no spots caused by other microorganisms and no sour smell. There should be no young fruitbodies growing out of the container.

In contrast, long stored spawn may have a gap between the spawn mass and container wall and may have yellow colored mycelia skins. Yellow or brown water droplets may form inside the container due to an oxygen shortage. Contaminated spawn may have green or orange stains on the surface, a bad, sour smell, and yellowish brown or reddish brown water drops.

Cultural methods to check apparent contamination of spawn

Moist culture

This method uses a moist chamber that provides conditions preferred by the fungi. A transparent plastic jar with a large mouth is filled with clean gauze or a kitchen towel to one third of the height. Enough water is poured to drench the filling materials, and then a dish shaped aluminum foil is put on top of it. Place about 10g of crushed spawn on top of the foil and cap the bottle. Place the bottle where direct sun light can not reach it, and maintain it at 20-30℃ for 3-5 days. Observe the re-growth of the mycelia.

Healthy white spawn will resume their growth and form a white mycelial mass. But, unhealthy spawn will not begin re-growth or, if ever, re-grow very slowly. Mycelia, contaminated by pathogenic fungi such as green mold (green, blue, greenish yellow, bluish green), red bread mold (*Neurospora*, orange, pink) or black mold (black, dark gray), show the specific color of contaminant's spore. And, the mycelia contaminated by bacteria secrete sticky mucous fluid without re-growth.

Distilled water culture

This is a still culture (stagnant culture) wherein mycelia are incubated in distilled water. Fill 100㎖ of distilled water in an Erlenmeyer flask, cap and sterilize it at 121℃ for 15-20 min. After finishing sterilization, cool the flask to 25℃, put 10g of sawdust spawn inside and incubate it at 25-30℃ for 3-5 days.

In healthy mycelia, a white mycelial colony will float on the clean and transparent water. In fungi contaminated mycelia, specific colored pathogenic spores float on the clear and transparent water. In bacteria infected mycelia, mucous membrane forms on the tainted water. In this test, contamination should be determined by the clarity of water rather than just the color of water, because the water color changes into tan or brown by the fermentation of the sawdust itself (Hyun-wook Lee, 1999).

Case Study: Mr. Lentinula

Mr. Lentinula, a virtual shiitake grower, hopes to produce 100kg of fresh shiitake every month. He knows how to make his own strain (tissue culture) from shiitake fruitbodies but he decided to acquire a superior strain from an extension service center and brought some strain in tubes, and transferred them into petri dishes. Now, they are almost incubated and seem to ready to make spawn.

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<Calculation #1: He produces 100kg shiitake. How much spawn does he need?>
Saying biological efficiency (B.E.) is 50%, 200kg of dry substrate is needed to produce 100kg of fresh shiitake.

B.E.=fresh weight of mushroom/dry weight of substrate

Assuming water contents of substrate is 65%, 371kg of water will be added to the 200kg dry substrate above.

water contents = water/(dry substrate+water)

Then, wet weight of substrate is 571kg.

wet weight = dry substrate+water

Saying the inoculation rate is 1.05%, he needs *ca*. 6kg of shiitake spawn.

inoculation rate = spawn/wet weight of substrate

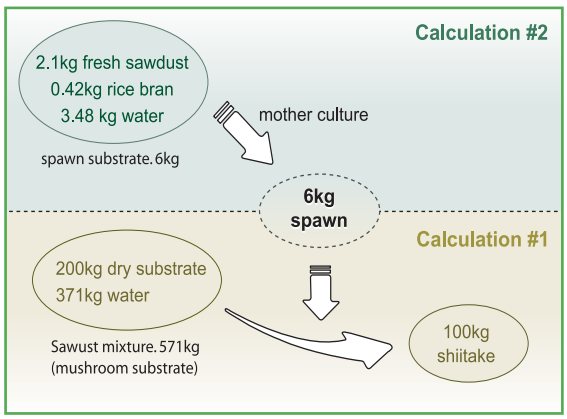

Figure 8. Mr. Lentinula's calculation

<Calculation #2: He needs 6kg spawn. How much each materials does he need?>
As for spawn substrate, Mr. Lentinula uses 80% dry sawdust (main material) and 20% dry rice bran (nutritious supplement); water content is 65%. From the three equations below, now he knows that he needs 3.9kg water, 1.68kg sawdust and 0.42kg rice bran.

dry sawdust+rice bran+water=6kg

water/6kg=0.65

dry sawdust=4 × rice bran

The supplier told him that water content of fresh sawdust was 20% and the bran was completely dried. Therefore, he ordered 2.1kg fresh sawdust which is 1.68kg dry sawdust & 0.42kg water, and 0.42kg rice bran. Then, already having 0.42kg water in the sawdust, he needs to add only 3.48kg water. Mixing them, he makes 6kg spawn substrate.

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