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Oriental Food Uses of *Aspergillus*

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INTRODUCTION

In the Western World, uses of fungi as food are few in number and bounded by a mixture of legend and taboos which often impinge only peripherally upon reality. Even in continental Europe, more macro—fungi are consumed than in the British Isles. It is automatically assumed that filamentous micro—fungi are necessarily harmful — with the sole exception of the blue and green moulds found in certain types of cheese. The modern discovery of aflatoxins offers some justification for this traditional distrust of the filamentous fungi, but the long—term nature of the effects of these toxins makes it unlikely that there is any causal connection.

In the Orient, on the other hand, there has long been a clear understanding that processes closely allied to putrefaction and biodegradation can, by exercising proper control and the selection of appropriate conditions, convert rather unappetising and distinctly indigestible ingredients, into palatable and nutritious food whose rich and varied flavours contribute much to a largely vegetable diet with a tendency to blandness. The origin of these processes is lost in antiquity, presaging by three millenia our modern use of enzymes in food processing, for it is in their ability to produce substantial amounts of extra—cellular hydrolases when growing on comparatively dry substrates that we find the explanation for what must have seemed the near magical power of these moulds to transform foodstuffs. To the Occidental observer, the range of foods made with the aid of moulds is a further remarkable aspect of this fascinating story. Hesseltine (1965) in his seminal study “A Millenium of Fungi, Food and Fermentation” has listed over a hundred such fermentations, commenting that “this list offers a wealth of subjects for future investigation”. Although fungi belonging to several genera are employed in the various fermentations, the genus *Aspergillus* is pre—eminent both in the number of products made through its activities, and in the commercial importance of certain of these products, notably soy sauce, already familiar to most Westerners, and miso, the generic term for a range of soy— based pastes now becoming available in this country.

At Strathclyde University a series of studies have examined the chemical and biochemical changes which occur during soy sauce (shoyu) fermentation, and an account of these changes and of the art of koji production will be given. This will be followed with a comparative examination of some other fermentations, including miso, tamari, saké and tempeh, and with some discussion of the subsequent processing steps and the properties of the finished products.

KOJI

This is a generic term of great antiquity, sometimes used in a rather confusing manner to encompass the mould, the starter culture, and the main fermentation mass. To avoid such confusion, the term ‘koji—mould’ will be used for the organisms, ‘seed—koji’ for the starter culture, and ‘koji’ for the main fermentation mass. The production and use of koji has certain similarities with the production and use of malted cereal grains in the West, and I shall start by briefly summarising the latter process. Malt is produced by soaking sound grain (normally barley, although wheat is occasionally employed) then subjecting it to conditions of warmth, moisture and adequate aeration. Uniform conditions are provided and local overheating and anaerobiosis are prevented by turning the heaps of grain from time to time, a process which also helps to prevent the rootlets of the seeds matting together, and thus simplifies the subsequent handling of the finished malt. When the process of germination has reached the desired stage, it is halted, either by drying the grain, or by wet—grinding it for immediate use. The timing of this step iscrucial to the success of the whole operation; if done too soon, both enzyme formation and degradation of the protein and starch reserves of the seeds will be insufficient to meet subsequent requirements; if left too late, then losses of reserves through respiration and through conversion to unavailable materials such as cellulose, will be too great with adverse effects on process economics. Regulating and timing the malting process demands a subtle combination of scientific analysis and shrewd observation based on long practical experience. All of these considerations apply in similar fashion to the quite different process of koji production. In both cases the objective is to effect maximum enzyme production and hydrolysis of the raw materials, while minimising undesirable changes. These objectives are to some extent in conflict, and must be met by careful regulation of moisture, temperature and aeration, and by halting the process just short of the initiation of undesirable changes i.e. shoot (acrospire) emergence in malting, sporulation in koji.

A number of moulds may be employed in koji production, the most commonly employed being strains of *Aspergillus oryzae* or *A. soyae.*

Depending on the process involved, many different substrates may be used, for example, rice, soybeans, beans mixed with rice, wheat or barley, and so forth. In soy sauce production, a mixture of soybeans and wheat flour is commonly used. Beans are first washed, then soaked for 16 to 24h in water, which is changed every few hours to prevent excessive bacterial growth and consequent souring of the mixture. The prime purpose of this soaking is to permit the beans to take up their full quota of water. Little work seems to have been done on the nature and extent of biochemical changes occurring in the bean during this phase of the process, or on the influence of such changes on subsequent stages and on the quality of the final product. However, it would seem reasonable to postulate significant differences between freshly harvested beans and those held in storage for long periods.

The soaked beans are then cooked, either by boiling at ambient pressure until soft, or in a pressure vessel at 10 p.s.i.g. for lh. There seems to be some conflict of opinion over the desirability of pressure—cooking the beans, but most modern authorities seem to agree that these mild conditions are quite acceptable, and have the additional advantage of sterilising the beans; we have used these conditions throughout our work.

Meanwhile, the wheat is prepared. Soft (i.e. low—protein) wheat is normally preferred. Whole wheat or wheat flour may be taken. Whole wheat is toasted until crisp, then crushed while wheat flour may be steamed or be subjected to dry heat ('parched'). In the present studies we have used wheat flour heated to 150° for 12h.

Wheat and beans are then thoroughly mixed, using roughly equal quantities on an original weight basis. The beans should remain separate and have an even coating of flour, giving a rather dry surface. The mould inoculum is then mixed in; this can be either a portion of a previous koji, or a specially prepared seed—koji, or a spore suspension. A spore suspension or seed—koji are preferred since they permit more exact microbiological control. In our work the mould is maintained on rice agar or malt wort agar slopes. Good sporulation occurs in 4 to 5 days at 30° under these conditions, and the spores are washed off with water containing a small amount of Tween detergent, and this suspension used to inoculate the substrate. Inoculated material is then spread into shallow trays, traditionally made of woven bamboo, although we have found expanded aluminium to be satisfactory. An open mesh structure for the trays ie essential to permit adequate aeration. Trays are then placed in a humid room at 30°. Spores germinate rapidly and hyphae permeate the substrate. Exactly as in malting, it is necessary to turn the koji at intervals in order to prevent local overheating, and even so a layer one inch deep will attain a temperature in excess of 35°. Turning also prevents the development of excessively dry conditions, conducive to spor—ulation, on the outside of the mass, helps promote aeration of the entire koji, and keeps the beans reasonably separate. Indeed, if mixing is inadequate the whole trayful soon becomes one solid mass, bound tightly together by the mould myceliurn. Mixing is normally effected at 24 and 48h after inoculation, and the koji is terminated after 72h. Unlike malt, the finished koji is never dried but is immediately used in the next stage of the process, in this respect resembling whisky distillery rather than brewery practice. If the koji stage is not terminated after about 72h, then sporulation rapidly sets in. No detailed study of the reasons why sporulation is initiated at this stage seems so far to have been carried out. There is ample food present in the form of the hydrolysis products which the mould enzymes have formed from the starches and proteins in the substrate, most of which are therefore wasted from the point of view of the mould. A possible explanation would seem to be that the koji experiences considerable water loss due to evaporation induced by the metabolic heat produced by the mould, and that it is this reduction in available water which induces sporulation.

If significant sporulation does occur then the soy sauce subsequently produced has a mouldy off—flavour, and an excessive level of ammonia. This latter seems a most remarkable state of affairs. In liquid culture, *Aspergillus* species normally start to sporulate only when the ammonia concentration has reached a very low level, yet, in koji, ammonia is produced in some quantity at the onset of sporulation. It seems unlikely that the organism should need to deaminate amino acids to obtain organic carbon, when it is surrounded by assimilable carbohydrates. Clearly, the behaviour of *Aspergillus oryzae* in koji has much to interest the microbial physiologist.

Having described the basic process, let us now examine in greater detail the biochemical changes involved. Yong (1971) and

Yong & Wood (1976) have shown that there is a precise sequence of changes in experimental kojis. After about 20h incubation, the temperature begins to rise from the initial value of 300, reaching a maximum of 38° to 40° some l0h later, thereafter declining slowly to 30° . It appears that spore germination and mycelial growth begins after about 20h incubation and that all other biochemical changes therefore begin at that time. The pH rises fairly steadily from then on, initially being around pH 6.5, reaching pH 7.3 after 72h and pH 7.5 after 96h. As has been previously mentioned, the moisture content declines from an initial value around 50%, to a final (72h) value between 30 to 35% depending on the strain.

The pattern of change in the level of reducing sugars is particularly interesting. From a low initial value (around 3 ng/g dry weight koji as glucose) it rises very rapidly to a value of 20 mg/g in the middle of the fermentation, then a secondary peak around 20 to 25 mg/g toward the end of the fermentation, thereafter holding steady or showing a slight downward drift if the fermentation is prolonged beyond 72h. The sharp initial rise occurs some time before significant amounts of amylase can be detected in the koji, due to the release of a sucrose—hydrolysing enzyme (Yong & Wood, 1975) which later work (Goel, 1974) has shown to be a fructofuranoside transferase. The enzyme produces reducing sugars from the sucrose and related saccharides which constitute up to 20% of the dry weight of the bean.

The latter rise will represent the hydrolysis of starch from the wheat flour by amylases. Both alpha — and beta —amylases are produced by *A. oryzae* and increase in amount steadily during the fermentation (Yong, 1971; Goel, 1974), whereas the sucrose—hydrolysing enzyme reaches a maximum after 30 to 50h, thereafter declining in concentration (Yong, 1971) although if the vegetative mycelium be challenged by transfer to fresh sucrose—containing medium it will release further enzyme (Goel, 1974).

Cellulase is also released into the koji (Goel, 1974) and we believe that this enzyme may play a significant role in aiding the disintegration of the beans during the secondary fermentation in brine (the moromi stage).

The fact that reducing sugar levels decline but little during the late phases of the koji fermentation contributes to the view that the appearance of ammonia during sporulation is not due to a simple lack of assimilable carbon necessitating the de—amimation of amino acids.

Total soluble nitrogen increases around three—fold during the course of the fermentation of koji, to a final value around 14 to 16 mg N/g (Yong, 1971). In fermentations prolonged beyond 72h there is no significant further change in this parameter. Simultaneous measurement of amino—nitrogen by the Sorensen Formol method revealed a more complex picture. Initially scarcely detectable, this material increased very rapidly between 20 to 30h of the fermentation to around 3 mg nitrogen / g dry weight of koji (further increasing to 5 mg N/g in one strain of *A. oryzae* being examined), them dropping almost as rapidly to half its peak value, rising to a minor peak at 65 to 70h, a second decrease and thereafter a steady increase until the fermentation was terminated at around l00h. It is clear from the great deal of work that has been published on the proteases and peptidases of the *Aspergilli* that these moulds generate a complex battery of these enzymes, with a range of pH optima and substrate specificities. Unfortunately, it has not been possible to carry out a detailed study of the pattern of protein—hydrolysing enzyme production by these moulds, and we have been compelled as a compromise to employ a general assay measuring casein hydrolysis at pH 6.5. This showed a pattern of changes quite similar to, although not identical with that exhibited by the amino—nitrogen level. There is clearly a relationship here, although at present there is not sufficient data to say to what extent a causal relationship exists, and one would like to know far more about the uptake of amino acids and simple peptides by the mould.

In the production of soy sauce, the 'mature' (72h old) koji is mixed with 18% brine, and this mixture (called moromi) undergoes a prolonged fermentation in which first lactic acid bacteria and then yeasts are involved. The finished product is pressed, clarified and pasteurised, bottled and marketed either alone or in a mixture with caramel, molasses and other additives, depending on the preferences of the market being served. We think that the enzymes produced by the mould during the koji stage remain active in the moromi, slowly hydrolysing the proteins and polysaccharides present in the mash, and so enhancing the quality of the finished product.

Soy—sauce fermentation is clearly a very complex process, and this is reflected in the very diverse studies which are carried out in Japan. Thus a recent review of the fermentation of soy sauce, miso, glutamic acid and nucleic acid related substances by Natsuno & Okada (1974) cited 604 references, while the review by Yong & Wood (1974) of the soy sauce fermentation cited 259. A further complication for the Occidental worker is the amount of material which —not surprisingly — is published in the Japanese language. English—language abstracts, no matter how carefully prepared, cannot possibly answer all the questions which will strike someone active in the field. For example the paper by Yamamoto, Yangida & Suminoe (1972) reports on studies of changes in enzyme levels during the preparation of the koji for soy sauce. Their general conclusions resemble those of Yong (1971) and Goel (1974), as judged from the abstract in "Chemical Abstracts". However, there is an intriguing reference to the enzyme phosphatase. Within the confines of an abstract it would be impossible to explain this fully, but the significance of the presence of this enzyme in koji is not readily apparent. Again, Oba (1974) reports that the enzyme tyrosinase was produced in a rice koji, and reached a maximum value after 40h incubation. In contrast, Yong (1971) could not detect tyrosinase activity at any stage of the soy sauce koji fermentation.

What of future developments in the shoyu fermentation? One obvious line for study is the possibility of using artificial mixtures of industrial enzymes produced in bulk to replace the traditional koji. Work on miso (Kitaoka, 1972) suggests that the entire replacement of koji by enzymes would result in an unsatisfactory produce with an inferior flavour. On the other hand, a combination of koji and enzymes could be very beneficial (Sato, Ito, Kawano & Endo, 1973; Takeuchi & Yokoo, 1974).

A recent patent (Beatrice Foods Co. , 1973), the review by Yokotsuka (1973) and the work by Goel (1974) indicate that more elaborate microbiological control of the processes will both accelerate the production of shoyu and miso, and also enhance the quality of the final product (Shibasaki & Hesseltine, 1962).

Finally, to those who live in countries where soybeans cannot be grown commercially, producing products similar to shoyu and miso from indigenous crops is attractive both in the potential saving on imports, and in the chance to upgrade materials of marginal utility in human nutrition. A report from Kansas State University (Kao, 1974) shows success in producing miso and tempeh from chick pea and horsebean, both of which seeds gave satisfactory products. There is evident scope for similar research using seeds produced in the United Kingdom.

The importance of the soy sauce market to the East is clear from the production in Japan alone of about 110 million kilolitres per year (Yokotsuka, 1972).

TAMARI

In the course of discussions with commercial concerns who distribute and market soy sauce, I became aware that the term tamari' is sometimes used interchangeably with 'shoyu'. At first I dismissed this as a quirk of the 'macrobiotic' food cult, but more detailed study has revealed a rather interesting situation. Hesseltine (1965) citing Yokotsuka (1960) describes tamari as a "type of soy sauce especially common in China in which a greater amount of soybeans is used than wheat". The latter author states that three types of soy sauce are available in Japan; tamari, a type more common in China, where it accounts for most of the soy sauce, raw material mainly soybeans; koikuchi, accounting for 90% of the Japanese soy sauce, consisting of a blend of fermented soy sauce and a chemical hydrolysate of defatted soybeans; and usukuchi, made from soybeans, wheat and a small amount of rice (Yokotsuka, 1972). Prescott & Dunn (1959) describe tamari as "a sauce prepared from soybeans, often with the addition of other materials, such as rice". They continue "the flavour of tamari sauce differs from that of soy sauce. This difference in flavour is due to the use of *A. tamarii,* this mould being the dominant microorganism in this process. The fermentation period is shorter than that of the soy sauce fermentation".

Murakami (1971) examining the classification of koji moulds by a computer—generated cluster analysis of 20 characters of each of 659 strains of *Aspergilli* recognised two groups, the *A. flavus* group and the *A. oryzae* group. *A. tamarii* was placed alongside *A. oryzae* and *A. sojae* and in the *A. oryzae* group, which included all the koji moulds examined. He states that *A. tamarii* is now isolated from koji.

This information suggests that it would be interesting to compare the behaviour of *A. oryzae* and *A. tamarii* in controlled koji fermentations, since there is clearly an area of confusion present, at least in the English language literature on the subject.

MISO

This is a preparation of equal importance with shoyu in Japan, although it is at present little known in Europe. Basic raw materials are soybeans, grain and salts and it is marketed as a thick paste, and is used as a source of flavour and salt in cooking and food preparation. Shibasaki & Hesseltine (1962) have described the making of this product, and its characteristics, although it is rather misleading to use the term 'product', since they describe several varieties of miso, and when even the United Kingdom suppliers carry three types of miso. There is evidence to suggest that the miso fermentation predates the production of soy sauce, and it is perhaps not surprising therefore that it encompasses a more diverse range both of substrates and of fermentation conditions, leading to products that are readily differentiated by even the untrained Occidental palate.

Three basic types of miso can be recognised (Hesseltine, 1965; Shibasaki & Hesseltine, 1962, on whose excellent reports the following summary is based). There is kome miso, made from beams and rice; mugi miso, made from beans and barley; and mame miso (sold as hatcho miso in the United Kingdom) made from soybeans alone. For the production of kome and mugi misos, the koji is produced by soaking polished rice overnight in water, steaming it for about an hour, then inoculating with mould spores. Incubation is for 50h at 35° with thorough stirring at least twice. The

A. oryzae strains used for these fermentations should be higher in amylolytic activity than is necessary for shoyu production.

The koji is then mixed with an appropriate mixture of soaked, cooked soybeans, barley or rice, and salt. Thus the process differs significantly from shoyu production in that the mould does not grow on the beans. Even in the case of mame miso, only a portion of the beans are moulded. The precise proportions of beans and grain used, and also the extent to which the beans are cooked, depends on the type of miso being made. Thus rice—based misos (kome miso) are further subdivided by Shibasaki & Hesseltine (1962) into 5 types.

The mixture of koji, beans, rice or barley, and salt is then inoculated. Traditionally, this is done with a portion of an old miso, but Shibasaki & Hesseltine (1962) obtained better results by using a pure culture of *Saccharomyces rouxii*. After fermentation at 28°, followed by a period at 35°, the finished miso if further blended and packaged for sale.

As illustration of the range of conditions which can be used, Shibasaki & Hesseltine (1962) give the details summarised in Table 1. Naturally, the lighter, less salty miso types have a shorter shelf—life than do the dark and salty varieties.

Table 1. Condition for producing different types of rice miso

Type	Colour	Nature	Fermentation time	Ratio of beans: rice: salt v/v
White		Sweet	1 week	10:10 to 20: 2.5 to 3.5
Edo	Pale red—brown	Sweet	2 weeks	10: 8 to 10: 0.25 to 0.4
Sendai	Red—brown	Sweet, salty	1 year or more	10: 4: 0.4 to 0.5
Shinshu	Light yellow—brown	Salty	1 year or more	10: 5: 4.5
Mame	Deep red—brown	Salty	2 years	Beans and salt only

Shibasaki & Hesseltine (1962) report fairly high microbial counts in the early stages of miso fermentation, and a decline in the later stages. They comment on the contribution of lactic acid bacteria to the sour taste of miso, and to the prevention of spoilage during storage, yet made very good miso without these organisms. In a preliminary examination of three miso samples on sale in the United Kingdom, I found rather a low microbial count, with the name miso having rather more organisms than did the kome or mugi miso samples.

The remarks about future developments in the soy sauce fermentation apply with at least equal force to the miso fermentation. In addition, miso is still a rather rare item on the United Kingdom market, and probably also on the European market. As with yoghurt, its primary incursions seem to be through the 'Health Food' trade, and there is no reason why it should not progress to a wider market in the same successful manner.

TEMPEH

Tempeh is a foodstuff common in Indonesia and some other countries in that part of South East Asia. Most authorities agree that the mould concerned belongs to the genus *Rhizopus*. Most of the strains examined belong to the species *R. oligosporus*, although 5 other species have been demonstrated (Hesseltine, 1965). However, the same author cites an early report (Burkill, 1935) which states that *Aspergillus oryzae* may be involved in the fermentation. In the early part of our own studies on soy sauce, we obtained three strains of *Aspergillus*. Two were soy sauce strains from China supplied to us by Dr. Hesseltine, the third was a strain procured from Singapore and described by Yong (1971) as follows; "*A. oryzae* strain AO1 was isolated from tempeh obtained in Singapore. The identity of this fungus is still doubtful but Tan (Miss S. K. Tan of the Singapore Institute of Standards and Industrial Research) (personal communication, 1970), has managed to produce soy sauce of good quality using this organism

We subjected this strain to the same tests on a typical sauce koji substrate, as were used for the two soy sauce strains of *Aspergillus*, and described above. The results of Yong (1971) show significant differences between strain AO1 and the two Chinese strains. Whereas the soy sauce strains gave a fairly steady increase in pH over the whole duration of the fermentation, AO1 gave a very rapid increase from pH 6.55 at 22h incubation, to 7.5 at 29.5h, a pH only reached by the soy sauce strains after 90h of fermentation. From the 20h to 65h, the pH remained fairly steady, then actually decreased to 7.2, a behaviour which was never observed in soy sauce mould kojis. Strain AO1 produced nearly three times as much alpha—amylase as did the other strains, but its overall protease level was rather lower, while lipase level was much lower.

The AO1 koji showed a rather similar pattern of changes in reducing sugar level to that shown by the other two moulds, except that the second maximum was both earlier and greater in the AO1 koji, as would be expected from the higher amylase levels which were observed. Invertase production by strain AO1, on the other hand, was very much lower than that by the other strains. Total soluble nitrogen levels followed those in the other two kojis, and amino nitrogen levels were close to those of the less productive soy sauce strain. Final ammonia levels were however slightly higher in the koji made with strain AO1.

If strain AO1 is typical of tempeh moulds, it is not particularly obvious what advantages the observed differences in biochemical activities would confer. It is however desirable that further comparative work be carried out, using conditions appropriate to tempeh production, before any firm conclusions be drawn.

SAKÉ

Saké is the traditional alcoholic beverage made from rice, known as ‘rice wine’ in the West because its alcoholic strength approximates that of a wine. Amerine & Cruess (1960) quote comparative Japanese production figures for 1952 as Grape Wine, 25 million U.S. gallons; Saké, 100 million U.S. gallons. Murakami (1972) reports that in 1970 about 1.6 million kilolitres of sake’ were made.

Saké production depends upon the hydrolysis of rice by a koji produced by growing *Aspergillus oryzae* on a portion of the rice. We may see analogies with the production of Grain Whisky in Scotland, where malted barley is used to hydrolyse much larger quantities of unmalted grains, or indeed with the sort of modern beer production where adjuncts such as maize are hydrolysed by malted barley assisted by mould enzymes. Sake’ production does however have many points of interest, and it is clear from the numerous references to Japanese language papers, that it is in a phase of rapid technical development, for example into continuous culture fermentation at the alcohol production stage.

Arima (1957) has given a detailed account of saké production. He shows that it is but one member of a family of such ‘wines’ (and spiritous distillates made therefrom) to be found throughout the East. Rice is the cereal most commonly used, although he reports that distilled drinks are made from sorghum and sweet potatoes. Variety may be introduced by employing adjuncts; for example a red wine is obtained by adding Ang—kak, which is rice moulded with *Monascus purpureus* which produces a very attractive red pigment.

When producing sake’, conditions are so arranged that, as with the fermentation stage of Scotch whisky production, enzymic hydrolysis of starch continues during fermentation. An important difference from whisky production is that rather low temperatures (around 10°) are favoured for the initial stages of saké. The presence of lactic acid bacteria in controlled amounts is essential for a satisfactory saké fermentation, since the pH of the basic mash is too high for good yeast growth, or for a satisfactory flavour in the finished product. It is interesting to note that there is a school of thought in the whisky industry which regards a controlled lactic fermentation as desirable for optimum flavour development in their finished product. It is of course essential that the lactic fermentation should not be excessive, since a very acid mash will inhibit yeast growth.

According to Arima (1957), saké production is conducted as follows. First the rice is highly polished, to remove some 12 to 15% of the original weight (even more for the finest grades of saké). This is because rice bran has adverse effects on the colour and flavour of saké. The polished rice is then steeped in water for several hours, steamed at atmospheric pressure for one hour, then cooled and the moisture content adjusted to 40 to 50% w/w.

For koji production, steamed rice is cooled to 40°; inoculated with *Aspergillus oryzae* spores; left in piles in a room at 28° until the spores germinate (12 to l5h); spread in shallow boxes and incubated for a further 30 to 36h, with stirring every 5 to 7h. As with some other kojis, mould growth is enhanced if a small proportion of wood ash made from selected timbers, is added to the rice; 0.5% is a suitable rate of addition. This procedure presumably represents supplementation with potash and possibly phosphates.

The short incubation period means that little, if any, sporulation will have taken place and that extracellular enzyme levels will be maximal. For best results in the subsequent fermentation, it is desirable that the koji he made with mould strains which give high amylase titres, a difference from soy sauce strains which are selected for proteinase production.

The koji is now used in the alcoholic fermentation. Steamed rice (90L) koji (36L) and water of medium hardness and chloride content of 30—70 mg per litre (110L) are mixed, and held at a temperature of 5 to 60 for 6 to 7 days with occasional stirring. Starch hydrolysis and a lactic fermentation occur. The mash is next inoculated with *Saccharornyces saké* and a vigorous fermentation ensues, with the temperature rising to 18° to 20°. Arima (1957) shows that the interactions between bacteria and yeasts are considerably more complex than this simplified account might indicate, although in more modern processes the lactic fermentation may be replaced by addition of commercial lactic acid.

The mash is then ‘fed’ with three successive additions of increasing amounts of steamed rice, koji and water, and after about 20 days the fermentation comes to an end, with a final alcoholic content between 20 to 22%. After filtration and a period of storage to permit maturation and clarification, the saké is diluted to an alcohol content of 15 to 16%, pasteurised and dispensed for sale.

A noteworthy aspect of the fermentation is the very high alcohol level which the yeast tolerates, which may be explained in part by the way in which the process operates to keep the sugar concentration low at all times.

As Hayashida, Kamachi & Hongo (1972) observe, saké's unusual feature of involving a fermentation of a thick mash high in solids, prepared without a sterilisation step, generally fermented by natural mixtures of bacteria and yeasts in an open vessel, create special problems in any attempt at a continuous fermentation process. However they have succeeded in operating a semi— continuous process, involving passage of the mash through a series of vessels arranged as a cascade.

SOME MINOR FERMENTATIONS

Throughout the East there is a wealth of fermentations, many of which are admittedly but small variations on the important fermentations already discussed. The information on them is frequently contained in unusual, obscure or very old sources, and it is difficult adequately to describe the importance of Hesseltine's contribution in securing and examining these references, and in collating his results in such a convenient form (Hesseltine, 1965). For the sake of brevity, therefore, an annotated list of these fermentations are set out; unless otherwise specified, the information given is drawn from Hesseltine (1965), where the original references may be found. It is worth noting that many authors claim that these mould fermentations represent a significant addition of B—group vitamins to the diet in addition to their more obvious roles in increasing the palat-ability and digestibility of their substrates.

Chee—fan. A type of soy—curd cheese (sufu) in which *A. glaucus* may participate, as well as the more usual *Mucor* and *Actinomucor.*

Cheese. Nakanishi (1973) has published a review of his patents on Japanese cheese production utilising *A. oryzee.* Unfortunately for the present author, this interesting—sounding paper is written in Japanese. He is presumably not concerned with the ordinary Chinese soy—bean curd cheese, called 'sufu' and fermented with *Actinomucor elegans*.

Fermented Minchin. A fermentation of wheat gluten, practiced in Northern China. The gluten is allowed to mould in a closed container, then mixed with 10% of salt and further aged. At least seven fungal genera, including *Aspergillus* spp. are thought to participate.

Hamanatto. Intact soybeans are soaked, cooked, and moulded with *A. oryzae* in the usual manner. After mycelium development is complete, the beans are dried in the sun to a moisture content of 12%, then steeped in l8% brine, along with strips of ginger, for up to a year. The beans retain their integrity, in marked contrast to their behaviour in the shoyu or miso fermentations; this is presumably due to their retaining the seed—coat, and to effects of the drying process. After ageing in brine, the beans are again dried. They are said to be soft and sweet, with a flavour otherwise like that of soy sauce or miso. Hesseltine (1965) states, and conversations with Japanese students confirm, that this interesting fermentation is of extremely local occurrence in Japan. Commercial concerns in the United Kingdom have expressed interest in it as a possible premium product. Some preliminary work in our laboratories suggests that air—drying may be a suitable alternative to sun—drying on completion of mould growth. For a fuller discussion of the published information on this fermentation see Wood & Yong (1975).

Kaffir Beer. Although an African product, this is of interest here because the grains used (sorghum, maize, etc.) are saccharified through the action of *Aspergillus flavus* and *Mucor rouxii.* After the starch has been hydrolysed, the mixture undergoes a mixed lactic acid bacteria plus yeast fermentation. Hesseltine (1965) stresses the importance of this beverage as a source of dietary niacin and riboflavin.

Katsoubushi. This is an extraordinary fermentation of fish by *Aspergillus ochraceus* and possibly *A. glancus.*

Ketjap. This is a form of soy sauce made in Indonesia, using *A. oryzae.*

Mirin. A mixture of distilled ethanolic liquor, rice koji and steamed glutinous rice, is allowed to age. The alcohol content is around 20%. The resulting produce is described as an extremely fragrant condiment.

Ragi. An Indonesian product made from rice fermented by yeasts, bacteria and moulds, and used to hydrolyse the starch in rice or cassava. Hesseltine (1965) states that the moulds present are *Rhizopus oryzae* and *Chlamydomucor oryzae,* but Stanton & Wallbridge (1969) list *Aspergillus oryzae* among the organisms present in this product.

Tao—Cho. Yet another *Aspergillus* fermentation of soybeans and rice flour reported from the Malay Peninsula.

Tao—Si. A Phillipine version of soy sauce made with *A. oryzae.*

FERMENTED FOODS AND MYCOTOXINS

The modern scientist, confronted with accounts of these traditional fermentations is at once driven to think of aflatoxins and similar products of mould fermentation. That secondary metabolite formation does occur is strongly suggested by the many references to the beneficial effects of moulded foodstuffs on digestive disorders and other diseases. Indeed it has been suggested that the health of the inhabitants of the Japanese town of Nods (which seems to be soy sauce production as Burton—on—Trent is to beer brewing) benefits from the high concentration of mould spores in the atmosphere.

Japanese workers have naturally been very concerned with the problem of aflatoxins in these traditional foodstuffs which are so important to their diet. The general conclusion of their many studies is that there is no evidence at all of the presence of toxic materials in properly produced foods fermented with *Aspergilli.* This is a most remarkable situation when we consider the great antiquity of these fermentations, far pre—dating the development of modern microbiology, with its range of pure culture techniques, isolation and identification procedures, etc. The real danger from many mycotoxins lies in their carcinogenicity, which may take many years to manifest itself. Acute poisoning is easy to detect and even primitive people rapidly learn the appropriate lessons. But how did the soy sauce and miso makers select out the beneficial strains of *A. oryzae* from all the superficially similar but ultimately deadly moulds?

It is not simply that the conditions found in Koji do not permit harmful organisms to grow, or that they prevent toxin formation. Shotwell *et al.* (1966) have obtained high yields of aflatoxin by growing *Aspergillus flavus* on solid rice. Manabe & Matsuura (1972) have shown that aflatoxins are stable under conditions found in miso, yet Manabe, Onuma & Matsura (1972) were unable to find any aflatoxins in the commercial and home—made miso samples which they examined. A few of the samples showed a fluorescent

Mizaume. Prescott & Dunn (1959) describe this as a sugar syrupmade in Japan through the action of *A. oryzac* on rice.

substance when extracts were examined by thin layer chromatography, but this material was not toxic to chick embryos. Similarly, in their examination of 214 Japanese industrial strains of *Aspergillus,* Murakami, Takase & Ishii (1967) and Murakami, Takase & Kuwabara (1968) found that while some organisms produced chloroform— extractable, fluorescent compounds, none produced detectable amounts of material possessing the spectral properties of afla— toxins.

Yokotsuka *at al.* (1968) have examined shoyu, miso and mirin for pyrazine compounds. On prolonged cultivation, 26 of 68 industrial *Aspergillus* strains produced aspergillic acid, but if incubation was restricted to only 2 or 3 days, then contamination with this material could be avoided. They point out that some strains produce it earlier than do others, and suggest that the latter be used for food production. They also adduse evidence that ether extracts of shoyu at dose rates corresponding to a year's consumption of shoyu by a Japanese adult (12L of shoyu) were not lethal to mice upon intraperitoneal injection.

It is therefore clear that the available evidence all tends to the conclusion that properly prepared foodstuffs made by controlled fermentations with species of *Aspergillus* are as safe and wholesome as they are appetising and enjoyable.

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