Proc. Natl. Sci. Counc. ROC(B)
Vol. 23, No. 2, 1999. pp. 45-61

(Review Paper)

Development and/or Reclamation of Bioresources with Solid State Fermentation

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(Received October 20, 1998; Accepted December 16, 1998)

ABSTRACT

Based on basic principles of solid state fermentation (SSF), we have recently conducted the following experiments in order to have further bioresource development. The cellophane membrane lying on organic C and N deficient mineral solid medium was successfully established in order to resynthesize lichen and could be used to screen adequate lichens as well as to examine the conditional factors in the laboratory. The colonization of lichen on stones, such as flat rock fragments or sands, offers the possibility of reclaiming areas of flat rock fragments or deserted non-cultivable land. We have propose synchronized culture technology which focuses on the first peak harvest for mushroom growing. Synchronized culture technology will significantly reduce the loss of substrate moisture, the size of mushroom farms and the capital investment required. A technology mimic of SSF has also been proposed to prevent the loss of cultivable lands by reducing nitrate pollution. We have found that detection of the appearance of geosmin can be used as an index for detection of the maturation of hog waste compost, which is a useful bioresource. We have also detected aromatic volatiles from sorghum mashes during sorghum brewing (a SSF) and a tasty oligopeptide produced during sweet wheat paste (Tieng-Meng Chiang) brewing (another SSF) using facultative anaerobic fungi. Both have potential economic value as natural flavors. All these approaches will eventually lead to the development of new bioresources or possible reclamation of bioresources. On the other hand, head space gas analysis and geosmin have enabled this laboratory to develop gas sensors and to realize the importance of breath fingerprints or the distribution of volatile fermentation products of SSF and even those of human breath if breath is used as head space gas. This evidence has motivated us to search for one or some particular volatile(s) to define human health. The developed or proposed technology of SSF presented here will change the nature of SSF from appropriate technology to high technology.

Key Words: solid state fermentation, lichen, mushroom growing, *Rhodococcus*, geosmin, natural flavors, breath fingerprints, rock fragments, membrane cultures, nitrate pollution, delicious oligopeptide

I. Introduction

A typical traditional example of solid state fermentation (SSF) is waste-based composting, which has been practiced worldwide. Grain-based koji (starter) making is another example and is common in the orient. There is no or little cost involved for raw materials, facilities or labor. Products are hand-made, and the technology is simple and appropriate as the resources are locally available as mentioned by Josephson (1977). However, scientific understanding of the basic principles of SSF is lacking. This has hampered the development of these technologies.

We have defined the state of the solid substrate used in SSF based on the water holding capacity (WHC) and discovered the importance of microbial adhesion on solid substrate for proper fermentation to take place (Wang, 1987, 1989). We have also described the major parameters of SSF, including the initial substrate moisture content in connection with WHC, water activity (Aw), inoculum density, microbial mass, anaerobic growth, and heat and mass transfer in the fermentation mashes, which could be used to design processes for spawn or koji-making. We have also investigated the production of secondary metabolites, storage of grains, preparation of the intermediate moisture food (IMF), and microbial adhesion on solid substrate granules in sorghum brewing (Wang, 1989). These fundamental parameters are important for successful management of SSF, particularly field site operations.

Three international symposia on SSF have been held during the last decade. The first one was organized by

Maurice Raimbault of the Institute Francais de la Recherché Scientifique pour le Development en Cooperation (ORSTOM) and was held in Montpellier, France, in 1988, and a proceeding was published in the same year. R. Matsuno, R.W. Silman, R. Tanner and the author were involved in organizing the second and third symposia, which took place in 1989 and 1992, respectively. The second symposium was held in Honolulu, Hawaii, U.S.A. as a section of the International Chemical Congress of Pacific Basin Societies. We held the Pre-conference Symposium on the Application of Solid State Fermentation for Food and Environmental Technology, Dec. 12-15, 1989, in Taipei before the 2nd symposium in Honolulu. The papers presented in the 2nd symposium and the pre-conference symposium were finally collected as a special issue of *Fungal Sciences* (formerly called the Transaction of Mycological Society of the Republic of China) in 1995. The third symposium was held as a section of the Ninth International Biotechnology Symposium, and the papers were published in *Biotechnology Advances* Vol.11 in 1993. These symposia covered many subjects, including environmental, brewing, industrial and economic microbiological aspects of SSF. The traditional SSF processes of (1) primary products for koji, enzymes and cheese (Antier *et al*., 1993; Raimbault, 1988; Thakur *et al*., 1990; Chahal, 1991; Tochikura and Y;no, 1991; Leatham, 1991; Karanth and Lonsane, 1988; Lin, 1995; Lin *et al*., 1993; Ortiz-Vazquez *et al*., 1993; Villegas *et al*., 1993; Ziffer and Shelef, 1988), (2) cellulosic wastes for composting, mushroom growing and protein enhanced feeds or foods (Srivastava, 1993; Moo-Young *et al*., 1993; Bisping *et al*., 1993; Yang, 1993; Aquiahualt *et al*., 1988; Martinez and Tamayo, 1988; Moo-Young, 1991), (3) bioreactors and fermentation kinetics, including environmental factors (Gutierrez *et al*., 1988; Durand and Blachere, 1988; Cochet and Lebeault, 1988; Tanner, 1988; Tanner *et al*., 1993; Ryoo *et al*., 1990, 1991; Greene *et al*., 1991; Huang, 1995; Kobayashi *et al*., 1991; Durand *et al*., 1993; Gumbira Sa'id *et al*., 1993; Gowthaman *et al*., 1993; Laszlo and Silman, 1993; Tengerdy, 1991; Effler and Tanner, 1995; Huang and Hsu, 1995; Matsuno *et al*., 1991), (4) analytical aspects (Wang *et al*., 1991; Guilbert, 1988; Wang, 1993; Wang and Hsu, 1991; Gordon *et al*., 1993; Roche *et al*., 1993; Huang and Hsu, 1993; Chang, 1993) and biopulping (Wall *et al*., 1991, 1993) and (5) production of agrochemicals and bulk chemicals (including natural flavors) (Matsuno *et al*., 1993; Christen *et al*., 1993; Silman *et al*., 1993; Desgranges *et al*., 1993; Barrios-Gonzalez *et al*., 1988, 1993a, 1993b; Chou and Hwang, 1995; Porres *et al*., 1993; Revah and Lebeault, 1988; Larroche and Gros, 1988) were discussed in these symposia.

The SSFs discussed in the 3 symposia did not in general cover to field site operations but, rather, were limited to laboratory work. This paper reviews our recent work, which offers the potential development of new resources and reclamation of bioresources, including processes for the development of (1) cultivable lands from deserts or areas of rock fragments by means of lichen growing (improved primary production), (2) short term production of edible mushrooms using synchronized culture technology and (3) the protection of cultivable lands from areas of nitrate pollution as well as the processing of primary products (natural flavor) and wastes (quantification of stable composts). The SSFs described here include those related to conceptual and laboratory work which have potential to be extended to field site operations.

II. Cultivable from Non-cultivable Land through Lichen Growing.

The Taiwan Sugar Corporation has tried to improve the fertility of gravel soils in southern Taiwan through the application of manures and composts (Yeh *et al*., 1969). There have been successful instances, but the manure application techniques can not be extended to windy dessert areas in southern Taiwan or some areas of flat rock fragments in eastern Taiwan, which suffer from yearly river flooding.

We intended to develop an alternate approach by growing lichens on the surface of stones in rock fragment or desert areas in order to increase the amounts of organic carbons and nitrogens, which are essential for the fertility of the soil. Such an attempt has never been made before. A strain of lichen, *Lepraria* sp., isolated on the campus of National Taiwan University (Wang *et al*., 1997; Liang, 1996) was used here. Since lichens are generally slow growers in fields, and quantification of the growth of lichens is difficult, we initially aimed to develop a method to quantitatively determine the growth of lichens on solid substrate in the laboratory, and to study the conditional factors which would allow lichens to colonize on non-cultivable sands or flat rock fragments.

First, we resynthesized lichen by inoculating the hyphal fragments of lichen mycobiont and phycobiont cells separated from the isolated lichen on the center of a cellophane membrane over-lying medium in a plate (Fig. 1), and incubated it under proper conditions (Wang *et al*., 1997; Liang, 1996). It was found that it took 2 weeks for direct

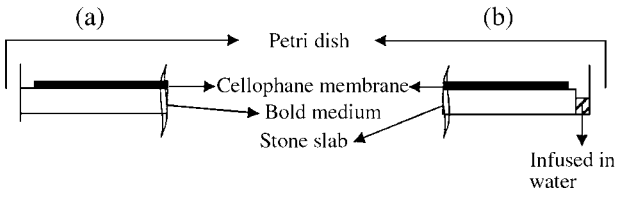


Fig. 1. Diagram of membrane culture. Cellophane membrane overlying (a) N depleted Bold medium and (b) stone slab infused in water.

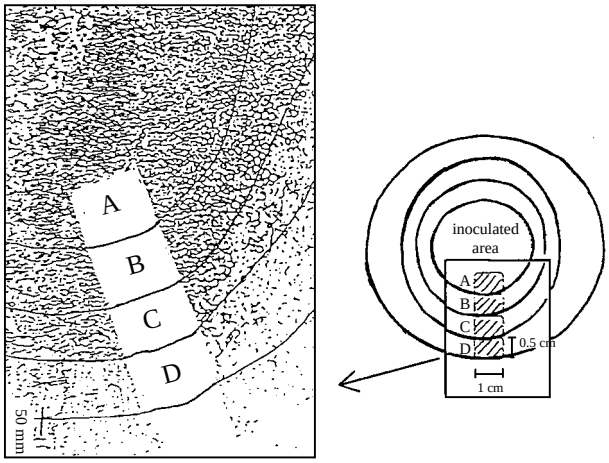


Fig. 2. (a) Sampling areas of the lichen colonies grown on cellophane membrane overlying solid Bold's basal medium without nitrogen sources from the inoculated area to the edge of the colony. (b) The mixed inoculates were inoculated at 20 ºC, 2000-3000 lux, under a 12:12 hour light-dark cycle. Sixteen weeks after inoculation, a strip (0.5×1.0 cm) of cellophane membrane was cut with a knife from the inoculated area to the edge of the colony. The strip was cut into four pieces for biomass determination as described, and each piece equaled a quarter.

A 0-0.5 cm: inoculated area, dense structure formed after sixteen weeks.
B 0-5-1.0 cm: mainly soredial layer formed after six weeks.
C 1.0-1.5 cm: mainly encircled layer formed after three weeks.
D 1.5-2.0 cm: colonial edge, mainly the layer of contact between the fungal and algal cells formed after two weeks.

contact between the fungal hyphal and algal cells to occur, 2-4 weeks for algal cells to be encircled by fungal cells, 4-16 weeks to form soredia, and approximately 16 weeks to form a distinct small scale-like structure (Fig. 2). The biomass of both the fungal and algal cells in the lichen was measured using DNA probes. We found that the algal cells biomass was thick on the inoculated area but thinned out farther away whereas the fungal biomass was rather thin in the center of the inoculated area and increased in thickness toward the edges of the colony (Fig. 3).

It should be noted that the lichen was grown on the nitrogen-depleted Bold's mineral medium (Ahmadjian, 1967) in the experiment described above. This means that the mineral nutrients except nitrogen were not limiting factors at the beginning of growth. The growth pattern of lichens in the laboratory were different from that observed in fields, but it was easy to measure the biomasses of lichen mycobiont and phycobiont separately using DNA probes (Liang, 1996). Nevertheless, the growth modes (Fig. 3) were similar to the first cycle of growth found in schematic periodical solutions for prey-predation relationships in the Lotka-Voluterra systems cited by Murray (1989). The prey-predation relationships expressed by the Lotka-Voluterra system can perhaps be used to describe the growth kinetics of lichens extended to more than 16 weeks. The maximum biomass of lichens could, thus, be predicted.

Lichen growing in fields needs further laboratory work, including study on the management of water supply and illumination as conditioned factors. Water supply in lichen growing is very important as it is in SSF. The main source of water in lichen growing in desert areas may be dewfall which condenses at night. In the future, lichen might be inoculated in desert areas, and then grown using of dewfall and some protection technique. The experimental results of Hsu *et al*. (1980) and Sharma (1976) are good references for this purpose; 25 g/cm^2 in the dry season from November to May in 1978-1979 in Ping-Tong soil in southern Taiwan (Hsu *et al*., 1980), and 15.53 g/cm^2 from March to November in 1974 in the semi-arid grassland of southeastern Australia (Sharma, 1976). Such factors will be examined in the laboratory with slight modification of the membrane culture as proposed in the present study, for example, the use of a stone slab instead of Bold medium as mentioned above (Fig. 1). In addition, the organic C and N of the biomass of lichens are released so slowly in soils that the lickens could be good biofertilizers for the prevention of nitrate pollution caused by the heavy use of chemical fertilizers.

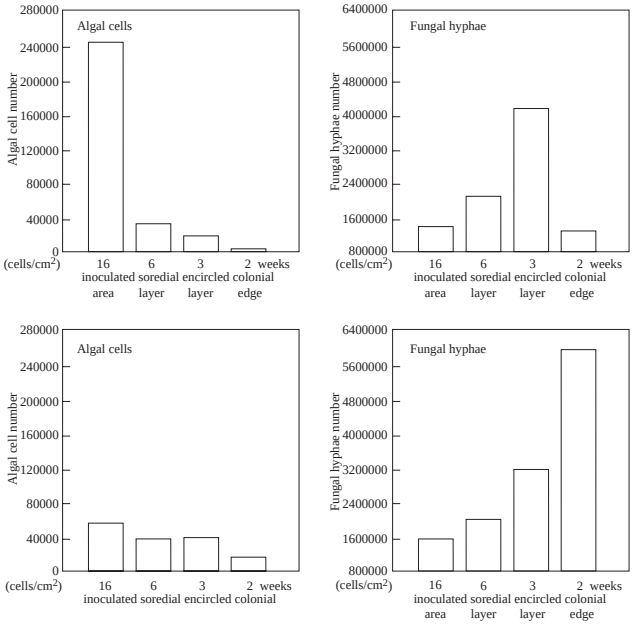


Fig. 3. Growth patterns of lichen mycobiot cells (estimated as *Zygosaccharomyces fermentati*) and phycobiont cells grown in the inoculated area on cellophane membrane overlying N depleted Bold medium.

III. Geosmin as an Indicator of Compost Stability or Maturity

Composting is a classic SSF, but there has been no convenient indicator for measuring the maturity of hog waste-based composting. Wang (1978) employed immobilized nitrogen as an indicator to measure straw-based composts, which have been widely used in mushroom (*Agaricus bisporus*) growing in this laboratory. However, measurement of immobilized N-content can not be applied to hog waste composting (Fig. 4) because the immobilized nitrogen content is much too high to begin with. Generally, as composting proceeds, fermentation heat accumulates inside compost heaps and reaches thermophilic conditions. When the oxygen inside the heap is used up, the temperature drops, and one has to aerate the heap. Then, fermentation proceeds, and the heap temperature rises again. When the fermentable matter in the compost substrate is used up, the heap temperature drops down to mesophilic conditions, which continue until composting finishes. This latter stage (mesophilic conditions) is called curing (Fig. 4). Also, with the change of the heap temperature, the microflora and metabolic products of the heap change. Therefore, we attempted to measure the breath (metabolic) fingerprints of gaseous metabolites, which were collected and concentrated as the extract of hog waste compost. The breath fingerprints were determined by means of gas-chromatography/MS (Wang and Chiou, 1998; Chiou, 1996) (Fig. 5). The breath fingerprints indicated the presence of geosmin (earthy odor) only in the cured compost (Fig. 5). Using a biosensor, an Arma Scan (Crewe, BK), the presence of geosmin in the cured hog waste compost was also detected. One kg (wet weight) of cured hog waste compost produced approximately 18.6 mcg of geosmin, which could stimulate the germination of cabbage seed (*Brassica chinesis*). Many strains of actinomycetes were isolated from cured hog waste composts (Chiou, 1996). Laboratory incubation of these strains in pure culture also confirmed the production of an earthy odor under mesophilic conditions but not under thermophilic conditions. Experimental results strongly suggest that the presence of geosmin can be used as an indicator in examining the maturation of hog waste composts, which are useful bioresources for organic farming and/or sustainable agriculture. It is worth pointing out that Gerber and Lechevalier (1965) isolated geosmin from metabolic products of several species of *Streptomyces*.

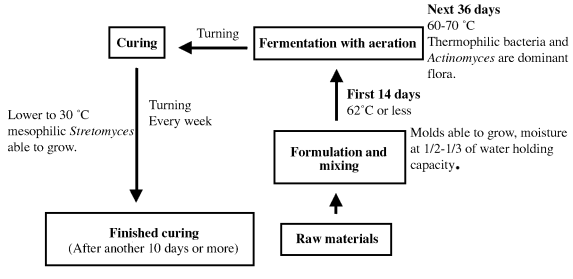


Fig. 4. A flow chart of hog waste composting.

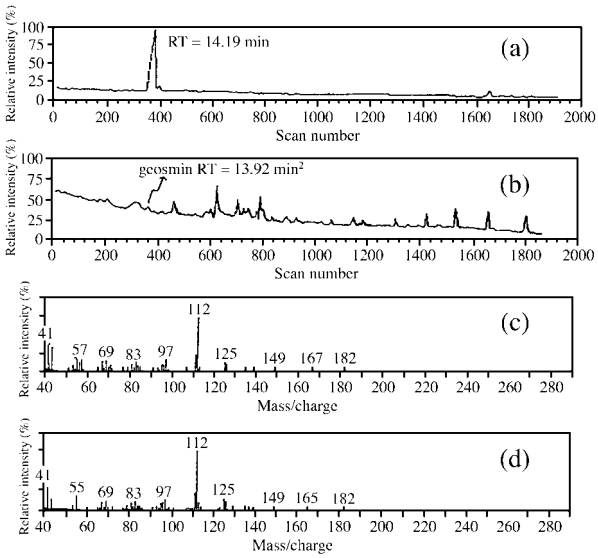


Fig. 5. (a) Total ion chromatogram of authentic geosmin, (b) total ion chromatogram of 50-day hog manure compost, (c) mass spectrum at the peak of retention time 14.19 min of authentic geosmin and (d) mass spectrum at retention time 13.92 min of 50-day cured hog manure compost.

IV. Mushroom Growing Using Synchronized Culture Technology

Commercial production of mushroom can be divided into three stages: (1) spawn production, (2) substrate preparation (composting), and (3) mushroom growing (Wang, 1978). All of these processes are SSFs and have been developed on large farms. Consequently, considerable capital investment is required. In the late 1960s and the early 1970s, large mushroom farms operated by canneries could not compete with small farms owned by growers in Taiwan. In 1996, Wang proposed reducing the farm size and capital investment needed for mushroom growing and using synchronized culture technology, which he intended to develop for any mushroom, even for medicinal mushrooms. This synchronized culture technology will greatly aid the mushroom business, which is an important agricultural industry in Taiwan.

Generally, in a synchronized culture of *Escherichia coli*, the number of cells in the culture remains approximately constant for one hour while the number of newly formed cells doubles. In the second division cycle, the plateau is less distinct and the population rise extends over a

longer period than in the first division cycle. In the third division cycle, each individual cell grows randomly, and almost no indication of synchrony remains (Stainer *et al*., 1986). Synchronous cultures rapidly loose their synchrony because various cells in a population do not divide at the same size, age, or time following the previous division. Messing (1980) in his review indicated that *E. coli* cells immobilized by adsorption on ion-change resins displayed a periodicity in releasing free cells, and that the released cells (daughter cells) divided synchronously in the medium. Messing (1980) also stated that (1) *E. coli* which were adsorbed on a filter and subjected to a flowing nutrient medium exhibited a shorter doubling time than did freely suspended cells, and that (2) similarly, the generation time of the yeast, *Saccharomyces uvarum*, in the non-immobilized state was longer than that of the yeast cells immobilized by adsorption on an adequate supporter. He concluded that a greater number of synchronized cycles could be obtained by immobilization of cells than by using cells that were not immobilized. During cultivation of the mushroom *Agaricus bisporus*, the mycelium grows on/in composts, which serve as a solid support. This is an immobilized state. The periodical production of fruiting bodies (fructifications) observed during cultivation seems to be due to synchronous division of mycelial nuclei (Wang, 1972).

Haecken's mushroom farm in the Netherlands has harvested mushrooms using a specialized machine while on traditional mushroom farms, growers pick mushrooms by hand. The harvest of the first peak on Haecken's farm was 19.2 kg (per unit area) while the second peak harvest was 5.7 kg and the third was only 3.0 kg (Chiang *et al*., 1983) . The first peak harvest was significantly larger than the second and third harvests. Under traditional mushroom cultivation, Vedder (1978) reported that the first peak harvest was 4.9 kg, and that it was 5.9 kg for the second, 3. 3 kg for the third, 2.4 kg for the fourth, and 1.5 kg for the final harvest. These results are in accordance with the hypothesis that synchronized nuclear division is probably associated with the initiation of synchronous fructification of mushrooms grown on solid state composts (Wang, 1972).

Therefore, we propose to develop a technology. The characteristic of this technology is to focuses the harvest of mushroom growing in the first peak. This would significantly shorten the time needed for cultivation and reduce the space needed for mushroom growing. Growers could cultivate many cycles of mushrooms in a year with the use of adequate strains. In the others words, growers could continue mushroom production through a year. This seems to be economically feasible for managing a small family farm rather than a large-scale mushroom farm, which requires much more labor and involves large costs for air circulation and ventilation. Air circulation and ventilation accelerate the loss of substrate moisture and eventually lower production.

Table 1. Estimation of Probable Maximal Production of *Shiitake*

Assumption	Estimate (fresh weight/bag)
1. 7 caps in a plane[a],	200 g ≒ (30 × 7)
2. 14 caps in two planes[b],	400 g ≒ (30 × 14)
3. 1 cap ≒ 30 g fresh weight.	

Source: Wang (1996)

[a] Maximal number of mushrooms projected on the upper-surface of a bag (7 caps in a plane) as shown in Fig. 6.

[b] During the first peak, two planes of mushrooms were assumed.

The probable maximal production of *Lentinus edodes* (*shiitake*) using the proposed synchronized culture technology is estimated to be up to 400 g fresh mushroom per bag (1500 g) on the basis of space and time (Table 1) (Fig. 6), based on experiences in Taiwan growing *shiitake* and in Ireland growing *Agaricus bisporus*. In both cases, the bags are placed in rows on the flour of the growing house (Fig. 7) (Wang, 1996). The probable maximal production of 400 g per bag using the proposed method is much higher than the 300-350 g per bag using the current method. The spent substrate after mushroom growing will contribute to sustainable agriculture (Wang, 1992).

V. Protection of Nitrate Pollution by SSF

The application rates of nitrogen fertilizers used in soils are much higher than those recommended (Moffat, 1998). The distribution of hog farms is much denser than that allowed by soil assimilative capacities of neighboring

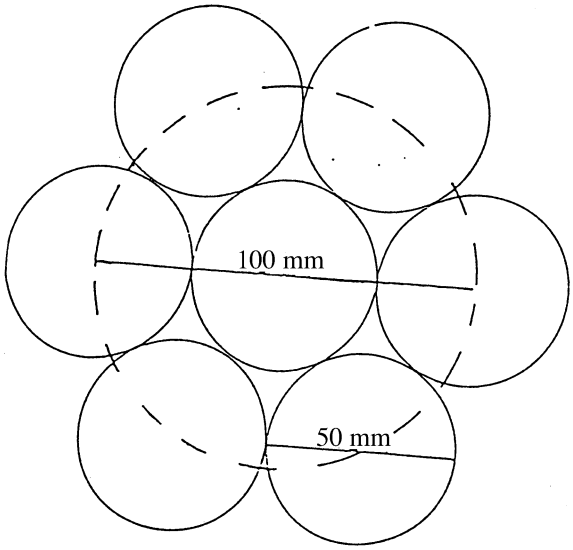
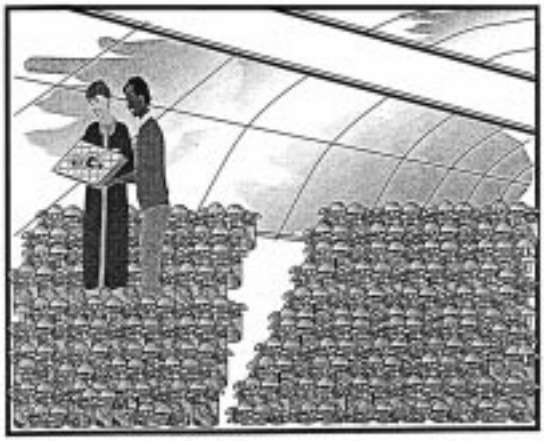
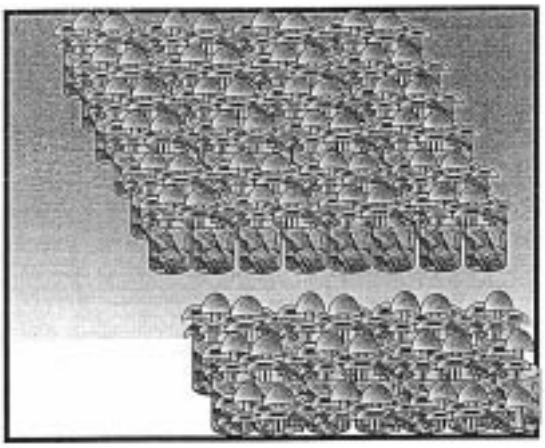


Fig. 6. The schematic pattern of the maximum number of mushrooms (dia. 50 mm) produced on the surface of a bag (dia. 100 mm) in a plane.



(a)



(b)

Fig. 7. (a) Mushroom cultivation in Ireland and (b) *shiitake* growing in Taiwan. One layer of bags placed in the ground instead of multiple layers of shelves or beds used; less or no loss of substrate moisture, less air circulation and ventilation.

soils in Taiwan (Wu and Wang, in preparation). Therefore, the level of nitrate in the water at the inlets of water plants in Taiwan has increased (Wang, 1992). In order to prevent such pollution, a packed-bed type process (Fig. 8) can be considered to operate nitrification (down-flow) and denitrification (up-flow) in order to remove NH_3-N and eventually to end the loss of land resources. The proposed process mimics a packed-bed reactor. Generally, packed-bed reactors are operated using a continuous gas atmosphere. The packing materials in the up-flow area are usually soils or landfills in practice (Fig. 8). For example, the (ammonia rich) effluents of methane fermentation of hog waste are introduced through a down-flow column with air to enable the oxidation of ammonia (nitrification). The outlet of the down-flow device reaches the bottom of the packing of the up-flow area, in which the reduction of nitrate to gaseous N (N_2 and/or N_2O) (denitrification) occurs. The surfaces of the particulated materials in both the down-flow column and up-flow area can be colonized by nitrifiers and by denitrifiers, respectively. Quastel and Schofield (1951) described this phenomenon as the colonization of nitirifers on soil particles. In this way, the packed-bed type process mimics SSF. Nitrification is a property of both chemolithotrophic and heterotrophic bacteria. The expression "heterophic nitrification" is used to describe the oxidation of ammonia associated with organotrophic metabolism in contrast with the better-known variant of autotrophic nitrification (Zumft, 1997). Traditionally, chemolithotrophic or autotrophic nitrifiers have been easily quantified by the most probable number (MPN) index with an organic carbon depleted medium, but heterotrophic nitrifiers can not be quantified using this method. It was found in this laboratory that 12 cultures of *Rhodococcus* spp. obtained from two culture collection centers showed heterotrophic nitrification with the use of acetamide or pyruvic oxime. The rate of heterotrophic nitrification of *Rhodococcus rhodochrous* DSM 363 was 766 μg N/day/g cells (Cheng, 1996; Cheng and Wang, 1995). The fate of nitrification of *Rhodococcus rhodochrous* DSM 363 inoculated in a small bioreactor ($19 \times 11 \times 22.5$ cm) containing 3.5 L of hog waste water was traced, and the biomasses of *Rhodococcus* spp. were measured using quantitative PCR (Cheng, 1996). Nitrification evaluated based on the ammonia decrement and the increment of nitrite and nitrate showed little difference between inoculated and uninoculated wastewater. Fewer than 10^5 cells/ml of *Rhodococcus* were found in the bioreactor not inoculated with waste water and 4×10^5 cells/ml in that inoculated with waste water at the beginning, but little difference was found after 14 hrs, fewer than 5×10^5 cells/ml in the inoculated case and fewer than 4×10^5 cells/ml in the uninoculated case. *Rhodococcus* spp. are widely distributed and particularly abundant in soil and herbivore dung (Goodfellow, 1986). The genus *Rhodococcus* is a unique taxon consisting of microbes that exhibit broad metabolic diversity (Finnerty, 1992; Cheng, 1996) (Table 2). Therefore, it seems that heterotrophic nitrification by *Rhodococcu*s is common in the waste water of hog wastes as well as in soils. The search for oligonucleotides to probe the existence of heterotrophic nitrifiers was followed using the 12 cultures of *Rhodococcus*, but no consistent result was

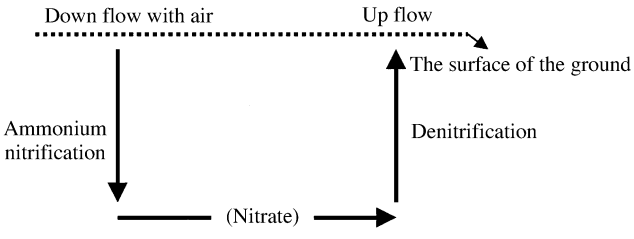


Fig. 8. A conceptual packed bed-like bioreactor mimicing SSF and performing nitrification and denitrification.

Table 2. Metabolic Diversity of *Rhodococcus* spp.

	Substrate	Product	Species	References
Hydrocarbon	acetylene	acetaldehyde, ethanol, acetate and CO_2	*R. rhodochrous*	Germon and Knowles (1998)
	ethylene, pro-1-ene, but-1-ene	1,2-epoy alkane	*Rhodococcus* sp.	Woods and Murrell (1990)
	propane	propionate acetone, and acetate	*R. rhodochrous* PNKB	Woods and Murrell (1989)
	alkane esterified f.a. cis-unsaturated alkene	unsaturated f.a.	*Rhodococcus* sp. KMS-B-MT66	Takeuchi *et al*. (1990)
	1 ˚ alcohol (C4-C14) tetradecan-2-ol	acid tetradecan-2-one	*R. equi*	Ueda *et al*. (1986)
	2-nitro-1-phenyl-prop-1-ene	2-nitro-1-phenyl-propane	*R. rhodochrous* IFO3338	Sakai *et al.* (1985)
	Pristane	pristanol, pritanic acid and pritanate	*Rhodococcus* sp. BPM1613	Nakajima *et al*. (1985)
Surface-active lipid production	sodenncane	Steroid, monoglycerol and glycolipid	*R. rhodochrous*	Sorkhoh *et al.* (1990)
	n-alkane	Glycolipid	*R. aurantiacus*	Ramsay *et al*. (1988)
	trehalose 2,3,6-mycolate	Trehalose-2,2',3,4-tetraester	*R. erythropolis*	Kim *et al*. (1991)
Complex lipid	gaglio-, lacto, globo-glycosphigolipid	Oligosaccharide and ceramide	*Rhodococcus* sp.	Ito and Yamagata (1986, 1989)
	gala-glycosphigolipid	Oligosaccharide and ceramide	*Rhodococcus* sp.	Ito and Yamagata (1986, 1989)
	N-acyl 1 ˚ aromatic Amine	1 ˚ Amine and aryl acyl-fatty acid	*R. erytthropolis* NCIB 12273	Watanabe *et al*. (1987)
Steroid oxidation	squalene	α-, β-unsaturated ketone	*Rhodococcus* sp.	Straube *et al.* (1990)
	δ5-3-sitosteroid	20-carboxypregna 1,4-diene-3-one	*Rhodococcus* sp.	Iida *et al.* (1985, 1987)
	cholesteroid	4-choleston-2-one	*Rhodococcus* sp.	Aihara *et al*. (1988)
Environmental application	Phenol			Straube *et al.* (1990)
	aromatic acid		*R. rhodochrous*	Bruce and Cain (1990)
	trifluoromethyl-benzoate		*R. erythropolis* Y2	Salis *et al*. (1990)
	tetrachloro-hydroquinone	1,2,4-trihydroxyl-bezene	*R. chloropheno-licus*	Haggblom *et al*. (1988)
Nitrogen-containing compounds	aniline quinoline	anthranilic acid	*Rhodococcus* sp.	Aoki *et al*. (1985)
	ntrile 3-cynopyridine	acrylamide nicotinamide	*Rhodococcus* sp. B1	Nagasawa *et al*. (1990)
	aliphatic ntrile, aromatic nitrile	acid, amide	*Rhodococcus* sp.	Bengis-Garber and Gutman (1988)
Sulfur-containing compounds	dibezothiophene	2-hydroxybiphenyl and sulfate	*R. rhodochrous*	Kilbane (1990)
	dibezothiophene, dimethyl sulfide	sulfoxide, elemental sulfur CS2	*Corynebacterium* sp. *SY1*[a]	Omori *et al*. (1992)

Source: Cheng (1996)

[a] reidentified as *Rhodococcus* sp. (Omori, personal communication)

found. However, the nitrification capacity of one cell of *Rhodococcus* may represent a certain fraction of the heterotrophic nitrification capacity of a target soil under a defined environment. *Rhodococcus rhodochrous* DSM 363 was also found to show denitrification with the production of N_2O under anaerobic conditions (Cheng, 1996). Denitrifiers have been measured using the time-consuming MPN method. Therefore, oligonucleotide probes to qualify or/and quantify denitrifiers have been screened by various workers based on molecular denitrification (Ye *et al*., 1993; Wu, 1995). The findings of this laboratory are summarized below. Using the homology of amino acid sequences of the copper-type nitrite reductase between *Achromobacter cycloclasters* ATCC 21921 (Fenderson *et al*., 1991) and *Pseudomonas* sp. strain G-179 (Ye *et al*., 1993), this laboratory synthesized a pair of primers, Cu-dNir1 and Cu-dNir2. The polymerase chain reaction was used to amplify the regions of copper-type nitrite reductase gene of 14 strains of denitrifiers, including *Achromobacter cycloclasters* ATCC 21921, 5 of *Pseudomonas* spp. and *Alcaligenes eutrophus* CCRC 13036. The following amplification conditions, 1.2 mM Mg^{2+} and 42.5 ºC annealing temperature for the primers, were required. The amplified fragments were used directly as probes. The probes were labeled by digoxigenin-dUTP. Further confirmation of specificity to denitrifying bacteria for probes was performed by means of dot blotting hybridization. The results showed that the 781-bp fragment of *A. eutrophus* CCTC 13036 detected more genera of denitrifiers containing copper-type or cytochrome cd_1 type nitrite reductase than did the other probes. A linear relationship for the quantitation of *A. eutrophus* between 1.522×10^6 and 3.805×10^7 cell number ml^{-1} by means of densitometric measurement was found. Direct extraction of DNA from Shoufeng, Erhshui or Lungkang soils was applied for the quantification of denitrifiers using the prepared probes. The indigenous microorganisms in the soils were lysed using 0.4 M NaOH-10 mM EDTA and incubated at 100 ºC for 10 min. The lysate was extracted with 10% CTAB-0.7M NaCl and chloroform-isoamyl alcohol. The number of denitrifiers in the Erhshui soil detected by means of hybridization with the probe was equivalent to 3.01×10^6 cells of *A. eutrophus* g^{-1} soil. However, the probe could not determine the denitrifiers in the Shoufeng and Lungkang soils. The number of denitrifiers in the Erhshui soil was 1000 times that determined previously using the MPN method. MPN is the result of cultural methods. The community of grown microorganisms was selected based on the composition of the medium used. Therefore, the number of denitrifiers measured using the MPN method may be generally lower than that in the soil. However, one can determine the assimilative capacities of nitrifiers and denitrifiers on the basis of one cell in soils to estimate the N removal and assess the efficiency of the prevention of nitrate pollution to halt the loss of land resources. Then we may be able to calculate the soil assimilative capacities of ammonia and/or nitrate. Using these proposed assimilative capacities, we may be able to estimate a maximal achievable potential of hog farming in sustainable environment under given conditions. Then, we may be able to prevent the loss of natural resources.

VI. Production of Natural Flavor

Food and condiment/flavor products are flavored *in situ* whereas flavor chemicals and building blocks (bases) of complete food flavors are produced *per se* through SSF (Seitz, 1991). Seitz (1991) surveyed a broad spectrum of solid state systems, which have been used to provide a host of flavored products, such as the flavor chemicals produced in foods, condiments, microbially *per se* produced flavor materials and flavor base products. He also reviewed the work carried out in his laboratory on the commercial production of flavor bases. The primary achievements were in the areas of cheese, sausage and mushroom flavors. The products all complied with the definition in the U.S.A. Federal Register of being natural. The basic approach was to use SSFs in their entirety or extracts of such cultures (those rich in exocellular enzymes and flavor adjuncts) incubated together in an aqueous food substrate system. The cultures gave rise to growth-media by-products, catabolites and anabolites that proved to be valuable tastants and odorants. The cheese flavor products contained fatty acids, methyl ketones, carbonyls and amino acids. Key volatiles for sausage and mushroom flavors were vinyl guaicol and 1-octen-3-ol, produced, respectively, in these two systems. Preparation and processing conditions of foods and condiments set the stage for the growth and metabolism of certain desirable microorganisms. Certain parameters, such as moisture level, prior heat treatment (e.g., cooking), salt concentration, aeration, temperature of incubation etc., were most important for the correct microflara to be established and to prevent the growth of undesirable or harmful types. The desirable types even today are often adventitiously incorporated into the substrate(s). However, careful microbiological studies and modern technology have enabled isolation of the essential and most favorable species and strains, and permit fermentation for optimum production of flavor, color and texture in foods and condiments.

This laboratory has worked in two directions to find new resources for natural flavors. One has been to establish an analytical technique for the measurement of breath fingerprints or head space gas analysis (HSGA) on sorghum liquor or solid brewing mashes to find characteristic aroma compounds. HSGA was originally used for process control because the sampling of solid mashes establishes different environments in SSF. The mashes were made of steamed grain of sorghum (Lu, 1984) or rice (Yang, 1987). The other

approach has been to find tasty oligopeptides, such as beefy or meaty flavors in the mashes inoculated with facultative anaerobic *Rhizopus*. Some natural and synthetic tasty oligopeptides indicating delicious tastes were summarized by Spanier *et al*. (1993). Characteristic aroma and tastes found in this laboratory include fruity, pungent, charcol-like, acid, sweet and umani (beefy flavor) (Yang, 1987; Yu, 1995). Sampled headspace gas was concentra-ted and analyzed using gas chromatographic (GC) or GC-Mass spectrometric methods (Lu, 1984). The result of HSGA on sorghum mashes for solid brewing inoculated with different cultures showed significantly different distributions of volatiles as given below:

In the first three experiments, sorghum brewing was carried out in a flask, but in the following 2 experiments, sorghum brewing was performed on a commercial scale at the Chiagi distiller (Fig. 9). In short, the former was laboratory work while the latter included field site operations.

1. Sorghum Brewing Inoculated with Natural Starter

Glass precolumns packed with Tenax GC with a circulation gas path were used to trap and concentrate volatile compounds in headspace gas before HSGA. 97 compounds were identified in the HSGA of sorghum mashes inoculated with natural starters while only 58 compounds were identified in the HSGA of the mashes inoculated with pure-culture of *Rhizopus* (see next column). Among these compounds, 3-hydroxy-2-butanone (acetone), ethyl methoxy-acetate and 2-pentylfuran were found in the mashes inoculated with natural starter and in the resultant sorghum liquor, but these aroma constituents were not detected by HSGA on the mashes inoculated with the pure culture of *Rhizopus*.

2. HSGA on Seed Cultures for Sorghum Brewing

Sterile cooked sorghum grains were inoculated with pure culture of *Rhizopus* isolates or *Aspergillus oryzae* and incubated. Based on GC-Mass spectra and Kovats indices, 58 compounds were identified in the HSGA results of these seed cultures, and the components present in significant amounts were acetaldehyde, furfural, palmitic acid, isoamyl alcohol, hexyl alcohol, diethyl succinate, hexyl isovalerate, dibutyl phthalate and 1-methyl-5-(1-methylethenyl)-cyclohexene.

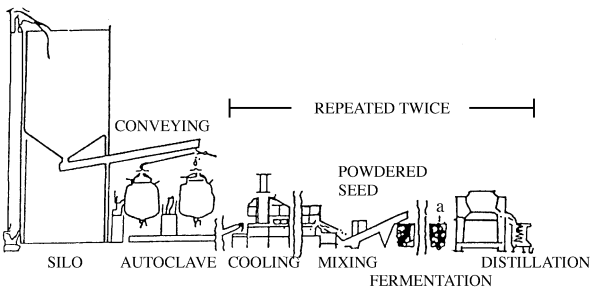


Fig. 9. Sorghum brewing process currently operated in the Chiayi Distillery.
[a] Stainless tanks covered with polyethylene sheets (Wang, 1986).

Compounds identified in the head space (HS) of *Rhizopus* culture but not detected in the *Aspergillus* culture were furfural, hexyl alcohol, 3-methoxy-1, 2-propanediol, 4-ethylphenol, methylpalmitate, 1-idethoxy-3-methylbutane and 1-methyl-5-(1-methylethenyl)-cyclohexene. Compounds identified only in the *Aspergillu*s culture were benzaldehyde, 2-methylbutanol, isoamylcaproate, hexyl acetate and hexyl phthalate.

The main volatile compounds of *Rhizopus* culture were isoamyl succinate, occupying a total of 61.2% of the compounds identified. *Aspergillus* produced large amounts of hexyl compounds, such as hexyl valerate. 1-octen-3-ol, the main aroma component commonly present in *Basidiomycetes*, was not found in either culture.

3. Effect of the Inoculation of Yeasts

Sterile cooked sorghum, inoculated with pure culture of *Rhizopus* alone or with yeast simultaneously and incubated in a closed vessel, was analyzed during the fermentation process. Higher concentrations of ethyl acetate and ethyl propionate, and lower concentrations of isoamyl alcohol and diethyl succinate were observed in the *Rhizopus*-Yeast mixed culture group. In the mixed culture group, higher pH and higher ethanol content, lower glucosidase and lower protease activities were observed. The free amino acid content of the mixed culture mashes was significantly lower than that of their *Rhizopus* counterparts. Yeast occupied a considerable portion of the biomass of the mixed culture group.

The compounds identified in the brewing mashes but not detected in the mashes of the pure culture of *Rhizopus* or *Aspergillus* were 3-methylbutanol, 3-hydroxy-2-butanone, caprylic acid, pentadecanoic acid, 3-methyl-1H-pyrazole-4-carboxylic acid, 2-lauryl alcohol, 3, 7-dimethyl acetal, nonanal diethyl acetal, ethyl 2-methyl-butyrate, ethyl valerate, ethyl heptanoate, ethyl caprylate, ethyl pelargonate, ethyl undecanoate, ethyl stearate, methyl myristate, methyl pentadecanoate, methyl heptadecanoate, isobutyl format, decyl thiobutyrate, gamma-palmitolactone, toluene, 3-ethyl-benzene, ethyltoluene, p-xylene, propylbenzene, 1, 2, 3-trimethylbenzene, 1, 2, 4-trimethylbenzene, 1, 3, 5-trimethylbenzene, ethanone-1-(2, 4-dihydroxyphenyl), 3, 4-dimethozyacetophenone, benzophenone and 2, 3-di-ethyl-5-methyl-pyrazine. Among these, remarkable concentrations of ethyl lactate, acetoin, ethyl heptano ethyl pelargonate, ethyl methoxyacetate and 2-pentyl-furan were found.

4. Sorghum Liquors Derived from Consecutive Fermentations

In the traditional brewing process, cooked sorghum is inoculated with natural starters and fermented and distilled in three consecutive cycles (Fig. 9) (Wang and Hsieh, 1972; Huang *et al*., 1985; Wang, 1986; Wang *et al*., 1975; Lai *et al*., 1991, 1995). The total aldehydes, total alcohol, and total esters in the distilled sorghum liquor increase with an increase in the number of fermentation cycles. The concentrations of isoamyl alcohol, ethyl acetate, ethyl palmitate, 3-methylbutanal, ethyl butyrate and hexyl acetate in the liquor increase with an increase in the number of fermentations cycles while the concentrations of ethyl valerate, ethyl isovalerate, ethyl caprylate, ethyl myristate, 1, 3, 5-trimethylbenzene and 2-pentylfuran decrease with an increase in the number of fermentation cycles.

HSGA analysis of the brewing mashes immediately before and after distillation revealed that most of the high boiling compounds, such as 4-ethylphenol, 2-methylnaphthalene, ethyllaurate, ethyl myristate, methyl myristate, ethyl oleate and myristic acid, remained in the HS of the solid mash after distillation while only a small portion of these compounds was found in the HS of the liquor.

5. The Effect of the Facultative Anaerobic *Rhizopus* on Sorghum Brewing

Sorghum grains are steamed and inoculated with natural starter, which is made of cracked raw wheat grains and contains undefined mixed cultures. The unit of the sorghum brewing process in Taiwan is 600 kg steamed sorghum grain (50% moisture), placed in a stainless steel box sealed with a polyethylene sheet (Fig. 9). The oxygen concentration in the HS of the mashes decreases rapidly in the early stage, finally becoming less than 5% (Wang *et al*.,1975; Lin and Wang, 1991; Lin *et al*.,1991). When the spores of *Rhizopus* isolated from colonies grown in conventional petri dishes are inoculated into the sorghum grains as a starter, there is neither germination nor hyphal growth in the mash. However, when a small portion of the mash is transferred into a flask or a jar, saccharification and alcohol fermentation occur. It seems that the development of low level oxygen by the natural starter may enrich the anaerobic saccharolytic molds during brewing. Traditionally, in sorghum brewing, the mash (600 kg of steamed sorghum grain in a batch) is covered with a polyethyle sheet to create semi-anaerobic condition and to avoid acetate fermentation, but the isolates (*Rhizopus* spp.) grown on conventional plates, that is, under aerobic conditions, do not grow in the mash. Therefore, a pure culture of facultative anaerobic *Rhizopus* (Lin, 1986; Lin *et al*., 1991; Wang and Tsao, 1994) (Fig. 10, Table 3) was chosen in this laboratory. The apparent fermentation heat generation rates were 3.3~12.4 × 10^{-2} kcal/kg • h, comparable to those of the conventional process: 1.6~7.4 × 10^{-2} kcal/kg • h. The higher the heat generation rate after turning of the mash, the higher the alcohol production found. Supplementary inoculation of pure cultures of facultative anaerobic *Rhizopus* (Lin and Wang, 1991; Wang and Tsao, 1994) enhanced the alcohol productivity, reduced the inoculation size of expensive natural starters and also helped to minimize change in the aroma of the sorghum liquor.

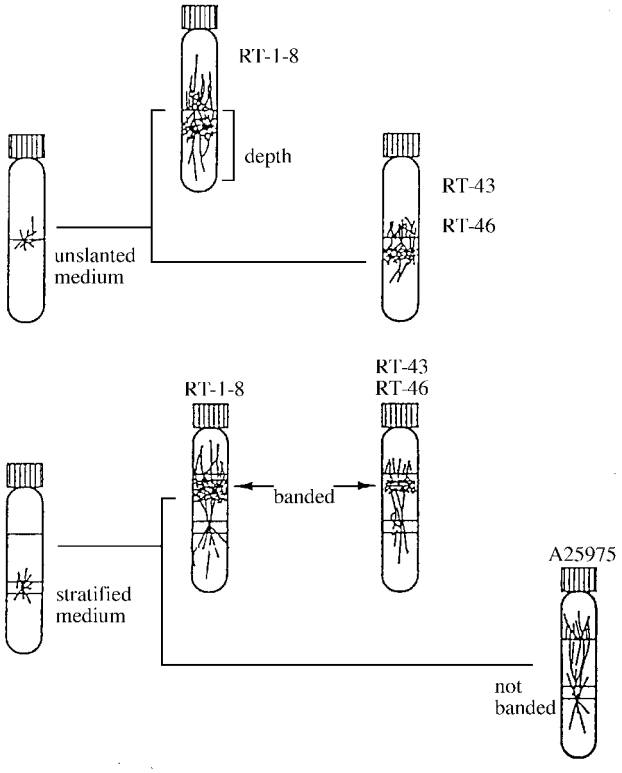


Fig. 10. Subsurface mycelial growth of facultative anaerobic *Rhizopus* in unslanted medium and stratified medium (Lin *et al*., 1991).

6. Delicious Oligopeptides

A tasty oligopeptide in the mashes of sweet wheat pastes (Tien-Mein Chiang) brewed with the facultative anaerobic *Rhizopus* was found in this laboratory (Yu, 1995). Chinese people popularly use sweet wheat pastes to season baked or fried chicken, duck and pork. The pastes have a very good fermented flavor and sweetness, and enhance the taste of baked or fried meat. Fermented sweet wheat pastes have been produced in Northern China for centuries. However, a very traditional process is still being used by Chinese people to produce this traditional product. The traditional process for wheat paste production of steamed bread with natural fermentation in brine water is laborious, and there is no quality control during SSF. The inoculation of *Aspergillus oryzae* takes the place of natural fermentation

Table 3. Brewing Data with the Use of Facultative Anaerobic *Rhizopus* RT-18

Starter (kg/car)[a]		Maximal temperature increment of mash (ºC)		Fermentation heat[e] Δ H/h (kcal/kg · h) ($x10^{-2}$)		Alcohol production (1/car)
		Before turning	After turning	Before turning	After turning	
1. Natural[b]	(18.0)	4.5	13	7.4	22.0	52.7
2. Natural[b]	(18.0)	4	13	6.6	22.0	57.29
3. Natural[b]	(18.0)	1	15	1.6	25.4	58.4
4. *Rhizopus* / yeast[c]	(15.0)	3.5	5.5	5.8	9.3	23.02
5. *Rhizopus* / yeast[c]	(15.0)	2	8	3.3	13.5	28.1
6. *Rhizopus* / yeast[c]	(15.0)	5	10.5	8.3	17.8	50.6
7. Natural	(9.0)	6.5	13	10.8	22.0	62.5
Rhizopus / yeast[d]	(3.0)					
8. Natural	(9.0)	3.5	13	5.8	22.0	73.8
Rhizopus / yeast[c]	(3.0)					
9. Natural	(9.0)	7.5	not turned	12.4	not turned	89.8
Rhizopus / yeast[c]	(3.0)					
10. Natural	(18.0)	2	12	3.3	20.3	76.6

Source: Wang and Tsao (1994) and Lai *et al*. (1991, 1995)

[a] for one stainless steel car having 600 kg of steamed sorghum grain.

[b] natural starter.

[c] *Rhizopus* RT-18 cultured in wheat flour was inoculated at the beginning, and the suspension of pure culture of *Saccharomyces* S-20 was sprayed when the mash was turned.

[d] Both *Rhizopus* RT-18 and *Saccharomyces* S-20 cultured in sterilized wheat flour were inoculated. The inoculum densities of the *Rhizopus* and the yeast were 10 spores and 10 cells, respectively.

[e] fermentation heat = heat increment × mass specific heat (Lai *et al*., 1991). (mass specific heat: 0.799 kcal/kg · ºC before turning, 0.815 kcal/kg · ºC after turning)

in Taiwan. In order to improve the quality and safety of the traditional process, the inoculation of facultative anaerobic *Rhizopus* isolates was used in this laboratory (Yu, 1995). Some cultures, RT-1-8, RT-43, RT-6, RT-7, RT-8, RT-10, RT-11, RT-14, and RT-46, were isolated from soysauce mashes in this laboratory. The cultures (31145, 31996 and 31631) were purchased from the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsin-Chu, Taiwan, R.O.C. The Research Institute of Wines, Taipei, also contributed one *Aspergillus oryzae* culture. We isolated the other twelve *Rhizopus* cultures which could survive when incubated under a 5% O_2 atmosphere. They showed definite mycelial growth in the subsurface of the stratified medium in the screwed tubes as in the case of sorghum brewing (Fig. 10). This result demonstrated that these twelve cultures are capable of growing under anaerobic conditions. From the results of the sensory evaluation on sweet wheat pastes obtained through solid fermentation of pure cultures, the twelve *Rhizopus* cultures showed scores better than 31145, 31996 and 31631, which did not indicate mycelial growth in the subsurface of the stratified medium (Fig. 10). Among the twelve *Rhizopus* cultures, RT-7 showed *umami* (beefy or meaty flavor) and sour tastes, and also sweet and salty flavors. The principle of *umami* in the mash was fractionated by means of column chromatography and identified as a tripeptide. The tripeptide has a molecular weight 300 to 400 daltons. Thin layer chromatography on the hydrolyzate of the peptide showed the presence of Glu, Lys and Asp. It is interesting that a *umami* principle of beef broth, Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala, had the same threshold value (1.41) as did the mixtures of synthetic component peptides Lys-Glu + Asp-Glu + Ser-Leu-Ala (Kuramitsu *et al*., 1993). From the results mentioned above, it can be seen that natural flavors (volatiles and oligopeptides) obtained through SSF using the cultures of facultative anaerobic *Rhizopus* constitute a new resource. It should pay attention to the importance of separation and purification which affects commercial usability, the measure of reasonable concentration in mash and probable toxicity of natural flavors as commented by Hausler and Munch (1997).

VII. Conclusion and Future Trends

The bioprocesses mentioned above for resource development through SSF were discussed from the viewpoint of the modification of solid substrates. Lichen growing, natural flavor production and HSGA were performed in our laboratory while short term mushroom growing and prevention of nitrate pollution were proposed by conceptual basis. This review has aimed to overcome the limitation of SSF as well as to extend the benefit of SSF to field site application in the near future. For example, the disadvantage of heat accumulation in the solid mash of SSF may be

overcome through intermittent mixing but not continuous mixing as is done in fluidized bed bioreactors, in which the microbe is detached from substrates. Breath fingerprints or the result of HSGA on solid mashes can be useful in characterizing the SSF process and in finding characteristic aroma compounds (Lu, 1984). For convenience of discussion, these bioprocesses for resource development can be divided into the following three categories.

1. Screening of Adequate Cultures and the Search for Adequate Water Management

Since lichens grow slowly, it is necessary to screen cultures adequately for growth in field sites. Deserts and flat rock fragment areas are very poor or null in water holding capacity, which plays a very important role in SSF. Therefore, the search for adequate water management is necessary. Based on the concepts of synchronized cultured technology, it has been concluded a short term mushroom growing is feasible, for example, s*hiitake* growing. Then, cultivation technology, particularly water management, adequate for field sites and environments and also selection of the cultures able for growth in field sites should be established.

2. Environmental Control and Bioreactors

Rhodococcus has been found to cause heterotrophic nitrification under aerobic conditions and denitrification under anaerobic conditions. The methods established in this laboratory for quantifying nitrifiers or denitrifiers can be extended to determine the fate of these microorganisms and the role of environmental conditions in field site or laboratory scale packed bed bioreactors. HSGA showed that different volatile metabolites were produced on different scales of SSF (flask or fermentor). Selection of the size of bioreactor is important for SSF in field site operation. Some facultative anaerobic *Rhizopus* were found to produce a characteristic aroma and a tasty peptide in this laboratory. Therefore, to make use of these cultures viable, an adequate bioreactor should be developed and conditional factors should be established in the near future.

3. Further Application of HSGA

This laboratory has developed a quartz crystal-based gas sensor for the quantification of volatiles metabolites in head space (Wang, 1993; Wu, 1990) but has encountered some difficulties, such as the effect of humidity on the adhesion of volatiles to the surface of quartz crystal (Wu *et al*., 1994). As mentioned above, the presence of one particular volatile metabolite, geosmin, was found in the breath fingerprints of hog-waste composts cured under mesophilic conditions but was not found in the process of main thermophilic composting (Chiou, 1996). Geosmin, an earthy odor, is the metabolite of mesophilic *Streptomyces* cultures and is not harmful to crops (Chiou, 1996). Therefore, the presence of geosmin could indicate compost stability. This suggests that some volatiles produced at the equilibrium point between solid state substrates and volatile metabolites in SSF could be quantified for some application. For example, if one considers that human bodies are in the state of SSF, the ability to find breath fingerprints would allow us to develop new gas sensors and new techniques to qualify or/and quantify the state of certain biotic reactions. Therefore, it is possible that SSF, originally an appropriate technology, will enable us to find new resources. For example, this laboratory has been preparing to work on identifying human health using breath fingerprints and HSGA. The primary candidate for a volatile metabolite is isoprene, a biogenic volatile, and its derivatives. A relatively high level of isoprene was found in human breath (Pleil and Lindstrom, 1995). Although the metabolic importance of isoprene remains to be determined, the isoprene derivatives, dolichol (a polyisoprenoid), the prenylated peptides of fungal sex pheromones and the prenylated proteins of the Ras superfamily may be good starting points for finding biogenic isoprene. The level of dolichol in tumors was found to decrease compared to that in control tissue whereas the concentration of dolichol phosphate did not exhibit any major change and the pattern of individual polyisopronoids in the free dolichol pool changed in several carcinomas with a relative increase in the shorter dolichols (Eggens *et al*., 1989). Many fungal mating (sexual) pheromones are highly hydrophobic due to modifications of C-terminal cysteine (of a peptide) by a farnesyl group (Bolker and Kahmann, 1993). Most of the known prenylated proteins are members of the Ras superfamily of low-molecular-weight guanine nucleotide-binding proteins. These proteins participate in a variety of cellular functions, including control of cell growth and differentiation, cytokinesis, and membrane trafficking (Schafer and Rine, 1992).

Acknowledgment

The author thanks the late Professor S. Sasaki for his advice to the author that he continue his research in soil microbiology in 1968. The author also thanks K. T. Chung for useful discussion, his colleagues C. M. Chiou and R. S. Hseu, and his graduate students for participation in this work and for help in preparation of the manuscript. Work in this laboratory has been supported by the National Science Council, R.O.C., the Council of Agriculture, R.O.C., the Departments of Environmental Protection and Public Health and the Taiwan Provincial Monopoly Bureau of Tobacco and Wine.

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Bioresource Development by SSF

以固態發酵開發生物資源

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摘　要

根據固態發酵(SSF)的理論，本研究室最近從事以下五項新生資源開發的研究。A建立薄膜培養法：即以玻璃紙薄膜覆蓋在不含碳、氮源的培養基，薄膜上接種地衣藻類及地衣真菌的菌體，而成功的合成地衣類，利用此方法在實驗室可篩選地衣類，將其培養在田間砂漠或河底岩石，可使之土壤化，即開發新土壤資源。B 以同步培養之理論，建議開發集中在第一週期採收的菇類栽培方法，此方法栽培期較短，基質水分損失少，菇場規模小，投資小，且一年之中可以栽培多次的適當菇類，適合本地的農民。C建議開發類似SSF的技術防治土壤的硝酸污染。D本研究室證實土臭味成分geosmin的生成可代表豬糞堆肥的熟成度，其中熟成堆肥是有機農業及永續農業的所需資材。E釀造高粱酒（一種SSF）的研究中發現，兼性嫌氣的*Rhizopus*菌株的接種時，酒膠之上層氣體有許多香氣。另外，用兼性嫌氣的*Rhizopus*菌株釀造甜麵醬（另一種SSF）時證明醬中含一種具鮮味的三酵類(tripeptides)，就是利用兼性嫌氣性*Rhizopus*菌株可開發天然調味料，是一種新的資源。香氣與geosmin的研究引起本研究室開發氣體感測器，而人的呼吸氣體視為SSF的上層氣體時，將可探討呼吸氣體指紋中特殊氣體表示一個人健康程度的可行性。上述開發研究可讓本來初級技術(Appropriate technology)的SSF昇級。