Journal of Industrial Microbiology & Biotechnology (1999) **22**, 482–499
© 1999 Society for Industrial Microbiology 1367-5435/99/$12.00
http://www.stockton-press.co.uk/jim

THOM AWARD ADDRESS

A Microbe's View of Fermentation*

C.W. HESSELTINE

Northern Regional Research Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Peoria, Illinois 61604

When I was notified of the great honor the Society had given me, I began to recall some recollections of my personal contacts with Dr. Charles Thom. He certainly had considerable influence on my career, even though I never worked with him.

In the fall of 1940, when I had just completed my first summer as a graduate research assistant at Wisconsin, my major professor told me that I was to go to the AAAS meeting in Dallas in December, but at my own expense. My 12-mo salary, as I recall, was $600. This was my first national scientific meeting, and I was to give a 12-min report on my research. Dr. Thom was chairman of the session in which I was to speak, and I was somewhat terrified because I already knew of his reputation. I am sure my report was quite poorly given, but afterward he looked me up and made some suggestions as to how better to present the information and how to conduct my research. Earlier, I had isolated a curious *Penicillium* and had sent it for his identification. I was very impressed when 10 d later, I received a letter which was the height of brevity "your strain is *Penicillium frequentans*," and that was that--not what I would expect from a bureaucrat! Dr. Thom was always in charge. At another meeting where he was presiding, one brash young man had 12 min for a presentation but he took 10 min beautifully giving an introduction to the subject. When his time was up, he had just launched into his work. Thom came up on the podium and, in a loud, authoritative voice, said "Young man, your time is up," and that ended the paper. My career was closely tied to Dr. Thom while I was at Lederle Laboratories. In 1952 he wrote me to ask if I would be interested in the position as head of the Culture Collection at Peoria. Frankly, I had long before said the only position I would ever accept in the government would be one in the Fermentation Laboratory at Peoria. I have often wondered what the odds were that I would be offered this position. I indicated by letter that I would definitely be interested in succeeding Dr. Ken Raper, who was going to the University of Wisconsin. Thom interviewed me in New York and the only thing I can remember that he said when we were talking about the advantages and disadvantages of going into government work at Peoria was the remark, "Well, every nest has its fleas." Dr. Thom was a consultant to the Peoria Laboratory; after I took the job, he made at least two visits of 2 or 3 d to look at what was being planned and what was being done. These were enjoyable times because he had many interesting stories to tell and he made it a

*Address given upon receipt of the 1980 Charles Thom Award.

point to talk to everyone in the laboratory regardless of rank.

He often talked about how important it is for those working with microorganisms to know what they are and how they look. He had a genuine interest in the Culture Collection; his mold collection, started in the early 1900s, and maintained on his own time for many years, was the nucleus of the present NRRL collection.

You can see that I am especially honored to receive the award since my career was so greatly influenced by Dr. Thom.

In my presentation, I want to touch on an aspect of microbiolgy, and especially mycology, that we have lost sight of in the United States, namely--the organism. the lack of appreciation of the numbers and biology of microorganisms is a factor, I believe, that has caused a decline in the position of the United States in the fermentation field. Since the topic is so large, I purposely have limited my paper to three areas that have been especially interesting to me--the number of microorganisms one has to choose from in developing a product, their biological interactions, and their interaction with the physical environment.

Number of Microorganisms

The number and diversity of activities of microorganisms that exist in the world are not appreciated by most of the people who use them. According to the *Dictionary of the Fungi* (Ainsworth 1971), there may be between 100,000 and 250,000 species of fungi alone. Furthermore, according to this reference, the current number of new species being reported in the *Index of Fungi* exceed 1,000 per year. The *Index of Fungi* is a publication listing all the new species and name changes in the fungi in the literature. It gives the name, the author(s) of the paper, the position of the new taxa in the classification of fungi, the reference to where the new taxa was described, and the location and substrate on which the fungus grew. Among other groups of microorganisms, the numbers of species will vary, but the number described and yet to be found is considerable. Among bacteria for instance, the latest *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons 1974) lists 245 genera and 1,609 species. *Streptomyces* has the most species (463). Similar large numbers of species are also found in algae and protozoa. A publication now 16 yr old (Benjamin et al. 1964) suggested that about 15-20,000 species of protozoa and 18,000 species of algae exist, with many more species still undescribed. Obviously, this represents a tremendous pool of microorganisms with many different abilities to grow on different substrates, to produce a myriad of enzyme combinations, and to form a multitude of different compounds. In fact, any compound on this earth can be attacked or modified by one or more microorganisms; and still, perhaps less than 2% of all the species of fungi have been examined to see what they can do. Microorganisms are like the 250,000 species of higher plants in that just a few have been domesticated and made useful. An example of what I mean is the recent discovery that *Cyathus stercoreus*, a fungus inhabiting old, well-decomposed cow pads, is an excellent destroyer of lignin. When one considers that the manure residue, after much microbial growth, would have left only the more recalcitrant material, it might be expected that lignin attackers would be there only because grass lignin would be the only material left to support growth. Yet no culture collection, including ours, had strains of this species, even though the fungus can

be isolated and grown on simple fungal nutrient media. It is not uncommon in nature, if one knows where and when to look for it. The point to be made is that there are thousands of microorganisms that do interesting things and make peculiar products. However, to exploit these fungi requires specialists who are broadly acquainted with microorganisms, where they grow in nature and how they can be grown, teamed with other scientists who can characterize the compounds and reactions. Considering the figure of 3,000 antibiotics isolated from the actinomycetes (Woodruff 1980) gives some idea of the number of compounds that might be available. This group and *Aspergillus* and *Penicillium* have been exploited perhaps more for what they can produce than any other group of microorganisms. To further increase the complexity of the problem is the fact that in a single species one can encounter strains that produce a specific compound and others that do not. Ramulosin is produced by only one nonsporulating strain of *Pestalotia ramulosa* NRRL 2826, whereas two other strains of the same species are completely negative (Hesseltine et al. 1963). When the ramulosin-producing strain is heat-treated, it loses its ability to produce product but again sporulates. What I am trying to say is that a tremendous number of microorganisms make thousands of as yet unknown compounds just waiting for the right team to exploit. Unfortunately, in this country the microbiologist, the biochemist, and the chemist are not wedded together to exploit the usefulness of microorganisms and fungi in particular. A second reason is the poor support of taxonomic-ecological research on microorganisms. This is reflected by the poor support of culture collections where only a very small number of the total microbial world is represented. This, in turn, is a reflection of the lack of support, both financial and moral, to sustain fundamental studies on the biology of microorganisms. Yet a great deal of money and time is directed toward certain fields, such as mycotoxins, in which certain aspects are repeatedly investigated over and over again.

As mentioned above, Dr. Thom emphasized the need for those working with molds to know intimately the microorganisms with which they worked--knowing the gross growth appearance, their place of occurrence in nature, their microscopic morphology, their classification, their variability, their growth needs, and what they make on a given substrate. One of the real constraints hindering the maximum utilization of microorganisms is the lack of trained persons with this understanding. I am reminded of a talk I heard a long time ago by Kettering. In his lecture, he told the story about the development of the first diesel railroad locomotive. When the first engines came out, someone asked him how his engineers came up with such a contraption that worked so well. His reply was that they fiddled around a lot and then did whatever made the engine run best and not what would have been a logical engineering design. I think the same thing can be said about the use of microorganisms. Too often research people do what they think is logical with microorganisms without being aware of the microorganism's requirements nor how it survives and thrives in nature.

I propose to discuss the microorganism's role in fermentation and applied microbiology. I purposely will emphasize the role of fungi and yeasts which behave physiologically more like filamentous fungi than bacteria, simply because I am more familiar with these groups of microorganisms. Microorganisms interact with other organisms, and this will be treated under biological interactions. The second major area is

the interaction of microorganisms with the physical environment. I will try to suggest some ideas that have been neglected in our handling of microorganisms to make them work for man.

Biological Interactions

Perhaps we should remind ourselves that it was just 100 yr ago that the first pure cultures of fungi were made by Brefeld, the results being published in 1881. However, fungi were utilized for practical purposes long before this time in such products as beer, spirits, cheese, bread, soy sauce, enzymes, and many others. These were successful because the operators knew from trial and error how to do the fermentation. Microbiologists today know a great deal about what microorganisms do and how they relate to their environment, and this information needs to be utilized in applied fermentation processes.

Social Interactions

By this, one means the interactions of organisms with each other. Mostly, we think in terms of pure cultures, because research people like to work in as simple a system as possible, with a single strain. However, in nature this condition does not exist except, perhaps, in a very few instances, such as in the invasive growth of a fungus into host tissue which, when attacked, is free of other microorganisms.

Ragi is an example of an inoculum sold in parts of Asia including Taiwan, China, Indonesia, Indochina, Malaysia, Singapore, and probably

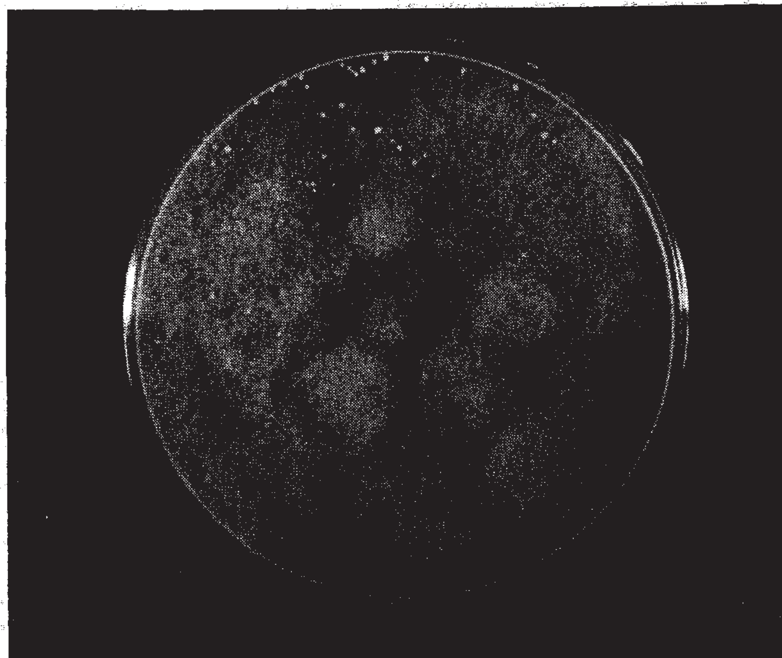


FIG. 1. Dilution plate of Chinese yeast showing yeast, *Amylomyces* and *Rhizopus*.

FIG. 2. Ragi balls.

other countries in the Central Asia area. Its purpose is to be used as an inoculum to make a number of foods (tape ketella, and tape ketan) based on starchy materials such as cassava and rice (Djien 1972). In China it is sold as Chinese Yeast with the same types of microorganisms (Fig. 1).

Recently, we have examined five fresh samples of ragi from Java (Fig. 2) supplied to us by Dr. F. G. Winarno, Food Technology and Development Center, Bogor Agricultural University, Jl. Raya Pajajaran, Bogor, Indonesia. Each sample was dry, hard, white, and varied somewhat in shape, depending on the ragi maker's style. Our interest was to examine fresh ragi sold on the open market. The counts of *Amylomyces rouxii*, yeast, and aerobic and anaerobic bacteria are shown in Table 1 using plate count agar (PCA) and incubating at 28 C for 1 wk.

It should be pointed out that the kinds of yeast and bacteria are very restricted. It is apparent that these samples contain three organisms: *Amylomyces rouxii*, a lactic acid bacterium; and a yeast, *Saccharomycopsis fibuliger*. It also should be noted that the pieces of ragi vary considerably in size. In two other samples examined from Indonesia, each contained *Amylomyces rouxii* and *Saccharomycopsis fibuliger*. In one of these samples, some *Rhizopus* was seen. In the five ragi samples studied above, only one sample showed *Rhizopus* growth. Besides the three dominant organisms found in every sample of ragi, we encountered several yeasts and, in low numbers, molds distinct for a particular ragi producer. One sample contained a black yeast, one sample had *Aspergillus clavatus*, and two samples had *Mucor*; but each was a distinct *Mucor* species. We believe each of these were contaminants and do not play a role in the activity of ragi. An interesting aspect of

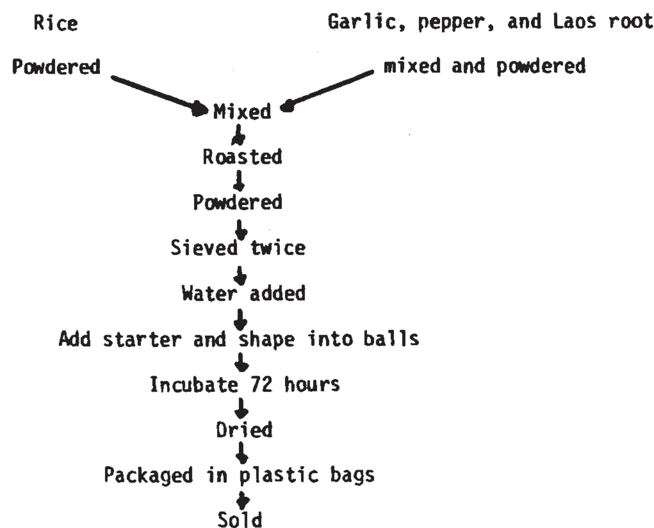
TABLE 1. The numbers of *Amylomyces rouxii*, yeast, and aerobic and anaerobic bacteria in ragi samples

Avg. Wt. of Each Starter (g)	Amylomyces	Bacterial Count Aerobic	Bacterial Count Anaerobic	Yeasts
4.47	6,000	1,350,000	240,000	5,100,000
	9,000	1,250,000	240,000	4,900,000
2.08	40,000	700,000	66,000	5,400,000
	80,000	820,000	50,000	5,800,000
6.67	70,000	1,350,000		68,000,000
	50,000	1,670,000		64,000.000
8.92	1,200	43,000		27,000,000
	2,200	26,000		33,000,000
6.06	280,000	460,000		64,000,000
	320,000	480,000		85,000,000

the ragi-making is the addition of powdered garlic, *Allium sativum* L.; pepper *Capsium annuum* L.; and Laos root, *Alpinia galanga*, to the rice flour before the ragi organisms are inoculated into the rice. We do not know, but we suspect, these spices are added to control contamination by undesirable microorganisms. Spices such as garlic and pepper are known to have antibacterial properties. The process, as I saw ragi prepared in Indonesia, is shown in Table 2.

Many other interactions of microorganisms involving yeast, bacteria, and fungi can be cited where they grow in association with each other in harmony. One situation that exists in nature is the growth of vesicular-arbuscular mycorrhiza that grow in association with many plants, including cultivated crops such as soybeans. In this case, the numerous genera and species serve the plant by extracting phosphorus from the soil and making it available to the plant. On the other hand, the fungus is benefited also, because it does not grow saprophytically but is dependent on some nutrients or growth factors supplied by the plant roots, the nature of which are not known. Numerous reports in the

TABLE 2. Flow sheet for making ragi



literature establish the beneficial influence of these specialized fungi on the plant (Gerdemann and Trappe 1979).

The Symba process (Wiken 1972) used to make single-cell protein from starchy wastes such as potatoes is an example of the use of two yeasts in series. The first yeast, *Endomycopsis fibuligera*, is grown in potato-starch solution; it produces enzymes for saccharification, resulting in glucose and maltose. After a proper holding time, the mash is pumped without treatment into a second tank in which a second yeast, *Candida utilis*, then uses the glucose and maltose to grow and produce biomass. This second yeast by itself cannot use starch but with the previous starch digestion, it can now grow to produce the single-cell protein desired, so the single-cell protein contains cells of both species.

Many other successful interactions of microorganisms can be cited. In my estimation, many other combinations of microorganisms could be developed by careful selection of the substrate and the conditions under which the fermentation is conducted. Before leaving the interaction of microorganisms, I would like to deal briefly with another example of use of multiple microorganisms in a single fermentation. This is the Chinese process for making alcohol from sorghum grain, referred to as the Kaoliang liquor process. Briefly, the inocula for this solid state fermentation involve wheat which is ground into flour and water is added to moisten the flour to a very specific moisture content. This is followed by an automated packing of the moist flour into cakes of several kilograms each, perforated by a central hole so that the solid cake resembles an angel food cake (Fig. 3). The cakes

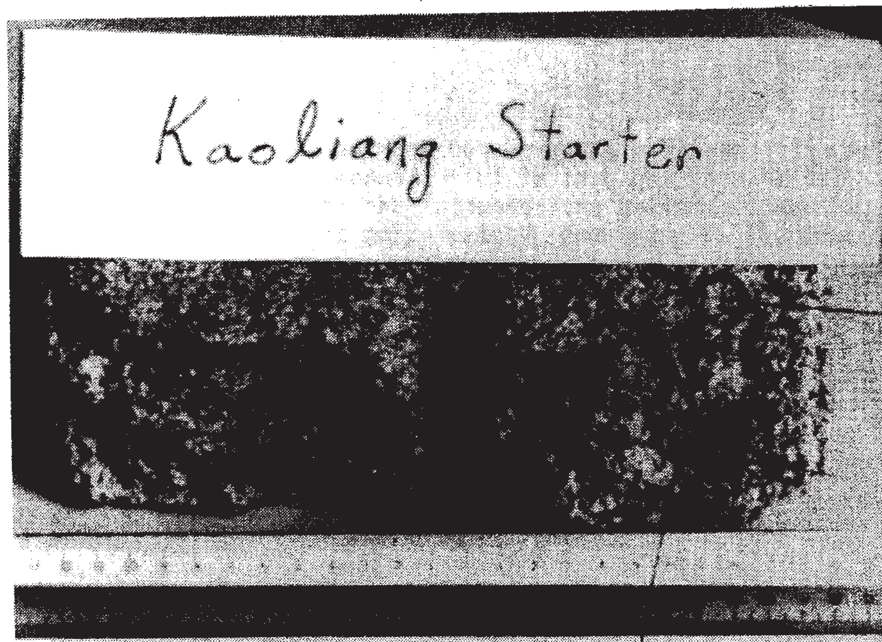


FIG. 3. Inoculum for the kaoliang process.

are conveyed automatically into incubator rooms where they are allowed to incubate. Under proper temperature (below 40 C), indigenous flora is allowed to be selected and this serves as the inoculum for the production of alcohol. The fermentation substrate is broken sorghum grain, which is cooked under pressure and cooled; the starter cake is broken into powder and mixed with the wet sorghum. This material is then loaded into solid-state fermentors that are on tracks for movement to the specially designed stills. The wet mash is covered with a suitable plastic cover to produce anaerobic conditions, and the fermentation proceeds. Saccharification and alcohol fermentation occur simultaneously. At the end of the primary fermentation (10 d), the fermentation tanks go to the distillation apparatus on tracks. After steam stripping, the sterile sorghum is again cooled and inoculated as before, and a secondary fermentation is carried out followed by distillation. Even a third fermentation and distillation may be conducted. This process has some very intriguing features: (1) there is no separate malting or enzymatic treatment of the starch; (2) there is no great quantity of liquid to dispose of; (3) the spent grain and yeast can be fed without drying or concentration, or with less drying than conventional fermentation mashes; (4) no inoculum needs to be developed as pure cultures; and (5) much of the process is automated. On the other hand, the process obviously has some disadvantages: (1) the time required to grow the inoculum is long, as well as the fermentation; and (2) the constituents of microflora probably vary from one lot to the next and, therefore, the ratio of the inoculum to fermentation substrate is high. H. H. Wang and T. C. Hsieh (1972) of the National Taiwan University have been studying the process using pure cultures. There is much to be learned from this process for producing alcohol and, obviously, there are many specific conditions that have to be met to make the process a successful one. In a recent visit to Taiwan, I was fortunate to visit one of these large producing distilleries which was somewhat automated in that the inoculum was produced and conveyed with automatic equipment. The interesting part of this alcohol process is that inoculum is produced by controlled environmental and physical factors, and alcohol is produced on an enormous scale, using a solid-state fermentation in mobile fermentors.

The use of microbial ecology to develop practical fermentations involving more than one microorganism has the following advantages. (1) Invariably, the dominant fermentative microorganisms are vigorous strains that will overcome chance contaminants. (2) In the case of bacteria, the problem of phage is overcome because some strains of dominant microorganisms will have resistance, and these will take over as the susceptible strains are destroyed. (3) Several fermentation steps will be accomplished at the same time or in succession. In making koji, two or three strains are usually blended together, each strain being used for a specific activity (Fig. 4). (4) In at least some instances, no sterilization is needed as in the fermentation step in storage of corn that prevents mold development and, consequently, prevents the formation of mold toxins. (5) Continuous fermentation is possible.

Genetics

Under biological interactions, one should not neglect some discussion

FIG. 4. Koji starter plated on nutrient agar showing different strains of *Aspergillus oryzae*.

of the latest development of a new technology that promises to bring a revolution in industrial fermentations as great as that which occurred in the 1940s with the development of the antibiotic industry based on agitated liquid media. This is the technique of transferring desirable genetic characters from microorganisms, plants, and animals into bacteria and yeasts or even into plants in which a plasmid is the transferring vehicle. A good discussion of the commercial possibilities may be found in a recent article in the *Wall Street Journal*, Tuesday, September 11, 1979 (DNA: Industrial Interest Grows). If we are to believe this and other articles in the popular press, insulin will soon be produced by bacteria in which the gene for insulin from an animal source has been transferred into a bacterium. Thus, insulin will no longer be obtained from animal sources but will be a fermentation product. It is probable that an alcohol-producing yeast will be constructed that will have the ability to utilize starch directly to form alcohol and, at the same time, have a protective factor inserted into its gene pool that will protect it from higher ethanol levels in the substrate. Several facts, however, need to be pointed out. (1) In all the processes of gene transfer between unrelated organisms, microorganisms such as yeast and bacteria must be used as the gene transfer

agent. Therefore, this work will be dependent on competent microbiologists. (2) In no instance is a gene created, but rather it is a transfer of existing genes from different sources into new combinations. (3) Recently, a landmark decision has been handed down by the U.S. Supreme Court that ruled, in a four to five decision, that microorganisms that are man-modified can be patented. Both the majority and minority opinions suggested that the Congress should pass legislation clarifying the patenting of living material, just as legislation has been passed governing the patenting of plants. The question of the patenting of a living organism that occurs in nature unmodified by man has not been decided. The arguments, both for and against patenting living microorganisms, can be found in a series of authored papers published in the *APLA Quarterly Journal* (Whale et al. 1979). (4) This new development will certainly add to the importance of culture collections because many new man-made strains will be deposited to fill patent requirements.

The purpose of culture collections is to maintain a gene pool of microorganisms and this is true from the time the first collections were formed. Functions of all culture collections are basically the same.

1. They are collections of reference strains, often the type strain on which the species is based. Currently, new taxa of bacteria must be deposited in a permanent recognized collection as part of the validation process.

2. Collections are places where unusual strains that have been isolated are kept; that is, strains which produce useful products and enzymes. For example, our collection contains a large number of species that produce a great variety of polymers. To reconstruct this group of microorganisms with their peculiar genes would require thousands of man hours and hundreds of thousands of dollars and, even then, one probably could not reconstruct the present gene pool. Curators of culture collections are asked why collections do not discard part of their holdings to keep costs down. Ten years ago, there was no interest in good nitrogen-fixing bacteria nor yeasts that would produce alcohol. Recently, because of the energy crisis due to the price of crude oil, a great interest developed in both fields. Who is to say what group of microorganisms will be needed 10 yr from now? With all the modern development in culture maintenance, there is less need for technicians and more need for highly trained scientists to carry out an active program in taxonomy and ecology. For anyone to study the interrelationship of microorganisms with other organisms, including plants and animals, one needs to actually know the proper names of the forms he is dealing with. In the United States the awareness of this kind of relationship by culture-collection personnel and the fermentation team is almost nil. This is not true in some other parts of the world. In looking at the U.S. patents issued this last May 15, I noted two Japanese patents that make use of fungi I suspect are completely unfamiliar to nearly all of you, let alone knowing where the species can be obtained in nature or characteristics of their growth. One patent deals with the genus *Coriolus* and its ability to produce a polysaccharide with antitumor activity (Ueno et al. 1980). The second (Misaki et al. 1980) describes the use of *Elsinoe* to produce a glucan. The point I am trying to make is that, in a complex undertaking such as development of a practical fermentation product, the team approach is

necessary and the team is no stronger than its weakest member. A football team with weak ends, no matter how good the rest of the team may be, will end up with a poor season. A fermentation team that does not have the expertise in ecology and biology of microorganisms will be in trouble. If it uses an organism already reported in the literature, it will always be behind. In my estimation, this is why the United States lost its leading role in industrial fermentation early in the sixties. Recently, I have seen the records on the commercialization of penicillin at the Northern Regional Research Center. The initial discovery of penicillin in *Penicillium notatum* was followed shortly by replacement of this species by *Penicillium chrysogenum*, because the eminent mycologists, Charles Thom and Kenneth Raper, knew that *P. chrysogenum* was a close relative and, furthermore, knew where to isolate new strains. This led to the discovery of a culture of the latter species that would produce penicillin under submerged conditions. From the initiation of the work at Peoria in June 1941 to December 1941, it was possible to increase the yields of this antibiotic 12-fold. But let us return to the value of culture collections to applied research.

3. Culture-collection holdings allow a great number of strains to be screened immediately without the time and effort required to isolate new strains. Once a species or group of species has been identified as being producers, the whole collection of strains in this species and related species can be screened so that one has the best genetic material to work with. Thus, one can take the highest alcohol producer and combine it with the highest starch saccharifier to produce a truly super alcohol producer.

4. Culture collections serve as a bank of strains used for testing or assay of various compounds. For example, cultures are designated as specific strains for testing antifungal chemicals.

5. Another function is as a depository of cultures that produce unique compounds in varying amounts. Often the compound may not have any particular known use, but someday it may. Strains of the patulin-producing mold laid dormant for years in our collection after it was shown to be too toxic for use as an antibiotic. With the advent of mycotoxins, patulin was found to be a carcinogen occurring naturally in some samples of apple juice; suddenly there was interest for producing some of this compound for study. Our culture collection had preserved the best-producing strains and these immediately became available.

6. A growing activity of certain collections is as a depository for strains deposited in connection with U.S. and foreign patents. The courts decided many years ago that a producing culture, not necessarily the best, had to be deposited in a public culture collection. The theory was that a patent could not be made to operate unless the proper microorganism was available. Hence, the patent had to identify the name of the microorganism, the location of the deposit, and the number assigned by the collection to this strain. With the recent court decision allowing the patenting of genetically engineered microorganisms, collections will need extra support because even more microorganisms will be deposited. It is even more important to preserve these strains since they will be man-made organisms one cannot find in nature. One can only conclude that culture collections will assume an even more important role in industrial microbiology than they now play, and they need more support.

Reproduction

Like other living things, sexual reproduction occurs in microorganisms, and sex may be exploited. Many years ago it was demonstrated that when plus and minus strains of certain Mucorales were combined in a suitable liquid medium and agitated, the resulting mixed mycelium became rich in β-carotene (Hesseltine and Anderson 1957; Barnett et al. 1956). Normally, when plus and minus strains were combined, the two colonies came together and formed β-carotene at the point of contact, with rapid development of black mature zygospores. However, in liquid media, this sexual process is frustrated and pigment accumulates. Hormones are involved in the process; hormones of a different nature are known to be produced in other groups of fungi and much is still to be learned.

However, I would like to dwell in more detail on a fundamental problem that requires a solution in reproduction in the fungi. The Basidiomycetes are a class of fungi with a great variety of forms, ranging from rusts and smuts to puff balls and gilled and pored fungi. Many of the Basidiomycetes produce large macroscopic fruiting bodies which, in time, form myriads of basidiospores. Among these are fungi that decay wood and all sorts of plant material containing cellulose and lignin. Besides degrading cellulosics, they make a number of possible useful compounds including pigments, antibacterials, enzymes, flavors, and from some, fruiting bodies are used for food. In culture, many species fail to produce any asexual spores. This causes difficulty in maintaining cultures and in production of inoculum. Because of the large size of the fruiting bodies in many species, it is not possible under usual conditions to produce basidiocarps and basidiospores within a suitable vessel. Maintenance problems were overcome with the advent of the use of liquid nitrogen as a means of preservation. However, the production of suitable inoculum for most Basidiomycetes that do not produce asexual spores still has not been accomplished. In two cases, extensive and expensive research has found ways of producing spawn for growing the commercial mushroom, *Agaricus bisporus*, and the paddy mushroom, *Volvariella volvacea* (Hesseltine et al. 1976) (Fig. 5). People who try to work with Basidiomycetes in a given fermentation are confronted with the problem of producing inoculum; usually they use blocks of agar containing mycelium or parts of colonies from liquid media. The result is that only a few pieces of mycelium are used to start liters of media or kilos of solid substrate. Even though the growth rate is rapid, as measured by the speed of expansion of the colony, the time required to produce product is long, often a matter of weeks. Should an actinomycete be inoculated at the same rate (a few spores per liter of medium), product formation would likewise be frustratingly slow. The obvious answer is to have a thousand sites of growth instead of just one. Since the fungus does not want to cooperate in producing asexual spores, a way must be found to circumvent the problem. The problem, as I see it, is what I like to call directed basic research, that is, research directed at a basic problem for which a solution must be found before one can successfully solve the applied research problem. Briefly, what is needed is research to develop very small amounts of viable mycelium in an organic or inorganic carrier, hopefully with a particle size of only 10 to 20 μm in diameter. Ideally, such inoculum would have the following characteristics: (1)

FIG. 5. *Agaricus bisporus* spawn.

it could be produced on larger masses of substrate, for instance, plant stems, which could be reduced to fine particles; (2) the fine particles containing the viable mycelium could be dried; (3) the particles containing the viable mycelium could be kept for several months with no reduction in viability; and (4) the inoculum would immediately begin growing when it was placed in a suitable substrate at an optimum temperature.

A fact that one must recognize and that is well known to many mycologists is that Basidiomycete mycelium can be dried and wetted repeatedly and still grow. Thus, one sees white oak trees on which the Basidiomycete *Aleurodiscus oakesii* lives for years only on the bark and, therefore, is repeatedly dried and wetted but continues to spread year after year. Obviously, the mycelium must remain viable during these periods of adversity.

The fairy ring mushroom, *Marasmius oreades*, which infects lawn grass, often is dried out; but when fall rains start, the mycelium begins to grow and, in a matter of a day or so, large masses of basidiocarps appear. Therefore, if this phenomenon occurs in nature, why can't a method be devised to produce quantities of small pieces of viable mycelium? Hopefully, by studying the nutrient needs, proper en-

vironmental conditions, and various carriers, relatively few materials could be found that could be used as starter culture for many species of Basidiomycetes, just as spawn for *Agaricus* was developed. Once this is accomplished, the limiting factor of inadequate numbers of fungus propules would vanish. The solution to this problem would make it possible to exploit Basidiomycetes to make a number of useful products. For example, in our laboratory, *Cyathus stercoreus* was found to be a good lignin and cellulose degrader (Wicklow et al. 1980). However, the fermentation time is far too long to be of any possible practical value as long as only a few pieces of mycelium are available for use as starter.

Physical Interactions

I have been discussing the interactions of microorganisms with other microorganisms. Now I would like to turn to the microorganism and its relation to the physical environment. That is, the relation of the microorganism to its habitat and, specifically, to the state of its food supply.

Fungi typically grow on the surface and within the substrate and not in a liquid menstrum. Of course, there are exceptions to this; large numbers of fungi classified as Oomycetes and Chrytrids complete their life cycle in aquatic situations, and some have even adjusted to nearly anaerobic conditions. Another exception is some yeasts which grow in fruit juices. However, the great majority of fungi grow on a solid substrate, such as seeds, leaves, stems, and other pieces of organic matter, with a great variation in moisture and nutrients. They are essentially solid-media dwellers rather than aquatic inhabitants. Under these conditions, mycelium is produced that invades the substrate, secreting enzymes to make available nutrients for growth and producing reproductive structures, which may be initiated in the substrate but ultimately must be present on the surface in order to release their spores into the atmosphere. It is this habit of growth in a moist, solid substrate with aeration that allows fungi to compete so well in nature.

Solid substrate fermentation refers to use of material that is fermented in a solid state. However, there are a number of conditions or states in which a solid material may be fermented. The following outline gives the various conditions referred to as a solid state and an example.

Solid material only:
1. Solid material allowed to mold in place; tempeh fermentation.
2. Solid material with an occasional stirring; typically, koji is made this way.
3. Solid material continuously agitated; method developed to produce aflatoxin using *Aspergillus parasiticus* or *A. flavus*.

Solid material in liquid:
1. Solid material in columns with liquid media circulated through it; ex-ferm process for alcohol fermentation.
2. Solid material suspended in a liquid medium with (a) agitation, (b) stationary; the kaffer beer fermentation used for food.

At the Northern Regional Research Center we have used solid sub-

strate fermentations to produce various mycotoxins, secondary metabolites, enzymes, foods, and feeds (Hesseltine 1972, 1977a,b). Most of this has been done on a laboratory scale. Usually, our aim has been to produce a small amount of product as soon as possible. Engineering aspects have received little attention. However, we have tried to relate the behavior of the organism (mostly fungi) to how they live in the wild state. Since water can be a limiting factor, the moisture of the substrate needs to be approximately the moisture level that the fungus likes. *Rhizopus oligosporus* prefers a high moisture level, whereas a mold such as *Aspergillus parasiticus* grows and produces product at a moisture level of 25 to 30%. Aspergilli in the *A. glaucus* series grow at moisture levels even as low as 13%. Since fungi are mostly aerobes, the particulate matter must be in a size range to allow free exchange of air and yet not be too large. Rice or pearled wheat is ideal, but corn and soybeans must be cracked into several pieces. Since the spore must germinate and enter the solid material, the surface of the solid particles should be invaded easily either through broken surfaces or by pearling of the outer grain layer. There may be advantages to inoculating the substrate and then allowing a short holding time, so that invasion of the substrate can occur and colonies can begin to develop before agitation begins. It also may be desirable to add additional nutrients or precursors to the fermentation to enhance growth or to produce more product.

Solid substrate fermentations using fungi are used widely to produce cheese, shoyu and miso kojis, tempeh, enzymes, and alcohol. Bacteria are also used in solid substrate fermentations to make silage, sauerkraut, and sausage.

According to Monteiro (1975), the potential pollution load from the sugar alcohol industry in the State of Sao Paulo in Brazil is equal to the biological oxygen demand from a population of about 51 million. The potential problem is made worse because most of the mills are located in rural areas, and cane processing coincides with the dry season. A unique concept in the alcohol production from sugar cane is described in a note published by Rolz et al. (1979) with a reference to a pending patent. The process employs sugar-cane pieces as the fermentation raw material. The sugar cane is chopped into pieces from 0.48 to 1.0 cm, presumably as cubes. These chips can be dried so the alcohol fermentation can be carried out all year. When fresh chips are used, the fermentor is charged with one part chips to 1.4 by weight of boiling water and inoculum is added. After fermentation, the yeast and ethanol are separated from the chips, and the chips are pressed. Both liquids are mixed and fresh cane is inoculated. The process is repeated until the ethanol inhibits the yeast or the ethanol yields become too low to recycle. The process has two advantages: Extraction of sucrose from cane is more complete (up to 99% of sucrose in the cane is fermented in the two-stage fermentation) and no organic nitrogen is required. It also appears that less liquid will have to be disposed of at the end of the two fermentation steps. The authors make reference to a 120,000-liter-per-day plant for which economic data show a lower initial investment and a greater sucrose conversion than with conventional processes. A cost reduction of 5 cents per liter is achieved.

Solid state fermentations have some distinct advantages over conventional stirred or aerated liquid media fermentations:

The medium may be simple such as a single whole grain. If necessary, other nutrients may be added.

The process may be scaled up to either batch or continuous fermentations. Like all continuous fermentations, the biggest problem is the mutation of the organisms so that the yield or nature of the product changes. This may be avoided by continuously adding new inoculum and removing the old.

The size of the equipment in relation to the product is less because less water is needed.

In some fermentations, the natural flora of the substrate may be used.

Because of the low moisture levels in most of the fungal fermentations, bacterial contamination is reduced.

The conditions under which the fungus grows are similar to those found in nature.

Sporulation in constant agitation fermentation is almost completely inhibited, thus reducing contamination of the fermentor area.

Since less substrate and water are used to produce the product, pollution problems are reduced.

Aeration is easier because of the spaces between the solid particles and especially so when the substrate is agitated.

Product yield is often quite high. In one of our current mycotoxin fermentations, we produce no yield with liquid media but good yields with solid substrate. *Amylomyces* appears to produce little amylase in liquid media but great amounts on solid media.

Yields are quite reproducible. An example of data can be found in Hesseltine (1977b).

Product made in solid substrate may be dried or freeze-dried and used directly as food or feed at less cost, because less water needs to be removed.

If the product is a chemical that must be removed, less solvent is required and extraction may be carried out directly in the fermentation vessel.

However, in all fairness, there are also some real disadvantages to solid substrate fermentations:

The microorganisms used must be those that can grow at reduced moisture levels, unless the solid substrate fermentation involves a liquid phase.

Even in small fermentors, the amount of heat is great and must be controlled by removal from the fermentation.

There is need for development of monitoring devices to determine temperature, pH, O_2, and CO_2.

For large fermentations requiring agitation, energy costs may be high.

The substrate may need to be treated prior to sterilization to get a good fermentation. For instance, corn needs to be cracked into pieces.

The amount of inoculum may be quite high, especially in a continuous inoculation of a continuous fermentation.

Additional sterile water may be required during the first 48 h of the fermentation.

In some instances, as in liquid fermentations, we do not get the desired product formed, or in small yields.

In conclusion, I would like to emphasize that we need more microbiologists who know the nature of microorganisms as they occur in nature. We should try and think like the "bugs" do in their wild state to make them useful servants for us.

Again, I would like to say to the Society--thank you for this honor bestowed on me. I will never forget it.

Finally, I would like particularly to acknowledge three long-time women colleagues. The first is Dr. Hwa L. Wang; her knowledge and interest in Oriental fermented foods and her background in nutritional biochemistry have complemented my interest in the same subject and also, for our many stimulating discussions on Oriental culture and foods. Dr. Odette L. Shotwell has been a long-time collaborator in the mycotoxin field, going back to the early sixties. Finally, Mrs. June Kestner, who has been my secretary and office manager since 1966, has been efficient in handling many administrative duties with intelligence and promptness so that my time could be devoted to things scientific, and also she has given valuable advice and criticism.

LITERATURE CITED

Ainsworth, G. C. 1971. *Ainsworth and Bisby's Dictionary of the Fungi.* Commonwealth Mycological Institute, Kew, Surrey, 663 p.

Benjamin, C. R., W. C. Haynes, and C. W. Hesseltine. 1964. Microorganisms: What they are, where they grow and what they do. *USDA Misc. Pub.* 955:1-35.

Barnett, H. L., V. G. Lilly, and R. F. Krause. 1956. Increased production of carotene by mixed + and - cultures of *Choanephora cucurbitarum*. *Science* 123:141.

Brefeld, A. 1881. Botanische Untersuchungen uber Schimmelpilze. Untersuchungen aus dem Gesammtgebiete der Mykologie. *Heft* 4:1-191.

Buchanan, R. E., and N. E. Gibbons. 1974. *Bergey's Manual of Determinative Bacteriology*, 8th ed. Williams and Wilkins Co., Baltimore, MD, 1246 p.

Djien, K. S. 1972. Tape fermentation. *Appl. Microbiol.* 23:976-978.
Gerdemann, J. W., and J. M. Trappe. 1979. The Endogonaceae in the Pacific Northwest. *Mycologia Memoirs* 5:1-76.
Hesseltine, C. W. 1972. Solid state fermentations. *Biotechnol. Bioeng.* 14:517-532.
______. 1977a. Solid state fermentation--Part 1. *Process Biochem.* 12(6):24-27.
______. 1977b. Solid state fermentation--Part 2. *Process Biochem.* 12 (9):29-30,32.
Hesseltine, C. W., C. R. Benjamin, Barbara Bradle, and W. F. Hendershot. 1963. Ramulosin fermentation. *Am. J. Bot.* 50:209-213.
Hesseltine, C. W., E. W. Swain, and H. L. Wang. 1976. Production of fungal spores as inocula for Oriental fermented foods. *Dev. Ind. Microbiol.* 17:101-115.
Hesseltine, C. W., and R. F. Anderson. 1957. Microbiological production of carotenoids. I. Zygospores and carotene produced by intraspecific and interspecific crosses of Choanephoraceae in liquid medium. *Mycologia* 49:449-452.
Misaki, A., S. Takaya, K. Yokobayashi, and Y. Tsuburaya. 1980. Glucan polysaccharide. U.S. Patent 4,202,966. May 13.
Monteiro, C. E. 1975. Brazilian experience with the disposal of waste water from the cane sugar and alcohol industry. *Process Biochem.* 10:33-41.
Rolz, C., S. de Cabreara, and R. Garcia. 1979. Ethanol from sugar cane: Ex-ferm concept. *Biotechnol. Bioeng.* 21:2347-2349.
Ueno, S., C. Yoshikumi, F. Hirose, Y. Omura, T. Wada, T. Fujii, and E. Takahashi. 1980. Method of producing nitrogen-containing polysaccharides. U.S. Patent 4,202,969. May 13.
Whale, A. R., H. W. Boyer, D. R. Dunner, C. E. Lipsey, T. D. Kiley, P. J. Luckern, C. W. Hesseltine, B. K. Zimmerman, F. Lederer, and I. Hayashi. 1979. The Pros and Cons on Patentability of Microorganisms *per se. APLA Quart. J.* 7:175-319.
Wang, H. H., and T. C. Hsieh. 1972. Kao-liang brewing by pure culture. Proc. Iv International Fermentation Symposium. *Ferment. Technol. Today* 651-658.
Wicklow, D. T., R. W. Detroy, and B. A. Jessee. 1980. Decomposition of lignocellulose by *Cyathus stercoreus* (Schw.) de Toni NRRL 6473, a "white rot" fungus from cattle dung. *Appl. Environ. Microbiol.* 40: 169-170.
Wiken, T. O. 1972. Utilization of agricultural and industrial wastes by cultivation of yeasts. Proc. IV International Fermentation Symposium. *Ferment. Technol. Today* 569-576.
Woodruff, H. B. 1980. Natural products from microorganisms. *Science* 208:1225-1229.