Rice-Grown *Rhizopus oligosporus* Inoculum for Tempeh Fermentation

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Received for publication 31 January 1974

A method of growing *Rhizopus oligosporus* on cooked rice as the inoculum for the fermentation of soybeans into tempeh was described and evaluated. Isolated *R. oligosporus* spores on glass beads survived best at low temperature and intermediate humidity. The activity of the rice-grown inoculum to ferment soybeans into tempeh did not decrease appreciably when stored desiccated for one year at 4°C or room temperature. Bacterial contaminants as high as 10⁸ counts per g of cooked soybeans did not seem to affect the fermentation.

Tempeh is a popular Indonesian food made from fermenting soybeans with the *Rhizopus* mold. Besides the academic interest in the fermentation process, the study of tempeh has been stimulated by the prospect of large-scale production that will be important in providing a low-cost protein diet.

Methods of tempeh production in a traditional cottage scale vary in several details (1, 7, 10, 11, 13). Essentially, air-dried soybeans are soaked in water and the seed coats are removed. The cotyledons are steamed or boiled in water, drained and cooled, and inoculated with one of the several traditional mold inocula. The beans are then packed in small parcels and incubated at room temperature (25°C) for approximately 40 h. Fermentation is considered complete when the beans have been bound tightly by the mold mycelium into compact white cakes, which are customarily consumed within a day or two.

Traditionally the inoculum is obtained in several ways. It may be the mold residue left over in the wrappings of the previous tempeh cake (1), or tempeh itself may be broken into pieces and mixed with the prepared soybeans (10). Pulverized dried tempeh can also be used (9). The surface of a tempeh, where most of the mold mycelium is found, may be sliced and sun-dried to be used as an inoculum. Another method is to place crushed leaves of *Musa* sp., *Erythrina* sp., *Hibiscus simillis*, or *Tectona grandis* inside the package of inoculated soybeans. The leaves, covered with the mycelium during fermentation, are sun-dried and stored for inoculum. Experimental inocula obtained from growing the mold on rice and cassava have also been reported (3).

Hesseltine (4) found *Rhizopus oligosporus* Saito to be the principal mold species responsible for the fermentation. S. D. Ko isolated molds from more than 80 tempeh samples collected throughout Java and Sumatra and found that this species was always present in tempeh of good quality (unpublished data). Pure cultures of this species have been used in a number of laboratory studies (5, 6, 8, 14) as well as in a pilot plant study (12).

In studies to improve traditional methods of tempeh production, our laboratory in Bandung, Indonesia, developed a simple but reliable method of inoculum preparation. In this method *R. oligosporus* was grown on cooked rice, which was then dried and used as the inoculum. The method was evaluated, the viability of isolated spores and spores in the inoculum was determined, and bacterial contamination during fermentation was examined.

**MATERIALS AND METHODS**

**Rice.** Locally bought sun-dried rice was used. Depending on the variety, the rice was cooked with 1 to 1.5 times its weight of water to obtain lumps that could be separated easily.

**Mold culture.** Throughout the experiments *Rhizopus oligosporus* NRRL 5865 was used. It was originally isolated from a tempeh sample bought in a market in Bandung, Indonesia.

**Culture media.** Ten grams of mungbean sprouts (*Phaseolus radiatus*) was extracted by boiling in water for 2 h, and the final volume of the extract was made to 100 ml. The medium, taoge sucrose agar, contained 6% sucrose and 2% agar in a 10% mungbean sprout extract. Taoge dextrose contained 1.5% dextrose in a 10% mungbean sprout extract.

**Preparation of the rice-grown mold inoculum.** Sterile water (5 ml) was added to a pure culture of *R.
oligosporus grown on a taoge sucrose agar slant for 7 days at 37 C. The spores were scraped off the agar with an inoculating wire.

One milliliter of the spore suspension was mixed with cooked rice obtained from every 100 g of sun-dried rice. The inoculated rice was spread to a loose layer approximately 1 cm thick in a covered aluminum tray (31 by 11 by 1.5 cm). The bottom and lid of the tray were perforated (1-mm-diameter holes, 15 mm apart) (Fig. 1). The tray was incubated at 37 C in a standard incubator without air circulation for 8 days to obtain a dry inoculum.

The inoculum so obtained could also be used to inoculate a new batch of preparation. For this purpose, 1 g of the inoculum was mixed with cooked rice obtained from every 100 g of sun-dried rice.

**Storage of spores.** Two spore preparations were stored in different combinations of temperature and humidity to test viability. These preparations were: (i) spores adhered on glass beads prepared by rolling 30 glass beads of 5-mm diameter (wetted aseptically with 50% sucrose solution) over a sporulating 3-day-old mold culture grown in a petri dish of taoge sucrose agar at 37 C; and (ii) the dried inoculum crushed in a mortar into pieces of about 5 mm.

Thirty glass beads or 20 g of inoculum pieces were placed in an uncapped, 60-ml cylindrical glass jar. The jar was placed inside a capped, 240-ml cylindrical glass jar. To provide a specific relative humidity (RH), the bigger jar contained 40 ml of water (100% RH), 40 ml of 44% H2SO4 (approximately 50% RH), or 20 g of granular CaCl2 (near 0% RH). These materials were replaced every 4 weeks. The jars were stored in a 45-C incubator, in a shelf in the laboratory (25 C), or in a 4-C refrigerator.

**Determining spore viability.** Germination of the spores was the criterion for spore viability. At 2-week intervals, one glass bead or one inoculum piece from each storage condition was shaken in a test tube containing 2 ml of taoge dextrose. The suspension from the inoculum piece was allowed to settle for 30 s, and the supernatant was decanted into another test tube containing 2 ml of taoge dextrose. The final suspensions were incubated at 37 C for 6.5 h. Germination percentage was calculated in triplicate from total spore count (approximately 500) and germinating spore count in 10 microscopic fields of the suspension placed in a hemacytometer. Calculated germination percentages fluctuated (within 5% germination) from one interval to the other. Best-fit curves were drawn through these fluctuations (Fig. 2 and 3).

**RESULTS AND DISCUSSION**

**Cooked rice consistency.** The rice should be cooked to obtain easily separated small lumps so that the mold can grow on as much substrate surface as possible. A common mistake is to use too much water. This results in poor spore production, since the mold can only grow on the outer surface of the sticky rice mass.

**Process development.** Before incubation, the inoculated rice contained 10⁶ to 10⁷ mold spores per g. During the first day of incubation, the spores germinated and the subsequent growth covered the rice lumps with a white, wooly mycelial layer. On the second day the color turned to light gray due to the formation of sporangia. During the following days more sporangia were formed and the color of the mass changed to dark gray. Concomitant with the darkening of the color, the initial substrate moisture content of 67% gradually decreased to 5% or less at the end of the 8-day incubation.

![Fig. 1. Cooked rice inoculated with R. oligosporus and placed in perforated aluminum trays at 37 C are shown after 0, 1, 3, and 9 days of inoculation. Note the darkening and shrinkage of the mass.](image)
Due to this dehydration process, the rice mass gradually shrank and became crumby (Fig. 1). The final product contained $10^7$ to $10^9$ mold spores per g.

**Application.** For every kilogram of the original air-dried soybeans, 1 g of the inoculum was used. The inoculum was crushed into small pieces or pulverized, and mixed with the cooked soybeans. The methods of inoculum preparation and application were successfully tested in a pilot plant producing 75 kg of tempeh daily for more than 1 year.

**Spore germination percentage during storage.** At the time the spores were prepared, the germination percentages were 72% for spores adhered on glass beads and 69% for spores in the inoculum pieces. These numbers decreased rapidly during the early storage period. Thereafter, in conditions favoring the survival of the spores, the germination percentage leveled off for some time, depending on the storage condition, and subsequently declined to very low points.

Changes in germination percentages of the spores on glass beads are shown in Fig. 2. The curves can be associated into three distinct groups, each belonging to the same temperature condition. The lower the temperature the longer the spores survived. At 45°C, survival of the spores was favored by 0% RH. At 25 and 4°C, however, intermediate humidity (25% RH) was more beneficial than the two humidity extremes (100 and 0% RH). Thus, the longevity of isolated *R. oligosporus* spores are maintained best at low temperature, as is true for most fungal spores, and the spores can be classified among those whose survival is greatest at intermediate relative humidity (2).

Changes in germination percentage of spores in the inoculum are shown in Fig. 3. Combinations of high temperature and/or high humidity (45 C-100% RH, 45 C-50% RH, 25 C-100% RH, 4 C-100% RH) rapidly decreased the germination percentages during the first few weeks. At high humidity the inoculum absorbed moisture and clumped together. High temperature blackened the clumped inoculum. Inocula stored in the other combinations maintained relatively high germination percentages. At the end of the 40th to 50th weeks, germination of spores stored

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**Fig. 2.** Changes in the germination percentages of *R. oligosporus* spores adhered on glass beads during storage in different combinations of temperature and humidity. The two numbers on each curve denote temperature in degrees centigrade and percent relative humidity, respectively.

**Fig. 3.** Changes in the germination percentages of *R. oligosporus* spores in the rice-grown inoculum during storage in different combinations of temperature and humidity. The numbers on the curves are explained in Fig. 2.
at 45 C-0% RH and 25 C-50% RH started to decline, whereas the germination of those stored at 25 C-0% RH, 4 C-50% RH, and 4 C-0% RH stayed almost at the same level of 30 to 40% germination to the end of the 60-week experiment.

**Keeping quality of the inoculum.** At the end of the above experiment, those inocula stored in the last three combinations were used to make tempeh. No discernible differences in the fermentation time or the quality of the tempeh were observed as compared with tempeh made with freshly prepared inoculum. This was also true for inocula stored for 1 year in a stoppered bottle and in sealed plastic bags.

The results indicate that the best storage conditions for the preservation of the inoculum are low temperature (4 C) and low relative humidity (near 0%). When refrigeration is not available, the inoculum can be stored in sealed dry containers at room temperature.

**Bacterial contaminants.** The rice and soybeans were cooked without pressure, and after cooking both showed bacterial counts of $10^4$ to $10^5$/g. Cooked rice inoculated with a previous batch of inoculum showed an increasing count during the first 2 days of incubation, when the moisture was still above 35%. Thereafter it decreased to $10^4$ to $10^5$/g in the final dried inoculum.

Notwithstanding the bacterial population, tempeh fermentation was not disturbed if normal hygienic procedures were followed. In separate experiments, cooked soybeans inoculated with pure cultures of *R. oligosporus* were deliberately contaminated with one of the following arbitrarily chosen bacteria: *Bacillus mycoides*, *Escherichia coli*, *Pseudomonas coccovenenans*, *Pseudomonas pyoceanea*, and *Proteus* sp. In spite of counts up to $2 \times 10^4$ of these bacteria per g, tempeh of regular quality was obtained.

An antibacterial agent has been found in tempeh extracts (15), and this may play a role during fermentation. Presumably, before the bacteria can proliferate, fermentation is complete and the tempeh is deep-fried or cooked in soups.

No attempts have yet been made to identify the bacterial species in cooked rice or cooked soybeans, nor have the development of these bacteria and their effects on tempeh fermentation been studied.

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