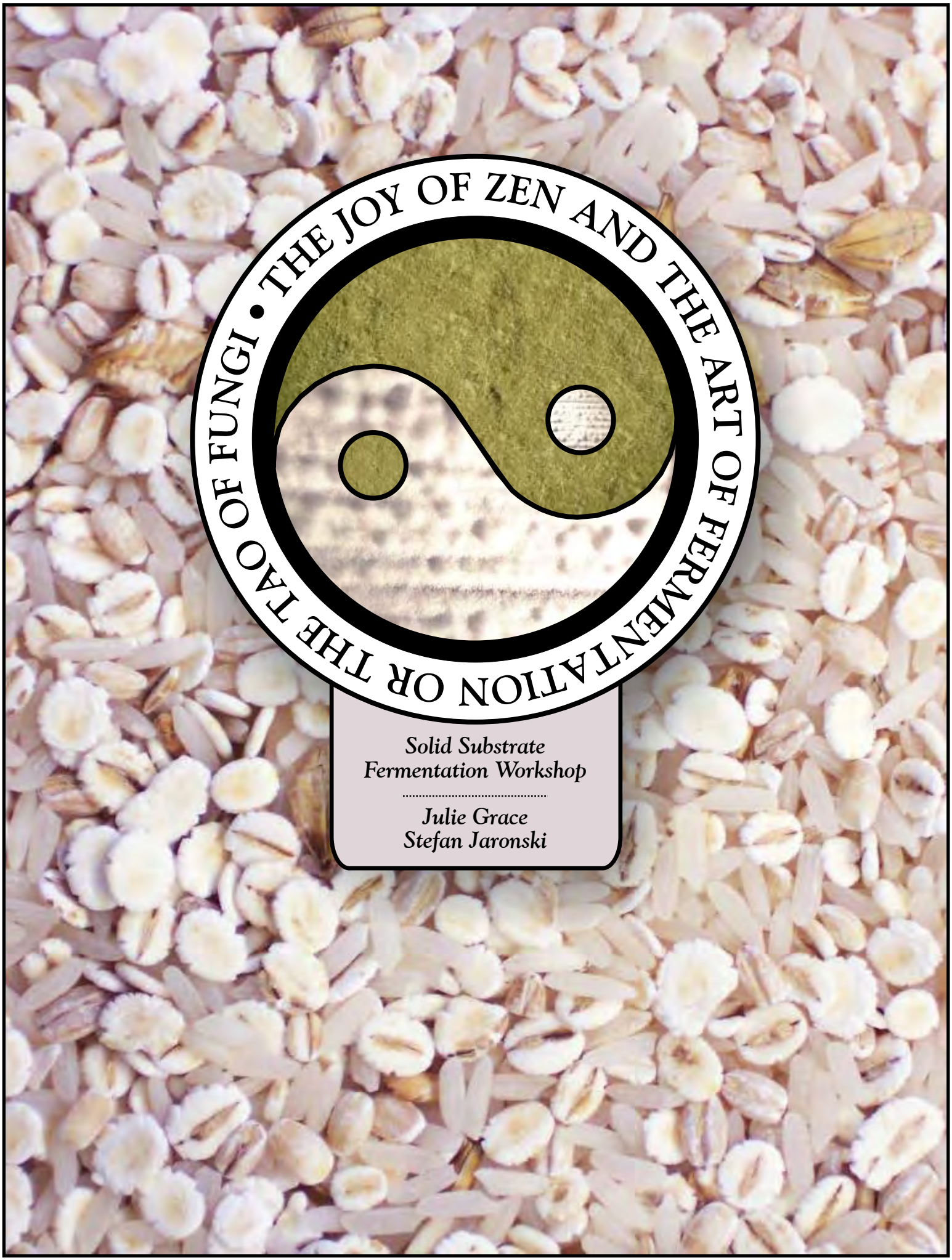


THE JOY OF ZEN AND THE ART OF FERMENTATION OR THE TAO OF FUNGI

Solid Substrate Fermentation Workshop

Julie Grace
Stefan Jaronski

SOLID SUBSTRATE FERMENTATION

of Beauveria bassiana and Metarhizium anisopliae

Workshop
February 16,17,18, 2005

USDA-ARS Northern Plains
Agricultural Research Laboratory
1500 North Central
Sidney, Montana 59270

Contact:
Stefan Jaronski

Tel: (406) 433-2020 (ext. 186)
Fax: (406) 433-5038
Email: sjaronski@sidney.ars.usda.gov

Written by: *Julie Grace* and *Stefan Jaronski*
Design by: *Bethany Redlin*

Commercial Endorsement Disclaimer:

The use of trade, firm, or corporation names in this web site is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

USDA Nondiscrimination Statement:

The U.S. Department of Agriculture (USDA) prohibits discrimination in all its programs and activities on the basis of race, color, national origin, age, disability, and where applicable, sex, marital status, familial status, parental status, religion, sexual orientation, genetic information, political beliefs, reprisal, or because all or part of an individual's income is derived from any public assistance program. (Not all prohibited bases apply to all programs.) Persons with disabilities who require alternative means for communication of program information (Braille, large print, audiotape, etc.) should contact USDA's TARGET Center at (202) 720-2600 (voice and TDD). To file a complaint of discrimination, write to USDA, Director, Office of Civil Rights, 1400 Independence Avenue, S.W., Washington, D.C. 20250-9410, or call (800) 795-3272 (voice) or (202) 720-6382 (TDD). USDA is an equal opportunity provider and employer.

United States Department of Agriculture

Agricultural Research Service

Northern Plains Agricultural Research Lab
Sidney, MT

Introduction *(By Julie Grace)*

While planning this "cooking class," I was asked a series of questions to which I answered, "Don't worry, you'll learn to feel the fungus." The response was, "Oh, good! A Zen cooking class!" And so, the Zen began. The use of Zen as an abstract concept of fermentation may seem, at first, on the side of absurd, but after giving it considerable thought, I came to understand how fermentation has become my mantra while fungus and media my "OM."

So, why NOT Zen? Why demand an organism to accept our terms and not to learn to accept theirs? Feel the fungus. As a fish swims but doesn't realize the water, a fungus grows but doesn't realize the medium. It's up to us to help them realize the medium...so...

Let the cooking class begin.....

The Substrates and How We Select Them

The basic requirements for healthy growth and conidia production of any filamentous fungi are adequate temperature, PH, water activity, gas exchange of CO_2 and O_2 and nutritional needs consisting primarily of carbon, phosphorous and nitrogen sources. There are several means of providing these requirements - the most common are liquid, agar and solid substrate fermentations. Why solid substrate fermentation? The Tao of fungal conidia is to be airborne. Solid substrate fermentation is the only means of acquiring a stable purified aerial product. It is produced relatively quickly in mass quantities with a simple recovery method.

Solid substrate fermentation is a very efficient process. The substrates are all products of agriculture grains, seeds, beans all sources of starch, cellulose and fiber. Once hydrated and sterilized, the grain readily absorbs nutrients from liquid culture providing the necessary requirements for healthy biomass production. Filamentous fungi decompose starch by excreting highly concentrate hydrolytic enzymes from their hyphal tip to penetrate the substrate and access nutrients - promoting metabolic activity and rapid development.

The heterogeneity of substrate beds present the only true drawback to solid substrate fermentation which is a challenge that can be modified by increasing surface area and daily mixing of granules to facilitate mycelia branching during first stages of vegetative growth in order to promote metabolism which optimizes spore production. Spawn bags offer low pressure air exchange, moderate water activity levels and osmotic pressure conducive to environmental factors for the physiological, enzymological and biochemical properties necessary to encourage microbial growth.

How do we select our substrate? There are two commonly used grains: barley and rice. We have experimented with many grains, beans, seeds, pulp, etc. Barley is our substrate of choice for B. *bassiana* and M. *anisopliae* because: 1) the species will penetrate and utilize the grain with ease 2)

(Continued next page)

Continued from previous page....

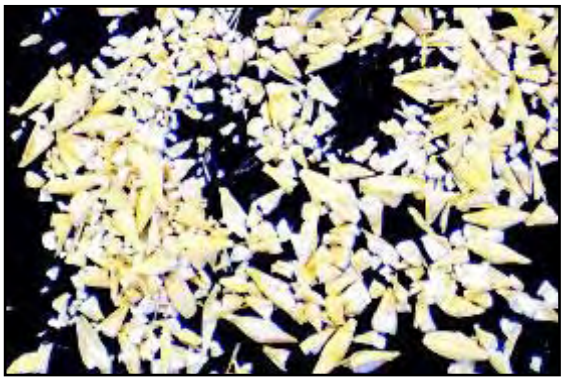
hydrated barley absorbs all liquid nutrients with great efficiency 3) barley crumbles well into individual granules that provide a large surface area 4) maintains a moisture gradient and 5) does not decompose into minute particulate that can mix with the end product. Ultimately, the decision is yours to make but why choose the path of most resistance?

There are strains of Beauveria and Metarhizium that do not do well in solid substrate fermentation. Fungi that display low growth rates are at risk from competitive microbes. Some isolates produce too much heat during fermentation which requires the installment of a cooling system to maintain the bed temperatures. Others produce a cement-like mycelium that binds the substrate together minimizing surface area and spore production. The latter situation can be remedied by adding an agent to "soften" the bed by increasing surface area - which discourages the binding of granules, and provides aeration throughout the substrate, which maintains a moisture gradient and even distribution of gas exchanges for metabolism. ■

Common Grains for Solid Substrate Fermentation - see next page.

Common Additives to Soften Beds - see page 7.

Common Grains for Solid Substrate Fermentation



Cracked Wheat: *Decomposes to tiny particulate.*
SUPPLIER: *Any grain vendor.*

Pearl barley: *Harder husk; not as easily penetrated.*
SUPPLIER: *Any grain vendor.*

Feed barley: *Hull on. Difficult to penetrate.*
SUPPLIER: *Any ranch and feed vendor.*

Rice: *Widely used; excellent grain.*
SUPPLIER: *Any grain vendor.*

Barley flake: *Very easily penetrated. Perfect for B. bassiana and M. anisopliae.*

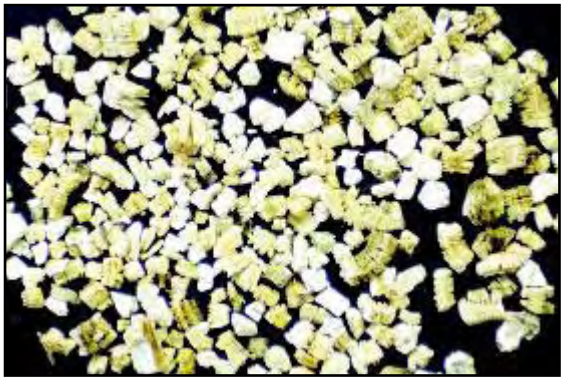
SUGGESTED SUPPLIER:

Minnesota Grain, Inc.
1380 Corporate Center Curve #105
Eagan, Mn 55121
Tel: (651) 681-1460,
(800) 535-7405 ext. 17 (Bonnie)
Fax: (651) 681-7975

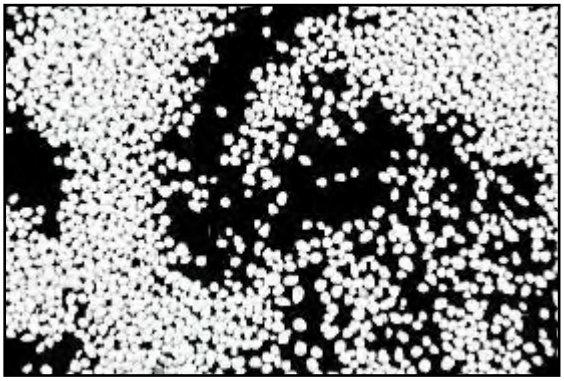
Common Additives to Soften Beds

Chick grit: *Works great except it gets very heavy.*
SUPPLIER: *Any feed, ranch supply.*

Peat moss: *Bad choice. Mixes with end product - clogs sieves during spore recovery.*
SUPPLIER: *Any garden center.*



Vermiculite and perlite: *Both work well, but perlite is the better choice because it holds up better, doesn't interfere with the end product.*
SUPPLIER: *Any garden center.*



Biodac: *A granule made of paper sludge - a material consisting of clays with short fibers. Clogs sieves during spore recovery.*

SUGGESTED SUPPLIER *(sole source)***:**
Kadant GranTek Inc.
607 Liberty Street
Green Bay, Wisconsin 54304
Tel: 414.454.2474
Fax: 414.258.9558

Corn granules (snack grits): *Excellent choice, but clogs sieves during spore recovery.*

SUGGESTED SUPPLIER:
Bunge North America
11720 Borman Drive
St. Louis, MO 63146
Tel: (314) 292-2000
Fax: (314) 292-2110
Email: bunge.info@bunge.com

Liquid Culture

The first step in solid substrate fermentation is to produce a hearty blastospore culture. This is the phase that introduces nutritional requirements of sugars, phosphorous, nitrogen, etc. dependent upon the needs of the strain you are producing. Appropriate recipes follow.

Materials needed:

CSYE+ broth in 500ml flasks w/ caps or plugs covered with aluminum foil
SPM+ broth in 500ml flasks w/ caps or plugs covered with aluminum foil
Plate cultures of B. *bassiana* and M. *anisopliae* (mature and well sporulated)
Sterile inoculating loops
Latex gloves
Clean lab coat or sterile Tyvek sleeves
Laminar flow hood, clean room, or biohazard hood

Procedure:

Prepare CSYE+ broth @ 300ml/sample in 500ml flasks (B. *bassiana*)

Recipe: add to 1 liter ROH_20 (reverse osmosis or deionized water)
40g glucose
10g KNO_3
5g KH_2PO_4
2g $MgSO_4$
0.05g $CaCl_2$
2.5g yeast extract
1ml gentomycin (our choice for antibiotic as it is autoclavable)

Prepare SPM+ broth @ 300ml/sample in 500ml flasks (M. *anisopliae* prefers a slightly enriched broth)

Recipe: add to 1 liter ROH_20
10g glucose
10g yeast
4g K_2HPO_4
2g KH_2PO_4
1g NH_4NO_3
0.2g $MgSO_4$
0.2g KCl
Trace minerals - 0.002g $FeCl_2$, 0.002g $MnSO_4$, 0.002g $ZnSO_4$
1ml gentomycin (again, our choice as it is autoclavable)

(Continued next page)

Continued from previous page....

Bring to a boil. Pour 300ml per flask; cap or place foam plugs on each lid cover plugs with aluminum foil; autoclave for 20 minutes @121°C. Cool.

Wear gloves and coat/Tyvek sleeves
Use Laminar flow hood, clean room, or biohazard hood
Inoculate each flask by removing cover, flame rim then add 1 full loop of conidia **(Fig.1 and 2)**
Replace lids; shake @ 150-170 rpm for 72 hours @ 27°C **(Fig. 3)**. ■

Fig. 1 *Loop is disposable 10ul*

Fig. 2 *Note amount of spores floating on surface*

Fig. 3 *Shaker table*

Inoculate Solid Substrate

The blastospore cultures are ready for substrate after 72 hours of rotation on a shaker table. Each culture must be negative for bacteria prior to inoculation. Under aseptic conditions (laminar flow hood works well) flame rim of flask, draw a droplet of culture and apply it to the center of a glass slide. Cover the slide then check under magnification (40x) for bacteria by scanning the area for any contaminants. If the culture is contaminated, discard by autoclaving. If the culture is pure, move on to the solid substrate phase.

Materials needed:

Spawn bags (1+kg) (***Note:*** select appropriate bag for the species. M. *anisopliae* has a definite gas exchange requirement that needs a bag with good ventilation from top to bottom and on both sides front and back. B. *bassiana* needs good ventilation but just enough to enable passive gas flow at the top and bottom of the bag.) (**Fig. 4 and 5**)

Barley flakes (1kg/sample)

ROH_20 (600ml/sample)

Autoclave bags (1/sample)

Autoclave tape

Autoclave pans (1/sample unless you have racks to add a layer)

Latex gloves

Clean lab coat/sterile Tyvek sleeves

Clean bag sealer

Procedure:

Place 1000g (1kg) of barley flake in each spawn bag. Add 600ml of ROH_20, let rest to absorb moisture, and massage well to mix. Flatten out the substrate to form a 1" to 1.5" bed. (**Fig. 6**) Seal spawn bag with autoclave tape. **Do not lay over air patches**. Place spawn bag into autoclave bag; open side first (this prevents any messy spills should the bag open during autoclaving). Tape autoclave bag only once to hold closed allow steam to escape during autoclave process.

Put bags in autoclave pans – do not stack – only one per pan unless you have a rack to place on top of the pan. Autoclave @ 121°C for 55 minutes on gravity exhaust as

(Continued next page)

Fig. 4 *Works well for M. anisopliae. Note the air patches front and back with gusseted bottom.*

SUPPLIER *(Sole Source)*
$SACO_2$ Microsac
Industriezone EKE
Rozenstraat 1A
B-9810 Eke-Nazareth (Belgium)
HRG. 171.697 BTW: BE-451.694.455
Tel: (32)9/280.09.80
Fax: (32)9/280.09.16
Email: info@saco2.com

Fig.5 *Works well for B. bassiana. Note the air patch in front. Can also use a bag with one small patch on the top and one small patch on the bottom.*

SUPPLIER *(Sole Source)*
Unicorn Imp & Mf Corp
113 Hwy 24, P.O. Box 272
Commerce, TX 75429
Tel: (903) 886-8282
Fax: (903) 886-8878
Email: UNICORNbag@aol.com

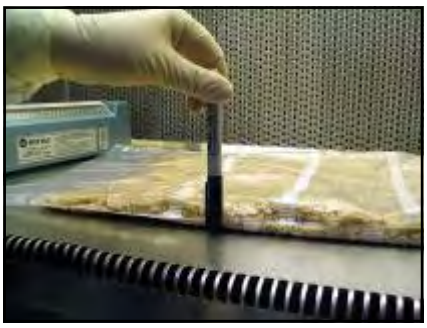


Fig. 6 *Bed should be between 1-1.5" deep*

Continued from previous page....

the vacuum exhaust tends to burn the substrate.

Place all bags in a laminar flow hood to cool. Allow the substrate to cool. completely before inoculating. If you leave it overnight in a laminar flow hood the barley will crumble much easier which increases surface area.

Clean bag sealer. Put in laminar flow hood and turn it on to 7.

Remove flasks from shaker table.

Remove spawn bags from autoclave bags. The spawn bag may stick to the autoclave bag, if so, gently separate the two and slide the spawn bag out.

Crumble substrate well you want it to be as close to individual flakes as possible. To do this you need to massage the bags continuously. **(Fig. 7 and 8)**

Open the bag and stand it up. Open flask, flame rim and pour inoculum into spawn bag. **(Fig. 9)** Let rest a moment to absorb moisture. Massage well to disperse blastospores. **(Fig. 10)** Flatten into 1-1.5 inch beds. **(Fig. 11)**

Seal **(Fig. 12 and 13)** by pressing lever down across the top of the bag then again at angles along both sides as seen in Fig. 12. This securely seals the bag against any possible leaks. Make sure all bags are labeled well and dated. Put bags in 27°C incubator **(Fig. 14)** Observe over 7-10 days.

If there are mushy areas on the substrate there is a bacterial contamination. ■

Fig. 7 *Massage substrate well to break up any clumps*

Fig. 8 *Note broken up substrate – individual granules and clumps*

Fig. 9 *Pouring inoculum into spawn bag.*

(More images next page)

Continued from previous page....

Fig. 10 *Massage inoculum evenly through the substrate.*

Fig. 11 *Flatten into 1-1.5 inch beds.*

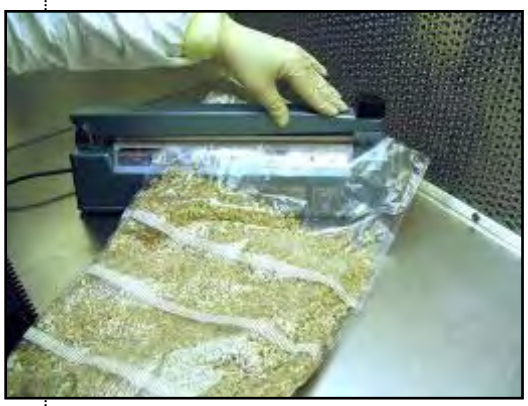


Fig. 12 *Seal at angles.*

Fig. 13 *Seal straight across the top of the bag, then at angles from the sides to the top.*

Fig. 14 *Lay bags on shelf in incubator.*

Drying Solid Substrate

Fermentation is complete within 7-10 days. Beauveria should never take any longer. However, Metarhizium tends to have a mind of its own and may take up to 14 days. Think in terms of Zen to remember some simple facts to unlocking the secrets of our two fungi. Picture the two flowing halves of the Yin/Yang symbol. Think of Beauveria as the masculine Yin side - light, hot, dry, disciplined and outgoing; Metarhizium the feminine Yang side - dark, cool, moist, shy, soft and passive.

There isn't a lot you can do to harm Beauveria. It grows in most broths and on most substrates, can handle a broad moisture range, doesn't flinch at temperature changes and has minimal gas exchange requirements. Metarhizium is quite delicate and has very specific nutrient, substrate, temperature, moisture and gas needs. One of the trickiest aspects of Metarhizium is the drying phase. Water activities need to be monitored daily.

Materials needed:

Scissors
Sterile brown paper bags or clean dry boxes in air chamber
Markers
Stapler
Ethanol or Lysol
Paper towels
Dry rack if using bags, e.g. old refrigerator racks
Latex gloves
Clean lab coat or sterile Tyvek sleeves
Laminar flow hood, clean room, or biohazard hood
Respirator

Procedure for bag drying:

Prepare paper bags by cutting 1/3 horizontally off the top. Use this quarter as a liner inside the bottom of the bag for strength. Label lower portion of the bag. Use two bags per kg of substrate, 500g for each. If weighing for yields, tare bags prior to adding substrate weigh again after substrate is added.

Put on lab coat, moon suit or other protective wear, as well as latex gloves and some form of respirator to avoid inhaling any aerial conidia.

Sterilize spore capture hood.

(Continued next page)

Continued from previous page....

Remove spawn bags from incubator cultures should be covered with spores. **(Fig. 15 and 16)** Be sure to put only one isolate at a time to dry.

Cut open the spawn bag pour ~500g in each paper bag. Crumble the substrate well then seal bag with staples and place on drying racks. **(Fig. 17)**

Autoclave spawn bags and other contaminated items.

Take random water activity samples throughout the drying phase.

Roll bags over daily to dry moisture and allow substrate to move around.

Fig. 15 *Sporulated Metarhizium on substrate.*

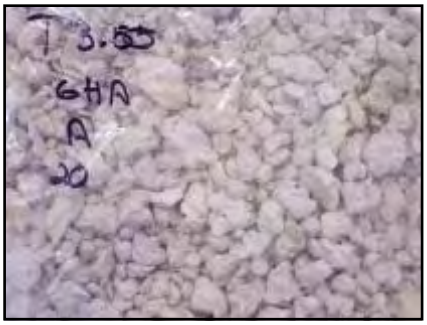


Fig. 16 *Sporulated Beauveria on substrate.*

Fig. 17 *Bags on dry racks.*

Procedure for alternate air chamber box drying:

Prepare boxes. Label lower portion of each. Use two boxes per kg of substrate 500g for each. If weighing for yields, tare boxes prior to adding substrate weigh again after substrate is added.

Put on lab coat or other protective wear, as well as latex gloves and some form of respirator to avoid inhaling any aerial conidia. Note: connidia maybe allergenic especially upon repeated inhalation exposures.

Sterilize spore capture hood.

Remove spawn bags from incubator cultures should be covered with spores. **(Fig. 15 and 16)** Be sure to put only one isolate at a time to dry.

Cut open the spawn bag pour ~500g in each box. Crumble the substrate well.

Use Gilmont flow meter to adjust L/min to equal 20-30 air exchanges/ hour for Beauveria and 4.6 /hour for Metarhizium. (need to calculate / area). Hook boxes up to air flow. **(Fig. 18, 19, 20 and 21)**

Autoclave spawn bags and other contaminated items.

Take water activity samples throughout the drying phase to monitor state of drying.

If the boxes begin to develop condensate, open lids and allow to dry overnight. ■

(More images next page)

Continued from previous page....

Fig. 18 *Air pressure reduction valve.*

Fig. 19 *Boxes in air chamber.*

Fig. 20 *Chamber and Gilmont flow meters.*

Fig. 21 *Substrate (Metarhizium) ready to go into dry chamber.*

Harvesting

The drying phase is complete for Beauveria in 9-10 days. Metarhizium is ready when water activity reaches 0.900. (**Table 1.**) Remember the Tao of Metarhizium is wet and cool. This is the critical phase for Metarhizium. If it dries too much there will be no viability or at best a short lived one. Harvesting presents challenges for Metarhizium as well. Excessive heat from the mechanical process will destroy it. Select methods that keep the species comfortable. As Stefan would say, "Harvest is a lot like a bank account, it's not how much you make, it's how much you keep." We look for quantity and quality (shelf life; purity).

Materials needed:

- Sieve pans 20 mesh, 100 mesh bottom and lid
- Ceramic beads or other material capable of loosening spores from substrate
- Electrical tape
- Scissors
- Funnel
- Balance
- Spatula
- Sample bottles – HDPE plastic preferred
- Sonicator, ultrasonic preferred
- Mycoharvesting unit in lieu of pans and sonicator
- Balance (up to 1kg)
- Latex gloves
- Clean lab coat or sterile Tyvek sleeves
- Respirator

Procedure for sieve and sonicator harvest:

In a laminar flow hood, clean room, or biohazard hood, set up sonicator.

Prepare sieves by putting a handful of beads into 20 mesh pan. (**Fig. 23 and 24**)

Open dry bags by cutting an end off the bottom and pour the substrate into sieve. (**Fig. 22**) If calculating yields, weigh bag with substrate first. Pour substrate into 20 mesh sieve until ~2/3 full. (**Fig. 25 and 26**) Put this pan on top of the 100 mesh sieve. Tape together to prevent conidia cloud from escaping. Put these on top of a bottom collecting pan. Tape together. Put lid on top. Tape it shut. (**Fig. 27**) Label sieve.

(Continued next page)

% Moisture	Water Activity
50	.988 - .991
47	.978 - .987
45	.975 - .978
41	.973 - .975
40	.967
39 (*Metarhizium*)	.958
38	.890 - .899
37	.843
11	.648
7	.302 - .482
4	.168 - .277
2	.138

Table 1 *If you do not have a water activity meter, use this guide for moisture equivalents.*

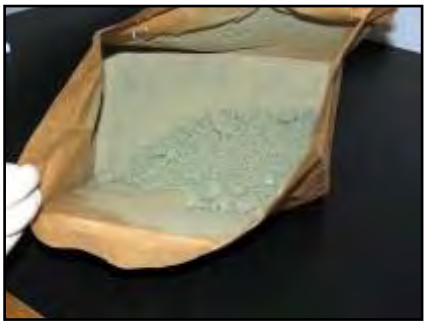


Fig. 22 *Metarhizium ready for harvest*

Fig. 23 *Pan set up. Diameter of sieve: 9 inches for this sonicator.*

Continued from previous page....

Connect the sieve stack to the sonicator make sure all handles are securely tightened. (**Fig. 28**) Turn on sonicator until the first two sections are solid white. (**Fig. 29**) Do not turn it on any higher as this will burn out the sonicator. Run for 20 minutes.

Remove pan stack form sonicator peel off tape. Remove beads from top substrate. If calculating yields, weigh +20 (initial substrate), -20 (substrate laying on top of 100 mesh screen) and 100 mesh (substrate lying in bottom collection pan) to get total substrate harvested. (**Fig. 30 and 31**) +20 and -20 mesh samples go in an autoclave bag. The 100 mesh conidia is funneled into a tared HDN plastic container using a spatula and funnel. Weigh conidia and place in frigeration until ready to do spore counts and viabilities.

Procedure for Mycoharvester:

In a laminar flow hood, clean room, or biohazard hood, set up Mycoharvester (*available from CABI Biosciences*).

Turn on machine. Pour ~1/3 of the bag of substrate at a time. (**Fig. 32**) Use handle below receiving arm to turn the substrate continuously as air sucks the spores into the unit. (**Fig. 33**) Stop when you can no longer see the conidia cloud raising up off the substrate. Repeat this process until all of the substrate for that strain has been harvested.

Retrieve spores in the collection pan located at the bottom of the collecting arm by lifting the collection arm away from the unit (**Fig. 34**) and gently pulling the collection pan off the arm. (**Fig. 35**) The yellow portion of the arm contains the -20 mesh (**Fig. 36 and 37**), the receiving arm contains the +20 mesh and the 100 mesh fraction is the conidia powder in the collection pan. (**Fig. 38 and 39**)

Remove conidia from collection pan into a tared HDPE plastic container using a spatula and funnel. Weigh conidia yields and refrigerate until ready to do spore counts and viabilities. ■

Fig. 24 *Sonicator*

Fig. 25 *Pour substrate in pans*

Fig. 26 *Substrate in pan sets*

(More images next page)

Fig. 27 *Tape pan seams tightly*

Fig. 28 *Pans on sonicator*

Fig. 29a *Sonicator turned off. First two sections appear black.*

Fig. 29b *Sonicator turned on. First two sections appear white.*

Fig. 30 *+20, -20 and 100 mesh samples.*

Fig. 31 *+20, -20 and 100 mesh samples.*

Fig. 32 *Pour substrate into receiving arm.*

(More images next page)

Fig. 33 *Rotate handle to move substrate.*

Fig. 34 *Pull collecting arm away from unit.*

Fig. 35 *Gently remove collecting pan from bottom of collecting arm.*

Fig. 36 *-20 mesh.*

Fig. 37 *+20, -20 and 100 mesh samples.*

Fig. 38 *Clean up!*

Spore Counts and Viabilities

Solid substrate fermentation is completed. The final step is to determine how many spores were recovered and whether or not they survived the process. There are two tests that need to be run: spore count and viability.

Viability Procedure

Materials needed:

Freshly harvested conidia
Sterile cotton tipped swabs
10ml test tubes
Test tube rack
Sterile 0.1% Tween -20 or Tween -80 solution
Sterile 0.05% Silwet L77solution
Vortex mixer
Marker
27° incubator
18 or 22mm glass cover slips
Lacto-fushin stain or lacto-phenol cotton blue
Phase contrast microscope
Counter
Latex gloves
Lab coat/Tyvek sleeves
Half strength Sabouraud dextrose agar (Beauveria) or full-strength potato dextrose agar (Metarhizium) plates

Fill one test tube/viability with 3-5ml sterile solution 0.05% Silwet or 0.1% Tween.

Roll a sterile swab in the conidia tap it off then place it in the appropriate test tube. Vortex sample for several seconds with swab in tube.

Throw swab out and place a fresh one into the solution if you use the same swab the plates get large amounts of spore coverage and viabilities are hard to count. Vortex the tube again with the fresh swab in it then smear the swab across a labeled section of an agar plate.

Keep plates in 27° incubator for 16-20 hours dependent upon the species (Beauveria 16-18 hours; Metarhizium 18-20 hours)

(Continued next page)

Continued from previous page....

Stain the plates with a drop stain to stop any further germination. Allow the plates to dry. Place a cover slip over the stained area and examine under a phase contrast microscope at 400x magnification.

Separately count the germinated and nongerminated spores at 400/sample. This allows for 10% precision 95% of the time based on Poisson distribution statistics. Change the area under coverslip being examined after counting every 100 spores.

A spore is germinated if the germ tube emerges from the spore. Swollen spores are not considered germinated.

Add together the total number of viable spores and divide by the total number of spores counted (viable and non viable). Report the percentage viability.

$$\frac{\textit{Total viable spores}}{\textit{Total counted spores}} \times 100 = \%\textit{Viability}$$

Spore Count Procedure

Materials needed:

Freshly harvested conidia
Balance (3 place)
Weigh paper
Metal spatula
1ml pipette
1ml tips
10ml test tubes
Test tube rack
30mm round glass beads
Sterile 0.1% Tween (-20 or -80) solution
Sterile 0.05% Silwet solution
Vortex mixer
Sonicator
Hemocytometer
Capillary tubes
Counter
Phase contrast microscope
Latex gloves

Fill one test tube/spore count, with 9.0 ml for residue and 9.9 ml for conidia, of sterile solution (0.05% Silwet, or

(Continued next page)

Continued from previous page....

0.1% Tween -20 or -80). Fill the second tube with 9 ml and third tube with 9 ml. This gives you three dilutions/sample.

Add 10 glass beads to each (-1) dilution tube; these help break up spore clumps for suspension. Weigh 0.100g/sample and pour it into the (-1) dilution tubes. Vortex for 1 minute; sonicate for 5 minutes; vortex for one minute.

Draw 1ml of (-1) suspension and add to the (-2) dilution tube; vortex 30 seconds then draw 1ml of suspension from the (-2) dilution tube and add it to the (-3) dilution tube; vortex 30 seconds.

The (-3) dilution is the one you will use. Fill a capillary tube with (-) 3 suspension. Fill the hemocytometer.

To count the spores: Count 5 of the blue squares (**Fig. 40**) (Any five squares you want to count.) from left to right, in a regular pattern to avoid counting the same square twice. Count only those spores that touch the top or right single rule line. Those touching the top or right triple ruled lines should be counted those touching the bottom or left triple ruled lines should NOT. * **NOTE**: When you see the grid under the microscope you see (**Fig. 40**) 25 squares/grid – these are blue on the diagram. Each of these 25 squares has 16 small squares inside it these are red on the diagram. You will be counting the entire area of 16 squares (red area inside the blue one). Count 5 blue squares.

If there are large clumps of spores, empty and clean the hemocytometer. Vortex the suspension and sonicate to break up the clumps. Reload the hemocytometer and try again. Count in triplicate (3 hemocytometers)/sample.

When the counts are complete, clean the hemocytometer with a mild soap solution, rinse with water and dry with a soft lens paper. To calculate results: formula =

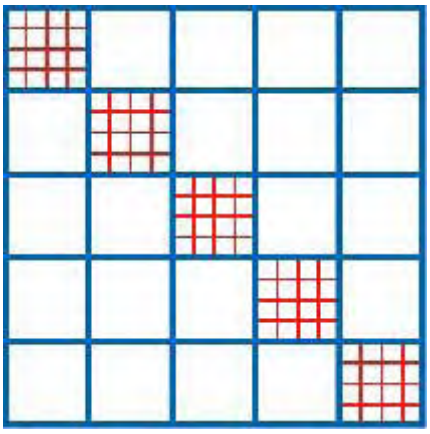


Fig. 40 *Hemocytometer Grid*

$$\frac{\text{(ave of the 3 spore counts/sample)}}{80} \times 4{,}000{,}000 \times \frac{\text{(9.9 or 9.0+ (sample weight))}}{\text{(sample weight)}} \times 10 \times 10 = \text{spores/ml}$$

This is the formula we use. You may use whatever formula you're accustomed to.

Zen Story

How Grass and Trees Become Enlightened

(*or, in our case, Fungi*)

During the Kamakura period, Shinkan studied Tendai six years and then studied Zen seven years; then he went to China and contemplated Zen for thirteen more years.

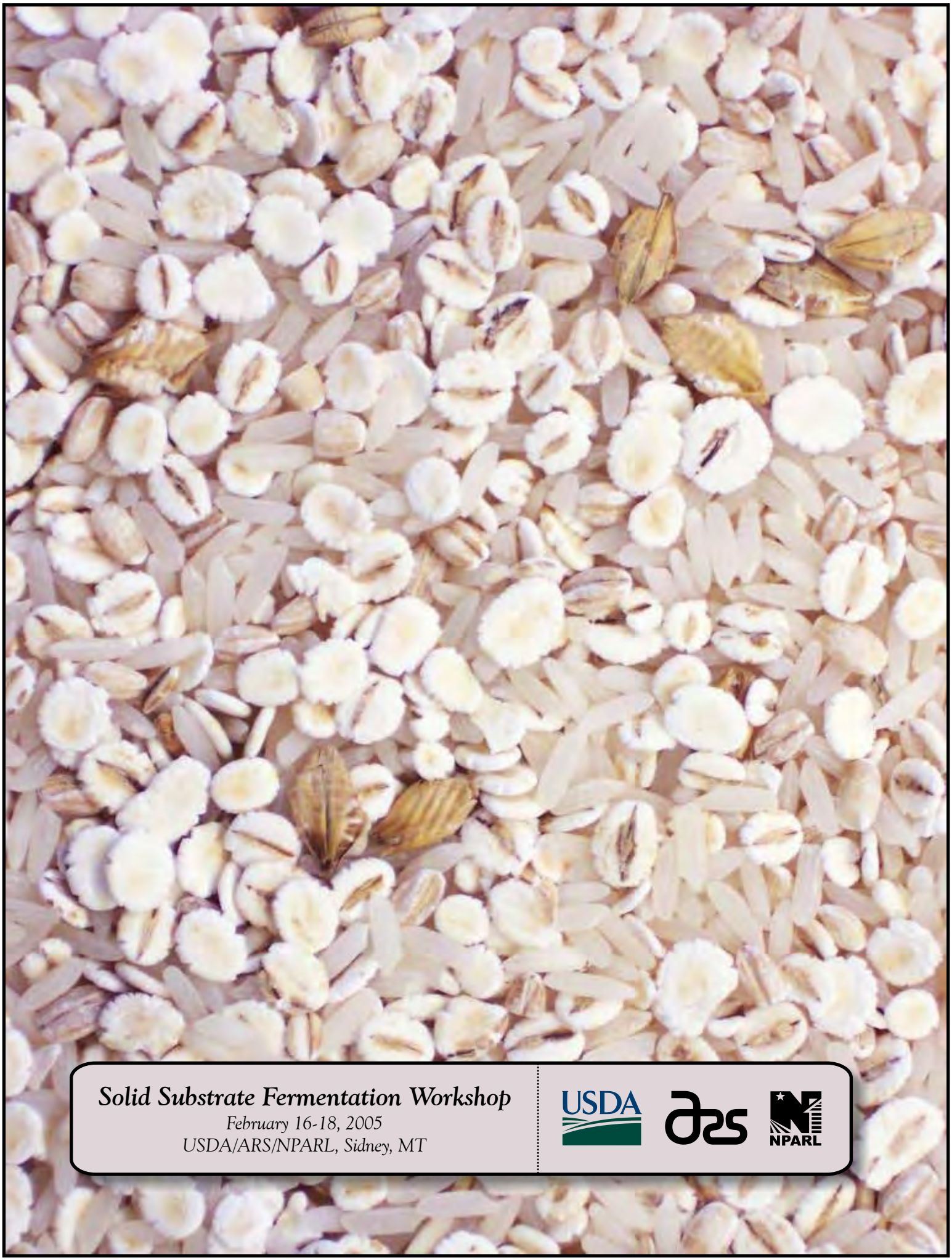
When he returned to Japan many desired to interview him and asked obscure questions. But when Shinkan received visitors, which was infrequently, he seldom answered them.

One day a fifty year old student of enlightenment said to shinkan: "I have studied the Tendai school of thought since I was a little boy, but one thing in it I cannot understand. Tendai claims that even the grass and trees will become enlightened. To me this seems very strange."

"Of what use is it to discuss how grass and trees become enlightened?" asked Shinkan. "The question is how you yourself have become so."

"I never thought of it that way," marveled the old man.

"Then go home and think it over," finished Shinkan. ■



Solid Substrate Fermentation Workshop

February 16-18, 2005

USDA/ARS/NPARL, Sidney, MT

USDA ars NPARL