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AN INSTANCE OF SYMBIOTIC FERMENTATION.

BY ALLAN MACFADYEN, M.D.

THE study of the biology of fermentation has made great strides within recent years, and this has been notably due to modern bacteriological methods and the facilities they afford for obtaining pure cultures of the organisms to be met with in such natural processes. Investigation has shown that the simultaneous presence of two or more organisms in given processes of fermentation is a frequent occurrence, and that in many instances this constitutes an essential factor for their success. The net result is brought about by a co-operation in the activities of different groups of organisms. This co-operation, as has been found on closer study, may be of a symbiotic or a metabiotic nature. The antagonistic action of the micro-organisms is a fact that attracted earlier and wider recognition. We have, for example, the inimical action of the saprophytic on the parasitic forms of bacteria. The greater reproductive power, or the more favourable conditions of soil, may favour the development of one organism at the expense of another, or it may be that the products of one form are fatal to certain other forms of life. The water bacteria rapidly crowd out any extraneous forms that may gain access to water.

As has been indicated, actions of a co-operative nature on the part of micro-organisms are attracting the attention they deserve. The phenomena of putrefaction in their various phases are due not to the action of one species, but to the action of various species of aerobic and anaerobic bacteria. The complete study of the agents of such putre-

factive changes has yet to be accomplished, though their diversity is beyond question.

An organism by its growth may create the conditions that favour the development of another on the same soil. Thus in many morbid processes mixed and secondary infections are constantly noted. The infection of purulent wounds with the bacillus pyocyaneus was at one time a familiar example, whilst in the course of a disease such mixed infections may give rise to grave symptoms, and are marked features in diphtheria and tetanus. Infection is frequently aided by the injection of mixed cultures of organisms, *e.g.*, symptomatic anthrax with the bacillus prodigiosus, or an infection may be prevented by the same means, *e.g.*, anthrax by means of the bacillus pyocyaneus. These questions, however, lie beyond the limits of the present paper.

When one organism in the course of its growth renders a soil suitable for the subsequent development of a second organism, the process is termed metabiosis. A good example is to be found in the changes that occur in a natural wine must. An alcoholic fermentation of the fluid occurs in the first instance, resulting in the production of alcohol and CO_2. When the alcoholic ferments have finished their work new agents appear, and an acetic acid fermentation occurs which lasts until the alcohol has been used up. A third group of organisms now appear, which convert the acetic acid into carbonic acid and water. When this has been accomplished the changed soil becomes finally a prey to putrefactive bacteria, which had hitherto been unable to develop owing to the presence of the alcohol and acid.

It has been found that when two or more organisms are simultaneously inoculated in the same soil, a symbiosis may ensue whereby products are formed that do not occur when the organisms are cultivated individually. Nencki found that the bacillus of symptomatic anthrax when grown in grape sugar solutions forms H_2, CO_2, normal butyric acid and inactive lactic acid, whilst the micrococcus acidi paralactici forms almost entirely the optically active lactic acid. If, however, both organisms are cultivated together a more

active fermentation ensues whereby not only the above substances are formed but also a *new* product—normal butyl alcohol.

Neither the bacillus coli communis nor the bacillus denitrificans can liberate nitrogen from nitrates, but when cultivated together they produce a complete reduction of nitric acid to nitrogen (Burri and Stutzer).[1]

We have familiar examples of spontaneous symbiotic fermentations in koumiss and kephir. These alcoholic beverages are apparently due to the joint action of lactic acid bacteria and yeasts on milk. The "ginger-beer plant" is another instance that has been worked out with great care by Prof. Marshall Ward.[2] It may be as well to state that "ginger-beer plant" is a popular name in many parts of the country for a substance used in the home manufacture of ginger beer. Marshall Ward found that the "plant" contained a mixture of organisms amongst which were two essential forms, viz., a species of saccharomyces and a schizomycete—which he named respectively Saccharomyces pyroformis and Bacterium vermiforme. The former inverts cane sugar and produces an alcoholic fermentation in the inverted cane-sugar solution—the latter produces a lactic acid fermentation. It is only when these two organisms are present that a result similar to that of the ordinary ferment is produced; and further, they must be simultaneously present. Their relationship is that of a symbiosis and the result a typical example of a symbiotic fermentation. The yeast and bacterium are probably introduced attached to the ginger and brown sugar obtained from the grocers' shops.

Marshall Ward cites likewise the affection of the grapes in some Rhine districts known as "Edelfäule." "These grapes are rendered mouldy and 'rotten' by a species of botrytis which so alters the constitution of the grapes that the proportion of acids, sugar and nitrogenous matter are altered before they go into the must—such grapes yield wines of higher quality and finer 'bouquet' than merely ripe healthy ones similarly fermented."

Reynolds Green[3] refers to a parasitic growth on the

sugar cane which is composed of a yeast and a bacterium and produces a fermentation resulting in the formation of alcohol, CO_2, acetic and succinic acid.

The action of mixed cultures of yeasts is of great importance in brewing operations. "Many of the best brews are known to be due to yeasts which are not of a pure strain in Hansen's sense, and it is not at all improbable that a better brew can be obtained by symbiotic ferments than by a pure one."[2]

As I have indicated, the whole subject is attracting increased attention. The large processes of nature, such as the re-distribution of cellulose and other forms of dead matter, are due to the co-operation of many living agents. This fruitful subject of inquiry is being gradually opened out, and results of great scientific and practical value may be anticipated. The following account does not lay claim to originality, as the results are simply a control of those obtained by others. They may perhaps serve to draw further attention to this promising line of investigation, dealing as they do with the successful applications of a pure symbiotic fermentation.

The production of alcoholic beverages, such as wine, beer, and spirits, is due to the action of living agents or "ferments" on sugars. The vegetable cells or yeasts employed for this purpose split up the sugar into alcohol and carbonic acid. The sugar may exist formed as in the case of the manufacture of wine from the grape, or it may have to be prepared as in the ordinary distilling operations. In the latter instance the sugar is generally prepared from starch by the addition of malt, which, in virtue of a saccharifying ferment it contains, dissolves and converts the starch in the grain employed into a fermentable sugar. To this sugary fluid the yeast is added, and an alcoholic fermentation ensues. We have, therefore, a twofold action :— (1) the saccharifying action by the malt ferment resulting in the production of sugar from starch ; (2) the ferment action by the yeast resulting in the production of alcohol from the sugar. The success of such processes depends upon the use of the right agents. The yeasts are a widely

distributed group of micro-organisms, and consist of a great variety of species. Of these, some species are specially adapted for employment in industrial fermentation processes, others are not. It is necessary to have the right kind of yeast to ensure a successful result. The technical advance made in bacteriological methods has enabled brewing chemists to cultivate the useful yeasts on suitable soils, and to conduct their processes with pure breeds of the right sort. In this respect the labours of Pasteur and Hansen led the way to the substitution of scientific for rule-of-thumb methods of work.

The alcoholic fermentation is a biological process, and an acquaintance with and a supervision of the living agents to which it is due are essential factors in its successful manipulation. The importance of this fact is now recognised by the establishment of zymotechnical laboratories for the determination of the organisms that can be most advantageously employed, and for the cultivation of the same for use in brewing and allied processes. This has led to a more accurate adaptation of the means to the end in view. But the selection of the right agents is not the only matter of importance ; care must also be taken to prevent the action of injurious organisms during a fermentation process. These, if present, will develop along with the essential factors in the fermentation and interfere with the yield and quality of the alcohol. This is one of the great troubles in connection with fermentation industries of the above nature, and has been partially combated by strict attention to cleanliness in all the operations with a view to preventing the intrusion of microparasites, which either hinder the fermentation or act injuriously on its products. The ubiquitous nature of the organisms in question renders this latter point a matter of great difficulty, and the attempt has been made to overcome it by the addition of antiseptics to the wort. The absolutely certain method of killing such infective agents by heat is impossible in the ordinary methods of distillation, inasmuch as the temperature necessary to effect this would destroy the saccharifying action of the malt upon the starch. An element of uncertainty as re-

gards results, therefore, remains in the distilling processes usually employed.

In the *Annales of the Pasteur Institute* M. Calmette[4] published a paper upon "La Levure Chinoise," or the Chinese Yeast. As already stated, the first step in the manufacture of alcohol in European countries is the conversion of starch into sugar by the action of an unorganised ferment, the malt diastase, or sometimes by the action of acids. In Eastern countries, however, organised or living ferments have long been used to saccharify the starch, *e.g.*, the Japanese Koji, which is used to prepare an alcoholic beverage from rice, called Sake. The agent in this instance is a mould, the *aspergillus orizæ*, and the saccharified fluid subsequently undergoes a spontaneous alcoholic fermentation in which yeasts take part. The Koji is also used by the Japanese for the preparation of the sauce "Soy" and in the making of bread. Takamine has devoted special attention to the study of this ferment and the utilisation of pure cultures of the mould as saccharifying agents.[5] Similarly in China and Indo-China a special ferment has been used, which M. Calmette found to be distinct from the Japanese Koji. This "Chinese Yeast" consists in the symbiosis of a mould and several varieties of alcoholic yeasts. It appears in commerce as flattened cakes. The recipe for its preparation includes about forty-six aromatic ingredients, but no mention is made of the living agents which were introduced into the cake with the rice grains used in its manufacture. The Chinese were, therefore, apparently ignorant of the essential factor in their process. As can be readily understood, such a mixture contained a number of impurities, which could only act deleteriously on the process, as the smell of a sample of the native spirit sufficiently testified. The Chinese add this "yeast" to a rice rich in starch, called "Nep." A saccharification of the starch occurs in three days and an alcoholic fermentation in two days. The liquid is then distilled in a primitive form of apparatus.

M. Calmette succeeded in isolating the essential factor from the crude "Chinese Yeast" by means of bacteriological methods, using a solid nutrient soil for this purpose, viz.,

beerwort gelatin. Growths were obtained of several yeasts and several species of moulds; one species of mould was particularly numerous, and its branching filaments rapidly covered the whole surface of the nutrient soil employed. This mould was also easily cultivated on other soils, such as potato, beerwort, rice, grains, &c. It also actively hydrolysed starchy media, producing dextrin and fermentable sugars. In this respect it was the most energetic fermentative constituent of the Chinese yeast, and undoubtedly the main factor in its action. The mould we are dealing with belongs to the group of the mucors, and was named by M. Calmette the *amylomyces rouxii*. The habitat of this mould appears to be the undecorticated rice grains, from which it can be readily isolated. The reproduction of the mould is brought about by means of spores, which are formed in special spore-sacs if the organism has free access to air, or they are formed in the continuity of its filaments if the access of air is limited. These spores preserve their vitality for a considerable time (two to three years), and if transplanted to a fresh soil reproduce the characteristic growth of the mould once more. There is little else to be seen in old cultures of the mould save the reproductive spores, and it is these spores which are utilised when it is desired to start a new growth and to set the special functions associated with the vegetative life of the mould at work. The useful functions of this mould vary with the amount of air to which it has access. If the amount of air be limited, or if growth takes place below the surface of a sugary fluid, the fermentative power of the organism is increased. The Chinese recognise this in a primitive fashion by placing a cover on their fermentation jars. A second point of practical importance is the temperature at which the mould is cultivated. The development and saccharifying action of the amylomyces rouxii occur best at 35° to 38° C.—*i.e.*, at temperatures approaching blood heat. The experiments of M. Calmette have thus shown that the "Chinese Yeast" contains a special ferment, and consists in the symbiosis of a saccharifying mould (amylomyces rouxii) with one or more wild alcoholic yeasts.

The amylomyces rouxii can also ferment the sugar it has formed, but experience has shown that the process works better when the action of a yeast is called in as well.

M. Sanguinetti[6] conducted a series of comparative experiments in M. Calmette's laboratory upon certain moulds which have the double property of fermenting starch and sugar, viz., the mucor alternans (Gayon), the aspergillus orizae (Japanese Sake ferment), and amylomyces rouxii (the Chinese ferment). It was found that the amylomyces rouxii was the best adapted for industrial use on account of its marked saccharifying and fermenting properties, as well as from the fact that its oxidising properties are feeble, and, consequently, it does not produce loss by the destruction of valuable products of fermentation.

Upon these observations of M. Calmette a process of distillation has been founded, which is far removed from the primitive methods employed in the East. Biologically the process consists in sowing the spores of amylomyces rouxii in a sterilised mash, allowing the organism to develop at a suitable temperature to saccharify the mass, and then adding a pure culture of a yeast to produce a complete alcoholic fermentation of the sugar so formed—the whole process being carried out under aseptic conditions in sterilised chambers or vats. The mash cannot be sterilised in the ordinary processes of fermentation, because the malt diastase which is used for saccharification is destroyed at temperatures approaching the boiling point. In this method complete sterilisation is possible, the saccharification being accomplished by the amylomyces rouxii that is added subsequent to the sterilisation of the mash. The conditions are entirely aseptic throughout, and represent the careful methods of the bacteriological laboratory carried out on a large scale. I have to thank M. Calmette for a pure culture of the amylomyces rouxii.

The organism is readily cultivated on the ordinary culture media of the bacteriological laboratory. It gives a good growth on beerwort agar at 22° C. and at 35° to 38° C. At the higher temperatures its development is particularly vigorous, and the whole surface of the medium

employed becomes covered with a silky growth in the space of twenty-four hours. Sown in liquid beerwort the growth is of an equally vigorous character. In sugar agar the organism also develops well, and the growth is accompanied by the development of gas bubbles, due to a fermentation of the sugar present. There is also a development of the "amylo" in the ordinary bouillon made from beef, and a felt-like growth occurs on the surface of slices of potato. On nutrient gelatin the "amylo" growth is accompanied by a liquefaction of the medium. In simple starch water the growth permeates the whole of the fluid in twenty-four hours.

When sown on slightly moistened rice grains the development is slower than on the above media. The growth when it does appear in 2 to 4 days consists of delicate aërial filaments or hyphæ, which spin themselves in a gossamer-like fashion round each individual rice grain. The sporulation of the organism on the rice grains proceeds quickly, and as the culture becomes older there is little else but these reproductive elements or spores to be seen. This soil is the best for obtaining stock cultures of the amylomyces rouxii, because the fructification that occurs is so complete, and the spores thus formed preserve their vitality for a long time. Such a culture can be used after many months for starting a fresh growth when required.

The following experiment, which can be readily carried out, is a happy illustration of a pure symbiotic fermentation: If the amylomyces rouxii be grown on boiled and moistened starch grains for about eight to ten days at a temperature of 33° C., a distinct, visible web-like growth appears on the surface of the individual rice grains. If this culture be added to sterilised distillers' wash, and the fluid kept at blood heat with free access of air, a very active growth occurs, and the whole liquid becomes filled with the thread-like filaments of the organism. The result is an active saccharification of the starchy material. If at an interval of about twenty-four hours a pure culture of a yeast be added to the flask, it undergoes a rapid development alongside of the mould in the now saccharine fluid. The yeast's growth appears to be

favoured and accelerated in the fluid which is interpenetrated with the filaments of the mould. If we may so express it, the two organisms join hands, the mould saccharifying the starch, and the yeast fermenting the sugar as quickly as it is formed into alcohol and carbonic acid. This symbiosis accomplishes more than if either organism were working alone, and at the same time their respective action is unhindered by foreign organisms, as is the case in the crude Chinese process of fermentation. The following experiments, suggested by Dr. Horace Brown, will serve to illustrate the results of the symbiosis I have endeavoured to describe. I am indebted to him and to M. Boidin for the analytical data that are given below.

A series of flasks are prepared containing well sterilised wash, and after treating the flasks in the manner described below, they are placed at a temperature of 32° C. for five days :—

Flask 1A, „ 1B — Seeded with yeast only.

„ 2A, „ 2B — Seeded with "amylo" only.

„ 3A, „ 3B — Seeded both with "amylo" and yeast, which were *added simultaneously*.

„ 4A, „ 4B — Seeded first with "amylo" and then, *twenty hours afterwards*, with yeast.

At the end of the five days the appearance of the flasks differed very much.

In 2A and 2B the growth of the "amylo" covered the entire surface of the liquid. In 3A and 3B, which had been seeded simultaneously with both yeast and "amylo," only a few small isolated patches of "amylo" appeared on the surface. The growth of "amylo" was meagre, the carbonic acid produced by the yeast having interfered with its development. In 4A and 4B, where the seeding with "amylo" was followed by the addition of the yeast twenty hours afterwards, there was a much larger growth of the "amylo" film.

The results of the chemical examination showing the extent of fermentation in each case are here tabulated :—

	Yeast Only.			"Amylo" Only.	
	Control.	1 A	1 B	A	2 B
Total acidity	0·029	0·068	0·068	0·147	0·156
Fixed acidity..	0·029	Lost	0·044	0·102	0·110
Iodine reaction	Violet	—	Violet	None	None
Alcohol per cent. weight	0	—	2·189	2·620	2·519
Apparent density	1031·3	—	1008·9	1008·8	1010·0
Real density after separation of alcohol	—	—	1013·1	1013·7	1013·9

	"Amylo" and Yeast.		"Amylo" with Yeast after Twenty Hours.	
	3 A	3 B	4 A	4 B
Total acidity	—	0·068	0·127	—
Fixed acidity..	—	0·058	0·080	—
Iodine reaction	—	None	None	—
Alcohol per cent. weight	3·16	3·920	4·135	4·085
Apparent density	1007	1006·0	999·4	999·6
Real density after separation of alcohol	1011·3	1010·6	1005	1005·4

It will be seen from the above experiments that the mould when grown alone can both saccharify starch and ferment the sugar formed, but that the combined action of the mould and yeast is greater than the action of either organism when grown alone. This symbiotic action is more active if the yeast is added subsequently to the mould.

These experiments illustrate facts of considerable biological interest, viz. :—

(1) The utilisation of pure cultures of a mould instead of diastase for purposes of saccharification and fermentation.

(2) The aseptic conditions that this procedure renders possible.

(3) The development of symbiotic activity that occurs when the mould and a yeast are cultivated together in an originally sterile fluid, the result being a pure symbiotic fermentation.

It cannot, of course, be supposed that these properties are peculiar to the amylomyces rouxii, and it remains for future research to determine in how far other species of moulds may be adapted for utilisation in a similar fashion.

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