THE CLOUDING OF WHITE WINE.

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Under the name of Chablis there is made in South Australia a variety of white wine which has a great tendency to develop a turbidity or cloudiness soon after it is bottled. It is a soft wine, and in consequence of the low acidity the fermentation is prolonged while a trace of sugar is left unfermented. In the 750 gallon storage casks, and in the smaller 100 gallon casks from which it is bottled the wine is absolutely brilliant. It is also perfectly bright immediately after bottling, but after an interval of some days or weeks—depending upon the air-temperature—the brilliancy disappears and the wine slowly becomes dull and then clouded. When undisturbed a deposit collects at the bottom of the bottle, and the wine becomes less clouded; a shake is, however, sufficient to disturb the sediment and distribute it throughout the wine. The change seems to have no effect upon the composition of the wine, for the flavour and bouquet remain unaltered. The trouble is of considerable importance to the wine industry, as many thousands of bottles may be annually rendered commercially useless, since the public will not buy a turbid wine, even although the wine is otherwise excellent.

At my request a sample bottle of wine was sent to the laboratory. It arrived in perfectly bright condition, but during the course of a fortnight it became dull and finally turbid. An investigation was started to determine the cause of the phenomenon. Obviously the first thing to be done was to grow any living organisms that might be in the wine, then having obtained them in pure condition to infect healthy wine, and so prove a particular organism as the

cause of the trouble or disease. Once the cause is obtained, experiment will soon suggest a cure. There is always the possibility, however, that the turbidity might not be due to the bodies of living organisms, but to the action of enzymes derived either from the original grapes or from the yeasts that played a part in the fermentation. But it is unlikely that an enzyme would coagulate some of the soluble constituents at so late a stage in the history of the wine.

Wortmann (Referat in Centralblatt für Bakt., ii. Abt., vi., 298) traced a turbidity of wine, which set in after the wine had been bottled, to the gradual disintegration of yeast cells. Sometimes, owing to a variety of causes, such as low acidity or too high a temperature during fermentation, wine may not be completely fermented. It is in such cases that slowly growing yeast cells may be present in greater amount than in the case of thoroughly fermented wines. Wortmann apparently came to his conclusion from a microscopical examination of the wine alone. Had I depended solely upon microscopical observations of the Chablis when the turbidity had made itself evident, I might have come to the same conclusion, for thin films of the wine showed nothing but what might be particles of organic matter in Brownian movement. However, to diagnose the exciting cause of any disease of food or animal from simple microscopical examination alone does not generally recommend itself to the zymo-technologist or the bacteriologist.

In separating the living cells ordinary nutrient agar was first used, and a series of plates were prepared from infected tubes of the molten medium. Although this medium favours the growth of most bacteria, yet yeasts can obtain enough nutriment from it to grow as small colonies. It is, therefore, an excellent medium for finding out what micro-organisms are probably present in any substance. The plates in due course grew several kinds of colonies, among which were a common mould, *Penicillium glaucum*, and two bacteria, *Sarcina lutea* and *Bacillus subtilis*. Besides these there were a number of yeast colonies, and to obtain more

vigorous cultures of these fresh plates of peptone-glucose-gelatine* were prepared. The examination of the colonies that developed upon these plates made it evident that there were two varieties of yeast. As passage through a solid medium tends to alter the characters of the yeast, the two kinds were isolated by the dilution method from a vigorous culture in Hansen's peptone-glucose fluid.

Pure cultures of the two yeasts obtained in this way showed that they both belonged to the group which has for its type *Saccharomyces membranifaciens*. The first of these yeasts consisted of round, oval or sausage-shaped cells, containing one or two refractile granules. In wine must it formed a strong crumpled film and a slight flocculent precipitate. Many cells of the film contained two, and occasionally three, round or flattened spores. The second of the yeasts formed in wine must a slight transparent film and a bulky white sediment. The cells were chiefly oval, indistinctly vacuolated, and contained one or two refractile granules. Only a few of the film cells contained two spores. Neither of the yeasts induced a visible fermentation.

I was informed that the turbidity which arises in the wine would probably not be found to have a bacterial origin since the cloudiness is never so pronounced as when the wine is attacked by bacterial diseases. From this it was to be inferred that the exciting agents would be yeasts, and of the two yeasts separated, one would probably be found to bring about a turbidity in experimental wine. For the purposes of experiment I had sent to the laboratory a few bottles of wine siphoned directly from the 750-gallon storage casks into sterilised bottles and closed with sterilised corks. The wine had been allowed to run for some time in order to clean the siphon. A portion of this wine was filled into small bottles and pasteurised at 75° C. for a quarter of an hour. These small experimental bottles were infected with the pure yeasts separately and together. At the

* Gelatine 10% added to Hansen's peptone-glucose fluid and neutralised to faint acidity with potassium hydrate.

same time two small bottles of unpasteurised wine were placed under observation. All the bottles were incubated at 22° C. for three weeks, when it was found that the pure yeasts, although still alive, had not grown, and it was evident that they were not responsible for the trouble. The unpasteurised wine was turbid, and had a thin surface film and a slight precipitate. During the experiment it was noted that a growth occurred first upon the surface of the unpasteurised wine and then spread downwards. An examination of the film revealed a zooglœa mass of bacteria. The same kind of bacteria were obtained in the body of the wine. On re-examining the original bottle of wine from which the yeast had been obtained, it was seen that a film had by this time formed, and it consisted entirely of bacteria similar to those found in the experimental bottles. The film had probably been present at an earlier stage, but it had been so slight as to be obscured by the dark colour of the glass of the bottle.

The investigation had so far advanced as to indicate the infecting organism, and in order to obtain it in pure culture, plates of nutrient agar and peptone-glucose-gelatine were prepared, but no growth appeared in these. The fact that the organism does not grow upon these media explains how I did not obtain it in my earlier experiments. Neither did it grow on nutrient glucose-gelatine. Colonies were, however, obtained upon plates of nutrient agar to which about one-third of the volume of pasteurised wine had been added while the agar was fluid. The colonies appeared at the end of four days when grown at 22° C. as small white points. When magnified sixty-fold they appeared round, brownish-black, and finely granular; the edges were smooth and the colonies had each a dark central point (the starting point of the colony). Within a central zone of half the radius of the colony the granulation was darker than at the margin. The deep and subsurface colonies appeared irregular, rough and slightly moruloid. The colonies enlarged, becoming round, white and glistening like a drop of wax. The middle of the colony was raised. The bacterial colonies derived from the original bottle of wine and from the wine from the storage cask, were

identical in appearance and structure, while films showed the same organism.

When taken from the surface of the wine and examined in the fresh and moist unstained condition, the organisms are seen to be of two kinds. One is thin and refractile, the other broader and non-refractile. The thinner cells are generally divided into two and rarely three parts, while the broader cells appear homogeneous. The thinner cells are about 0·7 μ broad and vary from 2 to 3 μ in length; the broader cells are from 0·8 to 1·0 μ broad and 0·9 to 1·5 μ long. The parts of the thinner cells showed up well with aqueous eosin, and when so stained appeared as spheres within a common capsule. Little protuberances that suggested buds appeared attached to the end of a few of the broader cells. This would indicate that the organism is a yeast, although at first sight the minute size makes it probable that it is a bacterium. If it is a yeast, then the endocellular eosin-staining spheres are probably spores. The appearances of the stained and balsam mounted films, however, indicate that the organism is a bacterium. Although little prominences could be occasionally seen on a few cells in stained films, yet they were so few and of so doubtful a nature that they could not be taken to prove the organism to be a yeast. In one case a distinct bud appeared on a cell at the margin of a colony grown upon a wine-gelatine film. When, however, the film was hardened and stained the bud had not taken up the stain and could not be seen. Furthermore, no other bud-like appearances could be found.

Films of the organisms prepared from the surface growth upon wine and stained after fixation in the ordinary way with thionin-blue exhibit cells that appear as rounded rods staining darker at the ends than in the middle. The middle portion varies in its capacity for fixing the stain and may be as deeply stained as the poles, lightly stained or colourless. In the first case the organism appears as a rod with rounded ends, in the second as a bipolar staining bacterium, and in the third as a double coccus. The cells when grown in wine vary from 0·5 : 1·0 μ to 0·6 : 1·5 μ in

size, and may therefore be called short stout rods. In fluids other than wine, as for example glucose-yeast-water, the organisms are thinner and show stained parts of unequal size. A rounder form is obtained when cultivations are made in nutrient gelatine, to which a little wine, say one-fourth of the volume of gelatine, is added. In this medium they appear as coccobacteria, varying in size from $0{\cdot}5 : 0{\cdot}6\,\mu$ to $0{\cdot}6 : 0{\cdot}9\,\mu$. When stained with blue they appear like diplococci, the unstained central part being a narrow unstained line, on either side of which are hemispherical stained portions.

The organism grows best in media which contain the products of yeast activity, such as wine or yeast-water. When floated upon the surface of wine it grows freely, though slowly; on the other hand, when submerged in the mass of the wine it grows very slowly indeed. In the latter case a slight surface film appears before growth occurs in the fluid. This is reversed when, instead of wine, yeast-water with or without glucose is employed. In this medium a delicate film appears after the medium has become turbid and a white sediment has collected at the bottom of the liquid. During the course of the experiments it became evident that the greater the air surface of the wine in the experimental bottles the quicker did the turbidity appear. The inference from this is that the bacterium in wine behaves as an aerobe, and it is probable that the slight aeration which the wine receives in the process of bottling stimulates the growth of the organism which has been restricted by the anaerobic condition of the wine in the large casks. The aerobic character was proved by placing the organism under the practically anaerobic conditions that obtain in Buchner's tubes (from which the oxygen is removed by alkaline pyrogallate). Yeast-water inoculated with the bacterium and placed under these conditions failed to produce a growth.

The temperature best suited to its development would appear to be about 25° C., as growth was feeble at 17° and comparatively quick at 22°. At 37° the organism refused to grow.

In endeavouring to discover the constituents of wine which favour the development of the bacterium I employed nutrient agar, to which various substances had been added. In the first place, alcohol obtained by distilling wine produced a slight growth. A similar growth was also obtained by the addition of levulose and of lactose. Maltose, dextrose and sucrose were without influence, while the addition of a small quantity of tartaric acid (to make 0·3%) to the carbohydrates hindered the development of the organism. Dextrin, glycerol and succinic acid were likewise inoperative. No growth took place in Hansen's peptose-glucose with or without the addition of gelatine. From these experiments it would appear probable that the one particular constituent of wine that is active in stimulating the growth of the organism is alcohol.

The obligate aerobic character of the bacterium, and the tendency that it has for forming films upon the surface of fluids, are suggestive of the acetic bacteria. With the object of determining the production of acid a culture was smeared over a plate of wine-litmus-lactose-agar and incubated at 22°. The litmus in the vicinity of the culture was reddened. As this might be due to the formation of lactic acid from the lactose or acetic acid from the alcohol, a plate of nutrient agar, to which had been added alcohol (wine distillate) and chalk, was smeared with a culture of the organism. The chalk in the vicinity of the growth was dissolved, and the agar, when microscopically examined, was found to be transparent and to contain no crystals. A chalk plate without alcohol was unaffected. From these experiments the bacterium appears to belong to the group of acetic-acid-forming bacteria.

My first impression was that the trouble might have originated in the corks with which the bottles were fastened, a view engendered by the observation that the cork of the first bottle contained a number of minute crevices in which the microscope revealed the greenish colour of fruiting *Penicillium glaucum;* while plate cultures from fragments of the cork produced the above mentioned hyphomyces and a variety of yeasts. This idea was,

however, negatived by obtaining the infecting organism from the later samples which had been carried in bottles sealed with sterilised corks.

With regard to the remedy for the trouble, it is apparent that since the organism is in the bulk of the wine and does not obtain access during the process of bottling, the employment of sterile bottles, corks, etc., would be of no avail. The wine itself must be treated so as to kill off the organism, and the only legitimate method of doing this consists in the pasteurisation of the wine. According to an experiment conducted with infected yeast-water, the lethal temperature was found to be between 66° and 72° C. A further experiment showed that the organism was killed and the yeast-water remained bright after an exposure for 5 minutes to 66° C. But the organism may be killed at a lower temperature when it is present in wine, and in an experiment where the surface of wine was infected previous to pasteurisation a temperature of 43° C. when maintained for 5 minutes was found to be sufficient.

In the experimental bottles of wine which I exhibit, both bottles were filled to the shoulder with wine, and then pasteurised for 15 minutes at 70° C. One was inoculated by floating a small loop taken from a wine film upon the surface. The other bottle was kept as a contrast to show the differences brought about by the organism. In five days, at 22° C., a strong film had spread over the surface. On shaking, the film broke up and settled to the bottom of the wine. On the seventh day the wine was decidedly turbid; on the fourteenth day it was very turbid, and had a strong sediment and surface film. The contrast bottle remained clear.

Note.—The conditions under which the above pasteurisation experiments were performed are not precisely such as would obtain in ordinary practice where the bacteria are suspended throughout the body of the wine. A fresh sample was obtained for the purpose of ascertaining the temperature requisite to kill the organisms in naturally infected wine. The original wine (Chablis)

had, however, been blended and sent to the London market, but in its place another variety (Reisling, 1897) which undergoes a similar clouding, was forwarded to the laboratory. This wine was made at the same time and from grapes grown upon the same kind of soil as the Chablis, the only difference being in the variety of grape employed. On its arrival at the laboratory, the wine had begun to show a turbidity, but this did not affect the experiment, as a check unpasteurised flask was always kept for purposes of comparison. A preliminary experiment showed that the bacteria were killed when exposed to a temperature rising in five minutes from 40° to 45° C. In the second experiment, the temperature rose one degree in five minutes, and those flasks which were heated from 42° to 43° C. were subsequently found to have been sterilised. The flasks which had been heated at 40°-41° C. showed a growth-turbidity four days later than those heated at a lower temperature; at 41°-42° C. the turbidity appeared seven days later. In wine which had been heated below the lethal temperature, a surface film appeared as the original sediment deposited. The film increased, and the wine became turbid throughout its mass. Those flasks which were heated at and above the pasteurisation-temperature (43° C.) remained clear after depositing their sediment. It is, therefore, immaterial whether the bacteria are suspended in wine or floated upon the surface; the lethal temperature is the same in both cases. It is fortunate that this temperature is so low, since the brightness and bouquet of the wine are not likely to be influenced by the process of pasteurisation.—*March 4th, 1901.*